Solvent-Resistant Elastomeric Microfluidic Devices and Applications

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Robert Michael van Dam

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Abstract

Microfluidics is increasingly being used in many areas of biotechnology and chemistry to achieve reduced reagent volumes, improved performance, integration, and parallelism, among other advantages. Though early devices were based on rigid materials such as glass and silicon, elastomeric materials such as polydimethylsiloxane (PDMS) are rapidly emerging as a ubiquitous platform for applications in biotechnology. This is due, in part, to simpler fabrication procedures and to the ability to integrate mechanical microvalves at vastly greater densities. For many applications in the areas of chemical synthesis and analysis, however, PDMS cannot replace glass and silicon due to its incompatibility with many solvents and reagents.

Such areas could benefit tremendously from the development of an elastomeric microfluidic device technology that combines the advantages of PDMS with the property of solvent resistance. Simplified fabrication could increase the accessibility of microfluidics, and the possibility of dense valve integration could lead to significant advances in device sophistication. Applications could be more rapidly developed by design re-use due to the independence of mechanical valves on fluid properties (unlike electrokinetic pumping), and the property of permeability could enable novel fluidic functions for accessing a broader range of reactions than is possible in glass and silicon.

The first half of this thesis describes our strategies and efforts to develop this new enabling technology. Several approaches are presented in Chapter 3, and two particularly successful ones, based on new elastomers (FNB and PFPE), are described in Chapters 4 and 5. Chapter 6 describes a novel method of fabricating devices from 3D molds that could expand the range of useful elastomers.

The second half of this thesis discusses microfluidic combinatorial synthesis and high throughput screening—applications that take particular advantage of the ability to integrate thousands of individual valves and reaction chambers. Chapter 7 introduces several scalable device architectures and presents results of preliminary steps toward the synthesis of combinatorial DNA and peptide arrays. A novel method of performing universal gene expression analysis with combinatorial DNA arrays is described in Chapter 8 and an algorithm for predicting relationships among genes from gene expression array data is presented in Chapter 9.

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Chapter 1 Introduction

1.1 Background

Microfluidics is increasingly being used to scale down and automate laboratory procedures in the fields of biotechnology and chemistry [218, 205]. The small dimensions of microchannels tend to reduce reagent consumption and waste production, leading to cost savings and enabling precious samples to be divided up among larger numbers of screening assays [97, 25]. Furthermore, many identical reactions or assays can be replicated on a single microfluidic chip to harness parallelism and increase throughput [170, 112, 48], or many different stages in a complex process can be integrated into a single chip to improve ease of use and reduce human error—for example, in medical diagnostic devices. It has also been reported that microchannels can improve the speed and accuracy of chemical reactions [286, 62], as well as the speed, sensitivity, and repeatability of many assays.

Microfluidic devices based on elastomeric materials such as polydimethylsiloxane (PDMS) are rapidly becoming a ubiquitous platform for applications in biotechnology [218, 205]. Recent growth in the field of PDMS microfluidics has far outpaced that in alternative device technologies based on glass and silicon, due in large part to significantly simpler and less expensive fabrication procedures as well as the possibility of easily incorporating integrated mechanical microvalves at extremely high densities [272, 268].

This trend is limited to applications involving aqueous solutions, however. Glass and silicon devices are still preferable to PDMS devices in many areas of microfluidic chemical synthesis and analysis, where acids, bases, and organic solvents are frequently used. PDMS is incompatibile with many such solvents [160], and exposure can lead to adverse effects (including swelling) that are especially pronounced in microscale channels due to the high surface to volume ratio. On the other hand, glass, silicon, and other rigid materials, such as ceramics and metals, are relatively inert.

Despite this inertness, there are several drawbacks to the use of rigid materials in microfluidic devices. In particular, mechanical valves are difficult and expensive to fabricate [218], and devices of high complexity have been impossible due to the large size of these valves—typically several millimeters [305, 218]. To circumvent these problems, non-mechanical fluid manipulation techniques such as electrokinetic pumping have frequently been used in these devices. Such methods do not scale well to complex channel networks, however, and unlike mechanical valves and pumps, their operation depends sensitively on the physical and chemical properties of the fluid [134]. This latter disadvantage is particularly problematic for applications in organic chemistry due to the huge variety of solvents with different properties that are commonly used.

We believe that the field of chemistry could benefit tremendously from the development of an elastomeric microfluidic device technology that offers the same advantages as PDMS microfluidics with the additional feature of high solvent-resistance. Efforts to develop this new enabling technology are the subject of the first half of this thesis.

With the advantage of simplified device fabrication, it is expected that solvent-resistant elastomeric microfluidics will become more accessible to a greater number of chemists than glass and silicon fluidics will, therefore accelerating explorations in this field. In addition, the use of mechanical microvalves could eliminate the dependence of reactor and assay designs on fluid properties, leading to greater design re-use and more rapid development of new applications. Unlike their glass and silicon predecessors, devices based on solvent-resistant elastomers possess the property of gas permeability, which allows device designs to be simplified through the use of dead-end channels. Permeability also allows evaporation to be used as a means to dry out reagents or to exchange solvents on-chip, thus providing valuable new tools for accessing a broader range of reactions than was previously possible with microfluidics. Finally, the ability to fabricate solvent-resistant devices with thousands of individual valves and reaction chambers will likely lead to novel applications such as combinatorial organic synthesis and high throughput screening that were not possible in glass and silicon devices. Combinatorial techniques are widely used in industry to discover and screen novel compounds for properties such as catalytic activity or therapeutic effects in a high-throughput brute force manner.

Microfluidic combinatorial chemistry is the subject of the last half of this thesis, with particular emphasis paid to the synthesis of combinatorial peptide and DNA arrays, and their applications in the areas of genomics and bioinformatics.

1.2 Organization

This thesis is organized as follows. Chapter 2 provides a brief overview of microfluidics, with particular emphasis on PDMS device fabrication. The chapter concludes with a discussion of the advantages of PDMS microfluidic technology that have led to its rapid adoption for many sophisticated biotechnology applications.

In Chapter 3, I argue that highly-integrated applications in the areas of chemical synthesis and analysis have not yet been realized due to the lack of solvent-resistance of PDMS and due to the many limitations of alternative technologies. The bulk of this chapter describes our efforts to fill this gap by developing new microfluidic device technologies that combine the advantages of PDMS devices with the property of solvent-resistance. Results are discussed for many different directions of investigation, some of which met with moderate success. Two additional approaches are described in Chapters 4 and 5, both of which culminated in the successful demonstration of solvent-resistant devices with functional microvalves. Chapter 4 discusses the fabrication of devices from fluorinated norbornene polymers in collaboration with Materia Incorporated, and Chapter 5 discusses fabrication from perfluoropolyethers in collaboration with Joseph DeSimone's group at the University of North Carolina at Chapel Hill.

A novel technique for fabricating microfluidic devices from three-dimensional molds is described in Chapter 6. While the approach was originally pursued merely as a means to eliminate bonding steps during device fabrication—steps that proved particularly problematic in solvent-resistant materials our molding technique may find other uses in 3D device fabrication due to its many advantages compared with alternatives.

Chapter 7 deals with combinatorial synthesis, an important branch of chemistry that can benefit from the high integration densities that are possible with solvent-resistant elastomeric microfluidic devices. In particular, we have designed microfluidic devices that have the potential to synthesize *in situ* arrays of compounds at much higher densities and with greater purity than other methods. As examples, we demonstrated several principles of the synthesis of DNA and peptide arrays by solid-phase methods.

In Chapter 8, I argue that combinatorial arrays of DNA could be used for genome-wide expression analysis and could offer many advantages—such as universality—over the targeted arrays that are currently used for such studies. We developed a mathematical model to determine the required value of n such that an array of all possible DNA n-mers could provide meaningful results in experiments with complex organisms such as mouse or human. We show that the minimum useful value of nis technically feasible in terms of array fabrication and readout. It was this result, in fact, that originally motivated our pursuit of microfluidic array synthesis and our development of solventresistant microfluidic device fabrication technologies.

High-throughput gene expression studies have helped to deduce the functions of unknown genes and to identify the interconnections among genes in the complex genetic networks of many organisms. Chapter 9 motivates and describes a new algorithm that we developed for mining the vast wealth of published gene expression data to determine pairs of genes that are likely to be related. Our algorithm uses a non-metric probability measure that can in principle detect a wider variety of relationships than other approaches.

1.3 Contributions

The work described in this thesis represents significant original contributions in several fields.

First, we developed several novel materials and fabrication procedures for elastomeric microfluidic devices to confer the property of high solvent resistance (Chapters 3, 4, and 5). This new property will enable elastomeric microfluidic devices—with their intrinsic advantages—to be used in numerous new applications, including chemical synthesis and analysis. Devices with functional microvalves were demonstrated using a variety of resistant materials and coatings. The work on perfluoropolyether devices (Chapter 5) has been published [230], and a patent application has been filed.

Second, we devised a new method to fabricate three-dimensional microfluidic networks based on replication molding from sacrificial wax molds (Chapter 6). With this technique we have demonstrated the first complex 3D fluidic networks containing integrated elastomeric microvalves. A manuscript is in preparation.

Third, we designed microfluidic devices that can be used for high-density combinatorial solidphase synthesis and demonstrated several aspects of their operation for the synthesis of DNA and peptide arrays (Chapter 7). A patent has been granted on these concepts [274]. However, this work has not yet been published due to the scarcity of solvent-resistant materials, which has prevented the demonstration of a large scale microfluidic synthesis.

Fourth, to our knowledge, we were the first to contemplate the use and advantages of combinatorial *n*-mer arrays for universal gene expression analysis (Chapter 8). The algorithm we developed enabled us to quantify the minimum value of n that is theoretically necessary to construct a *useful* array and to show that it is within the realm of technical feasibility. This work has been published [275], and a patent application has been filed [220]. Gene expression analysis using (slightly different) universal arrays has recently be experimentally demonstrated by Roth *et al.* [233].

Fifth, our probabilistic analysis of gene expression ratio data (Chapter 9) is a significant extension to the approach of Walker *et al.* [282, 281, 280]. Our modification fundamentally changes the type of data that can be analyzed, opening up a vast wealth of published microarray data to analysis by this approach. A manuscript describing this work is in preparation, and a patent has recently been awarded [219]. We have implemented this algorithm in computer code and have made the computed probabilities available in an online database.

Finally, I helped Matthew Reese to demonstrate the effectiveness of novel microarray pens that he microfabricated from stainless steel foil. These pens can be used on standard microarrayers to deposit cDNA probes or other biomolecules onto arrays at significantly higher densities than conventional pens. This work is described briefly in Chapter 7. Our results have been published [225], and a patent application has been filed [276].

Chapter 2 Introduction to Microfluidics

2.1 Introduction

The earliest microfluidic devices demonstrated that fluidic components could be miniaturized and integrated together, leading to the idea that one could fit an entire "lab on a chip", in much the same way that a microelectronic circuit is an entire computer on a chip. Since then, there has been tremendous interest in harnessing the full potential of this approach and, consequently, the development of countless microfluidic devices and fabrication methods. Elastomeric materials such as poly(dimethylsiloxane) (PDMS) have emerged recently as excellent alternatives to the silicon and glass used in early devices fabricated by MEMS (microelectromechanical systems) processes [205, 218]. Simplified device fabrication and the possibility of incorporating densely integrated microvalves into designs [272, 268] have helped microfluidics to explode into a ubiquitous technology that has found applications in many diverse fields.

This chapter begins with a brief introduction to microfluidics, followed by a description of the PDMS-based microfluidic technology that was developed in our lab. Many factors taken together have contributed to the success of this technology, as discussed in the final section. In Chapter 3, these desirable properties are used as a guide for the development of microfluidic devices from new chemically-resistant materials. Such devices have the potential to serve as powerful tools in novel areas of research and industry that are currently inaccessible due to fundamental incompatibilities of PDMS with many organic solvents [160].

2.2 Microfluidics

As numerous investigators have pointed out, scaling down fluidic processes to the microscale offers many significant advantages [178, 181, 195, 222, 62, 132, 133, 44, 256], some stemming directly from the reduction in size and others a result of the ability to integrate at this scale.

2.2.1 Benefits of size reduction

One obvious advantage is that miniaturized components and processes use smaller volumes of fluid, thus leading to reduced reagent consumption. This decreases costs and permits small quantities of precious samples to be stretched further (for example, divided up into a much larger number of screening assays) [25]. Quantities of waste products are also reduced.

The low thermal mass and large surface to volume ratio of small components facilitates rapid heat transfer, enabling quick temperature changes and precise temperature control. In exothermic reactions, this feature can help to eliminate the buildup of heat or "hot spots" that could otherwise lead to undesired side reactions or even explosions [62]. The large surface to volume ratio is also an advantage in processes involving support-bound catalysts or enzymes, and in solid-phase synthesis.

At the small length scales of microfluidic devices, diffusive mixing is fast, often increasing the speed and accuracy of reactions. Dramatic performance improvements are often seen in microfluidic *assays* as well: reduced measurement times, improved sensitivity, higher selectivity, and greater repeatability, are common. For example, dispersion broadening is reduced in electrophoretic separations by the rapid dissipation of Joule heat. In some separations, sensitivity is improved simply because the reduced measurement time leads to a lower degree of peak broadening [236].

Microfluidic devices sometimes enable tasks to be accomplished in entirely new ways. For example, fluid temperature can be rapidly cycled by moving the fluid among chip regions with different temperatures rather than heating and cooling the fluid in place. A device to screen for protein crystallization conditions harnesses free-interface diffusion—a process that is practical only at the microscale—to explore a continuous range of conditions when protein and salt solutions are gradually mixed [97]. The laminar nature of fluid flow in microchannels permits new methods for performing solvent exchange, filtering, and two-phase reactions [270].

2.2.2 Benefits of automation and integration

Many microfluidic technologies permit the construction of devices containing multiple components with different functionalities. A single integrated chip could perform significant biological or chemical processing from beginning to end, for example the sampling, pre-processing, and measurement involved in an assay. This is the kind of vision that led to the terms "lab-on-a-chip" and "micro total analysis system (μ TAS)". Performing all fluid handling operations within a single chip saves time, reduces risk of sample loss or contamination, and can eliminate the need for bulky, expensive laboratory robots. Furthermore, operation of microfluidic devices can be fully automated, thus increasing throughput, improving ease of use, improving repeatability, and reducing the element of human error. Automation is also useful in applications requiring remote operation, such as devices performing continuous monitoring of chemical or enivornmental processes in inaccessible locations [77].

Another way to increase throughput is to exploit parallelism. Single chips have been demonstrated that perform hundreds or thousands of identical assays or reactions [112, 170, 48]. These chips utilize synchronization and control-sharing so that their operation is not significantly more complex than that of a non-parallel chip. They also feature on-chip distribution of a single input sample to thousands of microreactors—an interesting solution to the micro-to-macro interface problem [82, 170]. This problem refers to the mismatch between sample sizes that can be easily manipulated in the lab (μ L-mL) versus the volume of microreactors (pL-nL). The task of controlling thousands of individual valves with a much smaller number of off-chip control inputs is achieved by implementing multiplexers or other more complex logic *on-chip*, as is done in microelectronic chips.

Being planar and on the same scale as semiconductor integrated circuits, microfluidic devices are ideally poised to be integrated with electronic or optical components such as sensors, actuators, and control logic. On the sensing side, significant progress has been made: chemical, electrical, optical absoprtion, fluorescence, flow, temperature, and pressure sensors are just some examples that have been reported. Numerous actuators, such as valves, pumps, heating elements, and electrodes for electrophoresis or electrokinetic flow, have also been demonstrated. Beebe *et al.* [16] devised an interesting way to link sensing to actuation—specially tailored hydrogels respond to particular properties of the fluid by swelling and directly actuating a valve. In general, however, the potential of integrated control logic has been largely untapped. In the future, hybrid devices that perform sophisticated *in situ* monitoring and computation may emerge, perhaps to implement feedback control circuits that maintain optimum operating conditions or detect problems.

Small integrated microfluidic devices may also offer the feature of portability, enabling mobile applications in chemical analysis, point-of-care medicine, or forensics. The ability to perform integrated diagnostic tests where they are needed rather than in a centralized lab could reduce costs, improve turn-around time, and reduce the risk of sample mix-up. If manufactured cheaply, devices could be disposable, eliminating cross-contamination between tests. Microfluidic applications in drug delivery are also possible.

2.2.3 Application areas

The literature contains many thousands of reports of reactions and assays that have been carried out in microfluidics devices (see reviews in [8, 133, 111, 188, 69]). Some have shown significant improvements in performance compared with their macroscale counterparts and have successfully competed in the commercial marketplace. In some rare cases, microscale implementations have completely transformed the way that a certain type of experiment is performed or have enabled massively parallel experiments that previously could not even be contemplated.

Among the numerous biological and biochemical processes demonstrated are polymerase chain reaction (PCR) [170], immunoassays [290], drug screening, cell counting and sorting [84], electrophoretic separations, nucleic acid extraction [112], analysis of unpurified blood samples [290], DNA sequencing [142], screens for protein crystallization conditions [97], cell culture studies [9], and single cell manipulation [293]. In chemistry applications, dramatic improvements in synthetic yields and selectivities have been observed [286, 62]. In addition, microfluidic devices may make possible *novel* reactions or processing conditions by unprecedented control over surface chemistry, local heat and mass transfer [186, 132, 133, 44], or reagent concentrations in space and time (using electroosmotic flow) [286, 77]. The greater degree of control may help to design experiments to increase knowledge about many chemical processes [77].

Several investigators have also argued that microreactors could be used in industrial chemical production or waste treatment plants if volumetric processing requirements are low [181, 186, 62, 132]. Scaling up production can be achieved by bringing additional microreactors into service at a relatively low incremental cost rather than constructing a new higher-capacity reactor—an ability that would be especially useful in pilot plants or in industries with production demands that change with time or geographical location [133]. The ability to set up production when and where it is needed could decrease the need for storage and transportation of hazardous or short-lived chemical products. Furthermore, microreactors have the potential to increase the safety of dangerous processes such as the fluorination of aromatic compounds and the synthesis of organic peroxides from acid chlorides by accurate temperature control and prevention of thermal runaway [186, 133]. In case of microreactor failure, the consequences will be relatively minor due to the small mass of material present in the reactor at a given time.

Aside from assays and reactions, microfluidics has played an interesting role in numerous other areas. Examples include microchannels for cooling microelectronic circuits [53], greyscale photomasks consisting of channels filled with different dye concentrations [38], pressurized elastomeric chambers acting as tunable lenses [45], a tunable microfluidic dye laser [22], and fluidic circuits for implementing DNA computing [277].

2.3 PDMS microfluidics

Microfluidic devices have been fabricated from a variety of materials, including silicon, glass, metals, ceramics, hard plastics, and elastomers. Several reviews of microfluidic technologies have been published [244, 133, 25, 278, 69].

Sophisticated integrated microfluidic devices require a method to deliver fluid in a controlled manner between different on-chip components. While devices based entirely on passive flow mechanisms have been successful in research and in commercial products, only relatively simple assays have been possible to date. Active flow mechanisms are required for more sophisticated applications such as highly parallel arrays of reactors in which inlet and outlet ports must be shared among many chip components. In hard materials, electroosmotic flow has proven to be an effective and flexible low-dispersion means of controlling fluids; however, unlike mechanical valves and pumps, its operation depends sensitively on the physical and chemical properties of the fluids (pH, ionic strength, ionic content), and it is not effective in larger channels [134]. In addition, with electroosmotic flow it is not possible to completely isolate samples within a chip nor is it possible to carry out many simultaneous manipulations due to electrical cross-talk between different parts of the chip. Sophisticated "flow-through" devices have been fabricated, however, including some capable of multi-step synthesis [287].

Other physical phenomena have been successfully harnessed for fluid manipulation, but most suffer from disadvantages such as a dependence on details of fluid and surface properties [54], a lack of reconfigurability [307], or a lack of individual valve control [71]. Mechanical valves and pumps, on the other hand, are completely independent of fluid (liquid or gas) properties and are ideally suited as a generic means to manipulate fluids in nearly any application. Furthermore, they can be actuated individually and can orchestrate fluid manipulations such as closed loop flow that are not possible with other techniques.

Despite much effort, the fabrication of active mechanical components in microfluidic devices consisting of rigid materials remains a difficult, complex, and expensive procedure, hindering the pace of device development. Existing valve technologies include a molten wax piston valve [206], an *in* situ-polymerized polymer piston valve [102], and a check valve with a parylene membrane [285], as well as numerous diaphragm valves, such as a PDMS membrane actuated pneumatically [94] or thermopneumatically (by heated fluid vapour pressure) [300], a plastic membrane actuated by a piston [305], and a silicon nitride membrane actuated with pyroelectric or piezoelectric transducers [68]. (See Reference [305] for a summary.) Early valves using stiff silicon membranes required large surface areas to achieve reasonable deflections. For some reason, recent valves continue to have large sizes (several millimeters), and thus only a small number of valves can fit into a single device.

In contrast, very small and simple, integrated, mechanical values can be fabricated in PDMS devices, enabled by the elasticity and sealing properties of this material [272, 218]. Since the invention of these values in our lab, there has been tremendous progress in the field, and the complexity and capabilities of PDMS devices (measured in value densities) have improved exponentially [111], with current state-of-the-art devices boasting hundreds of thousands of microvalues.

2.3.1 Elastomeric microvalves

A simple metaphor for the operational mechanism of a PDMS microvalve is someone stepping on a garden hose. The pressure applied by the foot deforms the top surface of the hose until the hose is squeezed completely shut and fluid cannot flow. One could also envision the blockage of fluid flow by a hose clamp. PDMS valves contain a thin elastic membrane that can be deflected to block microchannels by a variety of mechanisms, including direct mechanical force [63, 96, 289], electrostatic force, magnetic force, force of an expanding hydrogel [16], and piezoelectric force, as well as pneumatic and hydraulic force [272]. Typically the latter are controlled by an external pressure supply but have also been demonstrated by electronically controlled on-chip electrolysis of water to generate gas [74]. Pneumatically and hydraulically actuated valves have a very small size (footprint) and have proven particularly practical.

Though many variations are possible, PDMS microvalves typically have one of the two architectures shown in Figure 2.1. Two microchannels are shown: one contains the fluid to be controlled; the other is the controlling channel. They are referred to as the "fluid channel" and the "control channel", respectively. When the control channel is pressurized, the thin membrane of PDMS existing between the two channels where they cross (when viewed from above) is deflected into the fluid channel, diminishing the size of the flow path. When sufficient pressure is applied to overcome the PDMS elasticity and the fluid pressure, the valve is fully actuated and closes completely. When the pressure in the control channel is relieved, the elasticity of the PDMS causes the valve membrane to spring back to its original position, opening the valve. A top-view photograph of an open and closed microvalve is shown in Figure 2.2. In this and later chapters, I sometimes refer to this microvalve design as the "crossed-channel" valve architecture. During operation, control channels are typically filled with pressurized water instead of air to prevent the introduction of air bubbles into the fluid stream due to air diffusing through the valve membrane. Since water vapour can also diffuse through the valve membrane, a low viscosity oil such as Krytox Fluorinated Lubricant (DuPont) is used as an alternative when manipulating water-sensitive fluids in the device.

In the "push-down" architecture [272], pressure is applied in the upper channels to deflect the membrane downwards. The "push-up" architecture [259] has control channels at the bottom and the membrane deflects upwards. Typically the latter configuration can be actuated at significantly lower pressure due to the membrane shape [259]. It has the additional advantage that there is more space above the fluid channels to implement tall fluid-containing features such as reaction chambers. Such features would not fit in the confined space of the bottom layer in a push-down device. Note that a valve is created simply where a control channel crosses a fluid channel (above or below). To allow crossing without creating a valve, the width of the control channel can be reduced. This restricts the amount of deformation of the valve membrane, preventing it from deflecting completely at the pressure that is sufficient to close (full width) valves.

In order for the value to close completely, the fluid channel must have a rounded profile, otherwise the corners will leak. A semicircular profile is common, but a bell-shaped profile has been shown by computer modelling to have a lower actuation pressure; it also has the additional advantage that part of its top surface is completely flat and thus is superior for optical detection and imaging [85]. By deliberately using a square channel profile and thus a leaky value, one can implement a sort of



Figure 2.1: Schematic of two common PDMS microvalve architectures. (Left) Three diagrams of a *push-down* elastomeric valve. A top-view of the valve is shown in the upper diagram and a side-view is shown below. The fluid channel with rounded profile is in the bottom thin layer and flows beneath the control channel in the thick layer. A dashed circle highlights the thin elastomeric membrane that separates these channels and that is deflected during actuation. The lower diagram shows the valve in the closed state: the control channel is pressurized and deflects the membrane downwards until it completely blocks the fluid channel. A reduced control channel pressure would deflect the membrane only part way, leaving a reduced size opening for the passage of fluid. (Right) Corresponding three diagrams for a *push-up* elastomeric valve. In this case the fluid channel is in the thick layer and flows over the control channel. When actuated, the control channel deflects the intervening elastic membrane upwards, closing off the fluid channel. Typically devices are fabricated from two bonded layers; in both sets of figures, light red indicates the layer with actuation channels and light blue indicates the layer with fluid channels. Note the different shape of the valve membrane in the two cases. The valve membrane in a push-up device is a uniform thickness and is easier to deflect, resulting in lower actuation pressures.



Figure 2.2: Photograph of an elastomeric microfluidic valve. (Left) Photograph of an open PDMS valve. (Right) Photograph of same valve closed by pressurizing the control channel. (Reproduced from http://www.fluidigm.com/nanoflex.htm with permission. Copyright Fluidigm Corporation.)

filter. The gaps at the incompletely closed corners are large enough to allow fluids to pass through but small enough to trap particles such as microbeads or biological cells [179]. By flowing a solution of beads through such a valve I have created packed columns of 0.7 μ m microbeads on the upstream side of the valve for solid phase synthesis.

Three or more adjacent values can be actuated in a cyclical fashion to act as a peristaltic pump [272], drawing or pushing fluids through a flow channel or circulating the fluid around a closed path to perform mixing [43]. Two adjacent values along a fluid channel can be closed simultaneously to isolate the contents of the intervening length of fluid channel, thus forming a tiny chamber or reactor. Large arrays of isolated chambers can be implemented in this manner [268].

It should be noted that other mechanical valve architectures have been considered in PDMS including check valves such as diaphragm and flap valves [134], and a biologically inspired "lymph" valve [188]. However, such valves tend to be somewhat large, and they are passive, preventing sophisticated fluid handling. Ismagilov *et al.* [126] reported an interesting microfluidic switch based on fluid channels in separate layers *meeting* tangentially. The flow pattern (straight through or turning a corner) is determined by the relative aspect ratios of the channels and the size of the opening between them, as well as the position of the input stream within the channel. Pressure-

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actuated control channels were shown to squeeze the tangential channels to alter their relative aspect ratios and dynamically switch the flow pattern. Such switch elements do not offer all of the flexibility of valves, however, but may be useful in flow-through applications.

2.3.2 Multilayer device fabrication

Devices containing microvalves are typically fabricated by the two-layer replication molding process depicted in Figure 2.3. Replication molding is the process by which a material is cast on a mold that contains a microfabricated relief pattern. Using standard photolithographic techniques, two relief molds are created, each consisting of a pattern of photoresist on a silicon wafer or glass slide. Ridges on the mold become microchannels in the cast PDMS. One mold represents a pattern of roundedprofile fluid channels and will create the "fluid layer"; the other represents control channels and will create the "control layer". Typical channel dimensions are 100–200 μ m in width by 10–50 μ m in depth. Ridges on the fluid-layer mold must have a rounded profile to allow complete valve closing. Molds are prepared by spin-coating photoresist on a wafer, performing a soft-bake to solidify the resist, exposing the resist through a photomask defining the channel pattern, then immersing in a developer solution to remove uncrosslinked resist. In many resists, rounding can be achieved by heating above the resist melting temperature, causing it to reflow into a profile determined by surface tension; in some resists (such as SU-8), rounding can be achieved during the exposure stage [85]. Molds are typically treated with a mold release agent such as trimethylchlorosilane (TMCS) vapour prior to casting. Details of the mold preparation depend on the desired dimensions of fluid and control channels.

Depending on the configuration (push-up or push-down), PDMS prepolymer is spin-coated onto the mold representing the thin layer. The difference between the PDMS thickness and height of photoresist on the molds determines the valve membrane thickness. On the other mold, PDMS prepolymer is poured to a thickness of 3–7 mm. The layers are then cured into solids.

Subsequently, the thick layer is removed from its mold and holes are punched completely through to serve as inlet/outlet ports for channels in the bottom surface. This layer is then aligned and



Figure 2.3: Fabrication of 2-layer microfluidic devices by replication molding. Molds are created for the lower- and upper-layer channel patterns. Typically these are silicon wafers patterned by photolithography with photoresist traces representing microchannels. In a push-down device, the lower layer contains the fluid channels; in a push-up device, the lower layer contains the control channels. A thin layer of elastomer is cured on the lower-channel-layer mold, while a thick layer is cured on the upper-channel-layer mold. Since the thin layer is generally too thin to be handled without experiencing wrinkling or other damage, the thick layer is first removed from its mold, aligned, and bonded to the thin layer. Once bonded, the 2-layer device can be removed from the mold and is adhered to a substrate to seal the bottom layer of channels. Not shown are inlet and outlet holes. These are typically punched through the thick layer before the first bonding step as a means to access upper-layer channels. In addition, holes are punched through the whole device prior to substrate-bonding to provide access to the lower-layer channels. (Reproduced from [272]. Copyright the American Association for the Advancement of Science, 2000.)

bonded (patterned side down) to the thin layer, still affixed to its mold. Note that the channels in the bottom surface of the thick layer become embedded entirely within polymer, and the valve membrane is made from material in the bottom layer. There are two common techniques for bonding PDMS layers, described below. When bonding is complete, the two-layer device is removed from the mold, and holes are punched completely through to access the microchannels in the thin layer. Holes are typically punched by hand using Luer stubs or by a hole-punching machine (Technical Innovations, Brazoria, TX). The device is then bonded to a substrate such as glass, a slab of PDMS, or PDMS-coated glass to seal the floor of the channels in the thin layer. Tubing is inserted into the punched holes for fluid delivery and pressurization of control channels.

Note that when the thick layer is released from the mold, it instantly shrinks by about 1.5% in each dimension—an empirically determined factor for PDMS. Because the thin layer initially is left on its mold, it does not shrink. Thus, the mold pattern for the thick layer must be enlarged by this factor to arrive at the correct final size to ensure that proper registration is possible during the alignment step.

Devices in our lab are typically made from one of two commercially-available silicone elastomers: RTV 615 (GE Silicones) or Sylgard 184 (Dow Corning). Each is supplied as two components—an oligomer mixture and a cross-linking agent—which are normally mixed in a 10:1 ratio. We achieve bonding by off-ratio mixing, wherein one layer is endowed with an excess of one type of functional group and the second layer with an excess of another [272]. Generally, the thin layer is mixed in a 20:1 ratio while the thick layer is mixed in a 5:1 ratio. The materials are mixed in an automatic mixer (HM-501 hybrid mixer, Keyence Corporation) and degassed in a vacuum desiccator prior to molding. The first casting step involves a partial cure of both layers by baking at 80°C. The bake time is typically 60 min for RTV 615 or 30 min for Sylgard 184. After alignment and stacking of the thick layer, the device is further baked at 80°C to complete the curing process (4 h for RTV; 2 h for Sylgard). During this time, excess functional groups in the two layers interact to form covalent bonds across the interface. Alignment and hole-punching time after the initial cure is limited to about 30 minutes—otherwise layer bonding can fail. An alternative method for bonding layers employs oxygen plasma treatment [67]. Each layer of the device is made from 10:1 PDMS and fully cured. Layers to be bonded are treated with oxygen plasma and then placed in contact with a drop of methanol between. This fluid provides lubrication for alignment and prevents the treated surfaces from reconstructing to a lower-energy state, thus lengthening the available working time. Once properly aligned, the device is heated to drive out the methanol and surface groups react to covalently bond the layers together.

Strong bonding is crucial to device fabrication. Otherwise the large localized forces generated when channels are pressurized can peel the layers apart, leading to device failure—a process called "delamination".

2.3.3 Advantages of PDMS devices

More than in other device technologies, interest and research in PDMS microfluidics has exploded in recent years, probably due in large part to two important factors. One is the ability to tinker. The low cost of PDMS and the simplicity of PDMS device fabrication allow nearly any research laboratory to explore ideas without prior microfabrication experience: once molds are prepared, no specialized equipment or facilities are needed. Ideas can be matured quickly, as rapid prototyping enables device improvements and optimizations to be made on very short times scales with minimal expense. Device fabrication from materials such as glass and silicon requires numerous processes such as chemical etching, reactive ion etching (RIE), and thermal bonding; each iteration takes considerable time and effort. Tinkering is limited to those with access to the needed equipment and expertise. For these reasons, PDMS is likely superior from a commercial manufacturing perspective as well.

The second important factor was the invention of the integrated microvalve [272]. Building two-layer devices does not introduce much additional complexity but provides tremendous power in the ability to manipulate fluids in controlled ways through the use of valves and pumps. Being mechanical, these valves are completely independent of fluid properties, unlike other mechanisms that have been used for flow control in other types of devices. This is a tremendous advantage in that the same fabrication technology and design parameters can be used for devices in a wide variety of applications. Indeed, current devices are often designed by plugging together standardized components [49].

PDMS crossed-channel values are exceptionally small, having a square or rectangular footprint comparable in size to the channel width, typically 100 μ m. They also have a very small dead volume (100 pL for a typical 100 μ m×10 μ m cross-section), resulting in low carryover and quick response time. Furthermore, PDMS values are durable: studies have shown no signs of wear or fatigue after millions of actuation cycles [272]. These characteristics enable the fabrication of reliable, high density, integrated fluidic circuits.

Aside from elasticity (which enables microvalve fabrication), many other properties of PDMS have proven well suited for microfluidic devices, including transparency, gas permeability, and ease of surface modification. Optical transparency allows for visual inspection of chip operations for troubleshooting or for performing bright field and fluorescent detection and imaging. High gas permeability enables several unique design features. For example, a microreservoir does not require separate inlet and outlet channels. In a process called "blind filling", (or "dead end filling"), fluid entering the chamber through a single channel forces trapped air to escape directly through the bulk PDMS. This feature can be used to several advantages: (i) device designs are simplified by reducing the number of fluid channels and valves; (ii) the risk of sample loss (due to incorrect valve timings) is eliminated if a chamber has no outlet; and (iii) fluid volumes can be accurately metered by filling chambers having precisely known volumes. Gas permeability also enables a convenient method for solvent exchange. A closed chamber containing the original solution can be heated to cause evaporation and escape of the vapour through the PDMS, eliminating the original solvent. The desired new solvent can then be introduced via an inlet whereupon the dry solute is redissolved. Including empty, open-ended channels nearby can accelerate evaporation by shortening the thickness of PDMS that must be crossed by the vapour. The permeability of PDMS also allows sufficient gas exchange for biological cells to be cultured in microchannels for extended periods. (Cell survival also depends on the native biocompatibility of PDMS; other device materials often require special treatments or coatings to avoid adverse interactions with biological materials.) However, gas permeability is not always desirable—unwanted escape of water vapour can lead to concentration increases or sample drying, and unwanted influx can lead to contamination of water-sensitive reagents. Engineering solutions do exist, however.

Another advantage of PDMS devices in comparison with rigid material devices is the simplicity of connections to the external world. In PDMS, holes are punched through the device and metal or plastic microbore tubing is inserted; tubing is held in place simply by friction since the punched hole is slightly smaller than the tubing outer diameter. Depending on various parameters, such connections are sufficient for pressures up to several atmospheres. With other chip technologies, interfaces are often quite elaborate, involving many fabrication steps or several separate components [82]. One advantage of MEMS fabrication with glass or silicon is that electronics and optics can more naturally be incorporated into devices. However, it has also been possible to integrate PDMS devices with such components due to the ability to seal PDMS reversibly or irreversibly with many substrates including silicon [1].

PDMS is suitable in a vast range of applications, but there are circumstances that dictate the use of alternative device materials. For example, very high temperatures preclude the use of polymers, instead requiring devices fabricated from inorganic materials such as glass, silicon, ceramic, or metal. High pressures would likely interfere with the operation of elastomeric valves and may lead to significant loss of fluids by diffusion or evaporation through the permeable channel walls. Hard inorganic materials or plastics should be used in devices operating under such conditions. Most importantly, PDMS is incompatible with many organic solvents [160] and cannot be used in most chemical synthesis and analysis applications. Solvent-resistant fluoroelastomers are preferable under such conditions, as are glass, inert metals such as stainless steel and titanium, and inelastic fluoropolymers such as Teflon. The advantage of fluoro*elastomers*, of course, is that device designs can incorporate the same microvalves and other features that have enabled highly integrated PDMS devices.

In the next several chapters, I describe our efforts to develop devices based on such materials that can be used as drop-in replacements for PDMS devices when solvents or harsh chemicals are required. These devices have the potential to expand the use of microfluidics to new areas, serving as a more generalized platform for rapid-prototypable highly-integrated solvent-resistant microfluidics.

Chapter 3 Solvent-Resistant Microfluidics

3.1 Introduction

PDMS microfluidic technology has advanced at an astonishing rate over the past several years, far outpacing progress in alternative microfluidic technologies. Where PDMS has not kept pace, however, is in the variety of solvents in which reactions and analyses are performed. Though many impressive devices have been demonstrated, fundamental incompatibilities of PDMS with many solvents [160] have limited this technology primarily to applications involving aqueous media [133]. Solvents can cause swelling, leading to disruption of microscale channel features, or can directly interact with the polymer. Glass, silicon, metal, ceramic, and even some plastic devices have fared far better with regards to solvent variety. However, these technologies suffer from the disadvantages outlined in Chapter 2. Simple manipulation of solvents and reactive species has been demonstrated in devices fabricated from hard materials, but it is difficult to imagine how these devices can be scaled up to the levels of integration seen in recent PDMS devices [268]. In addition, these devices are often designed from scratch for each new application—an indication of the lack of generality of the fabrication and fluid manipulation methods being employed.

It is this limitation that we strive to eliminate. Drawing inspiration from PDMS microfluidic device technology and the many qualities that have led to its success, we have developed several novel device technologies based on fluoroelastomer materials and demonstrated functional crossed-channel microvalves. Due to their elastomeric properties, these devices share many of the same advantages of PDMS devices with the added advantage that they are resistant to most solvents. These technologies have the potential to expand the field of highly integrated microfluidics to many new applications in chemical synthesis and analysis, currently of great interest for chemical production and drug discovery. In addition, they may be able to expand the range of fluids used in existing applications, including protein crystallization screens [97] and optofluidics. Using similar microvalve architectures, these systems can be used as drop-in replacements for PDMS, leveraging much of the experience that has been accumulated by the community over the years. The fact that the operation of these mechanical valves is completely independent of the solution properties is especially important in chemistry applications where a very wide variety of solvents are in common use. Because the fabrication remains simple, these technologies will allow the kind of tinkering that has led to a near ubiquity of PDMS devices for biochemical and biological microfluidics.

In this chapter, I first briefly describe other work in the field of solvent-resistant microfluidics where devices are fabricated from glass, silicon, and other inert, hard materials. This first section also serves to highlight the disparity in complexity of such devices compared with state-of-the-art PDMS devices. Next, I describe how the susceptibility of PDMS (or other polymers) to many solvents leads to difficulties in microfluidic device applications. In the last two sections, I discuss our general approach for fabricating resistant devices incorporating elastomers and give a brief account of many specific material systems and device architectures that we considered. Two of the most successful technologies—fluorinated norbornene and perfluoropolyether devices—are discussed in later chapters.

3.2 Prior work

Because the earliest microfluidic devices were fabricated from glass and silicon (both of which are resistant to most solvents and stable at high temperatures), it is not surprising that reactions and separations involving harsh conditions have been possible for many years. Glass and silicon devices are still in use today in such applications, as are microreactors fabricated from other materials such as metals, ceramics, and Teflon. Many impressive devices have been demonstrated over the years, some containing very sophisticated fluidic components such as micromachined filters and packed bed reactors, integrated electronic heaters and optical sensors, and reactors consisting of thousands of parallel microchannels (see reviews in [278, 133, 227]). The vast range of chemical processes that have been successfully implemented is equally impressive (see reviews in [133, 62, 8, 77, 286]).

In most of this work with microreactors, it has been observed that separations are generally more complete and more rapid, and that synthesis often has improved selectivity and yield, compared to bulk processes. For example, Greenway *et al.* [93] observed sustantial improvements in efficiency over the bulk reaction when performing synthesis of 4-cyanobiphenyl from 4-bromobenzonitrile and phenylboronic acid in a glass microreactor. By immobilizing the $PdSiO_2$ catalyst, the additional benefit of reduced contamination in the product was realized. It is postulated that the catalyst bed also provides an enhancement of electroosmotic flow via a localized concentration effect at the Pd surface and causes partial ionization of water to generate base (the addition of which has been observed to improve the bulk reaction). These secondary results underscore the fact that reactions are very sensitive to flow conditions as well as the channel and catalyst surfaces, and suggest that each new microfluidic reaction could require optimization of these conditions.

The synthesis of peptides in continuous flow¹ borosilicate glass microreactors has been reported by Watts *et al.* [288, 287]. Several input channels branch off from different points along the main reaction channel, allowing reagents to be introduced sequentially. Fluids were driven by pulsed electroosmotic flow with inlet voltages adjusted to optimize the relative flow rates in order to maximize the yield. Dipeptides were synthesized via numerous routes including the following: introducing an (Fmoc)Nprotected amino acid in the first channel, an activator in the second channel, and an (Dmab)Cprotected amino acid in the third channel to yield a dipeptide (Fmoc- and Dmab-protected) at the output. To synthesize tripeptides, an (Fmoc)N-protected/C-activated amino acid was introduced in the first inlet, a C-protected amino acid in the second, an Fmoc deprotection reagent in the third, and

¹In a closed reactor, reagents are brought in together and reacted to form the product. Thus, product is created all at once in a "batch". In continuous flow reactors, reagents are introduced continually side by side in a channel or as alternating plugs of reagents. The reagents mix and react as they flow together, allowing products to be collected in a continuous stream at the output. Another method of reaction is solid-phase synthesis, in which the products remain affixed to a substrate and are built-up by sequentially introducing the needed reagents one at a time. Once finished, they can be cleaved from the substrate.

an (Fmoc)N-protected/C-activated amino acid in the fourth. (The final tripeptide was Fmoc- and Dmab-protected.) Multi-step syntheses were observed to occur with much higher yields than bulk reactions, on much shorter time scales, and with much lower reagent concentrations. However, this work also touches on the difficulties of performing multi-step synthesis in continuous flow reactors. If two reagents are not completely converted to product by the time they reach the inlet for the third reagent (which is intended to react with the product), there can be direct cross-reactions of the third reagent with the first two reagents, as there is no means to flush away the excesses. To reduce byproducts in multistep peptide synthesis, one could use orthogonal protecting groups on subsequent amino acids; however, since only a couple of different deprotection conditions are known, this would severely limit the maximum peptide length. A further disadvantage of solution-phase synthesis of peptides is that both ends of the amino acid must be protected during synthesis to avoid unwanted reactions. In solid-phase synthesis, one end is bound to the solid-support and is not free to react so such protection is unnecessary.

Fletcher *et al.* [77] postulate that details of electrokinetic flow may be responsible for the high reaction rates and synthetic yields that are observed in many glass and silicon microdevices. In mechanically driven flow, when two slugs of fluid are brought together, reagents from each slug diffuse into the other across the interface and react. As diffusion proceeds, the concentration locally drops and molecules from one slug encounter lower and lower concentrations in the other upon crossing the interface. Simulations supported by experiments indicate that this is not the case in electrokinetic flow [77]. It is as if one slug passes *through* the other one. Because the "interface" between the slugs is moving, there is no local depletion by diffusion, and concentrations encountered by molecules crossing the interface remain high. For optimal reactions, series of several narrow slugs are injected rather than a single large one. Interestingly, pulsed electrokinetic flow appears to be more effective than introducing two laminar streams side by side in a fluid channel. It should be noted that the concentration effect seen in electrokinetic flow can be "simulated" in mechanical flows as well. For example, one can isolate a slug in a chamber and evaporate the solvent (if the device is permeable). The second slug can then be brought into this chamber and each reagent will encounter the other at the full original concentration.

Daridon *et al.* [55] report the fabrication of 3-layer glass microfluidic devices for integrated synthesis and analysis. The top and bottom glass plates contain microchannels facing the center plate—a thin glass layer containing holes. These holes (vias) connect channels from one layer to those in the other and can also act as optical cuvettes for analyzing the absorbance (for example) of the fluid inside. The glass device was sandwiched between molded PMMA layers that held the external tubing and ferrules in place and served as guides for optical fibers on either side of the microcuvettes. The authors demonstrated a two-step Wittig reaction in methanol and the Berthelot reaction, a three-step organic reaction involving basic solutions (up to pH 12.5) for the colorimetric detection of ammonium.

Kikutani et al. [148] report the fabrication of a three-dimensional glass microchannel network for 2×2 continuous-flow parallel combinatorial synthesis. A set of two different amines in the aqueous phase and a set of two different acid chlorides in the organic phase were reacted in four combinations to produce four different amide products. The reaction is hypothesized to proceed via a phase transfer mechanism, wherein the amine diffuses into the organic phase and reacts, and the product remains in the organic phase. No significant impurities were observed in the organic phase despite there being a competing side reaction (hydrolysis of the acid chlorides). It is believed that the rate of the amide formation reaction is enhanced more than that of acid hydrolysis due to the high specific surface area between the two phases. This observation highlights the importance in microreactor design of carefully considering how the rate of side reactions is affected by the scale-down, in addition to the reaction of interest. More recent enhancements of these devices include integration with an extraction step in a device for heavy metal ion analysis and the fabrication of glass devices with up to 10 layers [270]. Kikutani *et al.* reported difficulties equalizing the flow rates despite careful device design and fluid delivery via accurate syringe pumps, an effect that will likely hamper significant increases in integration density in continuous flow reactors. This problem could be eliminated by using systems with mechanical microvalves, with which fluid volumes can be accurately metered.

Martin *et al.* [181] at Pacific Northwest National Laboratory (PNNL) fabricated continuous flow solvent-exchange devices by stacking several hundred thin stainless steel laminates. The device consists of a very long serpentine pair of microchannels with extremely high aspect ratio separated by a porous membrane. Hexanol was transferred between hexane and an aqueous fluid in this device. The authors also demonstrated a plasma microreactor, fabricated from two milled ceramic blocks sealed together with a Viton gasket. Plasma is a harsh chemical processing environment where UV light, radical species, or photocatalytically active catalysts can facilitate interesting reactions. The reactor was designed to break down methane into ethylene and hydrogen, and convert methane and air to syngas. Ceramic devices have also been fabricated by lamination methods [182]. Janicke *et al.* [130] report the use of laminated stainless steel microreactors to perform the controlled formation of water from explosive mixtures of hydrogen and oxygen gas in fuel cell applications. The heat exchanger in the device was sufficient to remove heat from the exothermic reaction, thus preventing thermal runaway. The reaction takes place on an alumina coating impregnated with platinum on the walls of the reactor.

While significant advancements in solvent-resistant microfluidics have been made in individual device components such as microreactors and separation columns, only modest steps have been taken towards *integrating* multiple functionalities into MEMS fluidic devices [152]. One of only a few exceptions, Burns *et al.* [30] demonstrated a device for performing a multi-stage DNA analysis: sample loading and preparation, heating and reaction, gel electrophoresis and photodetection are all integrated on a single chip. However, the device density and degree of integration do not compare with recent PDMS devices boasting tens of thousands of valves and reaction chambers [48].

The lag of silicon and glass devices is likely due to the fact that fabrication is difficult and expensive as discussed in Chapter 2. Mechanical pumps and valves are particularly difficult to fabricate in rigid materials—those that have been demonstrated are typically quite large (millimeters) and do not lend themselves to dense integration in devices. This limits devices to relatively simple flow-through configurations using capillary or electrokinetic flow. Furthermore, not all fluids can be electrokinetically pumped, and some researchers have altered the solvents used in reactions to fit this pumping technology [62]. Clearly, fixing the microfluidic chip technology by incorporating a more generic pumping method (e.g., mechanical pumping) would be preferable.

Fabrication of devices from polymers has helped to simplify and reduce the cost of device fabrication [272, 25, 244]; however, the materials used are typically not resistant to solvents. (The majority of applications are currently in the area of biotechnology and involve aqueous chemistry.) Photopolymerization has emerged as a simple fabrication technique that can use a variety of polymers [147], and Harrison *et al.* [100] have fabricated devices by this method from a thiolene-based optical adhesive. This material is resistant to many solvents including toluene, tetrahydrofuran, and ethanol, but it is susceptible to others such as methylene chloride and therefore is not suitable as a generalized platform for all applications in solvent-resistant fludics. Furthermore, it is a rigid material and does not solve the valve and pump problem. Rather than the current situation, where the device material must be carefully selected for each new microfluidic application, or, worse, where the chemistry must be altered to be compatibile with the available device technologies [62], the field of microfluidics would benefit tremendously from a generalized microfluidics platform that is suitable for nearly all applications.

We believe solvent-resistant elastomeric microfluidic devices can solve all of these problems. Possessing all of the properties of PDMS that facilitate very high levels of integration and simple fabrication, and additionally providing resistance to solvents, these devices have the potential to serve as powerful new tools in organic chemistry. The generality achieved (by both the device material and the mechanical valve operation being insensitive to the fluid properties) should help to speed the advancement of the field by reducing the effort that is currently spent tailoring devices and chemistries to each application.

Densely integrated, solvent-resistant devices would be ideal for novel applications in combinatorial chemistry, high throughput screening, and parallel multi-sample multi-analysis chips, possibly integrated with sample preparation or purification steps. A highly parallel combinatorial chemistry chip could have dedicated reactors for every possible output product, obviating the need for current techniques such as mix and split synthesis [148], which add complexity to assays by requiring a probe identification step after performing a screen.

3.3 Organic solvents and elastomers

Many polymers, including elastomers such as PDMS, are susceptible to swelling or to chemical attack upon exposure to at least some organic solvents or acids and bases. Such adverse interactions can have considerable impact on the operation of polymeric microfluidic devices due to the fragility and high surface to volume ratio of microscale features. Interactions with the solvent or with impurities in the solvent can adversely impact not only the device integrity but also the reaction or analysis being performed inside the device.

3.3.1 Adverse interactions

In general, the following four problems can arise: (i) swelling of the polymer; (ii) extraction of impurities; (iii) partitioning between the polymer and solvent; and (iv) chemical reaction with the polymer. Each of these is discussed below.

Swelling can deform microchannels, altering their dimensions or even closing them completely [200]. For example, dichloromethane cannot be flowed through PDMS microchannels for this reason. Dichloromethane swells PDMS by 22% in each linear dimension [160]. In an elastomeric device that is several millimeters thick, this represents a substantial deformation compared with the channel depth—typically tens of microns. Channels can easily be plugged due to non-uniform swelling, arising as a result of the exposure occurring within a microchannel or input port (and gradually diffusing outward). Swelling can also create stresses that disrupt bonding, leading to leaks and cross-contamination in devices that lack covalent bonding at solvent-exposed interfaces. This might be the case, for example, in applications involving *in situ* synthesis on the substrate, which employs *reversible* bonding so the microfluidic device can be removed during or after synthesis. An additional possible effect of swelling is the alteration of elastic properties, impacting microvalve performance (such as a change in actuation pressure). Extraction of impurities such as unpolymerized monomers or oligomers from the polymer can impact mechanical properties in some materials, but more importantly, it can introduce contaminants into the fluid channels that interfere with reactions or are present as contaminants in the final products. To a certain extent, this problem can be eliminated by pre-extracting the device in the solvent(s) with which it will be used. High swelling solvents enhance extraction.

Partitioning is the effect whereby a solute can be divided between the solution in the fluid channel and the polymer adjacent to the fluid channel. This effect can alter reagent concentrations in the fluid channels. Furthermore, solute trapped in the polymer may be difficult to flush out of channels and may be released during a later stage in a multi-step process, causing unwanted contamination.

Finally, some polymers are susceptible to direct chemical or ionic interactions with solvents or solutes. Such reactions can have a wide variety of adverse effects including significant depletion of reagents in fluid channels, contamination of the desired reaction with byproducts of polymer interaction, or chemical modifications to microchannel surfaces that can affect wetting properties or leave functional groups that interfere in later stages of a microfluidic process. Furthermore, some reactions can uncrosslink the polymer, affecting elasticity and even destroying the device. For example, I observed that PDMS soaked in dichloromethane with 3% trichloroacetic acid for several days became brittle and crumbled apart.

Clearly, these interactions should be avoided in microfluidic devices by appropriate choice of device materials. As a first approximation, the material should exhibit low swelling in the solvent(s) of interest and be chemically inert. Further evaluation requires the fabrication of actual microfluidic devices to accurately determine the extent of other interactions. To avoid having to tailor the device material to each application, it is desirable to find a *universal* material.

3.3.2 The problem with PDMS

PDMS is incompatible with a wide range of solvents, as recently reported in depth by Lee *et al.* [160]. Swelling data from that study is reproduced in Figure 3.1. It should be noted that 20 of the solvents tested caused equal or greater swelling compared to methylene chloride—a solvent that we found completely blocks flow in channels—and thus would be unlikely to be usable in PDMS devices. The additional incompatible solvents include acyclic and cyclic hydrocarbons (pentanes, hexanes, heptane, cyclohexane), aromatic hydrocarbons (xylenes, toluene, benzene), halogenated compounds (chloroform, trichloroethylene), ethers (diethyl ether, dimethoxyethane, tetrahydrofuran), and amines (diisopropylamine, dipropylamine, triethylamine) [160].



Figure 3.1: Swelling of PDMS in various solvents. The logarithm of the linear swelling ratio after 1 day immersion, S, is plotted as a function of the Hildebrand solubility parameter, δ , for a wide variety of solvents. Qualitatively, as predicted by solubility theory, the greatest degree of swelling is observed for solvents having a solubility parameter closest to that of PDMS (dotted vertical line). (Reproduced from [160]. Copyright the American Chemical Society, 2003.)

It may be possible that PDMS devices are suitable in a narrow range of applications in synthetic or analytical chemistry involving non-swelling solvents. The range of solvents may be extended to some high-swelling solvents if the chemical process can tolerate dilution with a non-swelling solvent such solvent mixtures often cause reduced swelling. However, PDMS devices are not suitable as a *generalized* microfluidics platform for chemistry. Certainly PDMS is not compatible with our original aim of DNA synthesis chemistry (involving dichloromethane and tetrahydrofan among other solvents).

3.3.3 Alternative materials

To help determine which materials are compatible with particular solvents, a variety of sources provide tabulated data such as (i) quantitative swelling measurements, (ii) qualitative compatibility data (sometimes with a letter or number scale), and (iii) solubility parameters. Alternatively, one can perform experiments to determine these data.

Quantitative swelling data are available from several sources [167, 166]. Such sources indicate that most polymers are susceptible to at least some solvents. According to data in the Plastics Design Library (PDL) Chemical Resistance handbooks, several plastics and elastomers exhibit excellent resistance to a *wide variety* of solvents and may be suitable as materials for generalized solventresistant microfluidics. These plastics include PVDF (polyvinylidene fluoride), polyolefins (including polypropylene), PEEK (polyetheretherketone), Tefzel (ETFE, ethylene-tetrafluoroethylene), Teflon (PTFE, poly-tetrafluoroethylene; TFE, tetrafluorethylene; FEP, fluoro ethylene propylene; PFA, perfluoroalkoxy), and others [167], while the elastomers include tetrafluoroethylene propylene copolymer and terpolymer, FKM fluoroelastomers, and FFKM fluoroelastomers, among others.

Qualitative compatibility data are generally available from the manufacturers or suppliers of polymers. However, such data are of limited usefulness due to inconsistencies arising from the different rating systems common in different industries and from the different solvents commonly used in those industries. For example, when a manufacturer claims "high chemical resistance", this is often true only for a few classes of solvents. Inconsistencies may also arise due to different ways in which various factors (such as weight change, length change, and visible change such as colour) are combined into a single rating or due to the presence of differing quantities of additives (e.g., colourants, plasticizers, etc.) from one manufacturer to another. Furthermore, because most studies pertain to macroscopic sizes of polymers and quantities of solvents, the data are not immediately applicable to the conditions under which microfluidic device channels are exposed to solvents.

Solubility parameters are a third type of data to guide materials selection. In order to explain their relevance, it is necessary to briefly introduce the principles of solubility theory. Based on Flory-Huggins theory and the lattice model of mixing, one can calculate a free energy change that occurs when a solvent is "mixed" with a polymer and causes swelling. This energy contains terms for mixing (subscript "mix") and deformation due to swelling (subscript "def"):

$$\Delta G = \Delta G_{mix} + \Delta G_{def} = \Delta H_{mix} - T\Delta S_{mix} - T\Delta S_{def}$$
(3.1)

where

$$\Delta H_{\rm mix} = kT \chi n_{\rm s} \Phi_{\rm p} \tag{3.2}$$

$$\Delta S_{\rm mix} = -k[n_{\rm s}\ln\Phi_{\rm p} + n_{\rm p}\ln\Phi_{\rm p}]$$
(3.3)

$$\Delta S_{def} = -k(3/2)n_{p}(\alpha^{2} - 1)$$
(3.4)

where k is the Boltzmann factor, T is the temperature, n_p is the number of polymer segments, n_s is the number of solvent molecules, Φ_p is the volume fraction of polymer, Φ_s is the volume fraction of solvent, and α is the fractional length change due to swelling. The solvent can dissolve (and thus swell) the polymer if $\Delta G < 0$.

In practice, one makes predictions of *relative* solubilities based solely on the enthalpy term, ΔH_{mix} . This term depends on the Flory-Huggins parameter $\chi \sim (\delta_p - \delta_s)^2$ where δ_p and δ_s are the Hildebrand solubility parameters for the polymer and solvent, respectively. This factor is the average cohesive energy density difference. When the solvent and polymer have similar cohesive energies, this factor is small and swelling is more likely to occur. This is related to the well-known principle of "like dissolves like". When two species have similar cohesive energy densities, it is more likely that one can be mixed into the other with little energy penalty.

Other sets of solubility parameters distinguish among the proportions of different *types* of cohesive interactions such as dispersion forces (d), polar forces (p), and hydrogen-bonding forces (h) that make up the total cohesive energy density. For example, Hansen parameters are defined as

$$\delta^2 = \delta_d^2 + \delta_p^2 + \delta_h^2, \tag{3.5}$$

and fractional parameters are defined as

$$f_{d} = \frac{\delta_{d}}{\delta_{d} + \delta_{p} + \delta_{h}}.$$
(3.6)

These types parameters are often more accurate as they are only similar if both the solvent and polymer have similar contributions of each type of bonding to their cohesive energy density. This further emphasizes the need for solvent and polymer to be chemically similar for swelling to occur.

Hildebrand parameters are tabulated for many solvents and polymers. However, as shown in Figure 3.1, the parameters are only a very rough guide. In PDMS, two perfluorinated solvents have very similar solubility parameters to PDMS but cause no swelling; compare this to, say, dioxane, which causes significant swelling but has a solubility parameter further from that of PDMS. Other types of parameters would clearly be more predictive in this case, but these more informative parameters are not available for many polymers. For novel polymers, such as the perfluoropolyether (Chapter 5) and fluorinated norbornene (Chapter 4) polymers developed by our collaborators, pre-existing solubility data are not available at all.

Fluorcarbon polymers are widely known to have exceptional solvent-resistance, particularly the *per*fluorinated (fully fluorinated) ones. These polymers are particularly stable due to the strength of the carbon-fluorine bond and due to steric hindrance arising from the strong forces between hydrogen and fluorine atoms in the macromolecules [175]. Using the colloquial principle of "like dissolves like",

one can argue that fluoropolymers exhibit low swelling because they are chemically dissimilar to most solvents (other than fluorinated solvents) encountered in chemistry. Fluor*oelastomer* history and chemistry are reviewed in References [175] and [106]. Good elastic properties are exhibited primarily by those materials consisting of long, linear chain molecules, exhibiting functional groups such that strong intermolecular forces that lead to crystallinity and hardness are avoided. Crosslinking of the network ensures complete recovery after deformation. Elastomers are often made by first polymerizing long chains of monomers, then crosslinking or "curing" these chains into a three-dimensional network. Cure sites are often the most vulnerable point in solvent-resistant elastomers [106] and account for many of the differences in solvent-resistance exhibited by different fluoropolymers.

With our original goal of performing DNA synthesis in chips, our solvent-resistance requirements were quite stringent due to the broad range of different solvents involved. In effect, this drove us to find a material that was resistant to nearly everything and that could serve as a material in generalized solvent-resistant microfluidics (i.e., suitable for any application). Instead of attempting to make predictions of the single best material, our approach was to select materials such as fluoropolymers that looked promising according to the available solvent-resistance data and then perform relevant *in situ* evaluations by attempting to fabricate simple microfluidic devices. When no solvent-resistance data was available, we performed our own experiments to assess compatibility. Typically, in such cases, a polymer sample was first evaluated by a surface exposure test. Drops of several solvents (dichloromethane, tetrahydrofuran, acetonitrile, and others) were deposited on the surface and monitored for signs of swelling or chemical attack. Highly swelling solvents were immediately visible due to a raised bump at the droplet location. Chemical attack was inferred if the surface exhibited pitting, discoloration, or other effects after evaporation of the solvents. While not quantitative, such experiments more closely resemble the conditions within a microfluidic device than do bulk solvent immersion tests.

3.4 Solvent-resistant device principles

When considering the fabrication of microfluidic devices from solvent-resistant materials, it is instructive to carefully examine which parts of devices (in addition to the elastic valve membrane) are actually exposed to solvents.

3.4.1 Two-layer architectures

As illustrated in Figure 3.2, in a push-up device, the fluid channel in the thick layer is sealed by the thin layer. Thus, solvents in the fluid channel are in direct contact with both layers of the device, and solvent-resistant materials must be used throughout. In contrast, solvents contact only the thin layer and the substrate in a push-down device. In principle, one could fabricate devices that are resistant only in their bottom layer. This is useful when the resistant material is very expensive or scarce. To avoid flowing solvents through holes punched in an incompatible material in the upper layer of the device, holes can be drilled through the substrate for solvent delivery directly into fluid channels. In Chapter 4, I describe two methods for connecting tubing to a drilled glass substrate for delivering solvents in this manner—a custom-built fluid delivery jig and commercial fluidic connectors. Note that solvent-resistant tubing is required for solvent delivery.

3.4.2 Coated devices

As an alternative to making the whole device or a device layer out of a resistant material, solventresistance may be conferred by a protective coating. In a push-down device, it is sufficient to apply the coating to the bottom surface. In such cases, solvents must typically be delivered through the glass as we found hole punching to severely damage most coatings in a large area around the hole. Furthermore, it is difficult to apply a complete coating in the interior of the punched inlet holes. Bottom coating protects the device while also permitting solvent in the fluid channel to contact the substrate if desired for *in situ* solid-phase synthesis on the substrate, for example. This procedure can complicate device fabrication, however, as it is necessary to find a method for bonding the coated device to the desired substrate. Another way to apply coatings is to flow a coating solution through



Figure 3.2: Exposure of device layers to solvents in different valve architectures. (Left) In the push-down valve architecture, solvents are carried by the fluid channels in the thin layer. Solvents (dark blue) contact the channel walls, consisting of the material in the lower layer (light blue) as well as the substrate. To deliver solvents to the fluid channels, holes can be punched through the whole device (top diagram) or holes can be drilled through the substrate (bottom). The latter is preferred for devices in which only the bottom layer is solvent-resistant or if solvent resistance is conferred by a protective coating. (Right) In the push-up architecture, solvents (dark blue) come into contact with both layers of the device. Thus, the device must be constructed entirely from materials that are compatible with the solvent or the fluid channel must be coated on all surfaces. In all diagrams, dark red represents the contents of the control channels, which may be air or a hydraulic fluid such as water or oil.

microchannels after the device is fully assembled. However, this can often lead to non-uniform coatings or to clogging of channels. To avoid the bonding problem, it may be possible to coat only part of the surface (i.e., the inside of fluid channels, but not the bottom of walls between them), perhaps using a masking technique.

In a push-up device, one must coat all surfaces of the fluid channel. Options are to flow a coating solution through channels, or to coat both device layers (i.e., the top of the thin layer and the bottom of the thick layer) prior to device assembly. In the latter case, a method for producing a strong coating-coating bond is needed. A subtle difference between push-up and push-down devices is that a coating on the valve membrane will be stretched in the former but compressed in the latter. This is an important consideration for plastic coatings (which do not stretch) or weak coatings (which can break if stretched).

It is important that the coating adhere well to the elastomeric device material and that the coating provide a barrier to diffusion of the solvents of interest. Coatings with high permeability or pin-hole defects are not sufficient as they allow solvents and reagents to rapidly reach the nonresistant material underneath.

3.4.3 Membrane architecture

We devised an additional novel architecture for crossed-channel microvalves, shown in Figure 3.3. It consists of a fluid- and control-channel layer separated by a thin uniform elastic membrane. Valve operation is identical to push-down or push-up valves. The main difference is that the elastic valve membrane is no longer part of the bottom molded device layer, but is contained in a separate nonpatterned layer. This architecture was invented after learning that several promising fluoroelastomers could not be easily molded at the micron scale but were commercially available as flat sheets. Coated membranes are also an option and may enable superior coating quality compared to coated 2-layer devices in which the negative relief pattern of the microchannels interferes with the coating process. Because the fluid layer must be resistant to solvents, we frequently fabricated it from glass, which can be chemically etched to give rounded microchannels. However, the use of glass eliminates permeability and complicates connections for solvent delivery. The control layer need not be resistant to solvents.



Figure 3.3: Novel membrane architecture for crossed-channel microvalves. (a) Top-down schematic of a membrane device illustrating orientation of fluid and control channels. (b) Side view schematic of the device. The fluid and control channel layers are not in contact but are separated by a thin elastic membrane. The materials from which the fluid and control layers are made need not be elastic. Note that the deflecting membrane is simply a flat featureless layer, useful in cases where resistant materials are available as flat sheets but cannot be molded with micron scale channel features. (c) Schematic of the device with the valve closed. As usual in crossed-channel valves, pressurizing the control channel deflects the membrane further and further into the fluid channel until it completely blocks the flow as shown here. (d) Schematic showing the device filled with fluids. Solvent (dark blue) contacts the fluid layer material as well as the membrane. Fluids are delivered to each layer by drilled or punched holes as shown.

A membrane device constructed with fluid and control layers made from glass contains only a very small amount of elastomer. We believe the effects of swelling are therefore reduced and that this might allow even high-swelling solvents to be used in such devices. As a demonstration, we fabricated a device with a PDMS membrane and successfully flowed dichloromethane through the channels. Unlike in bulk PDMS, the channels did not swell shut and block the flow.

Membrane devices, like conventional 2-layer devices, require strong adhesion between all layers for proper operation. At first glance, it appears that chemical bonding is not necessary and that one could simply hold the device together by applying force. However, after fabricating several devices it became clear that this is not the case. When actuating a valve, the membrane intially deflects as expected; however, the membrane continues to peel free of the control layer surface along the fluid channel in both directions, greatly expanding the region of deflection. Eventually, the entire fluid channel is "closed" due to the actuation of a single valve. This also leads to cross-talk between any valves connected to that channel.

3.4.4 Summary

To summarize, solvent-resistance can be conferred by choosing resistant materials or by applying protective coatings or surface treatments. Push-down devices allow construction from two materials in which only the thin layer need be fabricated from a resistant material, an important feature when using expensive or scarce materials. The membrane valve architecture is an alternative to push-up and push-down valves with the primary difference that the elastic membrane is not part of any patterned device layer but rather is a flat uniform sheet. This has implications for certain elastomers that are not easily patterned.

One other approach to solvent resistance may be the use of a "sheath flow", whereby a sheath of one solvent surrounds the flow of the desired reagents. They do not mix (except slowly by diffusion) if in the laminar flow regime. Obviously, the sheath solvent must be compatible with the reagents, and the polymer must be compatible with the sheath solvent. The difficulty is to arrange for the reagents to flow as desired—to truly protect the fluid channel, the sheath must surround the reagent in all three dimensions. Furthermore, the flow distance is severely limited unless flow rates are extremely high; therefore, pursuit of other methods had priority.

3.5 Research results

Our research into solvent-resistant microfluidic devices proceeded in many different directions: (i) investigation of new materials; (ii) investigation of coatings and surface treatments; (iii) design and demonstration of the membrane device architecture; and (iv) development of a three-dimensional molding procedure.

To fabricate devices from new materials, many factors must be considered. Of course the polymer must be elastic and must be compatible with the desired applications. We initially considered room temperature DNA synthesis, which turned out to impose stringent conditions on compatibility due to the wide range of solvents involved. It must also be possible to pattern the polymer surface at the $10-100 \ \mu m$ scale by methods such as replication molding or etching, and it must be possible to bond polymer layers. Though not essential, it is convenient if it is possible to punch holes in the material for making simple off-chip connections and if the material is transparent or translucent such that fluid flow can be observed directly.

One of the most important issues is bonding—both between layers and between the device and the substrate. Strong, covalent bonding is needed in order to withstand the large local pressures generated inside control channels and the deformation stresses that arise when polymers swell (even slightly) in solvents. Weak bonding leads to delamination of layers, which can result in crosscontamination of fluids in different channels or in device failure. A lack of covalent bonding has been observed to permit proteins to migrate up to 5 μ m laterally in between layers despite no signs of delamination [57]. One other problem I have observed is that very weak bonding of the device to the substrate allows the device to lift from the surface when push-down valves are actuated, causing valve membranes to continue to extend downwards, eventually rupturing. This problem could be solved by gently clamping the device to the substrate. One must be aware of the relative strengths of layer-layer and substrate-device bonding when choosing whether to use the push-down or push-up valve architecture. The latter has the highest pressure requirements at the device-substrate interface, for example.

Bonding of cured layers can be achieved in many ways: gluing, modification of the polymer to allow covalent attachment of layers, preparing layers with different fractions of constituents [272], and surfaces treatments [67], among others. When working with off-the-shelf polymers or polymers with proprietary structures, we found it challenging to find reliable bonding procedures, especially with fluoropolymers that often exhibit non-stick surfaces. Even in collaborations where polymers were being specifically designed with microfluidics applications in mind (Chapters 4 and 5), determining and optimizing a bonding protocol took considerable time (sometimes more than a year). It was important to find a *reliable* method of adhesion, to avoid wasting rare material samples while trying to fabricate full devices and to enable the investigation of more complex fluidic networks. This search for a bonding process hinders the evaluation of new materials in microfluidic devices and was often our most significant bottleneck. One way to avoid this problem is to eliminate bonding steps altogether. For example we recently developed three-dimensional molding techniques (discussed in Chapter 6) to cure both layers simultaneously into a monolithic device. Another way to eliminate the need for bonding is to use a different valve actuation scheme such as mechanical pins [96] so that a second device layer is unnecessary; however many of the desirable properties of 2-layer PDMS microfluidics would then be lost.

For coatings, it is necessary to find a method for reliably covering the solvent-exposed surfaces without clogging microchannel features. In addition, it must be shown that the coating provides an effective barrier to the solvents of interest and that it does not interfere with valve actuation. The coating must also adhere strongly to the polymer.

In the remainder of this section, I describe our specific achievements with respect to the first three research directions. The work has been organized into three sections: modified PDMS devices, fabrication from other materials, and fabrication of membrane devices. For completeness, I have included materials and processes that looked promising initially but that ultimately did not lead to practical devices. Particularly successful and extensive work done with two novel polymer materials—fluorinated norbornene and perfluoropolyether polymers—is discussed separately in Chapters 4 and 5.

3.5.1 Modified PDMS devices

The simplest approach to fabricating solvent-resistant elastomeric devices is the modification of PDMS devices to confer solvent resistance, thus leveraging the existing device design and fabrication expertise. In this section, I describe several experiments to confer solvent-resistance by applying coatings and by performing surface treatments and chemical modifications.

Flexible fluoropolymer coatings such as Viton, CYTOP, and Chemraz seem to be the most promising approaches, but are likely suitable only in applications having moderate solvent-resistance requirements. Most coatings (up to several microns thick) do not seem to provide a complete barrier to solvents; rather, they just slow down adverse effects such as swelling or chemical attack. Perhaps the coating is too thin and the diffusion time of the solvent through the coating is very fast, even for low diffusivities. Another possibility is that the coatings are highly porous due to the fact that they are deposited from solutions with very low solids content and therefore shrink considerably upon drying. Lack of barrier protection was observed both in CYTOP, an uncrosslinked (but annealed) coating, and Viton, a crosslinked coating. Coatings may prove most useful in applications where the problem is chemical attack rather than swelling. For example, PDMS valves stick shut if exposed to heated hydrochloric acid [159]; a coating may not prevent the underlying attack of the PDMS but could provide a barrier to at least prevent the sticking.

3.5.1.1 Viton coating

Viton is a black liquid-castable FKM fluorelastomer. FKM elastomers provide good chemical resistance, though, due to some hydrogen content, are more susceptible to swelling and chemical attack than perfluoroelastomers. Samples of Viton coating material (PLV 2000 and Accelerator #4) were generously provided by PelSeal Technologies LLC (Newtown, PA). Coating resin was prepared by mixing 44:1 PLV 2000:accelerator.

The coating solvent is methyl ethyl ketone, which swells PDMS significantly. Attempts to coat by flowing through channels failed due to the rapid evaporation of solvent (or diffusion into the PDMS). Instead we coated device surfaces. The best results were obtained by first coating the mold, then curing 10:1 RTV 615 PDMS prepolymer onto the coated mold. Since Viton sticks to silicon wafers after curing, it was necessary to prepare a mold made from PDMS. Viton was coated onto this mold by spin-coating at 2000 RPM and allowed to dry, then fresh PDMS was poured on top, degassed, and cured by baking for 4 h at 80°C. (This bake simultaneously crosslinked the Viton coating.) Treatment of the Viton-coated mold with oxygen plasma for 1 min prior to casting resulted in greatly improved adhesion of the coating to the newly cast device. Once peeled from the mold, the coated device sealed to glass nearly as well as uncoated PDMS does. Though the coating was not transparent, it was possible to see through it sufficiently well to observe fluid flow within the channels. A typical device is shown in Figure 3.4.



Figure 3.4: Viton-coated PDMS microfluidic device. This photograph was taken through the 2×3 inch glass slide to which the device was sealed. In this particular device, inlet holes were made with a hole punch prior to spin-coating the device with Viton.

Coated devices were also fabricated from Ebecryl 3708 Acrylated Epoxy resin (courtesy of UCB Chemicals). The resin was mixed with 5 wt% Irgacure 500 (Ciba Specialty Chemicals), poured on the Viton-coated mold and cured by UV exposure (ELC-500 UV Curing Chamber, Electro-Lite Corporation) for 20 min under a nitrogen purge. The resulting device sealed very strongly to glass (even with the coating), but the coating was not well-adhered to the device.

Since hole-punching was found to destroy the Viton coating, fluids were delivered to the device through holes in the glass substrate. A special jig (see Figure 4.4) was created for this purpose. The jig also helps to hold the device onto the glass substrate, but only a small force can be applied before causing collapse of microchannels. Dichloromethane could be flowed only a few centimeters along a channel before it stopped, suggesting that perhaps the Viton was not preventing swelling of the PDMS by this solvent. Furthermore, the Viton coating itself is not resistant to certain solvents such as acetone: exposure initially caused cracking and then dissolved holes completely through it.

3.5.1.2 CYTOP coating

PDMS devices were also coated with CYTOP 809A (Sigma Aldrich), a solvent-resistant perfluoropolymer coating material consisting of a 9 wt% solution of poly(1,1,2,4,4,5,5,6,7,7-decafluoro-3oxa-1,6-heptadiene) ($M_n \approx 100000$) in perfluorotributylamine (Figure 3.5). Curing the CYTOP coating is achieved by baking at a moderate temperature (80°C) to evaporate the solvent then baking at a high temperature (above the glass transition temperature, $T_g = 108^{\circ}$ C) to anneal the coating. No crosslinking occurs. However, the CYTOP contains additives to improve adhesion to substrates.



Figure 3.5: Structure of CYTOP perfluoropolymer coating.

Kanai *et al.* [140] reported the passivation of PDMS microfluidic channels with a CYTOP coating of 0.2–5 μ m thickness. Passivation successfully protected PDMS features from attack by the PDMS solvent tetrabutyl ammonium fluoride (TBAF) and prevented fluorescently labeled λ DNA and bovine serum albumin (BSA) from sticking to the surface. Devices were fabricated from two PDMS layers that were first treated with oxygen plasma then CYTOP coated by dip- or spincoating. Each layer was prebaked at 75°C, then the layers were bonded (with CYTOP coatings in contact) by baking at 115°C under a pressure of 40 kPa (6 psi). Actuation (complete closure) of a coated millimeter-sized diaphragm valve was also demonstrated. This bonding and annealing process solves an important problem we encountered earlier—CYTOP forms a very corrugated texture when coated and annealed on an isolated PDMS surface.

Mike Toepke (of Paul Kenis' lab at the University of Illinois at Urbana-Champaign) and I sought to duplicate this work and apply this principle to the fabrication of crossed-channel valves in PDMS microfluidic devices with the goal of demonstrating more sophisticated solvent handling applications. Initially, we bonded unpatterned slabs (2–3 mm thick) of Sylgard 184 PDMS after CYTOP coating. Holes were punched prior to coating in each slab to allow testing of the pressure that could be withstood by the layer bond. Slabs of PDMS were prepared from PDMS mixed in ratios of 20:1, 10:1, and 5:1, and were cured for times ranging from 30–90 min at 80°C. CYTOP was diluted 1:10 (w:w) in Fluorinert FC-43 (courtesy of 3M Corporation) and spin-coated onto the PDMS slabs after treating them for 1.5 min with oxygen plasma. PDMS slabs were spin coated by first sealing to a glass slide. Dirty glass slides were used so that the slabs could easily be removed without distortion (and possible damage) of the CYTOP coating. Samples were prebaked for 30 min at 75°C, then placed into contact with CYTOP coated surfaces, and baked for 45 min at 115°C. Among several methods considered for applying pressure during baking, sandwiching the layers between glass slides and clamping them together with standard office binder clips (3/4 inch size) resulted in the strongest and most uniform bond. Furthermore, bonding to a CYTOP-coated PDMS substrate rather than a CYTOP-coated glass substrate resulted in a stronger bond (15–20 psi vs. 4–7 psi). Note that when adhesion failed, usually the two CYTOP layers were stuck together, indicating a superior CYTOP-CYTOP than CYTOP-PDMS bond.

The CYTOP thickness was measured to be 0.05–0.1 μ m thick by profilometry. Swelling of the PDMS surface was not observed when exposed to dichloromethane droplets, provided the CYTOP had been annealed at 115°C.

Sylgard push-down devices containing a simple valve test pattern (100–500 μ m wide fluid channels at 90° to 100–500 μ m wide control channels) were fabricated, coated with CYTOP, and bonded to CYTOP-coated PDMS slabs. Since the push-down devices had more mass than the slabs originally used for testing, we found the binder clips to be unnecessary. Note that holes for the fluid channel were punched after the CYTOP coating was applied. Solvents (dyed dichloromethane) could be flowed through the channels at low pressure, and valves could be actuated at 25–30 psi (Figure 3.6). When operated with empty fluid channels, the surface of the fluid channel appeared wrinkled during and after valve actuation. Perhaps this is due to the high stiffness of CYTOP (1–2 GPa): the coating may buckle rather than deform uniformly.

In a later effort, we examined the effect of bake temperature on the CYTOP adhesion. We fabricated PDMS slabs with punched holes, coated them with CYTOP, and bonded them to PDMS-coated glass with a CYTOP coating on top. Devices bonded at 115°C with a 40 g weight for 24 h delaminated within about 30 min when injected with solvent (acetonitrile, dichloromethane, and methanol) at 5 psi. Devices baked at 165°C for 24 h with a 40 g weight withstood these conditions for at least 48 h.

We also tried to apply CYTOP coatings to push-up devices by flowing dilute CYTOP (1:10 in Fluorinert FC-75) through microchannels. Devices were fabricated and adhered to RCA-cleaned glass (Appendix A.2.1) by baking overnight at 80°C with a droplet of 3.7% HCl. CYTOP solution was then flowed at 10–12 psi for approximately 30 min and appeared to apply a uniform coating. With tubing left in place at chip inlets, the coated device was baked at 80°C for 20 min and then at 160°C for 60 min. Solvent flowed several centimeters through the device before stopping, in contrast to uncoated PDMS, where solvent stops flowing after only a few millimeters. Unfortunately every exit channel was clogged, presumably by CYTOP. By carefully watching the coating solution during the baking process, we observed that this problem arises as the solvent evaporates: the CYTOP coalesces—perhaps due to poor wetting of the PDMS—into larger and larger droplets that become solidified. In attempts to fix this problem, we tried: (i) turning devices upside-down during drying to encourage CYTOP to flow out along edges of punched holes rather than pooling in the



Control Channel

Figure 3.6: Microvalve actuation in solvent-resistant CYTOP-coated PDMS devices. (a,b) Micrographs of a CYTOP-coated PDMS device in (a) open and (b) closed states. The 100 μ m wide fluid channel is oriented left to right and the 300 μ m wide control channel is oriented top to bottom. The CY-TOP coating is approximately 50–100 nm thick. Note that because CYTOP is not an elastomer, wrinkling and other effects were observed during actuation (white arrows). The persistent wrinkles in the middle of the channel in the open state appeared after the valve was actuated for the first time. (c,d) Micrographs of another valve in the same device (100 μ m wide fluid channel, 100 μ m wide control channel). In this case, the valve only partially closed. (e,f) Microvalve (200 μ m fluid, 300 μ m control) in open and closed states when solvent (dichloromethane with acetonitrile and methanol to dissolve the blue dye xylene cyanol FF) is flowing in the fluid channel. Wrinkles are not apparent, perhaps due to optical effects. The valve was successfully actuated repeatedly over a period of several hours with no apparent degradation in performance. bottom; (ii) reducing the CYTOP concentration to 1:50 and 1:100; (iii) flowing a continuous stream of air or liquid through the channel after coating, attempting to maintain an open passage during drying; and (iv) fabricating push-up devices with fluid layer holes punched all the way through both layers, thus creating a small cylindrical volume at the bottom of the inlet holes where excess CYTOP could theoretically collect without interfering with the fluid path. The last was partly successful. In devices having a few open channels, we were able to properly test dichloromethane flow. We still observed the flow to stop after several centimeters.

We performed swelling tests (by immersion), to determine if CYTOP coated PDMS was providing a sufficient barrier to solvents. These tests revealed that CYTOP indeed provides a temporary barrier, but eventually the solvent swells the PDMS. Petri dishes were filled with 5:1, 10:1, and 20:1 Sylgard 184 and cured at 80°C overnight. Small PDMS samples (5 mm \times 5 mm \times 4 cm) were cut out. A batch of uncoated samples was evaluated as well as a batch coated in the following manner. Samples were dip coated three times in CYTOP diluted 1:10 in Fluorinert FC-75. Between coats, the samples were baked for 10 min at 80° C to evaporate solvent. To prevent holes in the coating, samples were supported on two parallel wooden sticks during baking and repositioned after each coat. Samples were then weighed, placed in glass vials, baked for 30 min at 80°C, baked for 60 min at 160 °C, and then slowly cooled down to room temperature. Dichloromethane was added to each vial. To determine the progress of swelling, samples were re-weighed after different lengths of exposure. (Due to the rapid evaporation of dichloromethane, each sample was weighed immediately after removing it from the vial and patting it dry with a Kimwipe.) As shown in Figure 3.7, the CYTOP coating leads to a small reduction (or delay) in swelling; however, the magnitude of swelling in dichloromethane is still quite large in all cases. This experiment was repeated with a 9-day 160°C annealing bake with very similar results.

While not suitable for applications requiring long-term solvent resistance, CYTOP-coated devices may be useful in applications requiring passivated channels [140] or in applications involving only intermittent exposures to solvents.



Figure 3.7: Effect of CYTOP coating on the swelling of PDMS in dichloromethane. Swelling (weight %) of uncoated and coated Sylgard 184 PDMS was determined after immersion in dichloromethane for different periods of time. Data is shown for several PDMS mixing ratios (5:1, 10:1, and 20:1). In all cases, there is still significant swelling with CYTOP present, though the magnitude is reduced or delayed.
3.5.1.3 Chemraz coating

Chemraz is a perfluorinated elastomer well known for its elasticity and solvent resistance. PDMS push-down microfluidic devices were sent to Jiang Huang at Fluidigm Corporation to be coated with Chemraz by a proprietary process and bonded to glass substrates. Initial devices were not usable as the introduction of solvents (mixture of dichloromethane, methanol, and acetonitrile) caused the fluid channels to delaminate from the substrate at pressures less than 1 psi. However, valves appeared to function normally in these devices, suggesting that the coating does not impede valve membrane deflection.

Recently, Fluidigm has developed a new coating process that solves the bonding issue. The ability of this Chemraz coating to act as a solvent barrier to protect the PDMS needs to be carefully evaluated.

3.5.1.4 Teflon AF coating

Teflon AF is a form of Teflon in solution in a perfluorinated solvent that can be spin-coated then dried and annealed to form thin transparent coatings with extremely high solvent resistance. After testing that the perfluorinated solvent Fluorinert FC-75 (courtesy of 3M Corporation) acceptably wets the surface of PDMS, I attempted spin-coating Teflon AF (DuPont) at 1000 RPM onto 10:1 RTV 615 devices. The coated devices were left at room temperature for 20 min for solvent evaporation, heated for 15 min to 120°C and then for 15 min to 170°C, and finally ramped back down to room temperature.

Upon cooling, the Teflon AF coating was visibly cracked. In fact, under the microscope, it appeared as flakes of Teflon surrounded by uncoated PDMS. Furthermore, the coating can be easily peeled from the PDMS. The coating is very rigid, exhibiting no adhesion at all to substrates such as glass, and undergoes audible cracking when the PDMS device is flexed slightly. For elastomeric microfluidics this does not seem to be a promising solution.

3.5.1.5 Parylene coating

Parylene is a non-fluorinated molecule that can be polymerized from the vapour phase onto a surface, resulting in very uniform conformal coatings. It is frequently used in the microelectronics industry as a surface passivation layer but has also been used in a wide variety of additional applications, including fabrication of microvalves in silicon microfluidic devices [285]. PDMS samples were coated with a $1-2 \mu m$ parylene film by Matthieu Liger in Yu-Chong Tai's lab at Caltech. The result was a transparent and flexible coating, strongly bonded to the PDMS. Surface tests revealed that parylene does not provide a barrier to dichloromethane, which swells the underlying PDMS almost instantly upon exposure. Clouding of the parylene was observed after several minutes of exposure.

3.5.1.6 Plastic coating

Samples of powders of several solvent-resistant plastics—polyvinylchloride (PVC), isotactic polypropylene (PP), and poly(vinylidene fluoride) (PVDF)—were purchased from Scientific Polymer Products Inc. (Ontario, NY). We intended to apply plastic coatings to PDMS, but could not find appropriate solvents for these powders that do not cause extreme swelling of PDMS, nor could we heat the PDMS to a sufficiently high temperature to apply a molten plastic layer.

3.5.1.7 Metal coating

Scraps of PDMS with a gold coating prepared by Scott Driggs via evaporation were evaluated for solvent-resistance. Under the microscope, the gold appeared to have many fine cracks and creases, perhaps from flexing or bending of the PDMS. Exposure to droplets of dichloromethane caused local swelling. When swelled, spaces between the cracks in the coating were clearly visible. It is possible that valve actuation would be sufficient to cause such cracking.

Coating with silver from a silver nitrate solution was also attempted. Coating for 25 min resulted in a visible silver coating. Drops of dichloromethane immediately lifted the silver from the surface and swelled the device. The coating could be rubbed off quite easily suggesting it is not very robust and likely not bonded to the PDMS.

3.5.1.8 Teflon lubricant spray coating

Teflon lubricant was sprayed onto a silicon wafer and PDMS prepolymer cured on top of it. After baking for 4 h at 80°C, the sample was removed. No Teflon remained on the wafer, indicating that it had been incorporated into the PDMS surface. However, solvent exposure tests revealed swelling upon exposure to dichloromethane. Since Teflon sprays consist of suspensions of Teflon particles it is not likely that this method could achieve the needed complete surface coverage.

3.5.1.9 CF₄ plasma-treatment

Anecdotal evidence indicated that exposure of PDMS to a tetrafluoromethane (CF₄) plasma generated Teflon-like compounds on the surface. We sought to test whether this residue could serve as a solvent-resistant coating on PDMS. A sample of cured 10:1 Sylgard 184 in a petri dish was partly covered with a glass cover slip. The sample was exposed to a CF₄ plasma (100 W power, 100 cm³/min gas flow rate) for 15 min. Profilometry revealed that the exposed surface had been etched down approximately 0.5 μ m with a roughness of 50–100 nm. Unexpectedly, qualitative contact angle measurements showed the surface to be more hydrophilic (lower contact angle) after treatment, in contrast with the high contact angle that is common of flurocarbon materials, an effect that may be related to the roughness. Surface testing with droplets of solvents showed no difference in local surface swelling between treated and untreated areas upon exposure to dichloromethane and diisopropylethylamine.

3.5.1.10 Fluorosilanization of PDMS surface

Genzer and Efimenko [91] reported a technique for assembling extremely dense monolayers of fluorinated trichlorosilanes $(F(CF_2)_y(CH_2)_xSiCl_3)$ on cured PDMS. They accomplished this by stretching the PDMS by 60–70% during silanization to increase the hydroxyl sites available for attachment. When the stretching was released, the surface molecules packed extremely tightly together.

I treated samples of Sylgard 184 PDMS with (Tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane while in the stretched state. Upon release, the PDMS exhibited extremely high contact angles with

water (> 110°). However, the treatment appears not to provide a barrier to solvents such as dichloromethane, as the samples exhibited significant surface swelling upon contact with solvent droplets.

3.5.1.11 Incorporation of fluorinated additives during polymerization

We attempted to prepare surface-fluorinated PDMS by the method of Thanawala and Chaudhury [267]. Krytox oil (courtesy of DuPont) was added to RTV 615 or Sylgard 184 PDMS during mixing. Device samples prepared by Markus Enzelberger exhibited immediate surface swelling upon exposure to dichloromethane.

3.5.2 Alternative elastomeric device materials

In addition to PDMS modifications, significant effort was expended in developing protocols to fabricate devices from alternative elastomeric materials after preliminary evaluations indicated acceptable elastic properties and solvent-resistance.

Development of a fabrication protocol is a significant undertaking, requiring the following issues to be addressed:

- Ensuring release from silicon wafer molds after curing.
- Developing a technique for fabricating a thin layer (spin coating for viscous prepolymers; other methods for low viscosity materials).
- Devising a method to bond the material to itself sufficiently strongly to withstand pressure inside microchannels. Self-bonding is needed between layers or between the device and a coated-substrate.
- (Optionally) Devising a method to bond material to glass, in cases of fabricating membrane devices or when it is desired that the fluid channel be open to the substrate (e.g., for *in situ* solid-phase synthesis).

• Determining the polymer shrink factor to allow mold designs to be properly scaled for correct layer alignment.

The most successful materials were fluoronorbornene (FNB) and perfluoropolyether (PFPE) polymers developed by our collaborators. These materials were designed specifically with microfluidic applications in mind, and properties were tailored to address the above issues. Details of experiments and results are discussed in Chapters 4 and 5, respectively. Limited success was achieved with other materials as well. In particular, the commercial product SIFEL seems a promising candidate. Work with these other materials is described below.

3.5.2.1 SIFEL

SIFEL [279] is a perfluorinated elastomer (type FFKM) consisting of a perfluoropolyether backbone with terminal silicone crosslinking groups (Figure 3.8). Samples of several SIFEL formulations were generously provided by Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan).



Figure 3.8: Chemical structure of SIFEL perfluoroelastomer. SIFEL has a perfluoropolyether backbone with terminal silicone crosslinking groups.

Two samples of adhesives, SIFEL610 and X-71-0603, cured to milky white and milky brown elastomeric materials, respectively. Both had qualitatively good flexibility, and holes could be easily punched with our hole-punching machine (Technical Innovations, Brazoria, TX). Immersion of samples in dichloromethane for 3 days indicated swelling of 13.6 wt% and 10.5 wt% for SIFEL610 and X-71-0603, respectively.

Both materials adhered very strongly to silicon or glass surfaces upon curing. Coating silicon wafers with annealed CYTOP or treating them with fluorosilane permitted samples to be easily released, however. Preliminary bonding tests with these materials were not successful. Cured materials were stacked on one another and baked at 150°C overnight in all four possible combinations of the two materials. None exhibited any adhesion. The prepolymers were too viscous to attempt spinning a thin "glue" layer between layers. However, curing freshly poured material onto another fully cured sample resulted in significant adhesion, suggesting that a partial curing technique might work. Preliminary attempts indicated that undercuring (by shortening the bake time and/or reducing the bake temperature) left a liquid center inside the thick layer samples. Unfortunately, if baked just long enough for the liquid to disappear, the samples no longer adhered to thin layers. Partial curing may be impossible or may simply be very sensitive to timing. Being one of the few liquid castable perfluoroelastomers available, further investigation may prove fruitful. Our tests ended after initial successes with other materials: perfluoropolyether (PFPE) (Chapter 5) and fluorinated norbornene (FNB) (Chapter 4).

It should be noted that another, non-adhesive SIFEL product, SIFEL8070 ("potting gel"), was also obtained and evaluated. Parts A and B were mixed in a 1:1 ratio and baked for 1 h at 150°C, as per the manufacturer's protocol. The result was a sticky non-solidified gel that was strongly adhered to the fluorosilaned silicon wafer. Other ratios resulted in a similar lack of solidification.

3.5.2.2 New materials for CLiPP synthesis

Hutchison *et al.* [116] report the fabrication of microfluidic devices by a photopolymerization technique called contact liquid photolithographic photopolymerization (CLiPP). Microfluidic devices and other structures are fabricated in layers—each new layer is applied in liquid form then selectively polymerized by UV exposure through a mask. A sacrificial material is filled into the recesses of the previous layer when synthesizing a new layer on top. A unique aspect of this work is the inclusion of "iniferters" in the monomer solutions. These molecules are covalently attached to the layer during polymerization and serve as initiators for the polymerization of subsequent layers. This leads to covalent bonding between adjacent crosslinked layers.

We collaborated with Brian Hutchison and colleagues in Christopher Bowman's lab at the University of Colorado to develop solvent-resistant elastomeric devices by their approach [115]. To fabricate fluid channels with a rounded profile to allow complete closure by elastomeric microvalves, it was necessary to fabricate the first layer on a silicon mold patterned with rounded channel features. It is not possible to include rounded features at any other stage in the CLiPP fabrication process (except, perhaps, by underexposure techniques [85]). First the material for the (bottom) fluid layer is poured and photopolymerized by flood exposure. Next, a second monomer layer is poured on top of the first and exposed through a mask to define the pattern of control channels, which is then backfilled with sacrificial material. Finally a thick layer is poured on top and polymerized.

Hutchison *et al.* evaluated numerous existing monomers and newly synthesized fluorinated monomers in terms of elastic modulus and swelling in DNA synthesis solvents, among other properties. One of the new formulations, a mixture of PFPE2000-A and F-C10-A, exhibited a modulus of 8 MPa and mass swelling of 10% or less in all solvents [114]. Several stages of the CLiPP process were successfully demonstrated. In order to produce a functional microfluidic device, a couple of issues remain to be resolved: (i) adhesion between layers, and (ii) adhesion to glass [114]. To address the first, Hutchison *et al.* synthesized several fluorinated iniferters but found them to be insoluble in the fluornated monomer formulation. Adhesion to glass is also suspected to be difficult.

With further development, this may be a viable route to solvent-resistant elastomeric device fabrication. The method has the advantage of simple fabrication. Because the second device layer is fabricated in place, there is no need to account for shrinkage differences between layers, and alignment is performed by aligning photomasks rather than soft polymer layers. The problem of adhering layers becomes simply a problem of iniferter design.

3.5.2.3 Fluorosilicones

Fluorosilicones (type FVQM fluoroelastomers), in general, possess most of the physical properties of regular silicone (PDMS) but with enhanced resistance to solvents.

A sample of Q4-2817 fluorosilicone sealant was provided by Dow Corning (Midland, MI) for evaluation. This product is a thick red paste that cures at room temperature in about 24 h, releasing acetic acid in the process. Primitive devices were molded on a patterned silicon wafer, and holes were punched for fluid channel inlets and outlets. It should be noted that the curing time was about 2 weeks since the paste was sandwiched between the wafer and a petri dish (to form a flat top surface) and presumably acetic acid could not escape rapidly. Though ethanol and acetonitrile could be flowed through the channels, dichloromethane could not. Additional tests showed the fluorosilicone to exhibit surface swelling in the presence of droplets of this solvent.

Evaluation of a sample of Dow Corning 730 Solvent Resistant sealant (courtesy of Dow Corning) exhibited releatively little swelling in dichloromethane. However, the material did not cure to a useable consistency—it remained somewhat sticky and plastically deformable.

3.5.2.4 Other materials

Though plenty of highly solvent-resistant materials such as perfluoroelastomers (FFKM fluoroelastomers) are commercially available, most are unsuitable for microfluidic device fabrication by replication molding. Such materials include Kalrez (DuPont Dow Elastomers), Chemraz (Greene Tweed & Co.), Chemtex/PFR (UTEX Industries, Inc.), Parofluor (Parker Hannifin Corp.), Simriz (Simrit), among others. These materials require melt processing, and due to the extremely high viscosity of the melt, it is not possible to mold features on the scale of microfluidic device features, according to engineers in industry.

It is conceivable that chemical or dry etching methods might be suitable for fabrication of micron scale features in the surfaces of such materials. However, samples we received had high surface roughness (several microns), and it is not clear whether starting materials with a sufficiently smooth surface can be obtained. In addition, it is likely that bonding of layers would prove difficult. We focussed on the development of microfluidic devices from liquid castable materials.

3.5.3 Membrane devices

An early search of commercially available elastomers showed that the most solvent-resistant ones (perfluoroelastomers such as Chemraz, Kalrez, Parofluor, etc.) were not easily patternable by molding or other means. These materials can only be melt processed, requiring temperatures of 300– 400°C, very high pressures, and specialized equipment. According to several seal manufacturers, these molding processes cannot produce void-free casts of molds with features below 1 mil (about 25 μ m) due to the extremely high viscosity of the melt. Since microfluidic device features are typically comparable to this size, it is unlikely that devices could be reliably molded.

Thin, flat sheets of perfluoroelastomer materials, however, are commercially available. Because the membrane architecture that we devised requires only an *unpatterned* thin sheet of elastomer as the deflecting layer between two channel-containing layers (Section 3.4.3), it seemed ideally suited for such materials. For proper operation, the membrane must be covalently bonded to the two layers; however, to quickly evaluate whether a membrane could be deflected, we often just clamped membranes between two glass or PDMS layers.

The architecture was first validated using PDMS membranes. We also attempted to incorporate solvent-resistant elastomer membranes; however, useful devices were not fabricated since elastomer sheets were not available in sufficiently thin layers or were not bondable.

3.5.3.1 Architecture validation with PDMS membrane

As an initial proof of principle, we fabricated membrane devices with PDMS membranes. PDMS was an ideal material for testing because there is a known method (oxygen plasma treatment, Appendix A.2.4) for covalently bonding the membrane to two glass channel layers. A 5 μ m PDMS (10:1 Sylgard 184) membrane was spun (4000 RPM, 60 sec, 15 sec ramp) on a flat unpatterned silicon wafer treated with fluorosilane (see Appendix A.1.5) and cured by baking at 80°C for 2 h. Two glass layers were etched (see Appendix A.2.2) with a simple pattern of parallel channels (100– 1000 μ m wide by 35 μ m deep). One slide served as the control layer and the other (with pattern rotated by 90°) as the fluid layer. First, the PDMS membrane and control layer were treated with oxygen plasma and bonded together with dilute HCl as a lubrication layer. The glass and membrane were then peeled from the wafer and plasma bonded to the glass flow layer. The channels in both glass layers faced the membrane. To provide a means of pressurizing the microchannels, inlet/outlet holes were drilled in each glass layer (see Appendix A.2.3) prior to device assembly, and NanoPort connectors (see Figure 4.7) were adhered to the back sides at these positions. For simplicity, the channels in the fluid layer are linked together so only a single inlet and outlet are needed. Similarly, in the control layer, only a single inlet is needed to pressurize all channels.

In one device, nearly all 100 values closed by actuating the control channels to 10 psi. When dichloromethane was introduced into the channels it could be flowed easily, indicating that this architecture solved the swelling problem that leads to plugging of channels in thick elastomer devices. However, dichloromethane very rapidly diffused through the membrane—droplets of solvent condensation were visible at the other side of the membranes (i.e., in control channels) after a few minutes of flow. Allowing the solvent to flow overnight caused the valve membranes to rupture, perhaps due to local weakening of PDMS. In a macroscopic piece of PDMS, maximal swelling with dichloromethane is reached in just a few hours and embrittlement within days; in a thin membrane, these time scales are likely dramatically reduced.

3.5.3.2 CYTOP-coated PDMS membrane

I also attempted fabricating membrane devices with CYTOP-coated PDMS as the membrane. 5 μ m PDMS layers were fabricated as above. First the glass control layer was plasma bonded channel-side down onto the membrane, and the bonded structure peeled from the wafer. The membrane was then spin-coated with CYTOP (1:10 dilution in Fluorinert FC-75) at 1000 RPM and baked for 30 min at 80°C and for 2 h at 160°C. The glass flow layer was also CYTOP-coated, then aligned and clamped to the other layers with standard office binder clips, and baked at 160°C for several hours to promote bonding. When tested, fluid leaks were observed at very low pressures (1 psi) due to the poor adhesion of CYTOP-coated PDMS to the rigid glass layer. Successful device fabrication would require finding a solvent-resistant fluid layer material that can be strongly bonded to CYTOP.

3.5.3.3 PFPE membrane

PFPE is a photocurable solvent-resistant perfluoropolymer developed in a collaboration with Joseph DeSimone's group at the University of North Carolina. Details of this material and curing methods are discussed in Chapter 5. Membrane device fabrication was attempted with 20–30 μ m films of PFPE cured on silicon wafers after spin-coating. However, due to poor adhesion of PFPE to glass, there was insufficient bonding to the glass control layer to allow the membrane to be peeled from the mold. I also attempted membrane transfer by adhering the membrane to pressure-sensitive tape as reported by the Whitesides group for handling of PDMS membranes [134]. However, the adhesion of PFPE to glass was insufficient to allow transfer of the PFPE from the tape to the glass control layer.

3.5.3.4 Kalrez sheet

Kalrez is a commerically available perfluorinated elastomer that is resistant to a tremendous variety of solvents. We obtained the thinnest available sample of Kalrez compound 6375 (Standard Sheet K#5011) from DuPont Dow Elastomers. The sheet was opaque black in colour. Qualitative tests of solvent resistance upon exposure to acetonitrile, dichloromethane, and tetrahydrofuran did not cause swelling and left no trace of exposure once evaporated. Unfortunately the sample sheet was several hundred microns thick—far too large to serve as a deflectable valve membrane in a *micro*fluidic device. Qualitatively, it had a very high elastic modulus, which would further reduce its ability to be deflected. The sample also had a very high surface roughness, and it was not possible to seal it to a substrate for even preliminary membrane valve testing.

3.5.3.5 Chemraz sheet

Chemraz is a perfluoroelastomer similar to Kalrez in terms of chemical resistance and mechanical properties. We purchased custom fabricated Chemraz sheets $(0.005 \pm 0.001 \text{ inch thick})$ from Greene, Tweed, & Co. (Kulpsville, PA). Chemraz has a modulus of 2–4 MPa, comparable to PDMS. The thickness of the sheet was measured to be 135 μ m by profilometry (see Appendix A.3.1) with a

roughness of several microns. The roughness is presumably due to the fact that the sheet was molded between two metal plates with visible polishing marks in the surfaces. Chemraz sheets were opaque white in colour. We fabricated etched glass slides with attached NanoPort connectors as used in other membrane device testing. The Chemraz membrane was sandwiched between the two etched glass slides using standard office binder clips (3/4 inch wide). Without bonding we didn't expect proper functioning—we simply attempted to achieve membrane deflection. However, the device could not accept more than 2 psi of pressure without leaking, likely due to the surface roughness. If Chemraz membranes of higher surface smoothness and lower thickness should become available, such a device might be feasible, provided that one can determine a reliable bonding method.

3.5.3.6 Teflon PFA film

I obtained some samples of a thin (12.5 μ m) Teflon PFA Film (courtesy of DuPont) for attempted construction of membrane devices. While Teflon PFA is a rigid plastic, the film is quite flexible because it is so thin. Due to lack of adhesion, the film could not be sealed between glass plates. Instead, I used two 1-layer PDMS devices as the flow and control layers and clamped the PFA film between them. The Sylgard 184 PDMS layers sealed (reversibly) to the film such that about 5–10 psi could be introduced into the control channel to attempt membrane deflection. No deflection was observed. Because the membrane must lengthen in order to deflect completely into the fluid channel, it is likely that only elastomeric materials are suitable for membrane devices.

3.5.3.7 Teflon tape

Though also not elastomeric, we considered using Teflon tape as a deflectable membrane. This thin white tape is used for sealing gas fittings and can be thinned further by stretching. We evaluated Threadmaster PTFE Sealant Tape (Merco Company, Hackensack, NJ). Droplets of dichloromethane on a piece of tape covering a sample of PDMS rapidly discoloured the tape and soon led to surface swelling of the PDMS below. This indicates that the tape is highly permeable (due to its composition or the presence of pinholes) or that the plasticizers present in the tape reduce its solvent resistance. Attempts to fabricate membrane devices by sandwiching tape between PDMS layers failed due to the tendency of the tape to plastically deform and due to the difficulty in maintaining its flatness.

3.6 Summary

Solvent-resistant chips with mechanical valves have the potential to provide a *generalized* platform for highly integrated microfluidic chips in applications involving non-aqueous media, such as many areas of synthetic and analytical chemistry and other domains yet to be explored. Such chips could directly benefit those areas by enabling more accurate, rapid, and safe syntheses, and more sensitive and rapid analyses, or by allowing detailed studies of kinetics or reaction pathways [133]. The ability to perform on-chip solvent exchange very simply via evaporation may be particularly useful. As an example application, in Chapter 7, I describe microfluidic device designs suitable for performing combinatorial solid-phase synthesis and report some preliminary successes in the fabrication of DNA and peptide arrays. Solvent-resistant chips may also find use in the exploration of microfluidic phenemona in organic solvents, in expanding the variety of fluids used in existing applications, and perhaps in combining chemistry with biology or biochemistry in integrated synthesis and screening chips. It will be exciting to watch this field unfold.

Over several years, we expended considerable effort in evaluating elastomeric materials, coatings, and PDMS surface treatments as starting points for the fabrication of solvent-resistant elastomeric devices. Based on initial screening for solvent resistance and suitable elasticity, we attempted to devise procedures for fabricating working devices with candidate material systems.

Several promising results were reported in this chapter, such as coating PDMS devices with CYTOP to confer solvent resistance, fabricating devices entirely from fluorinated materials such as SIFEL, and fabricating devices from fluorinated monomers by the CLiPP method. The next two chapters describe additional work with materials developed in collaboration with polymer chemists specifically for microfluidics applications. Like the collaboration with Hutchison *et al.*, these collaborations helped to solve problems related to bonding and molding that hindered progress with commercially available perfluoroelastomers. Fully functional solvent-resistant devices containing microvalves were demonstrated with these two material systems. Constructed from permeable elastomers, they provide many of the advantages of PDMS devices (see Chapter 2) with the added advantage of chemical resistance, and can leverage the design expertise and experience garnered by the PDMS microfluidics community.

Acknowledgment

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Chapter 4

Solvent-Resistant Fluorinated-Norbornene (FNB) Microfluidic Devices

4.1 Introduction

In pursuit of the goal of fabricating solvent-resistant microfluidic devices from permeable elastic materials we first tapped into the vast range of polymers that are commercially available. Since these materials were not designed with microfluidics applications in mind, we found most solvent resistant materials to be lacking in terms of mechanical properties, such as elasticity, or in terms of processability for our purposes (see Chapter 3). Collaborations with polymer chemists to develop new materials turned out to be far more fruitful, as the interactive development process led to the invention of novel materials ideally suited to the fabrication of elastomeric solvent-resistant microfluidic devices.

In this chapter, I discuss the results of one such collaboration with chemists at Materia Incorporated (Pasadena, CA), which culminated in the successful fabrication of microfluidic devices containing functional push-down microvalves. The first section gives a brief overview of the material chemistry, followed by two sections giving accounts of important results and research directions for two initial "generations" of devices. First-generation devices were composed of non-solvent-resistant norbornene-based elastomers with a CYTOP coating to provide protection from solvents, while second-generation devices incorporated a solvent-resistant norbornene-based elastomer in their bottom layer and did not require this coating. Unfortunately, our supply of material was exhausted soon after successful demonstration of valves in these devices and work did not proceed further for some time. However, due to collaborations with new partners, results relating to a third generation of devices—composed entirely of solvent-resistant elastomer—have recently emerged. I briefly highlight these results and discuss prospects for Materia's fluorinated polymers in the last section.

4.2 Chemistry

Materia specializes in the development of polymers and catalysts based on ring opening metathesis polymerization (ROMP), a chemistry that became practical with the development of catalysts by Grubbs *et al.* [95]. The mechanism of this chemistry is shown in Figure 4.1. We worked primarily with polymers based on monomers derived from norbornene (NB) and dicyclopentadiene (DCPD).



Figure 4.1: **Ring-opening metathesis polymerization (ROMP)**. (a) Reaction mechanism. A strained ring structure (bottom structure in first stage) is opened by a metal alkylidene catalyst (top structure), driven by relief of ring strain. At the end of the reaction (third stage), the catalyst remains bound to the opened ring and is available for further reactions. Considering the strained ring as a monomer, one can see a polymer grows by the insertion of ring-opened monomers between the catalyst and the rest of the structure. (Reproduced from Rob Toreki's Organometallic HyperTextBook, http://www.ilpi.com/organomet/romp. html. Copyright Rob Toreki, 2005.) (b) The structure of norbornene, from which one class of monomers can be derived. Materia's solvent-resist polymers evaluated in this work are built from monomers that are norbornene derivatives.

Polymerization produces monodisperse chains that are intertwined into an amorphous uncrosslinked elastomeric material. These materials are living catalyst systems, which turns out to be convenient for bonding because polymerization can continue into the adjacent layer when two partially cured polymers are brought into contact. Solvent resistance is determined primarily by the particular monomer used, while mechanical properties are also affected by degree of cross-linking (if branched monomers are incorporated) as well as the rate of polymerization. Typically, polymers are prepared from a mixture of one or more monomers, a catalyst, a solvent to dissolve the catalyst, and additives such as inhibitors, antioxidants, and adhesion promoters. Polymerization is an exothermic process, proceeding first through a gel phase then to a solid material. One controls the rate and maximum temperature of the reaction by formulation as well as by controlling the ambient temperature, initial reagent temperature, and the total mass of material. If polymerization is too fast, a phenomenon called "worming" is observed, wherein high speed gas bubbles are generated that leave hollow paths in the cured polymer. A high exotherm temperature may also have the detrimental effect of damaging the catalyst and preventing bonding in later fabrication steps.

4.3 First-generation devices: CYTOP coating

Initially, Materia supplied us with materials polymerized from proprietary formulations of hexylnorbornene (HNB), decylnorbornene (DNB), ethylidene norbornene (ENB), and some dicyclopentadiene (DCPD) derivatives. Early evaluation indicated that these polymers were susceptible to attack by many solvents including dichloromethane, acetonitrile, and tetrahydrofuran—solvents that interested us due to their role in DNA synthesis chemistry (see Chapter 7). The polymers exhibited considerable swelling and discoloration immediately upon exposure to solvents, and in many cases, exposed areas were permanently damaged by pitting or conversion to a sticky residue. Some of the materials did however possess qualitatively good elastic modulus, though complete recovery from the deformed state was often slow—up to several seconds. The elastomers were robust and could withstand considerable elongation (up to 100–200%); samples could even be folded back on themselves without cracking or breaking as we have observed in many other materials.

Solvent-resistant fluoromonomers were not yet available in the early stages of this work, so we attempted to confer resistance by coating with CYTOP 809A (Sigma Aldrich), a fluoropolymer coating material we had tried in conjunction with PDMS (see Chapter 3). For simplicity, we fabricated devices with a push-down valve architecture; since all fluid channels are located in the bottom layer

in such devices, a protective CYTOP coating is only needed on the bottom surface (see Chapter 2). Push-up valves expose both layers to solvents in fluid channels and would require applying the coating inside channels or applying coatings to two layers—on the bottom of the top (fluid) layer and on the top of the bottom (control) layer—and fusing them together. Both approaches have proven problematic in past experience with other materials.

The next section describes our efforts to develop an effective protocol for coating polymer samples with CYTOP. Subsequent sections describe in detail the steps involved in actual microfluidic device fabrication: preparation of the coated thin layer and the uncoated thick layer, bonding of the layers, and connection of devices to the outside world. While in principle very similar to PDMS device fabrication, the details are very different due to particular properties of the polymers involved.

4.3.1 Development of CYTOP coating procedure

Initially, we tried spin-coating the CYTOP directly onto 1-layer fluidic devices. Polymer samples with a variety of proprietary formulations were molded on silicon wafers containing a dense pattern of microchannel features. To apply the coating, each polymer sample was mounted directly on the spin-coater chuck with channels facing upwards and spun immediately after depositing a small volume of CYTOP on the surface. Fortunately, CYTOP had good wetting properties on all materials evaluated. Undiluted CYTOP is very viscous and resulted in very irregular, incomplete coatings. Instead, we diluted CYTOP 1:3 in Fluorinert FC-75 (3M Corporation, St. Paul, MN) for spinning. Typically, we pipetted 400 μ L of this mixture for each device and spun at 500 RPM for 10 seconds followed by 3000 RPM for 20 seconds. It was critical to begin spinning immediately due to the high volatility of the solvent. Devices were then baked for 1 h at 80°C followed by 1 h at 170°C. The latter is an annealing step, required because the very low solids content (9% initially; 2.25% after dilution) of the CYTOP mixture otherwise leads to high shrinkage and stress, potentially resulting in incomplete coatings. Initially having a starbust appearance with about 1 μ m in thickness variation (determined by profilometry), the coating becomes uniform and smooth after this step.

The high temperature baking caused severe discolouration and rigidification in early DCPD materials due to oxidation. To mitigate this effect, antioxidants were included in subsequent formulations. In addition, Materia fabricated a gas-tight aluminum chamber that could be placed in the oven. Prior to baking, we placed the sample in this chamber and evacuated it for 5–10 min to remove the air (and oxygen). We also tried purging with helium gas but observed a large number of bubbles in samples after baking. Internal measurements with a thermocouple indicated that the chamber takes 75 min to heat up from 80°C to 170°C; therefore the duration of the annealing step was increased to 2 h.

The resulting coatings were observed to be well adhered to the underlying polymer in all samples. Coatings were evaluated for solvent resistance by placing droplets of solvents on the surface and then looking for qualitative effects such as swelling and distortion, discolouration, and residue. Several CYTOP-coated polymers exhibited excellent resistance to dichloromethane, acetonitrile, and tetrahydrofuran in flat regions; however, areas with microchannels were susceptible to attack in all samples. Profiling the surface immediately after dichloromethane exposure revealed significant swelling within 10–20 μ m of microchannel walls with less swelling further away (Figure 4.2). We hypothesized that the channel side-walls were not being completely coated, perhaps due to the CYTOP flowing off of walls and pooling in the lower features (microchannels) during coating or annealing. In one sample, we profiled a step edge of the CYTOP coating and determined its thickness to be 3–4 μ m. The profile of the channels in this sample, shown in Figure 4.2, indicates a channel depth of about 44 μ m. This result is consistent with CYTOP pooling as the height of channel features on the silicon mold from which the polymer was cast was 46 μ m, indicating a 2 μ m loss. On samples with thicker coatings, a greater depth discrepancy was observed.

Testing coated polymers is a tedious process, requiring exposure to several different solvents (as some polymers exhibited resistance to certain solvents even when uncoated) and observation at many exposed locations often over long periods of time. We developed a rapid method for evaluating coating integrity by exposure to droplets of 96% sulphuric acid. Upon contact with any of the polymer samples, the acid causes nearly immediate charring of unprotected areas. Annealed



Figure 4.2: Effect of dichloromethane exposure on surface profile of CYTOP-coated device. Surface profile over several 100 μ m-wide microchannels of a CYTOP-coated Materia device (a) before and (b) after exposure to droplets of dichloromethane. No swelling was observed in regions away from microchannels; however, swelling was visible by eye in regions with channel features. The profile shows that the highest swelling is at the edges of microchannels, suggesting that the side walls are not adequately coated. The height of channel features on the original mold was 46 μ m; thus the 44 μ m height in these profiles suggests that the CYTOP coating procedure results in the loss of 2 μ m in feature height, perhaps due to the CYTOP pooling at the bottom of channels. This particular coated device was made from proprietary formulation B-47-012-B.

CYTOP provides a complete barrier that in one experiment protected a sample for more than a year, exhibiting no visible damage other than a small change in wetting properties of the exposed CYTOP. Samples were tested by moving a large droplet around the surface. Pinholes or other defects in the CYTOP coating where the acid can seep through lead to charring, sometimes preceeded by a discolouration to red, brown, then black. The high contrast and rapidity of the colour change made this test particularly convenient. With this testing method, CYTOP layers applied by spin coating were confirmed to have discontinuities in the vicinity of the microchannel patterns on the surface.

A dip-coating procedure was evaluated next. Devices were dipped face down in a 1:30 CYTOP dilution and allowed to dry at 80°C. Multiple coatings were applied, with the sample in a different orientation during each drying step. It was hoped that vertical device orientations would allow channel side walls to be properly coated. However, after annealing, these samples did not exhibit improved resistance in regions of the surface containing microchannels. Flat regions were resistant, however, indicating that the much thinner CYTOP coating achieved by this method was a sufficient barrier.

Our best coatings were obtained by first coating the patterned wafer with CYTOP then transferring this coating to a polymer by polymerizing the resin directly on top of it. One significant advantage of this technique is that the coated device is an exact replica of the mold; with spin and dip coating, the added CYTOP affects critical device dimensions such as channel depth and width. To facilitate release of CYTOP from the mold, wafers were treated with a fluorosilane vapour (see Appendix A.1.5). Though the coating can be applied by many means, we found that spraying diluted CYTOP (1:30) with an airbrush resulted in superior coverage. Presumably due to the large surface to volume ratio resulting from atomization, droplets of CYTOP dry immediately or soon after impact, thus remaining in place on all parts of the mold surface, including channel walls. When spin or dip coated, the CYTOP remains in liquid form sufficiently long to flow off of walls and into valleys before drying. Wafers were thoroughly coated by spraying about 1.75 mL of CYTOP solution from a variety of directions and then dried by baking at 80°C. Profilometry indicated that the coatings had a thickness in the range 0.5–1.0 μ m with a roughness of about 20–50 nm. Altough other polymers (e.g., PDMS) exhibit poor adhesion to CYTOP even when polymerized in place on top of the coating, several Materia polymers had sufficient adhesion that they lifted the CYTOP layer from the wafer. We evaluated dozens of formulations for this specific ability in addition to high elasticity, eventually settling upon the following recipe: 5g HNB, 150 mg Ethanox (antioxidant), 50 mg KR55 (adhesion promoter), 5 mg C848 (catalyst), 5 mg TPP (inhibitor), 0.5 mL toluene. Since the resins tend not to wet the CYTOP surface, coated wafers were treated for 3 min with oxygen plasma prior to spraying, pouring, or spinning the resin.

In 2-layer device fabrication, the thin fluid layer remains on the wafer while the control layer is bonded to it. This implies that annealing of the CYTOP layer would either have to be performed with the thin layer alone on the wafer or after assembly of a completed 2-layer device. The latter was not possible, as the HNB resin exhibited signs of melting and channel collapse at temperatures below 108°C, the glass transition temperature of CYTOP [47], which is the minimum temperature at which annealing is effective. Melting is not important when baking the thin layer by itself; however the CYTOP was found to become permanently bonded to the wafer or photoresist when baked in place. Fortunately, solvent and sulphuric acid exposure indicated that annealing was not necessary—the unannealed coating provided a sufficient barrier, presumably due to the thorough coverage achieved by spraying.

4.3.2 Fabricating the thin (fluid) layer

Details of the CYTOP-coating procedure were next adapted to produce the thin, coated fluid layer for 2-layer microfluidic devices. One of the most significant obstacles we encountered in device fabrication was polymerizing the thin (fluid) layers with a repeatable thickness. The difficulty stems partly from the extremely low viscosity of the Materia resins. The viscosity of the HNB resin is 12 cP, compared with Sylgard 184 PDMS prepolymer, which has a viscosity of 7000–8000 cP as measured by the same instrument. Repeatability is also hindered by the short time over which the viscosity changes once the catalyst is mixed into the resin. However, incorporation of inhibitor and cooling of the resin was sufficient to maintain the low viscosity for an extended period. In our push-down devices, fluid channels were typically 100 μ m wide and 10–15 μ m deep. We strove for a total fluid layer thickness of 20–30 μ m to achieve thin valve membranes that could be actuated at reasonably low pressures. The best coating method we found for our first-generation devices was spin coating immediately after mixing the resin with catalyst. Spin-coating parameters were 180–200 RPM, 10 s spin time, and 3 s ramp time. This extremely low speed is not sufficient to expel excess material from the wafer, so it was necessary to blot the edge with a Kimwipe during spinning to prevent this liquid from reflowing across the wafer when spinning was stopped. Wafers were baked at 45°C for several minutes immediately after coating to polymerize the layer. Coating thickness was often but not always in the target range of 20–30 μ m. To avoid variations due to viscosity buildup, an individual 2 g batch of monomer was prepared for each wafer, prechilled to -20° C, and mixed with catalyst solution (containing all other ingredients) immediately prior to spinning.

We tried numerous other methods to achieve thin polymer layers including pouring and spraying but both resulted in very poorly controlled layer thicknesses, even with accurately measured resin volumes.

4.3.3 Fabricating the thick (control) layer

Control layers were fabricated by pouring catalyzed resin onto a control layer mold treated with fluorosilane. Molds were patterned with thick SU-8 photoresist so the resulting deep, square-profile channels could not easily collapse during the baking step required for layer-layer adhesion. To conserve material, a mold barrier or "dam" was placed around the patterned area. PDMS and urethane gaskets provided an inadequate seal for the low viscosity resin so aluminum barriers were fabricated. The dam was affixed to the mold with a small amount of catalyzed HNB resin. Aluminum foil wrapped around the bottom of the wafer and pressed into an appropriate shape is also sufficient as a barrier, though a small amount of material may leak under the foil, requiring device trimming (e.g., with scissors) prior to use. Wafers were placed on tinfoil directly on a hotplate set to approximately 40–50°C. Pre-cooled resin was catalyzed and poured into the barriers. Any visible air bubbles near channel features were dislodged by jets of resin squirted by a pipette. To ensure the cast device has a flat upper surface (instead of a curved meniscus), a 2×3 inch glass slide was placed over the filled mold and a weight placed on top. A small opening was left at one corner to allow the escape of air bubbles. One corner of the mold was gently warmed with a heat gun until a slow polymerization exotherm was initiated. Samples were then allowed to cool for several minutes before removal from the molds. This procedure is shown in Figure 4.3.



Figure 4.3: Fabrication of thick device layers from Materia resin. (a) A control layer mold (patterned 3-inch silicon wafer) is placed on a hot plate surface. (b) An aluminum barrier mold is affixed to the wafer by polymerizing HNB (as a glue). (c) The resin is pipetted onto the mold within the barrier, and a glass slide is placed on top. The resin then polymerizes. (d) A photograph of the aluminum barrier mold. (e) A device in a mold immediately after the polymerization exotherm. At this point, the glass slide would be removed, and the device would be cut out of the mold for hole punching and assembly with the fluid (thin) layer.

After many variations in formulation, the following formula for the thick layer was developed. 7 g HNB, 7 g ENB, 2.5 g DNB, and 500 mg Ethanox were heated to dissolve the antioxidant, filtered with a 0.2 μ m syringe filter¹, and then chilled to -20°C. The resin was catalyzed with 10 mg C848

¹Filtering eliminates undissolved antioxidant and contaminants that build up in the ENB over time.

catalyst and 11 mg TPP inhibitor dissolved in about 0.5 mL toluene. This quantity of material was sufficient to fill two aluminum barriers. HNB provides overall flexibility and elasticity that helps the thick layer to seal to the thin layer, while the presence of ENB in the formulation provides rigidity that prevents channel collapse during the baking step to bond layers. DNB confers some flexibility that is useful during mold release.

Due to the toughness of the surface layer of this polymer formulation, holes could not be punched easily without bending the punch tool or Luer stubs that are normally used for PDMS devices. By freezing the polymer, we could create consistent holes by drilling but we observed significant contamination by dust and oil. The best method we found was to melt holes through the polymer. A Luer stub is heated with a Bunsen burner until red hot and then rapidly plunged through the thick polymer layer from the channel side at the desired point. The layer is then immersed in ice water for a few seconds with the Luer stub still in place. After cooling, the stub is removed, leaving a smooth hole. The Luer stub can be reheated to burn out the polymer core that is taken from the hole. Note that because the thick layer exhibits only moderate elasticity, the punch size was matched exactly to the tubing size that will be inserted. (In PDMS devices, the punched hole is typically somewhat smaller than the tubing size.) Though not a concern for our experiments, in a production setting, a technique such as laser drilling could conceivably be used to create these holes.

4.3.4 Bonding layers

Because Materia polymers are a living catalyst system, fresh monomer sprayed or poured on a cured device will polymerize onto the previous surface into a monolithic structure. One possible microfluidic-device-bonding strategy is thus to use a thin layer of resin as a glue between the fluid and control layers; however, we were not able to coat the material sufficiently thinly that it did not clog channels in the control layer. A strategy that did work well was placing the two solidified surfaces into contact at an elevated temperature for a prolonged period. Best results were obtained if the layers were bonded immediately after they solidified. (Layers could be frozen under dry conditions to prolong the processing window.) It is our hypothesis that barely cured polymers contain monomers

or oligomers that can further react, thus forming polymer chains that extend between two layers to join them covalently. When fabricating the control layers, it is important that the exotherm be slow to avoid high temperatures that could destroy the catalyst thus preventing further chain elongation.

The thick layer was aligned to the thin layer (still affixed to the fluid layer mold) under a stereoscope. Care is required, as layers cannot be peeled apart in case of alignment error due to the high tackiness of these polymers. Since we were primarily interested in demonstrating values, our devices consisted of an easily aligned design: a dense pattern of parallel fluid channels crossed by five widely spaced control channels in the perpendicular direction. As long as some channels intersected, alignment was successful. Once the layers were in contact, they were baked at 45°C for 2–3 hours. Higher temperatures or longer bake times led to collapsing of the control channel features. Likely this is due to the incomplete polymerization in the first stage—perhaps a higher exotherm temperature could help. While significant adhesion could be obtained by putting clean surfaces in contact, we also found that spraying surfaces with catalyst dissolved in toluene (no TPP or antioxidant) can improve bonding. Using an airbrush at a distance of 10–20 cm, we applied a few very brief (less than 1 second) sprays of catalyst mist—just enough to uniformly cover the surface. Both surfaces were sprayed then degassed for 5–10 min to ensure the toluene had completely evaporated; otherwise, bubbles form between layers during the baking step and interfere with adhesion. Several devices were capable of being pressurized to 22–25 psi for sustained periods without delamination. One drawback of using the catalyst spray is that particles remain between the surfaces in the final device. Occasionally these are located in channels and could lead to contamination. In addition, some are quite large (up to 8 μ m) and could interfere with layer-layer bonding.

4.3.5 Testing microfluidic devices

For testing, bonded microfluidic devices were peeled from the fluid layer mold and affixed to glass slides. Since the CYTOP layer exhibits very poor adhesion to glass, I constructed a number of fluid delivery jigs to hold the device onto the glass. One is shown in Figures 4.4 and 4.5. In this setup, solvents are delivered to the fluid layer through holes drilled through the glass slide (see Appendix A.2.3). This eliminates the need to punch input ports through the whole device, therefore avoiding damage to the CYTOP coating and preventing exposure of the upper, non-resistant part of the device to solvents. The jig simultaneously held O-rings in place on one side of the glass slide while holding the microfluidic device in place on the other. The bottom plate accepted HPLC fittings aligned with the O-rings to allow solvents to be delivered by microbore tubing under syringe or regulated nitrogen pressure. Four thumbscrews provided fine adjustments to control the force applied to the whole "sandwich". Note that the top surface of the microfluidc device must be very flat in order for the force to be applied evenly.

Though we demonstrated working values and successfully delivered solvents at low pressures, this method was not very robust, and we nearly always observed delamination between the device and the glass slide. Presumably the problem stems from the poor adhesion of CYTOP to glass. Since the walls between channels are not adhered to the substrate, it is probably quite easy for pressurized fluid in a channel to slightly lift the channel upwards, creating a small locally delaminated area.

CYTOP-coated devices suffered from the additional problem that they could not easily be removed from the fluid layer mold. Removal frequently tore tiny pieces of CYTOP away from the thin layer (rendering these regions non-resistant) or resulted in severe stretching of the polymer that led to cracking of the coating. Making molds with different resists, different release coatings, or even out of different materials (such as DCPD and urethane) did not resolve this issue. Fortunately most of the lessons learned were transferable to the fabrication of second-generation devices when fluorinated monomers became available.

4.4 Second-generation devices: FNB fluid layer

A second generation of microfluidic device development began when Materia succeeded in producing several fluorinated norbornene (FNB) monomers. Polymers based on these monomers are resistant to solvents, so microfluidic devices fabricated from them do not require a protective CYTOP coating. In total we received five proprietary monomers in unpurified form, designated by the codes CH39nnn, where nnn was 176, 188, 189, 191, or 192. CH39-188 behaved much differently than the others



Figure 4.4: Schematic of fluid delivery jig. The microfluidic device is held on a glass substrate by sandwiching between bottom and top plates and tightening the thumbscrews. The force applied not only helps adhere the device to the glass, but also helps seal the glass to the O-rings below. Fluids are delivered from microbore tubing by connecting HPLC fittings to the bottom plate. The aluminum part is threaded, and the Teflon part contains only tiny holes; thus the fittings can be tightened against the Teflon to compress the ferrule and achieve a tight seal. Note that fluids are only in contact with the microbore tubing, the ferrule, the Teflon plate, the O-ring, the glass slide, and the channels in the bottom surface of the microfluidic device. The top plate contains large holes (not shown) where connections are made via tubing inserted into the upper surface to pressurize control channels. Also not shown in this figure is the attachment between the Teflon and aluminum plates.



Figure 4.5: Fluid delivery jig used for testing early Materia devices. (a) The bottom plate consists of a 3×3 inch Teflon block attached by 4 nylon screws to a matching aluminum block. The pattern of 8 holes in the central region is designed to line up with the fluid inlet ports drilled through the glass slide. A cone-shaped depression is machined at each site to hold in place a black Viton O-ring. The remaining 4 holes are threaded for top plate attachment. (b) A microfluidic device is aligned to a glass slide with holes drilled in positions to match fluid inlets and outlets. (All fluids are delivered through the glass since devices were resistant only on the bottom surface, which was coated with CYTOP or made from FNB.) The holes in the glass slide are aligned with the centers of the O-rings. (c) Next a transparent plastic top plate is attached via thumbscrews threaded into the holes of the bottom plate. The screws are adjusted to press everything together, both to seal the device to the glass slide and to seal the glass slide to the O-rings. Pressure must be applied gently and evenly to avoid deforming or collapsing the microchannel pattern. (d) The aluminum plate contains threaded holes aligned with the fluid delivery holes in the bottom Teflon plate. It is designed to accept 1/4"-28 HPLC fittings with the ferrules tightened against the Teflon block. Fluid is delivered by syringe or by regulated nitrogen pressure.

in terms of colour, wetting properties, and polymerization, suggesting perhaps a problem with the reaction to make the monomer. We worked with the remaining four.

These new experimental monomers were supplied to us in very small quantities, typically 10–20 g (5–10 mL) at a time. Since previous 2-layer devices consumed about 7 g of resin each, we couldn't afford to make entire devices out of fluorinated resin. Therefore, we developed an alternative two-layer approach, wherein the bottom thin layer is made with the solvent-resistant elastomer, while the thick layer is made from non-resistant resin. As with the CYTOP-coated devices, fluids were delivered through holes in the glass substrate to prevent exposure of the non-resistant material to solvents.

We first checked the solvent compatibility of polymers based on these new monomers and then fabricated 2-layer microfluidic devices containing valves and showed they could be operated in the presence of organic solvents.

4.4.1 Solvent compatibility tests

To qualitatively evaluate solvent resistance, we polymerized some thin (appproximately 1 mm) sheets with each of the FNB monomers. Droplets of dichloromethane did not visibly distort any of the materials; however, this solvent rapidly diffused through the sheet of CH39-176 FNB to attack the plastic Petri dish below. We decided not to use this monomer in microfluidic devices because its inability to provide a solvent barrier would likely allow solvents in fluid channels to diffuse through and attack the non-resistant thick layer in our two-material architecture. Sulphuric acid droplets caused no visible effects other than a slight change in surface-wetting properties in the exposed area after many hours (see Figure 4.6b). We attempted to perform long term immersion tests to quantitate polymer swelling in a variety of solvents, but the solvents evaporated so quickly from our small thin samples that accurate measurements could not be made.

Polymers of CH39-189, CH39-191, and CH39-192 seemed to develop significant plasticity and rigidity over time, becoming completely inelastic after about 2–3 days. (This same "aging" effect occurred in our first-generation HNB-ENB-DNB thick-layer formulation, but not in our HNB thinlayer formulation.) 189 was superior in terms of initial flexibility, but 191 and 192 exhibited superior adhesion to glass. For microfluidic device fabrication, however, we were simply limited to using whichever monomer was available at the time. Mixtures of these monomers with CH39-176 at up to 75% loading did not have enhanced flexibility despite the high flexibility and elasticity of polymers made entirely of CH39-176.

In order to confirm *in situ* solvent compatibility in the context of our device architecture, we initially fabricated simplified (1-layer) devices. First, we polymerized a thin layer of FNB sprayed onto a silicon fluid layer mold. We then affixed an aluminum barrier on top of this layer and poured a thick layer of non-resistant resin to fill it. This resin was the same formulation as used for thick layers in our CYTOP-coated devices. These devices contained no control layer and no valves, but were otherwise identical to 2-layer devices. The devices were mounted on drilled glass slides and held in place by our fluid delivery jig (see Figures 4.5 and 4.4). In one experiment, we flowed dichloromethane at 5 psi through a long serpentine channel for approximately 24 h, observing no distortion or deformation of channel features. Upon removal of the device from the wafer, we observed that the material in the vicinity (few hundred microns) of the channel had become somewhat lighter in colour, perhaps due to the extraction of some impurities. (The monomers were supplied to us in unpurified form.) It should be noted, however, that the polymer matrix did not absorb any of the blue dye (xylene cyanol FF) that was contained in our dichloromethane solution. We also applied droplets of sulphuric acid to the bottom of this device and observed that the FNB layer protected the non-resistant thick layer from attack (see Figure 4.6a).

One problem we identified in our fluid-delivery setup was the plastic deformation of the fluid layer near the inlet holes. That is, when we removed the device from the glass slide, a permanent imprint of the edges of the holes was clearly visible. In long-term flow studies this was observed to cause a reduction in flow rate. Since we had observed similar plastic deformation when overtightening the thumbscrews on the fluid delivery jig, we hypothesized that the O-rings (made of Viton) were being swelled by the dichloromethane flowing through them, thus pushing on the glass slide and increasing the pressure with which the microfluidic device and glass slide were pushed together.



Figure 4.6: Sulphuric acid resistance of FNB. (a) Comparison of Materia norbornene device with FNB surface coating (left) and without (right) after exposure to droplets of 96% sulphuric acid. The uncoated device charred immediately, while the coated device was unaffected after many hours. Even after more than 1 year of exposure, one coated device exhibited no visible damage other than a slight change in wetting properties in the exposed region on the surface. Note that the colour difference between devices is simply related to the speed of polymerization. The darker one polymerized slightly more quickly, which generally tends to produce a harder material with higher resistance. The non-resistant norbornene polymer is made with our usual thick-layer formulation (see text). (b) Thin film of FNB CH39-191 unaffected by a drop of acid. This photo courtesy Tony Stephen, Materia, Inc.

This problem was circumvented by devising a new system for fluid delivery using newly available commercial fluidic fittings called NanoPorts, as described below.

4.4.2 Device fabrication

2-layer devices with microvalves were fabricated by a similar procedure to that used for CYTOPcoated devices (except for the coating step). Since only the bottom thin layer was made from solvent-resistant FNB, a push-down valve architecture was again necessary.

Thin layers were prepared by spraying a mixture of catalyzed FNB on a fluorosilanized silicon wafer mold, patterned with fluid channel features. The spray solution was prepared by mixing 4 parts FNB with 1 part of a solution consisting of: 30 mg Irganox, 1 mg C848 catalyst, 5 mg TPP, and 4 g toluene. This formulation results in an initially very flexible and elastic polymer. Spray coating resulted in a wide range of layer thicknesses ranging from 10–100 μ m, with huge variation even in a single wafer. However, some spray-coated wafers happened to have the correct thickness and could be used to produce functional microfluidic devices. Unfortunately, the spin-coating method used with the HNB resin for CYTOP-coated devices did not work here: poor wetting of the silicon wafer

surface by the FNB resulted in incomplete coverage as the FNB contracted into a small thick pool within seconds after spinning. Thin layers were polymerized by baking for 30 min at 50°C and then for 30 min at 80°C.

One method we tried to better control the layer thickness was to create a barrier wall around the mold pattern with a fixed internal area and to pour a fixed volume of catalyzed resin into this barrier. We attempted this with an epoxy barrier, but observed that the required resin volume is difficult to predict due to the large fraction of resin that collects on the barrier walls. In one set of experiments with a particlar geometry, 500 μ L of resin led to layers between 20–80 μ m thick; the predicted volume needed for a 30 μ m layer was 75 μ L. Another difficulty is that an exceptionally level surface is needed inside the oven during polymerization to ensure a uniform coating thickness across the whole mold.

Thick layers were prepared in an identical manner as those for CYTOP-coated devices, differing only by a slight modification of the formulation to reduce the layer rigidity. The revised formulation consisted of 7 g HNB, 7 g ENB, 2.5 g DNB, 45 mg Irganox, 8 mg C848 catalyst, 11 mg TPP, and 0.5 mL toluene.

Bonding was also nearly identical. Catalyst dissolved in toluene was sprayed with an airbrush onto the thin layer (still affixed to the flow-layer mold). Since toluene does not wet the FNB surface, care was taken to allow the toluene to dry after one spray of mist before applying another. Otherwise tiny toluene droplets coalesce to form large droplets that can damage the thin layer by causing pitting. Both layers were then vacuum degassed for 5 min, prior to alignment of the thick layer onto the thin layer. Baking for 30 min at 50°C followed by 30 min at 80°C affords sufficient adhesion so that the 2-layer device can be removed from the fluid-layer mold. Chances of successful bonding are enhanced by synchronizing fabrication steps such that the thin and thick layers are ready for bonding simultaneously. Bonding is possible between these two different materials (FNB and HNB-ENB-DNB mixture) because they share the same catalyst.

The 2-layer device is then bonded to a glass slide containing holes for fluid delivery. Bonding directly to glass is possible, though it is reversible, and the degree of bonding is not the same for

all FNB polymers. However, far superior adhesion is achieved by bonding the device to a glass slide coated with FNB. The coating on the slide is applied and cured in exactly the same manner as the thin device layer. After curing, the holes in the glass slide must be re-opened by punching out the plugs of FNB that block them. The microfluidic device is placed onto the coated slide and then baked for 30 min at 50°C followed by 30 min at 80°C. The mounted device is then baked at 50°C overnight to complete the bonding at the two interfaces. It is baked upside-down (with the glass slide at the top) to avoid channel collapse. Note that the brief excursions to 80°C improve bonding by softening the material a little bit, allowing the components to settle together to release bubbles and stress.

4.4.3 Device testing

After device assembly, NanoPort fittings (Upchurch Scientific, Inc., Oak Harbor, WA) were affixed to the glass slide to allow pressurized delivery of fluids into the device. These fittings are glued to the glass surface with epoxy and connect to 1/32-inch microbore tubing via a threaded port and fitting as shown in Figure 4.7. The bottom surface of the NanoPort contains a Kalrez O-ring to protect the epoxy from solvent attack. Though the NanoPorts are supplied with custom cut epoxy rings, they must be baked at 120–140°C for proper adhesion. Since we generally mounted our devices (which cannot withstand this temperature) onto the glass first, we were forced to use 5-minute epoxy. We obtained best results by applying epoxy to the outer ring of the NanoPort shortly after mixing but waiting until it nearly hardened before adhering the ports to the glass. In operation, this new setup is identical to the fluid delivery jig, but the risk of over-compressing the device and causing channel deformation is eliminated.

With all fittings in place, we were able to deliver solvents into the fluid channels under pressure and to pressurize control channels to actuate valves. Though a variety of fabrication difficulties (such as inaccurate fluid-layer thickness and collapsed channels during assembly) resulted in imperfect devices, we successfully demonstrated valve actuation and dead-end channel filling in 2-layer FNB devices.



Figure 4.7: Schematic and photographs of NanoPort connectors. (a) Schematic drawing of the NanoPort connector. The port is aligned to a hole in the glass substrate and affixed with epoxy. Fluid delivered from microbore tubing contacts only a small part of the NanoPort (made of polyetheretherketone (PEEK) polymer), the Kalrez O-ring, the glass slide, and the FNB (solvent-resistant) layer of the microfluidic device. The control layer can be made of a non-resistant polymer as there is no fluid contact. (b) Photograph of a 2-layer microfluidic device affixed to a drilled glass slide with one NanoPort attached. (c) Photograph of a NanoPort attached to a glass slide with the threaded fitting removed. (d) Underside view of the NanoPort, showing the O-ring and (faintly) the epoxy ring.

In our first working device, containing a CH39-191 FNB fluid layer, we observed a series of valves (controlled by a single control channel) to close after several minutes under a pressure of 12 psi. Materia polymers tend to be quite tacky and most valves remained stuck in the closed position after the control channel pressure was released. Applying 7–8 psi air pressure to the *fluid* channel was sufficient to open all valves after several minutes. NanoPorts were not yet available so solvent delivery was not attempted in this device.

Experiments with another CH39-191 device are shown in Figure 4.8. Bonding of the device to the glass was observed to be sufficient to withstand 5–6 psi of dichloromethane flow for extended periods without signs of delamination or deformation. Though the fluid layer was particularly thick in this device (20–100 μ m gradient across the device), one series of valves was partially actuated at 12 psi. Additional pressure could not be applied without causing delamination at the control input pin. Valve actuation was extremely slow due to the thickness and rigidity (due to aging) of the FNB layer.

While this experiment demonstrated long-term *in situ* solvent compatibility and that valves could be actuated completely in FNB devices, the speed of actuation was impractically slow for many applications. With a batch of the more flexible CH39-189 monomer, I successfully fabricated one additional 2-layer device. Flow of dichloromethane at pressures of up to 15 psi was successfully blocked by valves pressurized to 23 psi. With pressurized fluid helping to re-open the valves after closing, I was able to oscillate the valve at rates around 1 Hz. 23 psi control channel pressure and 5 psi fluid pressure resulted in a maximum cyclic actuation rate of 1.3 Hz. The operation of this valve is shown in Figure 4.9. The device happened to contain a couple of collapsed fluid channels, allowing dead-end channel filling to be demonstrated. Due to the permeability of the FNB, the air trapped in a segment of channel several millimeters in length and 10 μ m deep was expelled in about 10 minutes by incoming dichloromethane pressurized to 5 psi. Several video frames of this experiment are shown in Figure 4.10. Dead-end filling against a closed valve was also demonstrated.


Figure 4.8: Dichloromethane compatibility and partially closing valves in early FNB microfluidic device. (a–c) Series of three micrographs illustrating the flow of solvent through a long serpentine channel in an early FNB device. The blue fluid is dichloromethane with some acetonitrile and methanol to dissolve the blue dye xylene cyanol FF. Driven by a pressure of 5–6 psi, it traversed the 16 channel segments (10 μ m deep) in several minutes. At the leading edge, the dye is more concentrated, presumably due to evaporation of dichloromethane into the open channel in front, as well as some diffusion into the FNB layer. (d) An enlarged view of the region highlighted in b. (e) Partial valve actuation at 12 psi control channel pressure. The control channel is oriented left to right. Valve actuation was incomplete due to the thickness of the FNB layer (20 μ m at one side but 100 μ m at the other), and was very slow (10 min) due to the aging effect whereby the FNB layer becomes rigid over time. The leftmost blue channel closed completely but periodically leaked. At higher control channel pressures, the device delaminated near the control inlet. The FNB layer was polymerized from monomer CH39-191.



Figure 4.9: Successful valve actuation in FNB device. (a) Micrograph of a push-down valve in an FNB device in the open state. The (unpressurized) control channel is oriented left to right, and two fluid channels are oriented top to bottom. The leftmost fluid channel is filled with dichloromethane containing acetonitrile and methanol to dissolve the blue dye xylene cyanol FF; the rightmost channel is filled with air. (b) Same valve in the closed state (control channel 23 psi). The valve was oscillated hundreds of times between these two states with no apparent degradation in performance. With 5 psi fluid pressure, the valve could be actuated at a maximum rate of 1.3 Hz. Up to 15 psi fluid pressure could be applied before bursting the valve open. Note that, due to valve stickiness, the adjacent air-filled channel did not re-open when the control channel pressure was released unless it was pressurized. The device was able to withstand over 30 psi, delaminating only when extremely high pressure was gradually reduced. Note that when re-opened, the fluid channel remains partly pinched off (smaller width). This is due to the aging effect in the FNB, which gives rise to some platic-like properties. This fluid layer of this device was fabricated from CH39-189 FNB.



Figure 4.10: **Dead-end channel filling in FNB device**. The device of Figure 4.9 contained some fluid channels that collapsed during device assembly. These provided a convenient means to demonstrate the permeability of FNB by filling a dead-end channel. (a) Series of 4 video images of dichloromethane (dyed blue) filling a collapsed fluid channel. The permeability of FNB is significantly lower than PDMS as it took 4 min for the fluid to fill this short channel segment at 5 psi input pressure. (b) Series of 3 images illustrating dead-end filling against a closed valve (the same as shown in Figure 4.9). (c) Continuation of this series focussed on the region inside the red box.

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4.5 Third-generation devices: All FNB

Fabrication of devices from two different materials was necessary to perform our experiments with such limited quantities of resistant materials. In spite of the demonstration of successful devices using this approach and the fact that this architecture minimizes the amount of solvent-resistant (expensive) material needed in a device, two-material devices introduce many complications. Given sufficient quantities of fluorinated monomers, it would be preferable to fabricate devices entirely from a single resistant material (FNB).

One significant advantage would be in making connections to the chip. While NanoPort connectors work, it is difficult and time consuming to drill good quality holes in the glass substrate and to properly adhere each fitting. The epoxy gluing method is not perfect and frequently results in clogging or in an inadequate O-ring seal. In addition, the NanoPorts have a large footprint compared to the hole punching method used in PDMS devices, severely limiting the number of fluid connections that can be made to a single chip. Furthermore, the connectors obstruct the view of the channels below and thus need to be moved to regions of the chip design where observation is not required. A second important advantage is that fabrication of wholly resistant devices permits push-up valve architectures to be used. (In this architecture, the fluid channel is in contact with both device layers.) Push-up valves typically exhibit considerably reduced actuation pressures compared with push-down valves and also enable the fabrication of devices where the fluid layer contains extremely tall features such as large reaction chambers (see [159] supplementary information).

Recently, we have made significant progress towards the goal of fabricating all-FNB devices. Though the supply of FNB is still very limited, experiments using HNB as a surrogate have led to a number of promising strategies to address the difficulty in producing layers with consistent thickness. Several other small improvements in the fabrication procedure have also been achieved.

4.5.1 Towards repeatable layer thickness

In the first two device generations, we had considerable difficulty in producing fluid (thin) layers with a repeatable thickness. Problems primarily stemmed from the extremely low viscosity of the monomer resin that prevents the use of established techniques, such as spin coating, for building sufficiently thick layers.

Recently we have contemplated alternative approaches. For example, the viscosity of the resin could be increased so that spin coating can be performed. One way to do so is simply to allow the resin to begin polymerizing to increase its molecular weight and viscosity and then spin once the viscosity reaches the desired level. However, it is difficult to know when the resin has arrived at a precise viscosity. The polymerization is very sensitive to temperature and, in the vicinity of the viscosity of interest, is also very sensitive to time (see Figure 4.11). Even with carefully controlled mixing times, temperatures, and pipetting and spinning protocols, we observed qualitatively and quantitatively different viscosities from batch to batch. Perhaps there is a way to quantitatively measure the viscosity in real time. If so, the resin can be rapidly cooled once it has arrived at the correct viscosity to maintain it at that level sufficiently long to perform spin coating. Another tactic that Materia has developed is an oligomerization procedure where the resin is polymerized for some time, and then the catalyst is quenched. The viscosity of the resulting oligomer solution is stable over long periods, and the material can be recatalyzed for spinning onto molds. This does seem like a promising approach; however, there is not yet a reliable method to rapidly quench the first polymerization and arrive at a precise desired viscosity. Thus for each new oligomer batch (having a new viscosity), a new spin curve must be generated. Perhaps batches with a desired viscosity can be mixed from batches of higher and lower viscosity. A third method of increasing the viscosity of the resin is to add thixotropic agents. In one experiment we added 50-75 wt% of Cab-O-Sil (Cabot Corporation, Boston, MA), a silica powder, to increase the viscosity of HNB to a level qualitatively similar to Sylgard 184 PDMS prepolymer. However, the resulting cured part had dramatically altered mechanical properties such as rigidity and brittleness, clearly heavily influenced by the high glass content. In principle, one could use microparticles of HNB instead of silica so that the final material properties would not be strongly affected; however, this has not yet been tried.

Another coating method that has been investigated is the "doctor blade" technique, a common industrial method for applying thick films to surfaces. Essentially a blade is moved across the



Figure 4.11: Viscosity profile of purified FNB. (a) Linear scale; (b) Logarithmic scale. Viscosity was measured as a function of time to monitor the viscosity buildup during polymerization at 25° C of FNB. Approximately 1 mL of catalyzed resin was used from a batch prepared according to the following recipe: 5 mg FNB, 20 mg Irganox, 0.22 mg catalyst C848, 0.67 mg TPP, 0.42 mL dichloromethane. Measurements were made using a cone and plate viscometer (Model AR2000 rheometer, TA Instruments, New Castle, DE) with a 60 mm 1 degree cone at a constant angular velocity of 1 rad/s and shear rate of 57 s⁻¹. One can observe from this data that the slope (rate of viscosity change) is quite high for viscosities of 1000 cP or more that can easily be spin-coated to the needed thickness of 10s of microns.

sample at a fixed height to spread a uniform layer of a viscous coating material over the surface. We had thought that this method might eliminate viscosity dependence and that we could coat from catalyzed monomer (after first allowing it to polymerize/thicken to a qualitatively suitable level), but this was not the case: the coating thickness depends in a complicated way upon the blade height, viscosity of coating material, and other parameters. We have also observed poor repeatability: when tested with PDMS prepolymer we observed 20% thickness variation within a 56 μ m coating on a single wafer and 10% variation between wafers. This is consistent with the 0.5 mil (13 μ m) tolerance of the unit we used (Universal Blade Applicator, Paul N. Gardner Company, Inc., Pompano Beach, FL) [89].

Lastly, one method that might work with low viscosity monomer resin or oligomer resin is casting between a wafer and a flat surface, a technique that has been used to make thin PDMS membranes [137, 134]. This approach is completely independent of viscosity and seems quite promising.

4.5.2 Additional improvements

Attempts to make devices entirely out of HNB have led to several simplifications in the device fabrication protocol. (i) Resin can be cast on silicon wafers without any surface treatment, eliminating the need for the lengthy fluorosilanization protocol. (ii) Holes can be punched in devices by the same punch machine (Technical Innovations Inc., Brazoria, TX) that is used for PDMS devices. However, we were unable to punch holes manually using Luer stubs. (iii) Use of dichloromethane instead of toluene as a solvent ensures that the antioxidant is rapidly dissolved; thus, no filtering of the resin is required. (iv) Adhesion without catalyst spray provides a strong bond. In one test, a 1-layer HNB device was bonded to an HNB-coated substrate by baking for 3 days at 50°C. The resulting bond showed no signs of delamination when channels were cyclically pressurized between 0–24 psi at 2 Hz continually for 48 h. The first three improvements have been demonstrated to be compatible with FNB; thus, aside from calibrations for shrink factor and parameters related to layer thickness, we expect a smooth transition to the fabrication of all-FNB devices.

4.6 Summary

Through a collaboration with Materia, Inc., we have developed solvent-resistant 2-layer microfluidic devices containing functional microvalves by two different methods. In the first, the thin layer is fabricated from a non-resistant hexylnorbornene polymer with a protective CYTOP coating on the bottom surface. In the second, the thin layer is fabricated from a resistant fluorinated norbornene polymer. Both must use a push-down valve architecture to prevent solvents from coming into contact with the non-resistant parts of the device. We demonstrated long-term solvent (dichloromethane) flow through microchannels, dead-end channel filling, and most importantly, valve actuation. The requirement for a coating on the glass substrate precludes the use of these devices for *in situ* synthesis of compounds on glass; however this affects only a small set of applications.

Given sufficient availability of monomers, it would be preferable to fabricate devices entirely from solvent-resistant FNB. This would greatly simplify the delivery of solvents to devices and expand their usefulness by enabling push-up valve architectures. Significant steps have been made in this direction.

Acknowledgment

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Chapter 5

Solvent-Resistant Perfluoropolyether (PFPE) Microfluidic Devices

5.1 Introduction

Poly(dimethylsiloxane) (PDMS) has rapidly become the material of choice for many microfluidic device applications due to its numerous attractive properties [218, 272, 188, 268, 170], as discussed in Chapter 2. Upon crosslinking, it becomes an elastomeric material with a low Young's modulus of about 750 kPa [272]. This enables it to conform to surfaces and form both reversible and irreversible seals. These properties have enabled the fabrication of very simple but effective microvalves that can be densely integrated to produce extremely sophisticated devices [272, 268]. PDMS has a low surface energy, usually around 20 erg/cm², which facilitates easy release from molds after patterning [218, 188]. Another important feature of PDMS is its high gas permeability. This allows trapped air within channels to permeate out of the device (e.g., for blind filling) and is also useful for sustaining cells and microorganisms inside channels, or for evaporating solvents.

Many current PDMS devices are based on Sylgard 184 (Dow Corning, Midland, MI), which cures thermally through a platinum-catalyzed hydrosilation reaction. With this material, complete curing

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can take up to 5 hours. To expedite this process, Rogers *et al.* have recently reported the synthesis of a photocurable PDMS material with mechanical properties similar to that of Sylgard 184 for use in soft lithography [42]. This material cures through the use of free radical photoinitiators in just a few minutes, yet still has a long pot life.

Despite the advantages of PDMS for microfluidics technology, this material suffers from a serious drawback in that it swells in most organic solvents (Chapter 3). Swelling can lead to plugging of microchannels or device delamination, and other incompatibilities with solvents can lead to contamination of reactions and assays or to device damage. Instead of reverting back to devices made from inert but rigid materials such as silicon and glass [160] with their numerous drawbacks, it would be preferable to fabricate devices from a solvent-resistant elastomer with PDMS-like properties.

In a collaboration with DeSimone's research group at the University of North Carolina (UNC), we have taken this approach and replaced PDMS with perfluoropolyethers (PFPEs). PFPEs are a unique class of fluoropolymers that are viscous liquids at room temperature prior to curing, and that exhibit low surface energy, low modulus, high durability and toughness, high gas permeability, and low toxicity, with the added feature of being extremely chemically resistant [237]. They are highly fluorinated polymers with the chemical unit $-(CF_2CF_2O)_m-(CF_2O)_n-$. DeSimone *et al.* have reported extensively on the synthesis and solubility of PFPEs in supercritical carbon dioxide [29]. These materials have the potential to greatly extend the use of microfluidic devices to a wide variety of new chemical applications. With the added advantage of photocuring capability, the production time of microfluidic devices can be reduced from several hours to a matter of minutes.

With materials designed at UNC specifically for microfluidic device applications, we demonstrated the first fabrication of devices based on photocurable PFPEs. The next section describes the preparation and characterization of first-generation PFPEs and our success in fabricating solventresistant microfluidic devices with functional valves. This served as a proof-of-principle, from which further generations of material were evolved to improve the robustness of fabrication and the reliability of device operation. These improvements are described in subsequent sections. Based partly on the successes described herein, DeSimone and others recently founded a company, Liquidia Technologies (Morrisville, NC), to manufacture these materials and develop, among other things, microfluidic applications.

5.2 **Proof of principle**

5.2.1 Materials synthesis and characterization

The synthesis and photocuring of these materials (Figure 5.1) is based on earlier work done by Bongiovanni *et al.* [217]. The reaction involves the methacrylate-functionalization of a commercially available PFPE diol ($M_n = 3800 \text{ g/mol}$) with isocyanato-ethyl methacrylate. Subsequent photocuring of the material is accomplished by blending it with 1 wt% of 2,2-dimethoxy-2-phenylacetophenone (DMPA) and exposing it to UV radiation ($\lambda = 365 \text{ nm}$).



Figure 5.1: Synthesis and crosslinking of photocurable PFPEs. PFPE diol is reacted with isocyanato-ethyl methacrylate to form a PFPE dimethacrylate (PFPE DMA) that can subsequently be cured into an elastomeric material. Polymerization occurs by free-radical polymerization when PFPE DMA is mixed with 1 wt% 2,2-dimethoxy-2-phenylacetophenone and exposed to UV light. (Reproduced from [230] with permission. Copyright the American Chemical Society, 2004.)

To evaluate solvent resistance, tests using classical swelling measurements [234] were performed on both the crosslinked PFPE DMA and Sylgard 184. Sample weight was compared before and after immersion in dichloromethane for several hours. The data show that after 94 h the PDMS network had swelled to 109% by weight, while the PFPE network showed negligible swelling (< 3%). Using the same technique, we quantitated PFPE swelling in a wide variety of additional solvents (Table 5.1).

Solvent	Swelling $(wt\%)$ after 7 days	Comment
acetone	4.1	
acetonitrile	2.0	
ammonium hydroxide (27%)	0.4	
chlorobenzene	2.6	
chloroform	8.3	spotting on surface
cyclohexane	1.6	
dichloromethane	< 3	data from [230]
diisopropylamine	2.3	darkened in colour
dimethylformamide	4.2	
dimethyl sulfoxide	2.3	data from 3-day measurement
ether	3.6	
Fluorinert FC-75	66.3	
formaldehyde	0.7	
hexane	0.8	
isopropanol	2.3	
nitric acid (70%)	4.5	
pyridine	5.2	slight discolouration
sulphuric acid (96%)	10.3	surface turned black
tetrahydrofuran	5.3	
toluene	2.0	
trichloroethylene	6.7	spotting on surface
triethylamine	2.6	darkened in colour
xylene	1.7	

Table 5.1: **Swelling of PFPE in various solvents**. Swelling was quantified by performing classical immersion tests. Most solvents do not swell PFPE significantly, except for perfluorinated solvents such as Fluorinert FC-75. A few cause discolouration, suggesting that more in-depth studies of the interaction must be conducted if those solvents are to be used in devices.

The PDMS and PFPE precursor materials and the fully cured networks have similar processing and mechanical properties. Rheology experiments showed the viscosity of the uncured PFPE DMA at 25°C to be 0.36 Pa·s, which is significantly lower than that of 3.74 Pa·s for the uncured Sylgard 184. However, because both materials are sufficiently viscous oils at room temperature, standard PDMS device fabrication methods can be employed.

Dynamic mechanical thermal analysis (DMTA) was performed on the fully cured materials. Both the PFPE and PDMS networks exhibited low temperature transitions (-112° C and -128° C respectively) as evidenced by maxima in the loss modulus E'' (Figure 5.2). This transition accounts for the similar elastic behavior of the two crosslinked materials at room temperature.



Figure 5.2: Dynamic mechanical thermal analysis of PDMS and PFPE. DMTA traces of crosslinked PDMS and PFPE materials showing maximum in the storage modulus as a function of temperature. (Reproduced from [230] with permission. Copyright the American Chemical Society, 2004.)

Static contact angle measurements were made on both elastomers (Table 5.2). The PFPE DMA elastomer showed a higher contact angle than Sylgard 184 for water and methanol. Toluene and dichloromethane instantly swelled Sylgard 184 on contact, which prevented measurements to be taken. However, values for these solvents were obtained for the PFPE DMA material, as no swelling occurred.

Material	water	methanol	toluene	dichloromethane
PFPE-DMA	107	35	40	43
Sylgard 184	101	22	-	-

Table 5.2: Static contact angles for PFPE and PDMS. All values are in degrees. Note that measurements were not possible for droplets of toluene and dichloromethane on PDMS due to swelling.

5.2.2 Device fabrication

Device fabrication was accomplished according to the procedure illustrated in Figure 5.3. This method was first reported by Quake *et al.* for PDMS devices and utilizes partial curing techniques to adhere the two layers without compromising feature sizes [272]. The PFPE DMA material was easily spin-coated and molded in a similar manner to Sylgard 184. As a result of viscosity differences, spin-coating rates used in PFPE device fabrication were lower than those employed for

PDMS materials (800 RPM vs. 2000 RPM). Channels in the thick layer were 50 μ m × 100 μ m while channels in the thin layer were 12 μ m × 100 μ m. Devices exhibited superior adhesion between layers than between the device and the substrate—thus, we fabricated devices with push-down valves due to the lower pressure requirements at the device-substrate interface in this architecture.



Figure 5.3: **PFPE device fabrication procedure**. (a) A small drop of PFPE DMA containing 1 wt% DMPA is spin coated onto a patterned silicon wafer to a height of 20 μ m. Separately, a thicker layer (roughly 5 mm) is formed by pouring PFPE DMA containing 1 wt% DMPA into a temporary PDMS barrier mold surrounding a patterned wafer. Both wafers are then exposed to UV light until barely solidified. (b) The thick layer is peeled from its wafer and aligned on top of the thin layer. The entire device is then exposed to UV light to adhere the two layers together. Later generations of PFPE material involved complete curing of the two layers followed by a distinct adhesion step. (c) The device is peeled from the wafer and adhered to a substrate for microfluidic operation. (Reproduced from [230] with permission. Copyright the American Chemical Society, 2004.)

To compare the solvent compatibility of devices made from the two materials, a solution containing dichloromethane, acetonitrile, methanol, and the blue dye xylene cyanol FF was introduced into both a PFPE and a PDMS channel by capillary action (Figure 5.4). The PFPE channels showed no evidence of swelling as the solution traveled easily through the channel. A pronounced reverse meniscus was observed indicating good wetting behavior. In contrast, no solution entered the PDMS device because the channel was plugged shut when it made contact with the droplet. As a control, a dyed methanol solution was easily introduced into the PDMS channel in the same manner.

Actuation of valves was accomplished by introducing pressurized air (about 25 psi) to small holes that were punched through the thick layer at the beginning of the channels. When dyed solvent was present in the channel, valve actuation was easily observed (Figure 5.5). While fluid easily flows into channels due to capillary action, it was necessary to pressurize fluid channels to at least 4–5 psi



Figure 5.4: **PFPE microchannels are not swelled shut by solvents**. (a) Dyed solution of methylene chloride, acetonitrile, and methanol entering the end of a microchannel that is open to the side of a PFPE device. (b) This solution did not enter a PDMS channel of the same size due to swelling. (c) As a control, dyed methanol (which does not swell PDMS) does enter the same PDMS channel. (Adapted from [230] with permission. Parts *a* and *b* copyright the American Chemical Society, 2004.)

to eliminate air bubbles that otherwise form due to the very high wettability of the PFPE channel surfaces (Figure 5.6). The CAD design and photographs of the test devices are shown in Figure 5.7.

Solvent resistance enables a wide variety of chemistry to be performed inside microfluidic devices. Figure 5.8 shows the design and fabrication of a prototype chip that could be used for solid phase combinatorial array synthesis on a glass surface. For example, a combinatorial DNA array could be synthesized. Unlike PDMS, PFPE is compatible with all solvents involved in DNA synthesis reactions. The channel features in this device design were widely spaced to simplify layer alignment.

5.2.3 Methods

5.2.3.1 Materials

Poly(tetrafluoroethylene oxide-*co*-difluoromethylene oxide) α, ω -diol (ZDOL, average M_n \approx 3800 g/mol, 95% Aldrich), 2-isocyanatoethyl methacrylate (EIM, 99% Aldrich), 2,2-dimethoxy-2-phenyl acetophenone (DMPA, 99% Aldrich), dibutyltin diacetate (DBTDA, 99% Aldrich), and 1,1,2-trichlorotrifluoroethane (Freon 113, 99% Aldrich) were used as received.

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Figure 5.5: Microvalve actuation in PFPE microchannels containing solvents. (a) Top-down view of channels containing no solvent. The channels in the thin layer (to carry fluid) run top to bottom, while those on the thick layer (to carry pressurized air) run left to right. (b) Thin layer channel filled with dyed solution of acetonitrile, methylene chloride, and methanol. The thick layer (control) channel is unpressurized, and the valve is open. (c) Valve actuated by introducing 25 psi of air into the control channel. Beneath each photograph, a drawing of the valve cross-section is shown. (e,f) Photographs of additional valves of different dimensions with solvents present in the fluid channels. Valves closed at 12–14 psi could generally block fluids pressurized to 4 psi. Increasing both pressures results in faster response times during closing and re-opening. (Adapted from [230] with permission. Parts a, b, and c copyright the American Chemical Society, 2004.)



Figure 5.6: Extreme wetting leads to air bubbles in fluid channels. In this sequence of five video frames, fluid is being pushed from left to right under low pressure (< 5 psi). The control channel (oriented top to bottom) is unpressurized. Due to the extreme wetting of PFPE by dichloromethane (and many other solvents), fluid prefers to flow in narrower parts of the channel. In frame 2, as the leading edge of the meniscus reaches the valve, it pulls the valve membrane downward (towards the fluid channel), causing a slight constriction of the fluid channel. Fluid prefers to collect here, bypassing an air bubble that forms upstream of the valve. In frames 3–5, the air bubble expands to the left, even though the net flow of fluid is still to the right. Only by flowing fluid at sufficient pressures to deflect the valve membrane *away* from the fluid channel can this be prevented. Similarly, flushing out channels with air is difficult as the air tends to flow down the middle (tallest) part of the channel, leaving stagnant fluid at the sides.



Figure 5.7: Valve test pattern used to evaluate PFPE valves. (a) CAD design for valve size test pattern. A series of parallel fluid channels (blue) ranging from 124–250 μ m in width is controlled by a series of control channels (red) in the perpendicular direction with the same size range. (A previous design used a wider range of channel widths, 30–500 μ m, but the largest channels tended to collapse, and poor wetting of such wide photoresist lines by the liquid PFPE precursor led to fabrication defects in the thin layer.) At the right, images of actuated valves are shown (12 psi control pressure; no fluid) for each valve in the column where the control channel is 200 μ m wide. (b) Top-view photograph of the PFPE device fabricated from this pattern with red and blue food colouring filling the control and fluid channels respectively. Note that the control channels are dead-end channels while the fluid channels have ports at both ends. In this particular device, one control channel had a defect and could not be filled. (c) The same device viewed from a different angle to illustrate how thin (2 mm) these devices were (to conserve material during testing). It is mounted on a 2×3 inch glass slide. (d) Photograph of the device during valve testing on a stereoscope.



Figure 5.8: Design of a primitive combinatorial array synthesizer. (a) CAD design of a 4×4 array synthesizer based on the solid-phase combinatorial synthesis principle introduced by Southern et al. [248]. The device is designed to synthesize all 2-mers of DNA on a glass surface, for example, though it can also be used for other combinatorial chemistry applications. Combinatorial synthesis proceeds by first flowing each of the four nucleotides in a dedicated row to couple single nucleotides to the surface in a stripe pattern. Next, each of the four nucleotides is flowed in a dedicated column, forming 2-mers where the columns intersect the originally synthesized row stripes. All 16 possible DNA 2-mers are synthesized at the 16 intersections. The device is designed to implement this technique as follows. Pressurizing the row flow selector closes a set of valves that allows flow in fluid channels (blue) only in the horizontal direction. Each of the four nucleotides is fed into the row fluid inlet in succession. The row/column valves are configured such that the nucleotide flows along the single desired row in each case. Similarly a bank of valves switches the flow path to the column direction for introducing reagents in the second step. (The design and details of operation are described in significantly more detail in Chapter 7.) The device has a push-down valve architecture so that the contents of fluid channels are in direct contact with the glass substrate, where the coupling reactions occur. In the inset is a micrograph of a portion of a PFPE device corresponding to the region inside the green square. (b) Photograph of a PFPE combinatorial synthesis chip. (c) Photograph of PFPE device mounted on a glass slide with off-chip connections in place. (d) Micrograph of corner of PFPE chip. The fluid channels have been filled with a blue dyed solution of dichloromethane, acetonitrile, and methanol.

5.2.3.2 Preparation of PFPE DMA

In a typical synthesis, ZDOL (5.7227 g, 1.5 mmol) was added to a dry 50 mL round bottom flask and purged with argon for 15 min. EIM (0.43 mL, 3.0 mmol) was then added via syringe along with Freon 113 (2 mL) and DBTDA (50 μ L). The solution was immersed in an oil bath and allowed to stir at 50°C for 24 h. The solution was then passed through a chromatographic column (alumina, Freon 113, 2×5 cm). Evaporation of the solvent yielded a clear, colourless, viscous oil that was further purified by passage through a 0.22 μ m polyethersulfone filter. 1H-NMR (ppm): 2.1, s (3H); 3.7, q (2H); 4.4, t (2H); 4.7, t (2H); 5.3, m (1H); 5.8, s (1H); 6.3, s (1H).

5.2.3.3 Photocuring of PFPE DMA

In a typical cure, 1 wt% of DMPA (0.05 g, 2.0 mmol) was added to PFPE DMA (5 g, 1.2 mmol) along with 2 mL Freon 113 until a clear solution was formed. After removal of the solvent by vacuum degassing, the cloudy viscous oil was passed through a 0.22 μ m polyethersulfone filter to remove any DMPA that did not disperse into the PFPE DMA. The filtered PFPE DMA was then irradiated with a UV source (Electro-Lite ELC-500 UV curing chamber, $\lambda = 365$ nm, intensity: 24–28 mW/cm²) while under a nitrogen purge for 10 min. This resulted in a clear, slightly yellow, rubbery material. Prior to exposure, samples were left in the UV curing oven for at least 5 min under nitrogen purge. All generations of PFPE material were photocured in this manner.

5.2.3.4 Device fabrication with PFPE DMA

In a typical fabrication, PFPE DMA containing photoinitiator (as described in Section 5.2.3.3) was spin coated to a thickness of 20 μ m (800 RPM) onto an untreated silicon wafer containing the desired photoresist pattern. This wafer was then placed into the UV curing chamber and irradiated for 6 s. Separately, a thick layer (roughly 5 mm) of the material was produced by pouring the PFPE DMA containing photoinitiator into a PDMS barrier mold surrounding the silicon wafer containing the desired photoresist pattern. This wafer was irradiated with UV light for 1 min. Following this, the thick layer was removed, and inlet holes were carefully punched in specific areas of the device. Punching was performed with a hole-punching press (Technical Innovations, Inc., Brazoria, TX), as holes punched manually with Luer stubs tended to have rough edges that did not completely seal around stainless steel tubing. The thick layer was then carefully aligned with and placed on top of the thin layer and the entire device was irradiated for 10 min. Once complete, the entire device was peeled from the wafer with both layers adhered together. Additional inlet holes were punched through the device for channels in the thin layer, and the device was sealed to a clean glass slide or slide coated with fully cured PFPE. The above curing times were determined to be the optimal exposure times to achieve a good balance between structure failure and proper adhesion of the two layers. It should be noted that the later generations of PFPE material employed a distinct bonding mechanism, such that curing and bonding processes were not intertwined in this way.

5.2.3.5 Swelling experiments

Swelling experiments were performed by soaking fully cured PFPE DMA and fully cured Sylgard 184 (Dow Corning) in dichloromethane. Percent swelling was determined using the following equation:

% swelling =
$$100\% \cdot \frac{(W_t - W_0)}{W_0}$$
 (5.1)

where W_t is the weight of the material after soaking in dichloromethane for time t (measured immediately after removing the sample from the solvent and patting the surface dry with a Kimwipe), and W_0 is the original weight of the material. The same procedure was used to determine swelling of crosslinked PFPE DMA in other solvents.

5.2.3.6 Rheometry

Viscosities of the two elastomer precursors (PFPE DMA and Sylgard 184) were measured on a TA Instruments AR2000 Rheometer. Measurements were made with approximately 3–5 mL of material. Measurements on the Sylgard 184 precursors were taken immediately after mixing the two components. The shear rate for Sylgard 184 was varied from 0.03 s^{-1} to 0.70 s^{-1} , and a constant viscosity was observed at all rates. The shear rate for PFPE DMA was varied from 0.28 s^{-1}

to 34.74 s^{-1} , also resulting in a constant viscosity. Viscosities were obtained by taking an average of the viscosity values over all measured shear rates. The raw data for these experiments is shown in Figure 5.9.



Figure 5.9: Viscosity vs. shear rate for PFPE DMA and Sylgard 184 precursors. In both materials, viscosity is constant over the range of shear rates. A single viscosity value was computed for each by taking an average of the raw data here. (Reproduced from [230] supplementary information with permission. Copyright the American Chemical Society, 2004.)

5.2.3.7 Dynamic mechanical analysis

Modulus measurements were taken on a Perkin Elmer DMA 7E Dynamic Mechanical Analyzer. Samples were cut into $4 \times 8 \times 0.5$ mm (width×length×thickness) rectangles. The initial static force on each of the two samples was 5 mN, and the load was increased at a rate of 500 mN/min until the sample ruptured or the load reached 6400 mN. The tensile moduli were obtained from the initial slope (up to about 20% strain) of the stress/strain curves shown in Figure 5.2.

5.2.3.8 Dynamic mechanical thermal analysis

Thermal transitions of the two elastomers were obtained on a Seiko DMS 210 Dynamic Mechanical Thermal Analyzer. Samples were cut into $4 \times 20 \times 0.5$ mm (width×length×thickness) rectangles. The following settings were used: L_{amp}: 10; minimum tension/compression force: -10.000 g; tension/compression correction: 1.2; and force amplitude: 100. The temperature sweep ranged from – 140°C to 50°C. T_g values were obtained from the corresponding temperature at the maxima in a plot of E'' (loss modulus) vs. temperature.

5.2.3.9 Contact angle measurements

Static contact angles were measured using a KSV Instruments, Ltd. CAM 200 Optical Contact Angle Meter. Droplets were placed on each of the fully cured elastomers using a 250 μ L screw-top syringe.

5.3 Improvements in mechanical properties

The first-generation PFPE polymer exhibited many of the desirable properties of PDMS including its elasticity, modulus, and precursor viscosity. However, it was somewhat brittle. Cracks were created during the hole punching step, preventing complete sealing of inserted stainless steel tubing connectors. In addition, devices were fragile and difficult to handle as bending the PFPE too far resulted in cracking or breaking. Furthermore, the material exhibited poor elongation (< 20%), making it unsuitable for push-up valves that, depending on channel dimensions, can require the membrane to be stretched much further.

We initially devised engineering solutions to these issues. For example, droplets of PFPE cured around inserted inlet pins acted as a sealant to create air-tight connections (see Figure 5.10). However, modification of the polymer material itself was a more flexible and effective long term solution. Rolland *et al.* synthesized a PFPE mono-methacrylate (PFPE MMA) to be mixed with PFPE DMA to reduce the crosslink density in the final polymer. A 1:1 mixture of these two monomers yielded polymers with significantly improved flexibility and toughness, yet sufficient rigidity to prevent collapse of channel features. Punching holes in the new material seldom resulted in crack formation.

5.4 Improvements in device bonding

In the construction of 2-layer microfluidic devices there are two bonding interfaces—between layers and between the device and the substrate. Each generation of material synthesized by Rolland *et al.*



Figure 5.10: **PFPE sealing of off-chip connections**. First-generation PFPE devices suffered from cracking near the inlet holes during punching that resulted in fluid or air pressure leaks. These cracks were sealed by depositing and curing droplets of PFPE after inserting stainless steel tubing. Due to the thinness of early devices (typically 2 mm, to conserve PFPE), the tubing could easily be dislodged by normal handling thus breaking the seals. One solution to this problem was to encapsulate the entire device in a thick layer of PDMS. Although PDMS does not adhere to PFPE, the layer holds tubing firmly in place, extending the life of the seals and thus the devices.

was developed to evaluate a new chemical bonding strategy. I discuss the evolution of bonding at both interfaces.

5.4.1 First-generation PFPE

5.4.1.1 Layer bonding

Bonding of devices fabricated from first-generation PFPE was achieved by a delicately balanced partial curing technique. The thin layer was undercured by using a very short UV exposure such that a very thin liquid layer remained on the upper surface to act as a glue. The thick layer was cured the minimum time necessary until it solidified. If either layer was cured for too long, we found the bonding to be very weak, and devices quickly delaminated at relatively low pressures (2–10 psi). Undercuring too severely resulted in an excessive amount of liquid between layers that clogged channels. To a certain extent, the clogging could be reduced by baking at 80°C for several minutes prior to the UV bonding step. Heating reduced the viscosity, allowing the excess material to spread out along channels, therefore reducing the accumulation at any one point.



Figure 5.11: Adjusting crosslinking density of PFPE. (a) Simplified structure of PFPE DMA, a long-chain molecule containing a methacrylate group (represented by a double line) at each end, and PFPE MMA, the same molecule with only a single methacrylate. At the far right is a further simplified view of each molecule: imagine each circle to be a coiled-up polymer chain. (b) Schematic of the formation of a PFPE MMA network. During the polymerization process, methacrylate groups are joined together to form a linear polymer (with PFPE as a side group). The final elastomer material is a tangle of these individual chains. When using di-functional PFPE DMA, one can imagine a similar picture but with the *other* ends of each PFPE group interconnected in complex ways to form a highly crosslinked network. (Adapted from the web, http://emu0.emu.uct.ac.za/EMforBiologists/lecture2/Lecture-2.htm.) (c) Mixing both PFPE MMA and PFPE DMA results in an intermediate polymer with greater average distance between crosslinks when compared with pure PFPE DMA. The material is more flexible as a result. (Adapted from Isaure *et al., Journal of Materials Chemistry* 13(11), 2701–2710, 2003. Copyright the Royal Society of Chemistry, 2003.)

The optimal curing time for the thin layer was determined to be 6 s. However, it was difficult to precisely reproduce this exposure time in our curing oven due to a relatively long bulb ignition delay and a timer that could be adjusted only in 0.1 min increments. The large uncertainties in timing led to poor reproducibility of layer-layer adhesion. To reduce the *relative* error, we increased the time needed for curing by reducing the UV exposure intensity. Two of the four UV lamps were removed, and UV absorbers such as platic sheets were inserted into the light path. However, even with improved exposure time accuracy, optimal bonding was not consistently achieved. Batch-tobatch and even day-to-day variation in quality was observed. The lack of repeatability resulted in a low yield of functional microfluidic devices.

Furthermore, we realized that even the optimal bond achieved by this technique is a purely mechanical one. Invariably, 2-layer devices could be peeled apart without damage to either surface, suggesting that devices would be likely to eventually fail under normal operating conditions. We suspected that adhesion was due primarily to van der Waals interactions between the two smooth surfaces and also to mechanical interactions between the thick layer features and the thin liquid layer that cures closely around it. The lack of chemical bonding can also be demonstrated simply by curing some prepolymer on a fully-cured layer: it seals, but the layers can be peeled cleanly apart.

In an initial attempt to achieve chemical bonding, we treated samples with FluoroEtch (Acton Technologies, Inc., Pittston, PA), a chemical that strips fluorine atoms from Teflon and other uncrosslinked fluorocarbon polymers [265]. Once stripped, surface groups reorganize into a variety of functional groups that are capable of bonding to conventional adhesives such as epoxy (or perhaps to one another). Though PFPE is crosslinked and did not visibly respond to treatment as Teflon does, a change in surface-wetting properties was observed after immersion for several hours at 55°C. We chose not to use conventional adhesives for layer bonding due to their susceptibility to chemical attack, but did observe that treatment of the thick layer with FluoroEtch led to qualitatively improved bonding to the partially cured thin layer.

5.4.1.2 Substrate bonding

Initial PFPE devices were simply sealed to a clean glass slide or to a slide coated with fully cured PFPE. The amount of adhesion provided in this manner was sufficient to pressurize fluid channels up to about 5 psi before delamination of the device from the substrate.

Eventually, a superior substrate adhesion method based on partial curing was developed. A two-layer device was fabricated as described in Section 5.2.3.4 with the exception that the final UV exposure to bond the layers together lasted only 5 min instead of 10 min. Next, PFPE was spin coated (800 RPM) onto a cleaned glass slide and cured for approximately 3 s under a nitrogen purge. After punching holes in the 2-layer device, it was placed on the coated slide and allowed to sit for 5 min. Bonding was completed by a 10 min UV exposure of the whole device. Typically, the bond strength was improved compared to simply sealing the device to the substrate; however, it was typically less than the strength of the first layer-layer bond.

Due to the relative weakness of the device-substrate bond, we generally fabricated push-down devices. This valve architecture exposes that bonding interface to less pressure than the layer-layer interface during device operation (see Section 2.3.1).

5.4.2 Second-generation PFPE

5.4.2.1 Layer bonding

Later generations of materials developed by Jason Rolland *et al.* at UNC incorporated functional moities specifically for chemical bonding between layers. In second-generation PFPE, the prepolymer mixture in each layer was "doped" with 5–10% of PFPE derivatives with different end groups: isocyanate and hydroxyl. These dopants, poly(tetrafluoroethylene oxide-*co*-diffuoromethylene oxide) α, ω -diisocyanate (PFPE-A) (average M_n \approx 3000) and poly(tetrafluoroethylene oxide-*co*-diffuoromethylene oxide) α, ω -diol (PFPE-B) (average M_n \approx 3800), were originally synthesized at UNC but in later experiments were purchased from Aldrich. The hypothetical bonding mechanism is illustrated in Figure 5.12. PFPE-A and PFPE-B do not participate in the UV curing process, instead becoming entangled in the elastomer matrix as it cures. A certain fraction will display functional groups near the PFPE surface. When two cured polymers (one containing PFPE-A, the other containing PFPE-B) are brought into contact under heated conditions (120 °C), hydroxyl and isocyanate groups react, forming covalent bonds between the two layers, joining the layers by "polymer stitching". In fact, two layers containing PFPE-A should be bondable due to the reaction between isocyanates.



Figure 5.12: Chemical layer-bonding mechanism in second-generation PFPE. (a) Schematic of layer-bonding procedure between two PFPE polymers. One contains a small fraction of PFPE-diisocyanate (PFPE-A) (top, red), while the other contains a small fraction of PFPE-diol (PFPE-B) (bottom, blue). Groups at the surface react with one another to covalently bond chains in one layer with those in the other to stitch the two polymers together. One might expect superior bonding if the dopants were bifunctionalized such that one end would be covalently linked to the PFPE elastic network while the other participated in interlayer bonding. (b) Reaction of hydroxyl group with isocyanate group.

The revised device fabrication procedure was as follows. Both the thin and the thick layers were prepared as usual, except that each was *fully* cured by a 10 min UV exposure with a nitrogen purge. After removal of the thick layer from its mold, holes were punched, and the layer was aligned to the thin layer still affixed to its mold. Baking for 2 h at 110–120°C provided sufficient adhesion that the 2-layer device could be peeled from the mold for hole punching.

5.4.2.2 Substrate bonding

Substrate bonding was hindered by two effects. First, the 2-layer device developed a substantial curvature (upward at the edges) after the 2 h baking step. We attempted to use weights to flatten

the device during subsequent processing. Second, the 2 h bake seemed to remove all bonding capability from the bottom layer. Even when sealed onto a freshly cured coated glass slide and baked up to 48 h at 120°C, no bonding occurred. Typically we attempted to fabricate devices as a B-A-B/A sandwich, such that the thick layer was doped with PFPE-B, the thin layer with PFPE-A, and the glass coating with PFPE-B or PFPE-A.

In another approach we sought to bond devices to uncoated glass. PFPE-A provides isocyanate groups that should in theory allow a doped PFPE sample to bond to a variety of substrates including glass derivatized with hydroxyl, epoxide, or amine groups. Some of these substrates are ideal for microfluidic applications involving *in situ* solid-phase synthesis of DNA or peptides (using a push-down valve architecture). However, numerous experiments did not result in successful bonding to these surfaces, even with PFPE-A samples that had not previously been baked. In a typical experiment, a sample of UV cured PFPE-A was placed on a clean, dry, derivatized substrate and baked at 120°C overnight or longer with one or two glass slides placed on top as weights. We observed that the sample could easily be peeled from the substrate after cooling. Three types of commercial derivatized slides were tested: SuperAmine and SuperEpoxy (TeleChem International, Inc., Sunnyvale, CA) and Xenoslide A (Xenopore Corp., Hawthorne, NJ). Glass slides that I treated with aminopropyltriethoxysilane (APTES) or N-(3-(triethoxysilyl)-propyl)-4-hydroxybutyramide were also evaluated (see Appendices A.2.5 and A.2.6, respectively).

Although channels in the upper layer (between the thick and thin layers) could routinely be pressurized up to 25 psi without delamination due to a strong initial bond, the lack of any adhesion of the device to the substrate allowed the push-down valves to overdeflect. As membranes deflected downwards, they weren't stopped by the substrate surface; rather, they continued deflecting, lifting the device from the surface in the process, until they ruptured.

5.4.3 Third-generation PFPE

Third- and fourth-generation PFPE were developed after the formation of Liquidia Technologies Inc. and structures of these materials are proprietary. However, both continue to use a heat-activated bonding mechanism that is orthogonal to the UV curing (crosslinking) process. Unlike the secondgeneration material, the third and fourth use an identical material composition in all device layers.

The device fabrication protocol was identical to that for second-generation PFPE. Layer-layer bonding reliably withstood 20–30 psi actuation pressure under static conditions. When valves were actuated repeatedly, however, layers were observed to delaminate rapidly (see Figure 5.13) and would not be suitable for operation of actual microfluidic devices. We observed very significant batch-tobatch and day-to-day variation in bonding that was eventually attributed to phase separation that occurs over time and sometimes occurred during transport of material samples from UNC to Caltech. Usually filtering and blending with fresh photoinitiator restored the bonding capability.

It was found that bonding could be significantly improved by curing third-generation PFPE in *liquid* form onto an already cured layer and baking at 120° C. Because our usual device fabrication process involves the bonding of two solidified layers, it was necessary to consider alternatives. One strategy is to cure a thin layer of PFPE on the fluid layer mold, then pattern a sacrificial material on top of that layer to define the pattern of channels in the second layer. Liquid PFPE is then poured over this sacrificial material and cured, and the sacrificial material is removed. To test this idea, I patterned SPR 220-7 photoresist (Shipley) on top of a cured PFPE layer. Spin coating, exposing, and developing were carried out according to the manufacturer's protocol and were observed to have no adverse effect on the existing PFPE layer. A PDMS mold barrier was placed around this pattern and filled with PFPE. The sample was exposed to UV light for 10 min to cure the top liquid layer, followed by baking at 120–130°C overnight. Unfortunately, the prolonged baking step hard-bakes the photoresist making it nearly impossible to remove. Immersion in a variety of organic solvents with sonication and heating for several days had almost no effect on the resist. Eventually, in some samples, valve intersections exhibited local delamination as if solvents had gradually accumulated underneath the PFPE. Fabrication in this manner might be successful if an alternative sacrificial material (e.g., wax, see Chapter 6) can be used.



Figure 5.13: **Delamination in third-generation PFPE devices**. (a,b) In an optimally bonded device, layers do not delaminate under static conditions. Shown here is a valve held in the closed state by a fixed pressure of 7 psi (top) and 30 psi (bottom). At high pressures, the first signs of delamination are evident as roughness at the edge of the pressurized control channel (clear channel). (Photographs courtesy Jason Rolland, Liquidia Technologies, Inc.) (c,d) In a non-optimally bonded device, the same signs of delamination are visble at lower pressures. Top: 0 psi; bottom: 8 psi. (e,f,g) Micrographs showing progressive delamination (peeling apart) when a valve pressure of 8 psi is oscillated on and off at about 1 Hz. The horizontal channel is an empty fluid channel. The vertical channel is a control channel in this push-down device. Note that the jagged-edged, rounded region in the middle of the valve intersection is simply a fabrication defect where the control channel partially collapsed. As the pressure is oscillated, the rough channel edges progressively expand in an intricate pattern as the layers gradually peel apart. In this case, complete separation of layers occurred in about 1 min.

5.4.4 Fourth-generation PFPE

A fourth generation of material was synthesized recently by Liquidia and evaluated in the context of fabricating microfluidic chips for the synthesis of [¹⁸F]-fluoro-2-deoxyglucose (FDG) [159].

Materials were mixed in proportions as specified by Liquidia and degassed in a vacuum chamber. The thin layer mold and plain or aminated glass substrate are spin coated with PFPE and additional PFPE is poured into a PDMS gasket around the thick layer mold pattern. All three components are photocured by exposure to UV light for 20 min under a nitrogen purge. The cured thick layer is removed from the mold and holes are punched for inlets. The thick layer is then aligned to the thin layer (still affixed to its mold), taking care to ensure none of the channels are collapsed. (If not re-opened prior to the adhesion step, collapsed channels become permanently bonded shut.) The layers are bonded by baking for 20 min at 105°C. The two-layer device is then peeled from the wafer, additional holes are punched, and the device is placed on the coated glass. The entire device is heated at 105°C for 1 h to perform substrate bonding and to complete the first layer-bonding reaction. After this step, the material yellows slightly and the initial cloudiness disappears.

Athough theoretically possible to bond directly to aminated glass [229], attempts to do so by baking were unsuccessful. For now, this will preclude the use of PFPE devices for the class of applications involving *in situ* synthesis on glass.

Due to the high reactivity of fourth-generation PFPE with various photoresists, the mold for the thin layer (on which the material is baked for 20 min) must be passivated by sputtering a metal layer such as palladium-gold several nanometers thick. CYTOP or possibly parylene coatings could be used instead.

Mechanical properties have been dramatically improved in this material generation compared with previous ones. Increased flexibility and elongation permit hole punching without cracking and permit the fabrication of devices with push-up valves.

We fabricated partially functional microfluidic devices designed for FDG synthesis. Control channels withstood in excess of 60 psi of pressure exhibiting no signs of delamination either between layers or between the device and the substrate. Permeability was sufficient to perform dead-end channel filling and solvent evaporation. The material surface is quite tacky and valves are slow to reopen if fluid channels are empty. However, the presence of fluids allows valves to be actuated quickly (tens of Hz).

5.5 Summary

With novel PFPE elastomer materials designed and synthesized by Rolland *et al.*, we have demonstrated successful fabrication of microfluidic devices with integrated microvalves. Device fabrication and valve actuation were accomplished using established procedures for PDMS devices. Due to the properties of PFPE, these devices offer most of the same advantages as PDMS devices (see Chapter 2) with the added benefits of photocurability (which reduces fabrication time from hours to minutes) and remarkably high solvent resistance. By solving the solvent-resistance problem in elastomeric microfluidics, these new devices have the potential to expand the field to many novel applications and should be of great interest to anyone wishing to perform chemistry in microfluidic devices.

Acknowledgment

I thank Jason Rolland and his colleagues at Liquidia and in Joe DeSimone's group at UNC for their tremendous contributions to this work. In particular, they were responsible for material design, synthesis, and characterization and were involved with the optimization of the device fabrication protocol. The supply of PFPE precursors (various generations) for performing bonding optimization experiments and device fabrication is greatly appreciated. I also thank Saurabh Vyawahare at Caltech for help with several experiments, in particular the solvent-resistance testing. Jason Rolland and Arkadij Elizarov fabricated and tested the fourth-generation chips discussed here.

Chapter 6 3D-Molding of Microfluidic Devices

6.1 Introduction

Multilayer elastomeric device fabrication by replication molding requires a method for bonding together layers. In our development of solvent-resistant microfluidics, the goal with each promising new material or coating was to fabricate multilayer chips to evaluate crossed-channel valve performance and ultimately to implement functional elastomeric devices. However, determining a reliable adhesion process was often a significant obstacle (see Chapters 3, 4, and 5). Because methods can rarely be re-used in different material systems, development of bonding protocols was time consuming, slowing progress and limiting the number of materials that could be thoroughly investigated. Generic bonding methods such as gluing are generally not useful due to the presence of easily clogged microfeatures on the bonding surfaces and due to the incompatibility of glues with many solvents that might be flowed through channels. Surface chemistry modification and partial curing techniques are usually required.

To speed up our investigations, we developed a novel replication molding procedure based on sacrificial 3D wax molds, which eliminates the need for layer bonding entirely. A single mold contains a 3D pattern of fluid and control channels and a complete multilayer elastomeric device can be cast in a *single* step. We have demonstrated functional crossed-channel microvalves in elastomeric devices cast from these molds. While the resulting devices are of lower quality than those obtained by silicon wafers patterned with photoresist, this technique accelerates the ability to evaluate operational parameters such as modulus, porosity, and chemical compatibility of novel polymers within the context of functional microvalve networks. At the same time, the range of possible device materials is potentially broadened since one can use materials for which adhesion may be impossible or impractical, due to the presence of particularly stable surfaces or due to the lack of sufficient information such as is the case for materials with unknown (often proprietary) structures.

Despite the original motivation of eliminating the need for layer bonding, the general principle of 3D fabrication offers a number of other advantages. The most obvious is more topological flexibility. Instead of having the network of fluid channels confined to a single 2D layer as in two-layer PDMS chips, fluid channels can be routed vertically, enabling fluid channels to pass over one another. Another benefit is the reduced number of fabrication steps for complex fluidic components. 3D fabrication techniques also enable structures that cannot simply be fabricated by other means those that are very tall, that have extremely high aspect ratios, or that have complex geometries and topologies. Expanding into the third dimension may also enable increases in chip densities.

This chapter begins with an introduction to a variety of existing methods for 3D microfluidic fabrication, followed by a description of our molding process. Next, details and results of our channel and integrated valve fabrication tests are presented. The chapter concludes with further discussion of some of the possible applications for 3D microfluidic fabrication.

6.2 Fabrication technologies for 3D microfluidics

Within the enormous literature of microfabrication, there are countless reports of fluidic networks constructed by almost every imaginable process. In this section, I provide an overview of these processes, deliberately limited to those methods having some intrinsic 3D capability. This includes the fabrication of channels having three-dimensional paths and networks having multiple layers of channels.

There are at least three general approaches for making 3D microfluidic devices: (i) layered fabrication (requiring bonding); (ii) direct 3D fabrication; and (iii) 3D molding. Whether fabricating device layers, whole devices, or molds, a wide range of tools are available, including stereolithogra-
phy, photolithography, solid-object printing, and mechanical or laser machining. The remainder of this section provides some introduction to the available techniques, including their capabilities and limitations.¹

6.2.1 Layered fabrication

Thin layers containing vias and two-dimensional channel patterns can be stacked and bonded to form three-dimensional microfluidic networks of arbitrary topological complexity. Generally each layer is made by a relatively simple machining or molding process. The main drawback is the large number of time-consuming precision alignment and bonding steps needed to assemble the layers.

The two-layer PDMS devices developed in our lab are a familiar example of the layered fabrication approach. In fact, the process can be repeated to stack additional channel layers [272]; however, it has not been practical to fabricate vias between specific layers. Variations of the process have been demonstrated that exhibit more flexibility in this regard. For example, Jo et al. [137] reported the fabrication of complex structures including a 5-layer cascading channel and reservoir network and a three-dimensional passive serpentine micromixer. Their method involves the creation of twodimensional PDMS membranes by curing prepolymer between a silicon wafer patterned with SU-8 photoresist clamped to a flat plastic sheet. After curing, the plastic sheet is removed and the membrane is peeled from the silicon wafer to be assembled into the 3D device. Each membrane has a thickness matching that of the resist (100 μ m) and contains vias (and other shaped openings) where photoresist was present on the wafer. Alignment within about 15 μ m was facilitated by: (i) fabricating membrane layers of identical shape and size and aligning corners; and (ii) including common holes through each layer to promote self-alignment by surface tension when methanol is placed between oxygen plasma treated layers. Jeon et al. [134] reported a slight variation of this method, perhaps capable of thinner layers because a special pressure-sensitive adhesive tape is applied to the membrane for ease of handling during plasma bonding to the previous layer in the

¹Numerous other physical processes have been used to generate microscale patterns, including fluidic self-assembly, colloidal sedimentation, polymer phase-separation, and templated growth. However, most currently permit only simple geometric patterns or coarse control over bulk properties and are not suitable for creating arbitrary microfluidic structures: they have therefore been omitted from the discussion.

device. Diaphragm and flap check valves were demonstrated, though more complex networks are also possible. An alignment accuracy of about 10 μ m was achieved using an *x-y-z* translation jig. A superior alignment jig permitting 1–2 μ m accuracy has been reported by Kim *et al.* [150].

Anderson et al. [5] reported another variation in which each single membrane contains three layers of features—channels in the upper and lower surfaces, and vias completely through the membrane. Membranes were fabricated by curing PDMS prepolymer between two facing molds, each having features of two different heights. The taller parts of the molds contact each other if sufficient force is applied and thus create vias in the membrane, while the short parts create channels. Proper registration is achieved by the use of mechnical alignment features. Mold release was facilitated by making one mold out of PDMS that can be easily peeled away, allowing one surface of the membrane to be bonded to another membrane before removal from the second mold. An 8×8 basketweave pattern of channels 70 μ m deep by 100 μ m wide and a helical channel surrounding a straight channel were fabricated. Note that the basketweave structure has features in 3 layers and is implemented entirely in a single membrane (though its floor and ceiling must be sealed); the helix has 5 layers of features and requires two membranes. This method reduces assembly steps at the expense of more complex molds. While not demonstrated, it should be possible to create 3D structures containing pressure-actuated crossed-channel valves in many layers. While technically not a lamination approach, another interesting variation was reported by Wu et al. [295]. In this method, a patterned membrane was sealed to another flat PDMS membrane, and individual channels were "cut out" as thin tubes that could be manually tied into interesting structures such as knots, helices, and weaves. In some cases the channels were held in place by threading through holes in a specially patterned PDMS membrane. The entire structure was then filled with PDMS to yield a final monolithic device. An additional technique was described in this paper: a PDMS layer containing embedded channels open to the side is slipped onto photoresist posts on a silicon mold pattern. When PDMS is poured over this assembly, the result is a monolithic device containing a 3D channel network composed of the channels in the original device connected to the channels defined by the silicon mold pattern in a perpendicular plane.

These procedures are in principle compatible with any material that can be molded and bonded, and have also been used in conjuction with melt processing of the biodegradable plastic, poly(DLlactic-co-glycolide) 85:15 (PLGA 85:15) [151]. Polymer pellets were melted between two molds to create membranes with channels 2 μ m in width. Pressure and heat were applied to membrane stacks for extended durations to induce bonding by polymer-chain interdiffusion. Also reported in this work is an interesting technique for extruding integrated annular stubs onto which external microtubing can be connected.

Lamination has also been widely used in metal and ceramic microreactors that must withstand harsh conditions such as high temperatures, high pressures, organic solvents, plasmas, or microexplosions. Layering is the only way to produce high aspect ratio channels in these materials due to the difficulty of machining deep microchannels from the surface. The large interfacial area provided by the high aspect ratio is useful in applications requiring rapid heat transfer to or from the walls, or requiring rapid mass transfer across a membrane. High aspect ratio channels are also effective in reducing dispersion in analyses where the fluid must follow a serpentine path [55]. Among other devices, Martin *et al.* [181] of PNNL fabricated a laminated solvent-exchange device from a diffusionbonded stack of several hundred layers of 100 μ m thick 304 stainless steel shimstock (foil). Each foil layer is patterned by photochemical etching or a stamping process. The device contains two layer patterns stacked alternately: (i) perforated layers to act as porous membranes; and (ii) layers containing 1×8 cm rectangular holes to act as very wide, shallow channels (channel depth is equal to the foil thickness). Lamination with ceramics was also reported, in which devices were made from stacks of laser patterned 125–250 μ m thick green (unfired) ceramic tape. The assembled stack is fired to eliminate the binder, leaving the all-ceramic device.

Glass is also somewhat difficult to machine, but has the desirable properties of stability, inertness, and transparency. Kikutani *et al.* [148] report the fabrication of layered 3D glass microfluidic chips for diffusively-mixed continuous flow combinatorial synthesis. The chip contained 4 inlet ports for two pairs of reagents and 4 outlet ports for all possible combinations of reactions between the two pairs of inputs. A three-dimensional chip was needed in order to accommodate several fluid channel



Figure 6.1: **3D** laminated ceramic microfluidic device. (a) Assembly diagram of a simple laminated device showing five layers. The slots cut from layers 2 and 4 will become microchannels, while the holes cut in layers 1, 3, and 5 will become headers (vias) to connect all the channels in parallel. The pattern of vias determines the flow pattern through the device, such as parallel flow (in this case) or serpentine flow. (b) Photograph of a ceramic device, with several thin channels visible through the header hole indicated by arrows. (Adapted from [182]. Copyright the American Institute of Chemical Engineers, 2000.)

crossings. It consists of three thermally bonded Pyrex glass plates—an upper and a lower layer containing etched channels (240 μ m wide by 60 μ m deep) and a middle layer containing vias. A 10-layer assembly was reported in [270].

6.2.2 Direct 3D fabrication

When alignment and bonding are undesirable, methods are available for direct monolithic 3D device fabrication, including stereolithography, micromachining, and solid-object printing.

A variety of other techniques, such as CNC (computer numeric control) machining [305] and micromilling [221], wire EDM (electrical discharge machining), ultrasonic machining, acousticallyencoded groove cutting [73], spark-assisted etching, and laser cutting and drilling have been used to cut microchannel structures in a wide variety of materials, including glass, ceramic [181], metals, and hard plastics. Automated machines are capable of complex 3D patterning with resolutions down to 10–20 μ m by cutting from multiple axes; however, because material can only be removed from the surface, complex 3D fluidic network geometries are not possible. Typically, fabricated devices are 2D networks of channels with channel depth variations, sometimes used in laminated devices. In the remainder of this section, fabrication by stereolithography and photolithography are discussed in more detail. Solid-object printing will be discussed in Section 6.2.3 since to our knowledge, this technique has never been used to fabricate devices directly.

6.2.2.1 Stereolithography



Figure 6.2: Schematic of stereolithography process. A monolithic 3D object is built up one thin "slice" at a time from a liquid photosensitive resin. A UV laser scans a 2D pattern representing one slice of the object in a thin layer of fresh resin at the surface, solidying the exposed regions. To build subsequent layers, the sample is lowered further into the resin, and a new thin resin layer covers the top surface. This process is repeated until the object is complete, whereupon a developing procedure removes the unpolymerized resin. Two-photon stereolithography is similar; however, it is the position of the laser focus that moves vertically rather than the platform. Resolution is improved because problematic surface tension effects are eliminated and because non-linearities result in a smaller polymerized voxel size. (Reproduced from http://www.proform.ch/en/t_sl.htm with permission. Copyright PROFORM AG, 2005.)

Stereolithography is generally an additive process in which selected regions within a vat of photosensitive liquid resin are solidified via polymerization or crosslinking upon exposure by a focussed UV laser beam (see Figure 6.2). Rapid prototyping stereolithography machines are commercially available, albeit very expensive, and can fabricate complex objects from 3D CAD (computer aided design) drawings entirely automatically. In one approach, the object is constructed by sequentially solidifying additional "slices" of the object at the surface. Depth resolution is limited by the layer thickness. In the related technique known as *selected laser sintering*, the object is built up from a thermoplastic powder, sometimes combined with ceramics or metals. In a variation of these methods, the position of the laser focus is controlled in *three* dimensions, allowing improved fidelity because polymerization occurs deep within the resin where there are no problematic surface tension effects. Non-linear effects are needed to ensure a small polymerization volume near the focus without polymerization of all material through which the beam passes. In the two-photon technique a photoinitiator is used having sensitivity only to twice the laser photon energy. Only at the focus of a pulsed femtosecond laser is the intensity sufficient for two-photon absorption to occur. With non-linear material response, voxel sizes of 100 nm (below the diffraction limit) have been reported under optimized exposure and development conditions.² Another non-linear process is based on a temperature sensitive resin that polymerizes only if a temperature threshold is reached. Yamakawa et al. [298] report a very inexpensive implementation of this technique, wherein a CD-player pickup laser is employed for polymerization. An extensive review and history of photopolymerization chemistry and technology can be found in [261].

Several investigators have fabricated microfluidic devices using stereolithography. Kang *et al.* [141] fabricated several design variations of a 3D blood-typing system and employed a computer solidification model and the concept of "unit" components to improve fabrication reliability. Ikuta *et al.* [118] fabricated a microfluidic device integrated with a silicon sensor and demonstrated an electrostatically actuated flap valve constructed from a conductive polymer. Employing photogenerated acids, Zhou *et al.* [308, 304] performed *subtractive* two-photon stereolithography by photo*de*polymerization. A simple device containing twelve 50 μ m–long 4×4 μ m square channels buried 10 μ m beneath the surface was fabricated. The main limitation is the risk of overdevelopment if the time to remove material from long narrow channels is too great. However, the subtractive method significantly

²Impressive demonstrations of complex objects produced by two-photon stereolithography include a 10 μ m sculpture of a bull [143] and a chain with 50 μ m links [154].

speeds construction as one needs only excavate the channel and reservoir regions within a large solid body rather than building up walls around every microfluidic channel. Sugioka *et al.* [260] describe a different subtractive two-photon method based on photo-etchable glass. Upon exposure to UV radiation and subsequent heat treatment, exposed areas form a crystalline phase within the amorphous glass that has a much faster etch rate in dilute HF. Several microfluidic structures were demonstrated, including a Y-shaped microchannel (20 μ m wide by 70 μ m deep) located 300 μ m below the sample surface, and a Y-shaped channel in a vertical configuration, extending 2 mm between front and back surfaces of the glass. The authors also fabricated a vertical structure containing a tiny movable glass plate that could be switched between two positions using compressed air and thus could serve as a microvalve. Despite some drawbacks (large valve size, two dedicated control inputs required, and possibly incomplete sealing), the valves could enable the fabrication of complex fluidhandling networks in a variety of hard, inert materials. However, it is not clear whether the etch rate difference (about 45×) is sufficient to reliably produce very long narrow channels. Note that this example illustrates an important capability of subtractive direct-writing methods—the ability to form freely moving components.

Stereolithography is an expensive method for fabricating 3D microfluidic devices but has very high resolution, has complete three-dimensional freedom, and can simultaneously accommodate microscopic and macroscopic features. It is compatible with any material having appropriate photoor thermosensitivity. The two-photon volume polymerization approach is only compatible with transparent resins. To our knowledge, elastomeric materials have not yet been patterned by stereolithography. However, it is possible that laser ablation could pattern cavities and microchannels in PDMS and other transparent materials in an analogous manner to subtractive two-photon stereolithography. Maltezos [177] observed that a focussed laser beam could ablate PDMS to generate cavities in the interior of a sample. Little is currently known about the fabrication characteristics of this approach. Stereolithography could also be used to print 3D molds.

While somewhat different than stereolithography, Hutchison *et al.* [116] report the fabrication of 3D devices by a photopolymerization technique called contact liquid photolithographic photopoly-

merization (CLiPP). A thin layer of liquid monomer is poured into a chamber, polymerized in desired regions by exposure through a photomask, and finally developed to remove the uncured monomer. Molten wax is then poured into channels and solidified, serving as a sacrificial material and providing a flat surface on which to build the next layer. A new layer of monomer is poured on the surface, and the process is repeated until the object is complete, whereupon the wax is removed. Compared with stereolithography, this technique offers considerable speed advantage due to the parallel exposure and also offers the opportunity to incorporate different materials in different layers. Surface modification is straightforward and can provide particular functionalities and covalent bonding between materials in different layers. The authors demonstrated the fabrication of a 3D microfluidic device containing multiple layers of channels, a freely rotating flow meter, and a device containing an integrated photopolymerized heating electrode. Elastomeric materials have been used with this method, and though rounded channels were not reported initially, such channels can be created by polymerizing the first layer on an appropriate mold (to make push-down microvalves) [115].

6.2.2.2 Micromachining and photolithography

Sophisticated microfluidic devices have been fabricated with silicon, glass, metal, and PDMS by a variety of micromachining processes common in the field of MEMS, including photolithographic patterning of resists, bulk micromachining, surface micromachining, and LIGA (Lithographie Galvanoformung Abformung). These tools can be used either to make molds or to make devices directly—both will be discussed here for the sake of continuity. Though most micromachining techniques are inherently 2D processes, 3D devices can be fabricated by combining them in clever ways. As a simple example, Liu *et al.* [171] fabricated a 3D serpentine mixer in silicon by etching from both the top and bottom. Regions where only one side is etched form channels; regions where both sides are etched become vias completely through the wafer, joining the two channel layers. In principle additional layers can be deposited and patterned to build up complex 3D networks; however, due to the large number of processing steps, this tends not to be a very practical approach. Aside from micromachining techniques, methods have been reported to fabricate 3D structures out of photoresist by carefully crafted exposures and often a single developing step. Such processing is in many ways a special case of stereolithography, but having different exposure mechanisms. To achieve some limited 3D control of the exposure, techniques such as multiple exposures, tilted exposures, controlled depth exposures, and exposure by 3D interference patterns have been used. Photolithography can be substantially faster than stereolithography, if (parallel) flood exposures are used. Mold patterns with multiple height features, as well as fully three-dimensional devices and molds, have been demonstrated.

Multiple exposures with different penetration depths were used by Kim *et al.* [149] to create structures with three distinct layers of features in 80 μ m thick positive photoresist. Shallow front and back exposures defined the pattern in the top and bottom 20 μ m layers, while a deep front exposure through the entire resist layer delineated boundaries between structures. This technique can be used for direct fabrication of microchannel structures or can be used to make molds. Fluidic networks with crossing channels, or channels with surface textures such as grooves, pillars, or pits are possible.

Romanato *et al.* [231] created microfluidic channels in PMMA photoresist via tilted X-ray exposures. Exposing twice through a mask at different tilt angles (differing by a 180° azimuthal rotation of the sample) generated two leaning walls of resist that intersected to form a long hollow 11 μ m deep microchannel parallel to the surface with a downward-pointing triangular cross-section. In addition, they demonstrated a "fence" structure (standing vertically on the surface) that could perhaps be used in size-selective filtering.

Kudryashov *et al.* [153] employed UV exposure through greyscale masks combined with e-beam writing to fabricate interesting structures is SU-8 photoresist. E-beam illumination penetrates only a few microns into the upper surface, while UV illumination generates structures in the bulk of the resist. The height of UV-exposed structures depends on dose, permitting molds or devices with carefully designed channel profiles (e.g., rounded profiles for closable fluid channels), or complex multi-depth features. An interesting demonstration was the construction of a series of 15 μ m tall

posts supporting a thin (several microns) flexible "net" structure with a square mesh. The authors suggest that the mesh size can be varied between 2–100 μ m and that the nets might be useful as traps for soft objects such as biological cells. One could also imagine using this technique to fabricate molds for 3D fluidic networks with crossed channels.

The use of multiple beam exposure, or exposing through phase masks, generates an interference pattern throughout the volume of the resist. Carefully designed interference patterns can selectively expose regions *inside* the resist layer, unlike conventional exposure that progresses through the material from the surface. Generally it is only possible to generate 3D structures that are mostly periodic with feature size on the scale of the illumination wavelength; therefore these methods are probably better suited to the fabrication of components such as integrated filters, gratings, and photonic crystals rather than complex 3D channel networks. Jeon *et al.* [135] report the fabrication of a Y-shaped channel in SU-8 having an integrated nanofilter using the combination of a conventional amplitude mask for the channel and a phase mask for the filter.

Control over exposure at different depths within the resist can also be achieved by using multiple resist layers. Exposure can occur in between deposition of subsequent layers, or resists can be selected that have orthogonal processing conditions and exposure radiation sensitivities. Romanato *et al.* [231] created molds from three layers of resist to create a network of microtanks connected by channels. A thin layer of SAL photoresist on a thick PMMA layer was exposed via e-beam lithography and developed, yielding a pattern of SAL microwires (0.2–1 μ m wide) on top of the unaffected PMMA. A second PMMA layer was deposited and then the entire structure exposed via X-ray lithography, leaving a pattern of double-height PMMA posts connected by SAL microwires at mid-level. The microfluidic device was then created by electroforming with gold and then removing the resist. Yoon *et al.* [302] used a sequential process to build up complex microfluidic networks in nickel. Each photoresist layer was patterned via controlled depth exposures to build structures with two heights and was then electroplated with nickel up to the same height, providing a level surface on which to spin the next resist layer. Once complete, the resist was removed, revealing a complex network of channels (from short resist features) and vias (from tall resist features). The above photoresist patterning methods and most micromachining techniques are performed with hard materials, in which mechanical valves have only been demonstrated by very elaborate fabrication methods [300, 285]. Fully elastomeric devices based on integrated crossed-channel valves could be fabricated by molding from microfabricated molds.

6.2.3 3D molding

Molding is a process whereby the desired three-dimensional pattern of empty spaces (microchannels, microreactors, and other fluidic components) is defined. The mold comprises the "inverse" of the desired structure. Thus, for example, a microfluidic channel would be represented in the mold by a long thin *beam*. The final microfluidic device is fabricated from the mold by embossing (if there are no suspended features), casting, or injection molding. If the mold contains suspended features, it must be sacrificed in order for the device to be removed.

Molds must have sufficient structural integrity to maintain their shape prior to casting. In microfluidic designs that are sensitive to the vertical distance between channels (e.g., those having crossed-channel valves), long thin beams that are prone to sagging must either be avoided or be supported by pillars near critical gaps. Since pillars will leave voids in the final microfluidic device, they should be positioned so as not to interfere with fluid flow or other aspects of device operation. When pillars are needed, usually only a few layers of microchannels are practical. In fact, we found it simplest to route most channels along the mold substrate in two dimensions, only utilizing the third dimension when channel crossings, valves, or inherently 3D fluidic components were needed. Due to this constraint, molding does not generally offer as much design flexibility as direct fabrication methods such as stereolithography.

The primary advantage of molding is the elimination of alignment and bonding steps that are needed in layered fabrication. 3D molding also has an advantage with respect to stereolithography in that a much wider range of potential device materials can be used—there is no need for temperature or photosensitivity nor transparency. With molding, there are only a few restrictions on the device material: it must be chemically compatible with the mold, and it must have sufficiently low viscosity to fill the smallest cavities in the mold. Of course, the curing conditions of the device material must be tolerable by the mold material, and the final device must be inert to the mold removal conditions in the case of sacrificial molding.

Molds for microfluidic devices are most frequently fabricated by micromachining as discussed in the previous section, but have also been fabricated by solid-object printing and several other interesting methods. As one example of these other methods, Dharmatilleke *et al.* [63] cast PDMS on sacrificial molds made by manually drawing molten wax into thin filaments 100–200 μ m in diameter. The wax was dissolved to leave circular profile microchannels. Branched networks were created by "soldering" filaments together, and complex 3D channel shapes such as helices were also demonstrated. Another example is the use of micromilling with toolbits of 20–25 μ m diameter to fabricate (non-sacrificial) brass molds for a 3D serpentine mixer [12].

Solid-object printing is perhaps the most attractive mold-making option. With the availability of commercial ink-jet and thermoplastic extrusion machines, an entire 3D mold structure can be fabricated directly from a CAD file in a single unattended run. These printers generally cost much less than stereolithography machines to own and operate and have the additional advantage that multiple materials can be incorporated into the 3D object. For example, use of an easily removable sacrificial material allows the construction of elaborate suspended or even freely moving structures from another material. When the final mold itself is to be sacrificial, the mold material is typically a polymer, but ink-jet printing is also capable of printing other materials such as fused powders and metals [34]. Compared with micromachining and photolithography, solid-object printing does not require a clean room or specialized equipment such as mask aligners, exposure systems, and spin coaters, nor are toxic chemicals such as photoresists and developers required during processing. Printers can accommodate structures with a wide range of sizes, including very tall features that are not possible with photolithography due to thickness limitations of available resists (0.5–1.0 mm). Drawbacks of solid-object printing include high surface roughness and relatively poor resolution.

Cooper *et al.* previously demonstrated the use of a ThermoJet solid-object printer to fabricate molds for PDMS microfluidic devices [187]. This particular printer cannot fabricate suspended struc-

tures, so cast devices were simply peeled from the mold and sealed to a substrate. The minimum feature size (in all dimensions) was about 250 μ m. High surface roughness (8 μ m) interfered with sealing of the PDMS device to substrates, particularly glass. The authors circumvented this problem by first fabricating the inverse of the desired mold, annealing its top surface against a flat transparency film, and then casting a PDMS replica that would serve as the actual mold. This process resulted in a flat bottom surface for bonding but did not improve roughness of channel walls. Several devices were fabricated, one consisting of 2 channels in the x - y plane plus a vertical channel of 5 mm length above their intersection designed to hold an optical fiber for imaging. Another device was a chaotic advective mixer consisting of a microchannel with a staggered series of groove patterns in the floor, previously fabricated by double photoresist [258] or laser photoablation techniques. An immunoassay device was implemented with ports compatible with a 12-channel pipettor, demonstrating simultaneous fabrication at micro and macro scales. Finally, using the lamination strategy described in Section 6.2.1, two molds were printed with alignment features and used to create a 3D basketweave pattern [5]. The poor resolution, especially in the vertical direction (250 μ m), prevents the fabrication of crossed-channel microvalves.

In the next section, I describe our own work to print molds for microfluidics using a similar approach. Significant differences in our work include: (i) much improved resolution, with features as small as 13 μ m in z and about 200–250 μ m in both x and y; (ii) the ability to print molds directly on a flat substrate, obviating the need for an inverse mold and annealing step; (iii) the ability to print two materials—one acting as a structural mold material and the other acting as a sacrificial material—thus enabling the construction of buried and crossed channels without the need for multiple molds or layer bonding; and (iv) the demonstration of functional valves, including a crossed-channel microvalve—a first step towards sophisticated 3D fluid handling. The channel network of an entire typical two-layer elastomeric device can be represented on a *single* sacrificial mold and can be embodied in a microfluidic device in a single casting step. This enables the rapid evaluation of the performance of new materials in active microvalve devices without the need to first develop a layer bonding procedure.

6.3 Device fabrication from 3D wax molds



6.3.1 Mold fabrication

Figure 6.3: Solidscape T66 3D ink-jet printer. (a) Top view of the Solidscape T66 with the cover open. The large central square is the vertically moving build platform. A mold is being printed on a 2×3 inch glass slide (barely visible) glued to a square piece of foam support that is affixed to the lower left corner of the build platform. After printing the layer the print head will move to the far right for ink-jet nozzle cleaning. After allowing time for wax cooling, the milling head at the left begins spinning and passes over the mold left to right and back. The entire build platform then moves downwards to make room for the next layer. (b) Wide angle view of the printer showing a few elements not visible from the top: the vacuum hose connected to the milling head and the reservoirs for the build wax (blue) and support wax (red) at the rear of the machine. (c) Close-up view of the two ink-jet nozzles on the print head during printing of a mold.

Three-dimensional sacrificial wax molds were printed with a Solidscape T66 high resolution solidobject printer (see Figure 6.3). Mold patterns representing the inverse of the desired channel network were designed in SolidWorks (a 3D CAD program), exported in STL file format, and processed by ModelWorks for translation into the printer-readable t6 file format. ModelWorks divides the design into layers of the selected thickness (13–76 μ m) and automatically adds support material to each layer as needed (e.g., for suspended features). Molds are printed one layer at a time on a 6-inchsquare "build platform" within the machine by a print head moving in the *x-y* plane. Ink-jet nozzles deposit tiny droplets of molten wax approximately 75 μ m in size [120]. Since droplets are deposited approximately every 5 μ m, they overlap and provide reasonably straight edges on features. To ensure a uniform thickness of new wax material and a flat surface on which the next layer is built, a milling head cuts across the entire model after each layer is printed and cooled. The platform is then lowered by the layer thickness to make room for the next layer, and the cycle is repeated until the object is complete.

At the start of a print run, a jet cleaning and calibration procedure is performed to ensure that droplet volumes are consistent and that no air bubbles are present in the nozzles. The temperature controlled chamber further improves consistency by ensuring repeatable impact behaviour of droplets. Next, a piece of rigid foam (provided by Solidscape) or balsa wood is affixed to the build platform. (Initially we had used a softer foam provided by Solidscape, but its ability to deform resulted in poor printing accuracy.) The foam support is milled down in progressively smaller increments until level and flat. The milling head is then carefully cleaned to prevent dust from falling on the pattern during printing. In normal operation, solid objects are printed directly on the foam surface. However, the foam is not suitable as a mold substrate for casting microfluidic devices from liquid prepolymers due to its roughness and porosity. Instead, we affixed a 2×3 inch glass slide or untreated silicon wafer on top of the foam (using a glue stick). To prevent the milling head from hitting this new substrate, the build platform was manually moved down by a distance equal to the added thickness. This distance was determined by the difference of two Vernier caliper measurements—one of the platform and foam thickness immediately after milling the foam, the other of the platform, foam, and new substrate thickness after gluing. Accuracy is critical: if underestimated, the milling head can contact the substrate, resulting in complete removal of the first few wax layers or in damage to the substrate; if overestimated, the first layer may be very thick and irregular and the increased jet-substrate distance can result in poor printing quality. Once the build platform is properly lowered, printing proceeds normally on this new substrate. We found pattern quality to be somewhat better on glass than silicon, perhaps due to the better adhesion of wax to the uncleaned glass surface. The use of a glass or silicon mold substrate leads to a smooth bottom surface of the microfluidic device cast from the mold, suitable for bonding the device to a flat, glass bottom plate. Prior to the development of this method, we incorporated a rectangular slab in our design files, resulting in the mold features being built on wax slab. However, we experienced device bonding problems due to the relatively high surface roughness of the wax–approximately 1–2 μ m (RMS), according to measurements with an Alpha-step 500 profilometer (KLA-Tencor), in agreement with the specifications provided by Solidscape [119]. Typical surface height variation is ±2.5 μ m with excursions up to ±7–8 μ m. Use of the flat glass or silicon substrate solved this problem, but it should be noted that other surfaces of mold features (e.g., channels) remain rough.

In constructing each layer of the 3D pattern, two types of wax are printed by dedicated ink-jet nozzles—one is "build" wax from which the final 3D mold is made; the other is "support" wax and serves the temporary functions during printing of providing a solid surface on which suspended mold features are built, and forming a wall around all features to provide lateral support during milling and to protect against contamination by dust particles. The ModelWorks output file specifies the movement path of the print head and the droplet firing positions. Paths are specified in a vector format, with outlines printed first and subsequently filled, to ensure high fidelity of edge positions and shapes, even on rounded features. Build wax is printed first, followed by support wax. To speed up printing and later support wax removal, the regions of support wax are printed as a widely spaced grid. This caused us some problems in early design iterations, as the grid size was larger than the area of our crossed-channel microvalves, occasionally resulting in the absence of support wax in the critical gap between the two channels on the mold. During printing, the channel beams were fused by build wax, resulting in the channels being directly connected when cast into a microfluidic device. This problem was solved with a modified configuration file provided by Solidscape that has reduced grid spacing. Note that because ModelWorks adds a fixed number of grid squares surrounding all features, the thickness of the protective support wax wall surrounding each feature was substantially reduced. Typical print time for a single layer is about 1-2 min including printing and cooling times.

Resolution of the mold in the z-direction is determined by the selected layer thickness, typically 12.7 μ m in our molds. Designed distances in this direction were accurately reproduced by the printer due to milling between layers. However, the presence of wax dust from milling that was not completely removed by the vacuum and brush system connected to the milling head caused some problems. Dust particles that managed to get locked into the support material in the gap of a crossed-channel valve led to an open fissure between fluid and control channels in the microfluidic device cast from the mold. For this reason, vertical separations were designed to be at least several layers thick. According to specifications [119], the minimum feature size in the lateral directions (x-y plane) is about 250 μ m. Based on test patterns we created (see Section 6.4.1), we found a minimum feature size of about 200–250 μ m, with some variation depending on the state of the ink-jet nozzles—over time, the nozzles seem to print less accurately. We also found that features had to be separated laterally by at least 65 μ m in the design to reliably be separated in the printed part. We had difficulty building very tall structures such as posts for inlet ports—these features were often distorted or toppled during milling steps, even when surrounded by substantial amounts of support wax.

Once the mold has been printed, it is removed from the foam block and immersed in a hydrocarbon solvent (BioAct VSO) to dissolve the support wax, which is no longer needed. The solvent is heated to 60–65°C and gently stirred to accelerate support wax removal. Progress is visible due to the contrasting colours of the support wax (orange/red) and build wax (blue). Generally the final few minutes of this "dewax" process are performed in fresh solvent to minimize residue remaining after solvent evaporation. The mold is dried overnight at 60–65 °C with the mold substrate tilted at an angle to encourage solvent to flow away from the pattern. If left flat, we have observed significant residue near mold features after drying. Incomplete support wax removal can lead to the appearance of a cloudy film over the substrate or to the appearance of sharp crystal shards on feature surfaces after drying, both interfering with the sealing of cast devices to flat substrates. It is critical that the VSO solvent be eliminated as thoroughly as possible as it interferes with the proper curing of PDMS and encourages bonding of PDMS to the glass or silicon substrate. Many of our early silicon wafers had small pieces of PDMS stuck after mold melting (described in the next section), which had been torn from the device as it was removed from the wafer. The problematic regions correlated well with regions where solvent would be expected to evaporate most slowly—tight corners. This and other problems encountered during development of the fabrication process are depicted in Figure 6.5.

6.3.2 Device fabrication

Microfluidic devices were fabricated by casting liquid prepolymer on a 3D wax mold, melting the mold, and then sealing the cured elastomer to a substrate such as glass, as summarized in Figure 6.4.



Figure 6.4: Fabrication of microfluidic devices from 3D wax molds. (a) After printing a 3D wax mold, support wax is removed by immersion in VSO solvent, after which the solvent is evaporated by heating. (b) Prepolymer is poured on the mold, degassed, and cured. (c) Once solidifed, the polymeric device is released by melting the sacrificial mold and cleaning with solvents. (d) Holes are punched and the device is bonded to a substrate. No layer-layer bonding is required as the entire network of microchannels is replicated in a single casting step.

Prepolymer is first poured over the 3D mold and degassed until no further bubbles are observed to emerge from the smallest confined spaces (valve membrane regions) in the mold. To conserve material and to prevent leaking beneath the wafer that would complicate mold removal, a rectangular PDMS gasket is sealed to the mold substrate surrounding the pattern and filled with the prepolymer. The polymer is then cured by its normal processing conditions, modified if necessary to avoid destroying the mold. PDMS and Sifel were heat cured by baking at 60–65°C, a temperature selected to avoid melting the wax mold prior to polymer solidification. PFPE was cured in an ELC-500 UV curing chamber (Electro-Lite Corporation). Exposure for 1 min solidified the elastomer, and then the mold and PFPE were exposed for an additional 40 min with the orientation changed every 5 min (normal cure time is 10 min). A wide variety of orientations were necessary to ensure UV exposure of all PFPE regions—the opaque blue wax structures prevent UV radiation from reaching the liquid between intersecting beams (valve membranes) if only top illumination is used.

Once cured, the device is removed by melting the wax mold. Above about 110–120°C, the wax rapidly melts to a low viscosity liquid and can freely flow out of the channels. With the wax in the liquid state, the whole device can be peeled from the substrate without risk of breaking entrapped polymer pieces. We did not have any difficulty removing PDMS or PFPE from the untreated glass slide or silicon wafer used as the mold substrate. The wax remaining in the channels can be further drained by continued baking in appropriate orientations and by subsequent immersion of the device in an organic solvent such as acetone or methanol. After drying the solvent by heating, holes are punched in the cleaned device to access both the fluid and actuation channels, and the device is bonded to a substrate to seal the "floor" of any channels or support pillars that were printed directly on the mold substrate. For example, a PDMS device can be covalently bonded to a cleaned glass slide by oxygen-plasma treatment (see Appendix A.2.4). Since both fluid and control channels may be in contact with the substrate, it is necessary that the bond strength be sufficient to withstand all pressures involved during device operation.

6.4 Results

My original goal was to produce devices from solvent resistant elastomers such as PFPE, FNB, and Sifel for further material evaluation and ultimately to perform chemical synthesis. However, due to the very short supply of solvent-resistant materials from our collaborators, PDMS was used as a surrogate during development and optimization of the 3D molding procedure. We first demonstrated the compatibility of the other materials with all aspects of the basic process³ and then put them to better use investigating other methods of device fabrication in parallel.

 $^{^{3}}$ Had we found incompatibilities, we would have tried printing a negative relief version of the desired mold and casting an intermediate sacrificial material to serve as the mold for the microfluidic device. Presumably, one could also tap into the vast range of materials that have been printed with ink-jet technology [34] to find an alternative mold material.



Figure 6.5: Fabrication defects during protocol development. (a) A micrograph of a mold after support wax removal illustrates the large amount of debris (dust and stray droplets) and the highly irregular edges of channel features that can lead to merging of mold features. Designs must include extra space between channels to account for this. (b) Bottom view of a PDMS device cast from an early mold. Due to adhesion of PDMS to the mold substrate and subsequent tearing during mold removal, nearly all crossedchannel valve membranes are missing (2 are circled in red). This problem was solved by ensuring very complete VSO solvent drying after support wax removal. (c) Comparison of an intact valve (left) and a torn membrane (right) viewed from the bottom. In the intact valve, the control channel, oriented top to bottom, crosses behind the fluid channel, oriented left to right. When the membrane is damaged, the channel interiors are physically connected. (d) Micrograph of PDMS fragments on the silicon wafer after melting of the wax mold. These fragments include the missing valve membranes in c. (e) Comparison of an intact valve (left) and one with a hole through the membrane. Such smooth-edged ruptures are believed to be caused by air bubbles not removed during degassing or perhaps by defects in the mold itself due to printing artifacts. (f) Micrograph of two valves, the right one having a small chunk of PDMS missing (circled in red), thus joining the fluid channel (running diagonally from bottom left to top right) to the adjacent control channel support post. This is a less severe form of the problem in b and is solved in the same manner. (g) Image of the silicon wafer after mold removal illustrating the presence of small PDMS fragments corresponding to the missing parts in f.

This section describes a number of the mold patterns that were designed to develop the molding protocol and ultimately to demonstrate pressure-actuated microvalves.

6.4.1 Test patterns

Noticing a discrepancy between our initial design files and the printed wax mold, we designed test patterns to explore three aspects of printer performance: (A) minimum lateral gap between features; (B) minimum lateral feature size (line width); and (C) minimum reliable vertical gap between features.

Pattern A (designed by George Maltezos) consisted of a series of small blocks separated from a wall by progressively smaller distances. Visual inspection of printed molds under a stereoscope revealed that separations of less than 65 μ m in the *designed* mold resulted in merging of features in the printed wax.

Pattern B consisted of a series of short walls protruding perpendicularly from a long wall, separated from one another by gaps of 400 μ m and gradually increasing in width from 100 μ m up to 300 μ m. In the vector mode of printing, designs are printed as outlines first; thus each of the short walls was printed in at least two passes (for the outer edges). For all feature widths of 130 μ m and less, these passes completely overlap (by inspection of the ModelWorks file) and not surprisingly the printed features are roughly the same size. We observed a minimum printed line width of about 200 μ m (up to 300 μ m depending on ink-jet nozzle condition), with widths of larger features consistently in excess of the designed size by about 70 μ m. This is roughly in agreement with the minimum gap test, as it suggests that each feature overflows its designed size by at least 35 μ m on all sides. Based on the *worst case* overflow (85 μ m each side when print quality is poorest), we separated non-contacting features on later designs by at least 200 μ m in the lateral direction to ensure separation in the actual mold. The test was performed with the pattern oriented in two different directions. We observed no significant difference in minimum width between the two cases; however, there was significantly less debris trapped between walls when they protruded in a direction



Figure 6.6: Test of minimum vertical gap in wax molds. (a) 3D design of test pattern used to determine the minimum vertical gap that could be used in wax molds. The bottom channels have a series of different heights, and the top channels have a series of different clearances through which the bottom channels pass; thus many different gap thicknesses are represented in the design. During casting, a gap between channel structures on the mold becomes a polymer membrane between two empty channels. (b) Photograph of an intact membrane in a PDMS device (bottom view). (c) Top view of the design indicating the thickness (in number of 12.7 μ m layers) of the gap between each pair of crossed channels. (d) Photograph (top view) of a printed mold after removal of the support wax. (e) Composite of three photographs of the PDMS device cast from the mold (bottom view). Note that membranes are broken or missing at gaps of 4 layers or less.

Based on the method of operation of the Solidscape printer, a 1-layer vertical gap between features in a design should in principle be faithfully reproduced in the printed mold. Pattern C is an array of push-down valves designed to test this (see Figure 6.6). It consists of 5 fluid channels in one direction crossed by 6 control channels in the perpendicular direction. The fluid channels have a circular arc profile and are printed directly on the substrate. Different channels have different heights, ranging from 3 to 7 layers, where each layer is 12.7 μ m thick. From the side, control channels appeared as a series of arches spanning the fluid channels, each control channel having arches of a different height in the range of 5 to 10 layers. Vertical gaps between channels thus ranged in thickness from 1 to 7 layers. Due to the difficulty in visualizing the gap between channels in the mold and because we were interested in the minimum reliable gap thickness in actual microfluidic devices, we assessed the results of this test by inspection of a PDMS cast from the mold. Valve membranes less than 4 layers thick were missing or damaged in all cases. Some 4-layer membranes and all thicker membranes remained intact. These results suggested that subsequent designs should have at least 5 layers $(64 \ \mu m)$ of clearance between crossing structures to ensure reliable separation in the cast device. This test was performed at a time when we were still having difficulty completely drying the VSO solvent after support wax removal; thus we suspected the reason for membrane breakage was damage during the wax removal stage due to small pieces of PDMS bonded to the mold substrate. However, later devices built with our optimized fabrication protocol were consistent with these results. The cause of the missing and broken membranes is not clear. It is possible that dust and debris is trapped between the channels during printing leading to a fragile, perforated membrane in the cast device, or perhaps the degassing process is not effective and tiny air bubbles remain trapped between the channels preventing PDMS prepolymer from flowing in to form the valve membrane when casting. The presence of trapped debris may help to stabilize such air bubbles. One additional possible cause is incomplete removal of support wax; however, this is unlikely since interchannel gaps viewed from the side under a stereoscope did not reveal any remaining support wax.

6.4.2 Microvalves

Two valve architectures were designed and tested to demonstrate the capability to implement active microfluidic devices with our molding process—a tube-like valve architecture and a crossed-channel architecture.

6.4.2.1 Tube valve architecture

George Maltezos designed and successfully actuated a PDMS valve with the architecture depicted in Figure 6.7. Fluid flows through a hollow PDMS tube (much like a short piece of silicone tubing) surrounded by a chamber of air. The tube has a tall narrow hexagonal cross-section that is pinched shut when the surrounding air chamber is pressurized, thus closing the valve and blocking fluid flow. The length of the tube was typically 1–2 mm, and the designed thickness of the tube walls was typically 100 μ m, resulting in less than 50 μ m walls in the cast part.



Figure 6.7: **3D** tube valve. (a,b) Design drawing (hidden line view and shaded view) for the mold for a 3D microfluidic tube valve. The central bar becomes the fluid channel in the cast device while the gap between this bar and the outer structure becomes the polymer wall of this channel. The outer structure becomes a hollow air chamber that is pressurized to close the valve. (c) Photograph of valve cast in PFPE. The roughness of the bottom surface is due to the use of a wax slab substrate for this particular mold. (d) Same valve with fluid channel filled with methanol (dyed blue with xylene cyanol FF). (e) Mechanism of valve operation. A cross-section of the tube inside the air chamber is shown. When the chamber is pressurized, the tube is squeezed shut to block the flow.

6.4.2.2 Crossed-channel valve architecture

Having proved that devices with functional valves could be fabricated via 3D wax molding, we sought to demonstrate a crossed-channel valve to achieve a smaller valve footprint and to take advantage of the higher accuracy of the wax printer in the vertical direction to better control the thickness of the deflectable valve membrane. One additional difficulty with the tube valve design is the difficulty in curing *photo* polymers within the small gap that ultimately forms the tube wall. A crossed-channel valve has less hidden material. For our tests, a push-down architecture was selected as, at the time, PFPE was not able to withstand the large deflections required in a push-up device.

An initial value test pattern was designed by making several modifications to the layer thickness test described above. Fluid channel depths were all increased to 100 μ m (8 layers) to decrease the relative jaggedness of rounded profiles. If more typical PDMS channel depths (10–50 μ m) were used, channel molds would be printed with just 1–4 layers, resulting in only a very crude approximation to a curved upper surface. Due to the uncertainty of the effects of jaggedness on valve performance, five different cross-sectional profiles were investigated in this design—one fluid channel was rectangular, three were trapezoidal, and one was bell-shaped. The latter has been shown theoretically to be the optimal shape in terms of minimal closing force [85]. Channel widths were increased to 300 μ m in the design (thus nearly 400 μ m in the actual device) to avoid the aspect ratio being too high. Control channels were supported on vertical posts such that when viewed from the side, they had a rectangular opening where they crossed fluid channels. Since we had been having problems with small pieces of PDMS being torn from the device at the edges of posts during mold removal, fluid channel spacing was increased and control channel posts were designed to be 400 μ m away from fluid channels. The six control channels crossed at different heights, such that vertical gaps (valve membrane thicknesses) ranged from 2 to 12 layers in 2-layer increments.

The printed mold and PDMS devices cast from the mold are shown in Figures 6.8 and 6.9. For redundancy, three copies of the pattern were printed on each mold—two at the designed size and one at twice this size. PDMS devices were cast on the molds and oxygen plasma bonded to cleaned glass slides. Numerous leaks prevented valves from being properly pressurized in all devices; however, we observed partial membrane deflection at 25 psi in one device. Despite this failure, several interesting observations could be made regarding the molds and PDMS devices. First, inspection of the devices confirmed the results of the thin-layer test, in that all valves with 2-layer membranes were broken, while some 4-layer membranes and all thicker membranes were intact. Curiously, on the double-sized mold, some of the valves with an 8-layer membrane had broken membranes. If failed membranes are caused by air bubbles, this result may suggest that degassing depends not only the gap thickness but also the gap width. Second, examination of molds under a microscope revealed a peculiar artifact: the fluid channels were not uniform along their length. Rather, they undulated in width and height, becoming largest when passing under control channels and smaller in regions in between. There is no evidence of this in the ModelWorks file, so it is unclear how this effect arises. Perhaps it is related to the failure of valve membranes less than 4 layers thick. Third, the large number of fluid channels that were merged with control channel posts, due to missing chunks of PDMS, indicated that the lateral spacing of 400–800 μm was not always sufficient to prevent such leaks. However, subsequent improvement in the wax removal procedure solved this problem, obviating the need for a change in design rules for the next design iteration. One final observation was the presence of thin PDMS flaps covering parts of fluid channels and control channel posts at the bottom surface of the cast device. These areas should be open since the wax features from which they are cast are in contact with the mold substrate. This artifact therefore indicates that the liquid PDMS prepolymer is sometimes able to flow underneath wax structures attached to the mold substrate. It is not known why this occurs—perhaps the wax-substrate adhesion is relatively poor, or surface tension forces dislodge features during immersion and removal of the mold from the VSO solvent, during evaporation of VSO, or during pouring or degassing of the PDMS prepolymer. For the most part, these flaps were not problematic since the bottom surface of the device was intended to be sealed anyway by bonding to a glass slide. However, they did occasionally interfere with bonding if they folded over the bottom surface, locally lifting the device from the substrate. The spurious flaps would also interfere if one wanted to perform *in situ* chemical synthesis on a derivatized glass surface, for example.

To deal with the problem of leaks and to facilitate valve testing, the design was again modified. Valve architecture was maintained, but the height and width of fluid channels was reduced to $65 \ \mu m$ and 200 μm (actual size), respectively. In addition, the layout was simplified to have only a single line of valves. The control channel was broken into short segments, isolating valves such that failure of one would not prevent all others from being pressurized. Multiple identical valves were included in case some failed. After observing many of the control channel segments fall off of the glass mold substrate during removal of support wax, the segments were enlarged to increase their surface



300 µm

Figure 6.8: **Design and printed molds for crossed-channel valve tests**. (a) Design of the valve array test chip. Six control channels (gold) cross five fluid channels (blue). Control channel gaps become progressively larger, from 2 layers (25 μ m) to 12 layers (152 μ m). (b) End view of the design. All fluid channels are 100 μ m tall but have different cross-sectional profiles. Note that all of the following photographs reflect molds printed with a very similar, but not identical, design—fluid channels are spaced more closely together. (c) Photograph of 3 molds printed on a silicon wafer. The largest was printed at twice the designed size. (d) Composite micrograph of the large mold (top view). Detail of a few channels is shown in the inset. (e) Tilted end view of the same mold, showing different channel profiles and the gradually decreasing gap thicknesses. (f) Photograph of one of the small molds (top view), scaled up for comparison with d, with detail shown in the inset. Since features on the small mold are close to the minimum feature size of the printer, printing artifacts are more prominent (debris and undulating fluid channel widths). (g) Tiled end view of the same mold. (h) Series of micrographs of the large mold illustrating gap sizes from 4 to 24 layers, in increments of 4 layers. (Same scale bar for all images.)



Figure 6.9: **PDMS devices cast from 3D crossed-channel valve test mold**. (a) Composite micrograph of PDMS device cast from large (double-sized) mold. Inset shows the detail of a valve viewed from below (bar: 400 μ m). The fluid channel is oriented top to bottom, while the control channel is oriented left to right. (b) Micrograph of PDMS device cast from normal-sized mold, at twice the magnification in *a*. Detail of a valve is shown in the inset (bar: 400 μ m). Note the pronounced non-uniformity in fluid channel width. (c) Tilted views of the large device from below (top) and above (bottom). (d) Micrograph of normalsized device from above. Fluid channels are filled with water dyed blue with xylene cyanol FF. (e) Bottom view through the glass substrate of a single valve in the unpressurized (top, 0 psi) and pressurized (bottom, 25 psi) configurations. The fluid channel is oriented left to right, and the control channel is oriented top to bottom. When pressurized, the entire control channel expands, and at the crossing, the membrane bulges into the fluid channel, squeezing it towards the glass and partially blocking the flow.

contact and ended up looking like "H"s. (The fact that the segments fell off may be indicative of poor wax-substrate adhesion and could explain how the PDMS is able to leak beneath features.) This design, along with corresponding molds and devices, is illustrated in Figure 6.10.

To operate the valves, control channels ("H" structures) were filled with mineral oil and pressurized. If air was used, production of bubbles was observed in the fluid channel, resulting from diffusion across the valve membrane. Water (dved blue with xylene cyanol FF) was introduced into the fluid channel at a fixed low pressure, typically 0-5 psi. Because the fluid is a better refractive index match to the PDMS than air, the surface roughness does not so severely obscure the valve, and its state can be observed visually. In one experiment, the valve was successfully closed at 27 psi, though there remained a significant leak flow rate, observed by watching the meniscus of the fluid move through the external tubing over a period of several hours. We could increase the fluid pressure to about 9-10 psi before the valve was forced open and the leak flow rate suddenly increased. In another experiment with a different PDMS device, we observed that 8 psi fluid pressure forced open a valve pressurized to 30 psi. Incomplete valve closure was presumably due to the approximately square profile of the fluid channel, which is difficult to close completely in any device, and due to the roughness (2–3 μ m bumps) of the top of the fluid channel due to the wax mold. The latter is the same effect that prevents the whole device from being sealed to a substrate, if the mold is printed on a wax slab support. The leak rate was quite slow at 30 psi control channel pressure. It may be possible to achieve more complete actuation simply by further increasing the pressure. Such over-pressure can also be achieved at the same external pressure by decreasing the valve membrane thickness. It may also be possible to improve valve sealing by decreasing the roughness of the channel features on molds. We attempted to achieve this by heating near the melting point; however, structures sagged and roughness was not decreased. Another attempt—prolonged exposure of molds to a solvent vapour (acetone)—resulted in significantly *increased* roughness. Lastly, we attempted to perform smoothing during mold fabrication by pressing a heated flat surface against the pattern after each milling step. Technically, this needs only to be done after the layer in which the uppermost part of the fluid channel is printed; however, smoothing all layers would improve



Figure 6.10: Design and testing of "H" valve. (a) Design drawing of the pattern of "H" valves. Each "H" is a short segment of a control channel crossing a fluid channel, as shown in the detailed inset. (b) Photograph of PDMS cast on a mold printed on a 2×3 inch glass microscope slide. (c) Photograph of same device after wax mold removal, hole punching, and plasma bonding to a 1×3 inch cleaned glass slide. (d) Micrograph of device showing several "H" valves and holes punched to access the channels. A closeup of two valves from above is shown in (e). In each, the path of the fluid channel is faintly visible under the center of the "H". (f) Bottom view of several valves. The fluid channels are printed at the minimum feature width and therefore show considerable irregularity due to individual droplet effects. (g) Micrograph of a single valve, taken through the glass substrate. The fluid channel runs left to right and is sealed by the glass, as are the sides of the "H". The central region is the valve—the control channel (oriented top to bottom) expands when pressurized and flattens the fluid channel against the glass to close the valve. The high surface roughness of the fluid channel surface is evident. (h) Single valve in the open configuration. The blue fluid is water dyed with xylene cyanol FF at 1 psi. The control channel ("H") is filled with mineral oil. (Same scale as q.) (i) The same value in the closed configuration with the mineral oil pressurized to 27 psi. Flow in the fluid channel is stopped. The circles are air bubbles in the mineral oil that disappeared about 30 minutes later.

visibility through the device. Unfortunately, using a silicon wafer and small weight heated to 60°C significantly damaged the support wax in the mold and had no affect on the build wax surface.

Additional modifications were also made to improve valve closure. We attempted printing shallower fluid channels (1–3 layers) but observed channels to be collapsed shut after plasma bonding. We have also fabricated devices with a quasi-rounded channel profile and with a push-up valve architecture. The push-up architecture not only gives potentially reduced actuation pressures, but the rough surface of the valve membrane should seal better to another PDMS surface than to glass [187]. However, only a marginal decrease in leak rate was observed in these push-up valve devices. An additional strategy is to first pattern the mold with rounded channels for the fluid channels by some other means (e.g., with photoresist) and then to print the suspended wax structures for the control channels on top of this. To attempt this will first require devising a means to align the printhead with the photoresist pattern.

The crossed-channel valve design is essentially the same as that used in multilayer PDMS microfluidic devices cast from photoresist molds. Once satisfactory valve fabrication and operation are achieved, this technology should therefore be suitable for any applications in which 2-layer architectures are already used. For example, Figure 6.11 shows a design for a 4×4 combinatorial array synthesizer (see Chapter 7) along with molds and devices that were fabricated. These particular devices were non-functional due to this early design not conforming to the design rules we later developed, but they give an approximate sense of the possible valve densities and device complexities.

6.4.3 Fully suspended structures

As described above, the polymer cast of a three-dimensional inverse channel network mold is not a finished microfluidic device—an adhesion step is still necessary to bond this polymer to a suitable flat substrate. This step seals the "floor" of all channel and support structures that are open at the bottom surface because the corresponding mold features were in direct contact with the mold substrate.



Figure 6.11: Microfluidic device for 4×4 combinatorial array synthesis. (a) 3D design for the central component of a microfluidic combinatorial array synthesizer (see Chapter 7). A grid of fluid channels (blue) is crossed by two sets (red and gold) of control channels in perpendicular directions. Actuating one set of control channels closes off all flow in one direction, forcing fluid flow through the device along 4 parallel fluid channels in the perpendicular direction. Carefully orchestrated delivery of reagents in fluid channels combined with alternation of flow direction allows for combinatorial synthesis of an array of compounds. (b) Overview of the entire design including posts that become inlet/outlet holes in the final device. Posts were later eliminated from the design due to their long mold fabrication time (many layers) and due to difficulties fabricating tall narrow structures. (c) Photograph (after support wax removal) of a wax mold with this design printed at double size on a silicon wafer. A detailed micrograph of the area inside the red box is shown in the inset. (d) Photograph of PDMS cured on the mold. (e) Photograph of PDMS device after melting and dissolving the wax mold.

In comparison with multilaver fabrication of PDMS microfluidic devices, we have demonstrated that three-dimensional molding eliminates the bonding step between device layers. In principle, it is also possible to eliminate the device to substrate bonding step as well. Microfluidic devices require inlet and outlet holes to connect to the outside world. On the mold, these could be represented by solid posts. With a sufficient number of carefully spaced posts, one could imagine fabricating the mold upside-down, supported entirely by these inlet and outlet posts. (Imagine an upside-down version of Figure 6.11b.) A thick polymer layer could be cast to completely encapsulate such a mold. thus forming a completely enclosed fluidic network after mold removal. The channel network must be carefully routed such that all beams (inverse channels) can be fully supported by posts without sagging. Sagging will result in altered channel shapes and, for crossed-channel valves, will affect the spacing between the fluid and actuation channels, resulting in unpredictable valve membrane thickness (and hence actuation pressure). Control channels pose a particularly difficult challenge since in multilayer PDMS devices these are typically implemented in a dead-end fashion with only one inlet and no outlet. Suspending an entire inverse control channel by a single post will be impossible in general; however, one could insert one or more extra posts into the design for mold fabrication and then plug these extraneous inlets in the final microfluidic device to allow the channels to be pressurized.

To reduce the possible adverse impacts of sagging, an alternative valve architecture could also be considered. A tall thin channel could possibly be actuated from one side [269], or a tube architecture could be used. In such designs, the critical dimension is in the lateral direction, and the vertical alignment is less critical.

I created several 3D designs to evaluate the ability of various channel cross-sections to avoid distortion when spanning long distances. However, the milling head of the Solidscape printer tends to topple tall thin posts and break long thin structures during printing if they are not attached to the mold substrate, and these patterns were never successfully fabricated. Since, by this time, bonding isues in solvent-resistant polymers had been resolved, efforts in this direction were suspended, and attention was focussed on development of working valves.

6.5 Discussion

6.5.1 Summary

In summary, we developed a method to fabricate microfluidic devices by replication molding in a single step from 3D wax molds using a commercial rapid prototyping machine. After numerous iterations of device designs and protocol modifications, we demonstrated devices having functional microvalves—both a tube architecture and the crossed-channel architecture commonly used in multi-layer PDMS devices—as a proof of principle. We also showed that other, solvent-resistant, polymers (PFPE and Sifel) are compatible with this technique.

Our 3D molding technique offers several significant advantages when compared with other fabrication methods. Fabrication is simplified as the mold itself is printed entirely automatically, and microfluidic device construction requires no alignment or layer-bonding steps. Elimination of layer bonding enables accelerated exploration of new elastomer materials, as valve performance can be evalutated to screen materials before undergoing the lengthy process of developing and optimizing a layer-bonding protocol. Compared with stereolithography, a much wider variety of device materials can be used since there is no requirement for photosensitivity or transparency. 3D molding also makes it very simple to implement topologically complex fluidic networks, many layers of valve control channels, or geometrically complex fluidic and optical structures.

There are a few drawbacks as well, perhaps the most serious at this time being the printing resolution. We found practical lower bounds of 200–250 μ m in channel width, 400 μ m in channel spacing, 4–5 layers (51–64 μ m) in valve membrane thickness to avoid breaks and leaks, and about 3–4 layers (38–51 μ m) in channel depth to avoid collapse due to the large width. There is probably some room for improvement of the ink-jet technology itself, perhaps by switching to other printing materials, as droplet sizes down to 20–30 μ m droplets have been demonstrated with other fluids, and sizes down to about 10 μ m are thought to be possible [34]. Pushing past 10 μ m has only been possible by lithographically patterning the substrate surface prior to printing, a process not suitable for three-dimensional objects since it only affects the first printed layer. To reduce the long printing time that

would be associated with such high-resolution printing, hybrid droplet schemes have been considered in which the outer edges are printed slowly with very tiny droplets while the internal regions are filled more quickly using much larger droplets. Resolution in the z-direction can presumably also be improved with the use of higher precision motors on the build platform. However, surface roughness and incomplete dust and debris removal will have to be addressed before additional resolution would be useful. An additional drawback is that sacrificial molds cannot be reused. Though printing and dewaxing a 3D mold of 1–2 mm height takes no longer than photolithographically patterning 2D molds for multilayer devices, the average microfluidic device fabrication time is much shorter for multilayer devices since a 2D mold can be reused many times. Average 3D mold fabrication time can be reduced by printing batches of multiple molds on the 6-inch build platform, as additional molds do not incur additional wax-cooling or milling time.

Many techniques exist for constructing three-dimensional microfluidic devices, each having particular capabilities and limitations, as reviewed in Section 6.2. As with any technology, one must weigh the benefits and drawbacks in the context of a particular application and choose accordingly among alternative fabrication methods.

6.5.2 The future of 3D fabrication

3D fabrication is inherently more complicated than 2D fabrication, and it is worthwhile to consider when the additional complexity is warranted. Indeed, for relatively simple assays and reactions, two dimensions are adequate, as several commercial products and the huge volume of literature illustrate. However, the third dimension can be exploited in a number of useful ways, sometimes enabling applications that would otherwise be impossible. I have already discussed the benefits of using 3D fabrication to eliminate layer-layer bonding steps in microfluidic devices. This section will elaborate on its other uses.

In Chapter 2, I discussed the many advantages of crossed-channel elastomeric valves over alternatives for fluid manipulation. These microvalves require 3D fabrication to implement an independent layer of control channels a small distance above or below the fluid channel network. The control channels provide the actuation mechanism of microvalves and the connections between these valves and ports that connect to off-chip pressure supplies. Sophisticated control of fluids has been demonstrated, incorporating components such as multiplexers to help reduce the number of off-chip connections required and improve scalability [268, 274]. Additional layers of control can provide additional flexibility and further reductions in number of connections to the outside world. For example, Thorsen et al. [268] demonstrated an individually addressable array, in which $N \times M$ chambers could be selectively purged using only one fluid input, one fluid output, and $\log(N) + \log(M)$ control inputs. This is clearly far more practical than having one control input per chamber or even one input for every row and column. To permit efficient addressing of a chamber by its row and column, two multiplexers were used: one acted on fluid channels to direct fluid from a single inlet to the selected row: the other acted on column control channels to select a single column of valves that would be opened. The more "processing" that can be performed on-chip, the fewer external control connections are required. Additional layers afforded by 3D fabrication could provide additional space for routing channels in dense networks or enable more complex control schemes (see Figure 6.12). Several interesting control schemes have also been reported that take advantage of three dimensions, including tangential channel microfluidic switches that can be dynamically reconfigured using air pressure [126].

The third dimension has also proven useful in expanding the topological flexibility of fluidic networks by allowing fluid channels to cross over one another. This flexibility has been used to perform combinatorial chemistry [148], to solve graph theory problems in computer science [41], and to pattern proteins and cells on surfaces in complex arrangements [40]. Microfluidic devices have been used extensively in cell culture studies (see [164] for a review), largely in the areas of evaluating drug effects and tissue engineering (growth and repair of tissues). Microfluidic devices have been used to create mimics of spatially organized biological tissues, such as *in vitro* mimics of blood vessel walls consisting of three layers of different cell types [264, 263]. Sophisticated 3D devices have also been used for establishing precisely controlled microenvironments (substrate topology and composition, type and position of neighbouring cells, etc.) to study cellular responses such as


Figure 6.12: Schematic of controlled control channel operation. Additional channel layers enable new types of fluid control. For example, with three layers of channels, one can control not only the fluid channels, but also the control channels. Blue represents fluid channels in the bottom layer; red represents hydraulic control channels in the second layer; green represents hydraulic control channels in the third layer. So that the green channel can control the red one, it must use a higher pressure or some kind of force amplifier [2, 10]. Control channel inputs are designated by small circles. The valve controlling the fluid channel is encircled by a dotted line: an arrow along the fluid channel indicates it is open; an X indicates it is closed. (a,b) A state-preserving control. Actuation of control channel C2 locks the valve in the last state of control channel C1. In a, C1 is initially pressurized and the valve is closed (top). When C2 is pressurized, the pressurized fluid in the rightmost segment of channel C1 is trapped (middle), such that the valve remains closed even if C1 is later released (bottom). Similarly, b shows the operation if C1 is initially unpressurized (top). When C2 is pressurized (middle), it blocks C1. Even if C1 is subsequently activated, the pressurized fluid cannot reach the valve and the fluid channel remains open, thus preserving the initial state prior to C2 activation. (c) A boolean "AND" control. In this arrangement, the valve is closed only if both control inputs (C1 and C2) are pressurized. C1 contains an open outlet port at right so that it is impossible to build up pressure in the channel. Only by also activating C2 is the outlet blocked, allowing sufficient pressure to build up inside channel C1 and close the fluid channel valve. Although not shown, if C1 is not activated, the valve remains open regardless of the state of C2. It may also be possible to construct an AND valve simply by having two control channels stacked above one another.

migration and remodeling [151]. Microfluidic devices have also been used to create and study living neural networks with controlled 2D architectures [108], and there is no reason to believe such studies couldn't be extended to 3D networks.

Certain microfluidic processes and devices rely on 3D geometrical variations for their efficient operation. For example, rapid mixing in the diffusion-limited turbulence-free laminar flow regime requires some technique to rapidly fold and elongate the fluid to reduce the diffusion distance. Chaotic advection has been used in passive mixers consisting of serpentine channels with flow alternating between perpendicular planes [171] or channels with a staggered groove pattern in one of the channel walls [258]. (The latter can also perform additional novel functions such as controlling plug dispersion and positioning narrow streams within a channel [257].) In addition to these passive designs, active mixers have been demonstrated [35], including a rotary mixer utilizing three microvalves (in a second channel layer) as a peristaltic pump [43]. 3D fabrication also offers flexibility in the design of other components such as filters for removing particular contaminants [62], or traps for beads (to perform separations or solid phase synthesis) or for biological cells. In our lab, we have demonstrated active filters/traps consisting of partially closed valves, with the unique feature that the "pore" size can be adjusted or removed by controlling the valve pressure. 3D fabrication techniques have also proven useful to fabricate channels with unprecedented aspect ratios and long lengths for rapid mass or heat exchange [181].

In the area of integration of microfluidics with electronics and optics, 3D microfabrication technologies have been used to fabricate fluidic networks in place on top of silicon circuits, eliminating the need for alignment and bonding steps, potentially decreasing manufacturing time and cost [151]. Stereolithography and other techniques may even permit *in situ* fabrication of complex shapes such as external fluidic connectors (possibly macroscopic) or receptacles for aligning optical fibers. Mizukami *et al.* [197] report the integration of a stereolithographically fabricated serpentine acrylic channel network onto a photosensor array microchip for real-time imaging of separations. Similarly, Tse *et al.* [271] reported a technique for stereolithographically fabricating a plastic microfluidic flow cell directly on top of a silicon microelectronic chemical impedence sensor. 3D fabrication may also benefit the emerging field of optofluidics, permitting construction of complex fluid-filled optical elements.

Finally, 3D fabrication may be exploited simply to increase chip densities as it enables vertical stacking of components and provides more space for routing interconnections. Expanding into the third dimension also provides additional space for large reactors, for example, without using up all the chip real estate.

For these, and undoubtedly many currently unimagined reasons, it is likely that 3D fabrication will play an increasing role in microfluidics as device complexity increases and as devices are applied to an ever-increasing range of applications.

Acknowledgment

This molding approach was first developed by George Maltezos in Axel Scherer's group at Caltech. I thank him for sharing the results of his original work, for helping to improve the device fabrication protocol and microfluidic device designs to the point where devices could be properly sealed to flat substrates and crossed-channel valves could be demonstrated, and also for taking the time to print numerous 3D wax molds for my work. I also acknowledge the assistance of Jaime Perez, formerly of Solidscape, and other Solidscape engineers in modifying the printing process to accommodate our needs.

Chapter 7

Microfluidic Combinatorial Chemistry

7.1 Introduction

Though one can imagine many applications for solvent-resistant microfluidic devices, combinatorial chemistry stands out as particularly suitable due to its need for high integration density as well as chemical inertness. Combinatorial chemistry is a powerful strategy for discovering new chemical substances. It is basically a brute force search strategy in which vast libraries of compounds are randomly or systematically created, then screened for desirable properties. This approach has been used for a wide variety of purposes including the discovery of new drugs, catalysts, and materials. A brief history and introduction to the field is given by DeLue [56].

Depending on the application, the library may consist of molecules ("probes") tethered to a flat substrate in an array format, molecules in solution in individual wells of microtiter plates, or molecules pooled together (in solution or tethered to beads). High throughput screening is necessary to evaluate large libraries of substances in a reasonable amount of time. Arrays are particularly suitable for performing high-throughput screens due to the ease of deconvolution—that is, once a positive result is detected, the successful molecule can be identified immediately by its position on the array rather than some more elaborate method to determine its identity. In "pool" libraries, deconvolution may be achieved by a tedious omission library approach, or molecules may be tagged during synthesis for instant identification. Arrays are also useful for collecting measurements for all arrayed substances in parallel under identical experimental conditions. In screens for binding affinity, the level of binding of each probe to a target may be detected by a wide variety of target-labelling schemes such as fluorescence or radioactivity, or other detectable property. Interactions have also been detected by label-free methods such as surface plasmon resonance or atomic force microscopy (AFM). Screens for enzyme activity often involve some detectable transformation of the probes by the analyte, or the localized generation of heat. Numerous ingeneous methods have been reported to screen for other desirable probe properties. Yet, the difficulty in developing screens has hindered the use of combinatorial chemistry in many areas [56].

To investigate some novel approaches for performing combinatorial synthesis and high throughput screening in microfluidic devices, we explored solid-phase synthesis of arrays of biopolymers such as DNA and peptides. DNA arrays have emerged recently for high-throughput analysis of gene expression at the whole-genome level [238] to determine gene function, mechanisms of disease or genetic disorders, and biological response to infection, drugs, or environmental toxins. Gene expression studies are generally *targeted* and contain only selected probes of interest, though the use of true combinatorial arrays (containing all possible DNA sequences of a certain length) could in principle provide many benefits (see Chapter 8). Some additional uses of targeted arrays include discovery of splice variants and polymorphisms [199], genotyping [204], discovery and analysis of transcription factors or other DNA-binding proteins [251], and characterization of the methylation state of the genome. Combinatorial arrays have been used for sequencing by hybridization [283], sequence "fingerprinting" [246], and studying the physics and specificity of DNA duplex formation [194, 248], among other applications. Many excellent reviews on DNA array applications have been published [174, 198].

Similarly, peptide arrays have been developed to enable high throughput studies of protein interactions. Arrays of whole proteins have also been studied, but short peptides can often capture the full functionality of the whole protein [80, 193], without suffering from problems related to degradation and misfolding. Combinatorial peptide arrays have been used to identify and map the sites of interaction between proteins, most commonly to determine the epitopes of antibodies and to determine the substrate specificity of enzymes such as kinases. They have also been used in metal-binding assays. *Targeted* peptide arrays and protein arrays have been used for a huge variety of applications such as: (i) protein expression profiling, (ii) screening for and studying protein-protein, protein-DNA, protein-drug, receptor-ligand, enzyme-substrate, etc. interactions, (iii) identifying posttranslational modifications and splice variants of proteins, (iv) determining the location of protein expression (intracellular or secreted), (v) studying mechanisms of diseases and disorders [228], and (vi) identifying secreted biological markers that may be used in diagnostic tests to screen for problems. Protein and peptide arrays have been reviewed extensively [72, 193, 80, 226, 236].

To synthesize arrays of specific compounds or combinatorial sets of compounds, we propose the use of microfluidic devices with dense networks of microvalves to reconfigure flow paths. These devices offer many advantages compared to alternative approaches such as ink-jet printing, robotic deposition, and light-directed synthesis. Microfluidic array synthesis uses conventional (optimized) reagent sets, can operate in a highly parallel fashion, and can potentially achieve very small feature sizes and therefore high densities of surface-bound products.

I begin this chapter with a brief review of the general principles of solid-phase synthesis and the chemistry of DNA and peptide synthesis. Current methods for array synthesis are described next, providing a context in which to argue the principles and advantages of microfluidic array synthesis. The ideas presented here are not specific to DNA and peptide arrays but could be extended to arrays of other biopolymers such as RNA, PNA, oligosaccharides, etc. or arrays of small molecules such as drugs. Subsequently, I report results of experiments applying the microfluidic approach to the *in situ* synthesis of DNA and peptide arrays. In the final section, I discuss microfluidic device designs for synthesis on trapped solid-support beads rather than on flat surfaces. Bead synthesis can give large quantities of products and can be used in situations where direct synthesis on a flat substrate is not practical.

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7.2 Introduction to solid-phase synthesis

Before describing methods for making combinatorial arrays, it is useful to review the principle of solid-phase synthesis. Synthesis begins at the end of a linker molecule attached to a solid surface. Building blocks are added one at a time to synthesize the desired molecule as depicted in Figure 7.1. The whole set of reactions needed to add a single building block is known as a "cycle". Solid-phase synthesis is particularly useful for long multi-step syntheses and is an easily automated technique. Because products are covalently tethered to the support, reagents from previous steps can easily be thoroughly washed away before continuing with the next step. However, this also requires that the reactions have nearly quantitative yield as tethered molecules that fail to react cannot be removed. Large excesses of reagents are frequently used to ensure rapid, high-yield reactions. The role of the linker molecule is both to tether the product and to distance the product from the substrate, as reactions (and subsequent assays) are often sterically hindered near surfaces [163, 184, 241, 250, 200].

Biopolymers including DNA (built from nucleotides), RNA, PNA, peptides (built from amino acids), and oligosaccharides are frequently synthesized by this method. Standard libraries of protected building blocks also exist for other classes of molecules. Building blocks need not be linear: they can contain multiple reaction sites to build branched and cyclic molecules.

When synthesizing a single compound, the solid support usually consists of tiny beads of controlledpore glass (CPG) or swellable polymer resin to enhance the surface area in contact with solution. The beads are trapped in a fritted chamber that allows reagents to be flushed through for each reaction step. Typically the product is cleaved from the substrate during the last step of synthesis. Combinatorial arrays of many tethered compounds, on the other hand, are generated by confining each synthesis reaction to a small region of a planar solid support such as a derivatized glass slide or silicon wafer. This can be achieved by physical masking, which only allows reagents to access the solid support in selected regions, or by another means such as masking the region of light-exposure in a photo-sensitive chemistry step. Many different compounds are synthesized in distinct regions on the same substrate and remain tethered even after the final deprotection.



Figure 7.1: Schematic of solid-phase synthesis. The desired molecules are built up one building block at a time. Each building block contains a terminal protecting group that ensures only a single building block can be attached in a given step. Building blocks also contain side-chain protecting groups that prevent other functional groups in the building block from reacting during synthesis. Synthesis proceeds in a cyclical fashion by the following steps: (1) removing the terminal protecting group; (2) coupling a new building block at the newly opened site; and (3) optionally capping the small percentage of molecules for which coupling was unsuccessful. Capping prevents those molecules from being extended in a later step; otherwise incomplete reactions would lead to "deletion" sequences in addition to truncations (due to capping). Finally, after all building blocks are assembled, all the protecting groups (side chain protecting groups and the terminal protecting group) are removed (step 4). Depending on the application, the product remains affixed to the solid support, or it may be cleaved off.

7.2.1 Cycle efficiency

In any synthesis reaction, a certain fraction of molecules do not react. For solid-phase synthesis, it is common to refer to an overall cycle efficiency, representing the average fraction of desired molecules that react with the new monomer during a complete cycle. When synthesizing polymers (e.g., DNA and peptides), the fraction of desired full-length sequences at the end of n synthesis cycles is E^n , where E is the cycle efficiency.

Imperfect synthesis results in the production of a "distribution" of sequences, including molecules with the desired full-length sequence as well as shorter, truncated molecules whose growth was terminated by an earlier capping step. In array applications, where the products remain covalently bound to the substrate, it is impossible to remove the erroneous molecules, and thus it is especially desirable to maximize the cycle efficiency—otherwise, the results of assays can be difficult to interpret.

Failures in the *coupling* reaction leave deprotected endgroups on molecules, but these can be reacted with a capping agent to immediately terminate the sequence. Typically the capping step is designed to have very high efficiency and can be considered to go to completion. Thus coupling failures lead to truncation errors, in which the erroneous sequences are subsequences of the desired sequence. In DNA hybridization experiments, such sequences can result in a slight loss of specificity: the truncated sequences are able to hybridize to targets that are similar to the desired target, though the binding is weaker due to the shorter sequence length. On the other hand, failures in the *deprotection* reaction can lead to "deletion sequences". When deprotection fails, the molecule will be unable to incorporate the monomer at the subsequent coupling step. Because it looks chemically identical to molecules that were successfully deprotected and coupled (or molecules that were not intended to be deprotected), there is no way it can be identified and terminated. It is likely that the molecule will be successfully deprotected during a later cycle and synthesis will resume. If this occurs, the molecule will contain a "deletion" where incorporation of the monomer failed. In DNA hybridizations, deletion sequences may bind to completely different target molecules, or alter the secondary structure of the probe in the bound or unbound state, leading to fundamental differences in hybridization kinetics. In addition, the majority of deletion sequences will have length n-1and will thus be capable of forming relatively stable duplexes with the "wrong" targets, further complicating the interpretation of assay results. Truncated sequences, on the other hand, have little impact because they are much shorter on average and do not exhibit significant binding to targets.

7.2.2 DNA synthesis chemistry

Unlike enzymatic DNA synthesis, which requires a pre-existing template in order to make new DNA, chemical synthesis methods can generate single-stranded DNA from scratch. The most prevalent chemistry, involving phosphoramidites, has been highly optimized for use in commercial DNA synthesizers over the many decades since its inception in the early 1980s [185]. A history of the development of the chemistry can be found in Reference [109] and details of practice can be found in References [86, 15, 6].

Synthesis of a desired sequence is achieved by coupling protected phosphoramidite nucleosides one at a time to a growing strand. Each nucleoside is added by a four-step room-temperature reaction cycle consisting of deblocking, coupling, capping, and oxidation steps as depicted in Figure 7.2. First, a detritylation reaction is performed to remove the dimethoxytrityl (DMT) group that serves as the terminal protecting group. This is accomplished with trichloroacetic acid (TCA) in dichloromethane (DCM). Next, a new DMT-protected nucleoside phosphoramidite is coupled to the end of the DNA molecule. The nucleoside is dissolved in dry acetonitrile, with tetrazole added to activate the phosphorus linkage, which binds to the active hydroxyl group exposed by the previous detritylation reaction. A capping reaction is performed next with acetic anhydride and N-methylimidazole in tetrahydrofuran (THF) to acetylate any unreacted hydroxyl groups. Finally, the newly formed phosphite linkage is oxidized to a more stable phosphate linkage with a solution of dilute iodine in water, pyridine, and THF. The desired oligonucleotide is built by repeating the cycle to couple the desired nucleosides in sequence. Synthesis proceeds in the $3' \rightarrow 5'$ direction, though, with modified reagents, the other direction is possible [3]. Several companies distribute pre-mixed reagents for each step of the synthesis cycle. Dry acetonitrile is used as a wash solvent.



Figure 7.2: Chemistry of DNA synthesis. A DNA synthesis cycle begins by removing the dimethyoxytrityl (DMT) protecting group on the previous nucleoside in the molecule being synthesized, leaving an active hydroxyl (OH) group at the 5' position. A DMT-protected activated phosphoramidite nucleoside is coupled by the phosphorus at its 3' position to this hydroxyl group, extending the chain by one. Synthesis thus proceeds in the 3' to 5' direction, with the 3' end tethered to the solid support. The newly formed products are stabilized by oxidizing the phophite linkage to a phosphate linkage, and unreacted molecules are capped by acetylating their hydroxyl groups. For each additional reaction cycle to extend the DNA molecule, the DMT group must first be removed from the previous nucleoside added. (Reproduced from http://www.abrf.org/JBT/2000/September00/sep00bintzler.html. Copyright the Association of Biomolecular Resource Facilities, 2000.)

In standard phosphoramidite chemistry, all steps have extremely high efficiency and are nearly quantitative. The cycle efficiency is limited by the coupling step. With standard nucleotides, coupling efficiencies are often 98–99.5%, though with modified bases (including spacers, amine linkers, fluorescent dyes, etc.), efficiencies can be somewhat lower. Note that the coupling reagents (phosphoramidites and activator) are extremely moisture sensitive so synthesis must use dry reagents and must often be performed in an inert environment to ensure high yields. Coupling efficiency can be monitored by measuring the optical absorbance of the deprotection solution, which contains the cleaved, orange-coloured DMT ion. Some commercial synthesizers are equipped to monitor this in real-time to give an estimate of the efficiency of the previous coupling step.

After synthesis, the cyanoethyl and other side-chain protecting groups must be removed from the synthesized DNA. When synthesizing a single DNA sequence (e.g., in a commercial oligonucleotide synthesizer), this is typically achieved by incubating the solid support material in 30% ammonium hydroxide for 1–2 h at 65°C. The solid support material is supplied with the first nucleotide already attached via a linkage that is cleavable under these same conditions; thus, this reaction simultaneously deprotects and cleaves the oligonucleotides from the support. To facilitate purification, the final DMT group is sometimes left on the DNA ("DMT-on").

In the fabrication of DNA *arrays*, surfaces are frequently derivatized with a linker molecule that provides a terminal hydroxyl group on which synthesis begins. The linker is designed to be stable with respect to the conditions in the final deprotection step so that oligonucleotides remain tethered to the surface. A popular combination is the use of glass substrates derivatized with N-(3-(triethoxysilyl)propyl)-4-hydroxybutyramide and a final deprotection reaction consisting of immersion in ethylene diamine (EDA) and ethanol (1:1, v/v) for 2 h at room temperature. Note that it is first necessary to remove the final DMT group by a detritylation step at the end of the synthesis ("DMT-off").

7.2.3 Peptide synthesis chemistry

Solid-phase peptide chemistry predates DNA synthesis chemistry and was introduced by Merrifield in 1963 [192]. A history of the development of the chemistry is provided in [180], and a good summary is provided in [98].

Two types of peptide chemistry are commonly used in current commercial peptide synthesizers: tBoc (t-butyloxycarbonyl) [201] and Fmoc (9-fluorenylmethoxycarbonyl) [209]. These names refer to the terminal (α -amino) protecting group used. The chemistries also differ in their choice of linker, side-chain protecting groups, and conditions for deprotection and cleavage. Fmoc chemistry often has higher yields and purity since the deprotection conditions are milder. In Fmoc chemistry, the Fmoc protecting group is cleaved by a weak base (20% piperidine in dimethylformamide (DMF), v/v), and the amino acid side-chain protecting groups (tButyl) can be removed by a weak acid (trifluoroacetic acid (TFA)) at the end of synthesis. In tBoc chemistry, a weak acid (50% TFA in DCM, v/v) is used for removal of tBoc on every cycle, and the removal of benzyl side-chain protecting groups is performed in a strong acid (hydrofluoric acid (HF)). Fmoc chemistry is often selected to avoid the hazards of working with this acid. The linkers used in commercial peptide synthesizers are designed to be cleaved under the conditions of the final deprotection. In the remainder of this chapter, the use of Fmoc chemistry is assumed.

Peptides are synthesized in the C- to N-terminal direction one amino acid at a time as depicted in Figure 7.3. First, the Fmoc protecting group is removed by incubation with piperidine (20% in DMF, v/v) to yield an active amine group at the end of the growing peptide chain. Next, a new Fmoc-protected amino acid is activated and coupled to this amine. Activation is achieved by dissolving the amino acid with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), and diisopropylethylamine (DIEA) in N-methyl pyrrolidinone (NMP) and DMF to produce an amino acid ester. Typically, the activated ester is reacted in a $4 \times$ molar excess. Next, a capping reaction is performed to block any unreacted amine groups. The cycle is repeated to build the desired peptide. After completion, the peptide is thoroughly washed in dichloromethane and dried. Side-chain protecting groups are then removed by treatment with 20% TFA in DCM with water as a scavenger. (Depending on amino acid sequence, more concentrated acid such as 95% TFA in water can be used for deprotection and cleavage.) DMF and NMP are used as solvents during reactions and wash steps due to their ability to solvate peptides.

In commercial synthesizers, the linker is designed to be cleaved during the final deprotection step. However, to build a tethered peptide array, it is necessary to use a non-cleavable linker. Several possibilities exist: one can treat glass slides with aminopropyltriethoxy silane, or one can purchase commercial aminated slides such as ArrayIt SuperAmine substrates (TeleChem International) and Xenoslide A substrates (Xenopore Corp.).

It should be noted that the synthesis conditions are much more forgiving when compared with DNA synthesis. In fact, reactions can be carried out at room temperature in air with no special conditions such as a dry atmosphere [79, 81]. However, one drawback is that the efficiency of the coupling and deblocking steps can depend tremendously on the amino acid sequence synthesized up to that point. (In constrast, the efficiency of DNA synthesis is relatively constant and independent of sequence.) The variation in efficiency is due to the secondary structure of certain peptide sequences that can "bury" the N-terminus, hindering the access of reagents. In a synthesized peptide *array*, such variations can lead to different peptide densities and purities in each array location. For this reason, to obtain high purity peptides, testing of completeness should be performed during each reaction cycle. Numerous test methods are reviewed by Sabatino *et al.* [235].

A ninhydrin test can be performed during manual synthesis to determine whether the coupling step has gone to completion. A small amount of solid support resin is removed from the support column and mixed with the 2–3 drops each of the following three solutions: ninhydrin (0.5 g) in ethanol (10 mL), phenol (80 g) in ethanol (20 mL), and aqueous 0.001 M KCN (0.4 mL) in pyridine (20 mL). After mixing, the solution is heated to 110°C for 5 min. If the solution turns blue, this signifies the presence of amine groups and thus an incomplete coupling reaction. The coupling step can be repeated immediately if necessary. Measurements of the optical absorbance at 570 nm can quantitate the degree of completeness. Note that the ninhydrin test can also be used after the



Figure 7.3: Fmoc peptide synthesis chemistry. In each synthesis cycle, the terminal Fmoc protecting group is removed from the growing peptide chain by piperidine, and an Fmoc-protected, activated amino acid is then coupled to the newly exposed amine. This cycle is repeated to build the desired peptide. Synthesis proceeds from the C-terminus to the N-terminus, with the C-terminus tethered to the solid support. Once the peptide is completed, the tButyl side chain protecting groups are removed and the peptide may be cleaved from the support. Note that "L" refers to the linker by which the peptide is attached to the solid support resin. (Reproduced from [7]. Copyright Applied Biosystems, 2004.)

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deprotection step to verify completion of Fmoc removal. For synthesis on a substrate, the ninhydrin test is not a practical method for obtaining reaction feedback because amine groups are destroyed in the test and are thus not available for re-coupling should incomplete coupling be indicated. In the technique known as SPOT synthesis, real-time monitoring of coupling is performed with bromophenol blue [79]. The indicator can be added with the coupling reagents and as the reaction proceeds to completion, the colour changes from blue to yellow. Alternatively, the test can also be performed at the end of the coupling step and coupling repeated if the test fails. This is a non-destructive test and can thus be incorporated into an *in situ* array synthesis.

In the deprotection step, Fmoc is removed by reacting two equivalents of piperidine. One equivalent acts as a general base to remove the base-labile Fmoc group from the N-terminus of the peptide, while the second covalently binds to the Fmoc group and forms a fulvene-piperidine adduct. The concentration of this adduct can be measured by its optical absorbance, A_{301} , at 301 nm. In a typical test, the absorbance is compared to a blank (consisting of the same solutions but without the Fmoc) and the amount of Fmoc is determined by an empirical formula. Commercial peptide synthesizers monitor the release of the Fmoc group during the deblocking step in real time. The removal of Fmoc by piperidine generates a conductive carbamate salt that can be detected by a conductivity measurement. Generally, the amount of Fmoc released is measured in several successive treatments with the deprotection agent. Only when the difference between successive measurements is below some threshold is the deprotection step complete. Often the difficulty of deprotection is related to the difficulty of coupling the next amino acid, so commercial synthesizers increase the coupling times accordingly.

7.3 Synthesizing DNA and peptide arrays

Arrays are convenient and powerful tools for many types of high throughput measurements. Performing parallel measurements on a single substrate reduces costs, increases convenience, and ensures identical experimental conditions among all measurements. High density arrays may also permit multiple replicates of each measurement in order to further increase data quality [136]. Currently, most DNA and peptide arrays are "targeted", containing molecule sequences carefully selected to probe the particular biology being studied. Because such arrays require prior knowledge of what to look for, they can serve only as a platform for hypothesis-driven research [193]. Combinatorial DNA and peptide arrays, on the other hand, contain *all possible sequences* of a certain length. Since no sequences are omitted, even completely unexpected interactions can be detected, potentially leading to novel discoveries [193] that would not have been made had sequences been hand-selected. The inclusion of all possible sequences has additional advantages, even in hypothesis-driven experiments. For example, once an experiment has been performed, it need not be repeated when new genes are discovered or when gene sequences are updated; instead, the existing data can simply be reanalyzed. Another possibility is that combinatorial arrays could form the basis of a standardized array design that can be used in any type of experiment with any organism—only the computer analysis would differ for each case. In Chapter 8, we argue that even experiments with the complexity of gene expression analysis can be performed with universal DNA *n*-mer arrays.

In a combinatorial array, the number of different probe sequences, (m^n) , increases exponentially with the sequence length, n, where m is the number of monomers. (m = 4 for DNA and m = 20for peptides, assuming only natural building blocks.) Current technologies are capable of printing arrays with sizes up to roughly a million spots, sufficient for a combinatorial 10-mer DNA array or 5-mer peptide array. While these sizes are useful for several applications, other areas will require significantly longer sequences. For example, we argue in Chapter 8 that universal gene expression analysis will require DNA arrays with at least all possible 13-mers. Due to the extremely large number of different probes, combinatorial arrays must be fabricated by *in situ* (in-place) synthesis. Methods such as robotic or ink-jet deposition of *pre-synthesized* DNA strands are not practical due to the enormous costs associated with synthesizing, storing, and handling all the individual probes. These problems would undoubtedly be exacerbated as array sizes increase further.

In the remainder of this section, I briefly review several large-scale *in situ* array synthesis technologies that have emerged during the past decade and discuss their merits and drawbacks in terms of minimum feature size, chemistry efficiency, and whether the method is serial or parallel in nature. We have developed a new microfluidic synthesis technology, described in the next section, that strives to address important shortcomings of the other methods.

7.3.1 Array replication

Before delving into technologies for array *fabrication*, it should be noted that several methods have been reported for array *replication*. Such methods provide a means to economically produce many copies from a single "master" array. The time and cost associated with fabrication of the original master array thus become less important than other factors such as fabrication density and quality. Replication occurs in parallel and is independent of the number of spots on the master.

Kumar *et al.* [155] report a method for replicating DNA arrays based on strand transfer. A "master chip" containing DNA attached by disulfide bonds is brought into contact with a "print chip" containing an acrylamide layer. When heated, some molecules are transferred from the master chip to the print chip; the copying process takes less than 1 min. Presumably libraries of other types of molecules could be replicated in a similar fashion. Note that the copies are not identical to the master—the density of molecules at each array site is lower. Since the master chip is depleted each time, the number of copies is limited.

The "nanostamping" technique reported by Yu *et al.* [303] could be used, in principle, to make unlimited copies of a single-stranded master array with identical molecular density. First, a set of oligonucleotides is hybridized to the master. Each oligo is linked to a functional group that forms a bond with the target substrate when it is brought into contact. Heating then denatures the DNA duplexes, leaving the original pattern on the master array and the copied (hybridized) pattern on the target substrate. Note that this method does not require any special attachment of oligos to the master array. In fact, the copies can easily be used as masters, permitting an exponentially increasing rate of array production.¹ Because the master array will selectively pull down the complementary strands to the proper parts of the array during hybridization, all of the oligos can be pooled together, greatly simplifying their storage and handling. In fact, it is even conceivable that the oligo mixture

 $^{^{1}}$ Of course, it is important to account for the fact that the copy contains sequences *complementary* to the originals. However, for a combinatorial array of all possible sequences, both the original and complementary arrays contain identical sets of compounds.

be generated by a simple pooled synthesis approach such as mix and split. (Any unneeded sequences are simply ignored.) This method seems quite practical for mass production, though it is not clear whether copies of copies would exhibit reductions in resolution or reductions in sequence purity and density (due to imperfect hybridization).

Mitra and Church [196] report a method for amplifying deposited DNA by performing PCR within a polyacrylamide film on the surface of a glass microscope slide. Products remain localized near the original spots. If the primers contain appropriate functional groups, the product molecules can bond to a target substrate brought into contact with the original array. Like the previous method, potentially unlimited numbers of copies can be made from a single master.

7.3.2 Array fabrication by deposition

In robotic deposition, a "pen" (or "pin") is dipped into a solution containing DNA or peptide molecules of a particular sequence and then briefly brought into contact with a substrate, leaving a small droplet of the solution behind. As the droplet dries, the molecules become immobilized on the substrate surface. Often, the pens contain special reservoirs such that the initial loading phase stores enough solution to print a spot on each of hundreds of substrates in succession. Robotic spotting machines are sold commercially or can be built relatively easily from parts [60]. As discussed above, it is not economical to individually synthesize each sequence in an oligonucleotide array, therefore deposition methods are typically reserved for printing isolated biological materials such as long cDNA strands or proteins.

The size of the printed droplet is determined by surface tension and the shape of the printing tip. With commercial tips, spot sizes are typically 100 μ m or greater, though 50–75 μ m spots are possible according to specifications from several manufacturers (Majer Precision MicroQuill pins [215], and ArrayIt 946 [121] and ArrayIt Stealth [122] pins). Pens are most frequently fabricated from stainless steel or titanium, though ceramic tips have been reported to be more durable and capable of printing smaller features [92]. Typically, they are cylindrical with a slot and reservoir cut into the pointed tip. In our lab, Matthew Reese microfabricated trench-shaped stainless steel pens by etching stainless 12.7 μ m thick steel foil from both surfaces (see Figure 7.4). At the tip, the pens were approximately 100 μ m wide by 13 μ m thick, containing a trench about 30 μ m wide by 7 μ m deep. We demonstrated printing of spots as small as 20×40 μ m (corresponding to densities up to 25000 spots/cm²) with dye [225, 276]. Furthermore, we demonstrated printing of two different DNA 10-mers in an alternating array pattern and showed that complementary oligos exhibited the correct specificity when hybridized to these arrays (Figure 7.5).



Figure 7.4: Microfabricated stainless steel trench pens. (a) Comparison of commercial slot pen (left) machined by conventional methods with our microfabricated trench pen (right). The tip sizes are similar; however, the trench pens are capable of smaller spot sizes. This is presumably due in part to the printing method, in which the flexible trench pens are tapped on the surface at an angle. We observed the spot size to be comparable to the trench size (30 μ m wide by 7 μ m deep), rather than the total tip surface area as is observed in conventional pens that are tapped on the surface in a perpendicular direction. The trench pen is shown in side view (top right) and overhead view (bottom right). (b) A collection of microfabricated stainless steel pens. The various pen designs incorporate features such as reservoirs, support struts, and trenches with different aspect ratios. The ability to fabricate pens using photolithography rather than conventional machining gives considerable design flexibility. Although our lithographic process at the time was limited to a lateral resolution of about 30 μ m, one could scale down the design to produce smaller pens and spot sizes. (Adapted from [225] with permission. Copyright Cold Spring Harbor Laboratory Press, 2003.)

Dip-pen nanolithography is a related technique that uses an atomic force microscope (AFM) tip to write thin lines or spots onto a surface. Liquid is transferred from the tip to the surface when brought into contact. Patterns produced by this method have extremely small features. Demers et al. [58] report the spotting of DNA onto gold and silicon dioxide surfaces at spot sizes down to about 50 nm. 130 nm protein spots have been demonstrated by Lim et al. [168], and lines of biotin 75 nm in width were reported by Jung et al. [139]. Due to the slow printing speed (up to several



Figure 7.5: Hybridization to an array printed by stainless steel trench pens. Two 10-mer probe sequences were spotted onto a glass substrate with our microfabricated trench pen in an alternating fashion. (Top) Complement 1 hybridizes selectively to probe 10mer-1 and does not hybridize to probe 10mer-2 as shown in this fluorescence image. (Bottom) After boiling and washing to remove the hybridized target, the array was re-hybridized with Complement 2, which similarly shows specificity in its binding only to probe 10mer-2. Note that two successive hybridizations were necessary because both targets were labelled with the same dye—Cy3. The hybridization protocol is described in Section 7.5. (Adapted from [225] with permission. Copyright Cold Spring Harbor Laboratory Press, 2003.)

seconds for a small feature), it is not likely that large ordered arrays could practically be fabricated by dip-pen nanolithography unless large tip arrays were available. Surfactants have been reported to improve wetting properties and to improve the reliability and speed of printing. Their use may even extend the range of "inks" that can be printed [139].

Additional techniques have been used for depositing pre-existing DNA and peptides into array patterns. For example, bubble jet technology was used to print arrays of oligos [204, 99]. The authors optimized the printing solvent and demonstrated that printing does not result in DNA damage, even for sequences up to 300 bp in length. Spot sizes on the order of 75 μ m were demonstrated.

Feng and Nerenberg [76] have developed a microelectronic deposition strategy in which the substrate is patterned with electrodes to which different voltages can be applied. When an electrode is positively charged, it attracts (negatively charged) DNA. With appropriate functional groups, the DNA can attach covalently to the electrode. In this manner, DNA in solution can be selectively pulled down to desired array locations. This method is not suitable for large arrays, however, because solutions containing each desired probe sequence must be applied to the chip in *sequence*. An advantage of the electrodes is that different voltages can be applied to each point during assays such as hybridization to locally control the stringency and provide optimal specificity at each site. This is important in applications where small differences in binding must be distinguished, such as SNP (single nucleotide polymorphism) and STR (short tandem repeat) analysis. Hybridization is also very rapid using these electrodes, occurring in just seconds. Livache *et al.* [172] report a similar method in which electrodes on a chip determine the location of electropolymerization of polypyrrole mixed with oligonucleotides or peptides grafted to pyrrole groups.

As discussed earlier, deposition methods all suffer from the drawback that sequences must be individually synthesized or isolated, stored, and manipulated. For very large arrays, this is prohibitively expensive, and methods must be based on *in situ* synthesis instead. Furthermore, molecules are printed *serially* so these methods are not scalable to very large collections of compounds. In addition, deposition arrays often require longer fabrication times than synthesis techniques—the need to load the print-head with each probe solution adds a considerable amount of time to the print run. The loading time can be amortized over many arrays, however, by printing spots on several arrays after each load. Stimpson *et al.* [253] report an additional interesting amortization strategy in which *lines* of oligonucleotides were printed on a membrane by thermal ink-jet printing. The membrane was subsequently rolled up (will lines parallel to the roll axis) and sliced into a large number of diskshaped arrays. Deposition methods have the additional drawback that an immobilization strategy is needed. Tagging biological materials such as RNA, DNA, or proteins with functional groups to promote tethering at specific sites can be tricky. On the other hand, *in situ* synthesis naturally incorporates a single well-controlled point of attachment.

One significant advantage of deposition techniques such as dip-pen nanolithography is the extremely high density that is theoretically possible. Another advantage is that higher sequence purity is possible. In *in situ* synthesis, all molecules—including those with truncation or deletion errors are covalently linked to the substrate and cannot be removed. When molecules are pre-synthesized, they can be purified prior to spotting. One clever technique is to incorporate a covalent attachment group as the last oligonucleotide synthesis step. Molecules that did not reach full length lack this group and are washed away rather than being immobilized when spotted on a substrate.

7.3.3 Ink-jet and robotic synthesis of arrays

Ink-jet and robotic *synthesis* are very similar to the deposition methods discussed previously, except that synthesis reagents—rather than pre-synthesized molecules—are deposited. Arbitrary patterns of probes can be fabricated by selecting the series of reagents delivered to each array location.

Hughes *et al.* [113] used ink-jet printing to deposit reagents for the synthesis of arrays of oligonucleotides as long as 60-mers with a stepwise yield of 94–98%. Arrays as large as 25000 spots on a 25×75 mm glass slide were demonstrated. Printing must be performed under a dry inert atmostphere. Butler *et al.* [31] report an improved technique wherein arrays are synthesized on a substrate patterned with regions of differing surface tension. Synthesis occurs within the boundaries of circular features treated with an amino-terminated organosilane (3-aminopropyltriethoxysilane). These features are surrounded by a perfluorosilanated surface. The difference in surface tension confines reagents to highly localized areas—in principle much smaller than the normal size of an ink-jet droplet. A mixed solvent system (10% acetonitrile, 90% adiponitrile) limits evaporation during reagent delivery and during coupling reactions, resulting in coupling efficiencies of 97–99%. A detailed design for building an inkjet synthesizer was published by Lausted *et al.* [158]. In ink-jet synthesis, only the phosphoramidites need be deposited by ink-jet printing—the remainder of the reactions in each synthesis cycle can be done in bulk on the whole slide. These methods are both examples of confining the *coupling* reagents to determine the synthesis location.

Synthesis of peptide arrays by the SPOT technique involves manual or automated pipetting of spots of reagents and Fmoc-protected amino acid monomers on a support surface such as a cellulose membrane [79]. Generally, the coupling reaction is performed by spotting, and additional synthesis reactions are performed by washing the whole membrane in a reagent bath. Spot size is determined by the droplet volume and properties of the membrane, with densities of hundreds of sequences per cm² possible. A unique feature of SPOT synthesis is that evaporation in the "open reactor" format leads to a maintenance of high reagent concentrations, improving yields [81]. Unlike for DNA synthesis, a dry inert gas environment is not required.

In addition to methods for creating arrays on flat substrates, automated methods have also been reported for synthesizing compounds in microtiter plates. Cheng *et al.* [39] demonstrated the synthesis of DNA in four 384-well plates (for a total of 1536 reaction sites) via a robotic pipetting system. Stepwise yields of up to 99.3% were observed. Each well contains a small amount of solidsupport resin that is trapped by a frit. A vacuum system draws reagents out of the bottom of wells through the frits after each reaction step. While not suitable for producing particularly large sets of compounds, this method provides a means to reduce the cost of DNA synthesis when the quantities of product required are significantly smaller than the 40 nmol lower limit of commercial synthesizers. The products could be used individually or assays could be performed directly in the microtiter plates.

Ink-jet and robotic synthesis solve many of the shortcomings of deposition methods, but they are still serial techniques, and synthesis of extremely large arrays would be prohibitively time-consuming.

7.3.4 Light-directed synthesis

In light-directed synthesis, the synthesis chemistry is sensitive to light during a particular step (usually deprotection), allowing photolithographic methods to be used for patterning regions of the surface in which synthesis occurs. The use of photolithographic techniques has the potential to reduce spot sizes by an order of magnitude or more compared with ink-jet printing and spotting methods. Light-directed chemistries for both DNA and peptide synthesis have been reported. Typically the substrate is mounted in a flow cell connected to conventional DNA or peptide synthesizer and is exposed to a pattern of light during the deprotection step in each synthesis cycle. Deprotection occurs only in the illuminated areas. When coupling reagents are flooded across the substrate, coupling will only occur in these deprotected regions. Light-directed methods offer a high degree of parallelism, because all molecules requiring the same monomer at a particular position in their sequence can be processed simultaneously. The selection of photomask pattern and monomer in each synthesis cycle determines the compounds that are generated on the array.

Fodor *et al.* [78] modified standard peptide synthesis chemistry to incorporate the photolabile blocking group nitroveratryloxycarbonyl (NVOC) instead of the standard blocking group. Peptide arrays with spot sizes of 50 μ m were demonstrated with cycle efficiencies of 85–95%. Arrays with features as small as 18 μ m have been reported in the literature [169]; however, the technology is thought to be capable of printing arrays with 10 μ m features, corresponding to a density of 10⁶ probes/cm² [11]. Illumination through a chrome photomask deblocks only selected areas of the substrate. Up to 20*n* photomasks are needed to synthesize an array of *n*-mers—one mask for each of the 20 natural amino acids in each position of the sequence. The synthesis of oligonucleotides using NVOC protecting groups was also reported. Pease *et al.* [207] later extended this oligonucleotide work and reported the synthesis of a 256-octanucleotide DNA array via standard phosphoramidite chemistry modified with the photolabile (*alpha*-methyl-2-nitropiperonyl)oxycarbonyl (MeNPOC) protecting group. Synthesis cycle efficiencies were reported to be 95–100% in one assay and 85– 98% in another. A more systematic study of deprotection efficiency by McGall *et al.* [189] suggests efficiencies are in the range 92–94%. With photolabile protecting groups such as NVOC and MeNPOC, the relatively low efficiency of the photodeprotection step dominates the cycle efficiency. Thus, array positions typically contain a much smaller fraction of full-length sequences compared with arrays synthesized by conventional phosphoramidite chemistry. For example, the fraction of full-length 25-mers is only 21% assuming 94% efficiency, while it is 88% assuming 99.5% efficiency. This limits the maximum sequence length that can be produced and also complicates the interpretation of hybridization results since the incomplete sequences cannot be removed from the array. Lower efficiencies have been reported to *increase* hybridization efficiency due to reduced molecular crowding [11], but it is preferable to achieve this by controlling the density of functional groups on the derivatized surface.

The relatively low efficiency of photodeprotection introduces another problem—the presence of deletion sequences—that further complicates the analysis of array assays. Affymetrix, a commercial manufacturer of DNA arrays fabricated using photodeprotection, typically incorporates several different dedicated sequences to detect *each* desired gene target. Proprietary calibrations and analyses are used to determine the concentration of the target molecule in the sample based on the combination of hybridization measurements. A better understanding and quantization of the synthesis errors may also help to interpret assays. For example, a method to monitor the quality of synthesis in real-time has been reported, in which cleavable fluorescent amidites are coupled in a final step, then measured and removed [17]. Garland and Serafinowski [90] studied the effects of stray light on synthesis quality, an effect that can lead to additional "contaminants" such as extra-long sequences due to unintended deprotection.

To increase the *flexibility* of array production by eliminating the up-front cost of chrome photomask fabrication for each new design, programmable digital micromirror arrays have been used to provide the illumination pattern during the deprotection step [243, 23, 14]. This is particularly useful in peptide chemistry, in which there are many monomers, each requiring a different illumination pattern for each cycle of synthesis. A micromirror array contains tiny mirrors that can be individually rotated to one of two positions: in one position, light is deflected away from the synthesis substrate; in the other, light is directed towards it. Micromirror fabrication is described in Reference [161]. Spot sizes as small as 14 μ m separated by a 3 μ m gap and array sizes as large as 200000 features have been demonstrated [203, 36]. Oligonucleotide arrays created with this technology were successfully used in gene expression studies (validated by quantitative PCR) and in tiling arrays to find optimal probes for a target gene. The chemistry involved the photolabile blocking group 2-nitrophenyl propoxycarbonyl (NPPOC) that exhibits average stepwise yields from 96–99%, depending on the nucleoside. Beier and Hoheisel [17] report the efficiency of the previously used protecting group MeNPOC to be only 88% that of NPPOC under optimized conditions for each blocking group.

Shin *et al.* [242] optimized the surface derivatization and linker chemistry for peptide arrays produced using micromirror arrays and NVOC chemistry. Glass treatment with 3-glycidoxypropyltrimethoxysilane, chitosan, and either the spacers N-NVOC-6-aminocaproic acid or N-NVOC-O,O'bis-(2-aminopropyl)polypropylene glycol 500-succinic acid resulted in the best signal-to-noise ratio in binding assays and did not require a BSA passivation treatment.

Another variant of light-directed DNA and peptide synthesis chemistry involves the use of a photogenerated acid (PGA) during deprotection. This allows standard, highly efficient, acid-cleavable protecting group chemistry to be used (e.g., DMT for DNA and tBoc for peptides). Barone *et al.* [11] report a method wherein the acid is generated by a photosensitive polymer film deposited over the array prior to each exposure step. Stepwise synthesis yields up to 98% were observed, and furthermore, the speed of deprotection was improved by an order of magnitude. A similar method (though not using PGA) is the use of a standard photoresist film covering the oligonucleotides or peptides. The photoresist is patterned by conventional photolithographic methods, leaving parts of the surface exposed. These open areas can then be treated with an acid for conventional deprotection whereas the covered areas remain protected. Feature sizes down to $10 \times 10 \ \mu$ m have been reported with this method [283]. One disadvantage of these methods is that the conditions for removing the overlayer may be harsh and lead to contamination [57].

Gao *et al.* [87] report the use of *solution* photogenerated acids for standard DMT deprotection of DNA oligonucleotides in a light-directed fashion. A photosensitive compound is added during the deprotection step that generates an acid in solution when illuminated. Average yields greater than 98% were observed—a significant improvement over direct photocleaving of NVOC, MeNPOC, and NPPOC. The use of a solution acid generator is more convenient than applying and removing a polymer layer in each step. Photoacid generators have also been used in light-directed peptide synthesis based on conventional tBoc chemistry [208, 88]. Acid diffusion between reaction sites must be prevented by a physical barrier such as a hydrophobic film that confines reactions to discrete droplets on the surface. Otherwise, acid can diffuse hundreds of microns during the deprotection time (minutes) preventing the fabrication of high density arrays. Gao *et al.* [88] report the use of a substrate containing microchannels to be an effective means to isolate reaction regions. One could also imagine adding other compounds to the deprotection cocktail, such as quenchers, which are used in photoresists and 2-photon stereolithography resins to maintain high contrast. An epitope binding assay was performed with PGA-deprotected peptide chips, as was a metal binding assay [88]. Preliminary results for photo-generated *base* deprotection of Fmoc were also reported.

Aside from higher cycle efficiencies, the use of a photoacid generator offers many other advantages. Since light-sensitivity is relegated to the photoacid generator, standard off-the-shelf chemicals can be used in all aspects of the synthesis. Light-directed synthesis can thus easily be extended to the synthesis of other biomolecules, for which monomers are not available with photolabile protecting groups. In addition, the inclusion of non-standard nucleotides or amino acids is simpler as it is not necessary to first devise a method to attach a photolabile protecting group. Furthermore, different photogenerated species (e.g., acids and bases) could be used at different stages of synthesis to incorporate a wider variety of monomer combinations. To achieve the same flexibility with photolabile protecting groups would require groups sensitive to different illumination wavelengths, for example. Finally, the non-linear response of photogenerated reagents gives sharper contrast (i.e., sharper array spot boundaries) than the linear response of direct photolabile-protecting-group removal [88].

The array densities that can be achieved with light-directed synthesis methods are limited by many factors: the resolution of the photomask or micromirror array, the diffraction limit of the light, and the diffusion of photogenerated acids, if used. Feature sizes as small as 10 μ m have been reported using photomasks [283], while feature sizes in arrays fabricated with digital micromirror arrays have reached 14 μ m [203]. Physical masking techniques (e.g., using microchannels) may enable smaller feature sizes.

As with ink-jet and other spotting methods, reaction sites are fully addressable in light-directed synthesis. Thus it is possible to generate arbitrary arrays of sequences. Of course, combinatorial arrays are also possible [78].

7.4 Microfluidic combinatorial synthesis

To address many of the issues raised in the previous sections, we developed a novel method to synthesize combinatorial arrays within microfluidic devices. The principles of operation, design details, and relationship to other work in the field are described here.

7.4.1 Principle of operation

Southern *et al.* [248] reported an elegant method for *in situ* synthesis of combinatorial sets of oligonucleotides. The procedure for making arrays is depicted schematically in Figure 7.6. The authors used a physical masking procedure to confine coupling reactions to parallel stripes along a flat derivatized solid support, with different nucleotides flowed in different stripes. In one "step" of the synthesis, stripes are oriented in one direction; in the next, they are oriented in the perpendicular direction. Compounds are built up at the points where stripes intersect. The set of sequences that are synthesized on the array is determined by the number of steps and by selection of which nucleotides flow in each stripe during each step. For example, an array of all possible 6-mers can be synthesized in 6 steps according to the scheme in Figure 7.7. Southern *et al.* synthesized an array of all possible octapurine DNA sequences (i.e., all possible DNA 8-mers composed of adenine (A) and guanine (G)) in eight synthesis steps [248]. Other combinatorial sets are possible: for example, reducing the size of the monomer set in certain synthesis steps to one (so all stripes carry the same nucleotide) generates

arrays where all oligonucleotides are identical at certain positions (e.g., fixed flanking sequences around a variable sequence).



Figure 7.6: Principle of *in situ* solid-phase synthesis by surface striping. Using microchannels or other means, one can confine reagents to flow in a thin stripe along the substrate surface. By flowing the appropriate reagents to perform coupling of a monomer (e.g., nucleotide, amino acid, etc.), one obtains a stripe along the substrate where that monomer has been coupled to the surface. In (a), two stripes are created: green 1-mers and blue 1-mers. If one now rotates the apparatus so that fluids flow along the surface in the perpendicular direction, one obtains new stripes of monomers. Where the new stripes cross old ones, the second monomer is added to the first, thus generating a 2-mer at the stripe intersections. In (b), two new stripes (red and yellow) are generated. At the intersections are green-yellow, blue-yellow, green-red, and blue-red 2-mers. In the third step, the orientation and stripe positions match those of the first step. As this process is continued, the desired products continue to be built up at the intersections. After *n* steps, one obtains *n*-mers. Molecules along other parts of the stripes (i.e., not at intersections) will consist of n/2-mers, but can be shortened to 1-mers if appropriate capping reactions are performed early in the synthesis.

It should be noted that a similar scheme of row and column patterning for synthesis of combinatorial arrays was reported by Pease *et al.* [207] in conjunction with *light-directed* synthesis. A 256-octanucleotide matrix was synthesized and a labelled oligo selectively hybridized to the correct spot. Patterning was achieved by light masking rather than physical confinement of reagent flow.



Figure 7.7: Pattern of nucleotide coupling steps to build all DNA 6-mers in 6 steps. There are 4 "monomers" from which DNA is synthesized: A, C, G, and T. To make all possible 6-mers by the stripe synthesis method, one requires an array with $4^6 = 4096$ spots, or 64 rows by 64 columns. In the first coupling step, 16 adjacent stripes are patterned with A, 16 with C, 16 with G, and 16 with T. In the second coupling step, the flow orientation is rotated 90° , and the same set of monomers is flowed. For the third step, each of the four initial groups of 16 channels having the same monomer is subdivided into 4 groups of 4 channels as shown. The fourth step is identical except rotated by 90° . The fifth step further subdivides each of the previous groups of 4 channels into four individual channels, and the sixth is simply a rotated version of the same flow pattern. After all 6-steps, one obtains all possible DNA 6-mers.

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7.4.2 Microfluidic architecture

Southern *et al.* used a macroscopic masking scheme to confine reagent flow to stripes [251]. Lines of silicone rubber or polyethylene tubing were glued to one glass plate which could be clamped to the substrate to confine flow to the spaces between adjacent lines of tubing. After each coupling step, the masking apparatus was removed from the substrate, rotated 90° , realigned, and reattached to the substrate.²

Masking could just as easily be achieved by reversibly sealing a *micro*fluidic device containing parallel channels to the substrate. The use of micron-scale channels reduces the spot size (size of stripe intersections) and permits a larger number of compounds to be synthesized in a given area. With the demonstration of nanoscale (100 nm) channels [50], the possibility exists for array densities far greater than those achieved by ink-jet or light-directed synthesis methods. In the simplest case, one could use a 1-layer microfluidic device containing a series of parallel fluid channels, each with dedicated input and ouput ports. However, for large array sizes, the microfluidic device would need an impractically large number of connections. Furthermore, these connections would need to be reconfigured for each step of the synthesis to deliver a different configuration of nucleotides to the various channels. Instead, one can have simply a dedicated pair of connections (input and output) for each of the four nucleotides, with the fluidic network taking care of routing the inputs to the proper subset of channels. Reconfiguration of which nucleotides are assigned to each channel can be achieved simply by using a different device design for each step of the synthesis. For example, I designed the set of three 1-layer microfluidic devices shown in Figure 7.8 to synthesize all possible 6-mers (Figure 7.7). Each device is used for two synthesis steps (once in each orientation) for a total of 6 steps. While probably not a useful array size for a DNA array, this 6-mer array synthesizer design serves as a non-trivial demonstration that issues such as the number of off-chip connections can be addressed in a scalable way.

 $^{^{2}}$ It should be noted that Southern *et al.* reported an additional interesting scheme using circular or diamondshaped flow cells to synthesize "scanning arrays" or "tiling arrays" consisting of all possible subsequences of a desired sequence [249, 247]. Each nucleotide of the sequence is coupled in turn in the flow cell, each time displacing the flow cell by a small amount in one direction such that its new position overlaps the old one. The choice of the amount of overlap determines the maximum size of n-mers produced.



Figure 7.8: Scheme for synthesizing all DNA 6-mers with passive microfluidic devices. Essentially the passive device consists of 64 parallel channels. To reduce the number of chip inlets, these channels are tied together such that all channels carrying nucleotide A are joined to a single inlet, etc. Channels are tied together in series (via a serpentine pattern) rather than parallel to ensure that the fluid passes through all the desired channels. A parallel design would allow much faster operation as all relevant stripes could be filled simultaneously. Because there are three different flow patterns (each used in two perpendicular orientations for a total of six, see Figure 7.7), three separate microfluidic devices were designed. One is used during steps 1 and 2 (with removal, rotation, realignment, and reattachment between these steps), one during steps 3 and 4, and the last during steps 5 and 6. In general, n/2 different devices are needed to synthesize an array of all possible *n*-mers. In each of the three channel patterns, the input pins are labelled with the nucleotide they carry. Note that, in practice, each device contained alignment marks to be aligned with matching marks etched or patterned onto the substrate before derivatization.

During operation, only the coupling step need be performed inside the microchannels to confine the reaction region. All other steps of DNA synthesis can be performed by immersing the substrate in reagent baths. An advantage of doing so is that the microfluidic device needs to be compatible with only a single solvent (acetonitrile), rather than the full set of solvents used during a complete DNA synthesis cycle (deprotection, coupling, capping, oxidation). Devices can possibly be made from PDMS, which reversibly seals to the substrate and exhibits relatively low swelling in acetonitrile. Alternatively, devices can be made from an inert non-elastic material such as glass or Teflon and simply sealed against the substrate with force.

The need to remove, rotate, realign, and reattach the device to the substrate between reaction steps in this approach complicates synthesis, introduces the possibility for contamination, and introduces the possibility of sequence errors due to misalignments. In our non-automated setup, it also significantly increased the overall synthesis time. By adding some complexity to the design of the microfluidic device, one can perform the 90° rotation of channels *virtually*. As shown in Figure 7.9 the device can contain a full grid of channels (parallel channels in two orientations). By appropriate placement of valves, one can confine fluids to flow in channels (stripes) only in one orientation or the other—hence the virtual rotation. This technique saves time, reduces the risk of contamination and human error, and simplifies device operation. Figure 7.10 shows the design of a single active microfluidic device that can be used for synthesizing arrays of all possible DNA 6-mers. Of course, the microfluidic device must now be compatible with the reagents involved in all reactions of the DNA synthesis cycle.

While the above microfluidic designs assume that synthesis occurs on the substrate, similar array designs could be used for synthesis on trapped solid support beads. This would be useful if a larger amount of each product is needed (enabled by the larger surface area of beads compared to the substrate surface) or when it is impossible to adhere the device to an appropriately derivatized substrate, as was the case with many solvent-resistant elastomeric device technologies we explored in earlier chapters. A simple way to perform synthesis on beads would be to use partially closing valves around each intersection position to confine solid support beads in tiny reaction chambers.



Figure 7.9: Switching the flow direction (row or column) in a grid of microchannels. (a) Design of a *passive* microfluidic device. Fluid channels are shown in light blue. Reagents are flowed in rows for one step of the synthesis, then rows are flushed and dried. The device must then be physically removed from the substrate, rotated 90°, and realigned and reattached to the substrate so that reagents can be flowed in the column direction. (b) Design of an *active* microfluidic device containing a grid of fluid channels. The device remains affixed to the substrate during the *entire* synthesis. Valves, actuated by microchannels in the control layer, perform a "virtual" rotation of the flow direction between synthesis steps. Virtual rotation saves time, reduces contamination and the risk of human error, and greatly simplifies device operation. Valves and control lines are shown in light red in (c) and (d). (c) One bank of valves, actuated by a single input, prevents flow in the column direction. Each point where a control channel crosses a fluid channel and creates a valve is marked by an X. Reagents can only flow in the row direction (shown by dotted arrows) while this bank of valves is closed. (d) A second bank of valves, again actuated by a single input, prevents flow in the row direction. Reagents can only flow along columns as shown. Where the control channels are narrow, crossing the fluid channel does not act as a valve; hence no Xs are shown in these locations. Note that the two sets of valves can be interdigitated to fit into a single control layer of a 2-layer microfluidic device.



Figure 7.10: Design of an active DNA 6-mer synthesis device. (a) Unlike the passive design of Figure 7.8, in which three different fluidic devices are needed, the active approach requires only a *single* device containing a fluid layer (blue) and a control layer (red). Rotation of flow direction is achieved via two banks of valves (dense region in middle), each controlled by a single inlet (row flow selector and column flow selector). Selection of which nucleotides pass through each of the channels is controlled by two multiplexers. Each multiplexer setting opens 16 of the 64 channels through which the current input reagent flows. In each of the 6 steps of 6-mer synthesis, the four nucleotides must be introduced sequentially. Each one will have a different configuration of multiplexer valves to flow the nucleotide through a specific set of 16 channels. However, capping, oxidation, and deprotection steps are performed in all 64 channels simultaneously. The central array is about 1.25 cm on a side. (b) View of the fluid layer alone, as it is obscured by the control layer in *a*. (c) Detailed view of the array region in the center. The fluid layer consists of a grid of channels (dark blue) crossed by two sets of valves to select row or column flow.

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Partially closed values allow fluid flow but prevent the escape of the beads. In a typical step of synthesis, values preventing flow in one direction (row or column) would be completely closed. The other value bank would remain partially closed to hold the beads while reagents were flowed through the columns or rows. (Alternatively, dedicated frit values or other structures could be incorporated to trap the beads.) Such a device could serve as a massively parallel DNA or peptide synthesizer and products could be cleaved from the beads after synthesis. Alternatively, the chip could be used in array assays by leaving the beads trapped in chambers and flowing the analyte through the microchannels.

7.4.3 Individually-addressable arrays

The microfluidic devices described above are suitable for the synthesis of *all* possible sequences of a set of monomers.³ It is not possible to synthesize an arbitrary subset of sequences. Flowing reagents along a row causes the same monomer to be added to all product sequences in the row. Therefore, for two sequences to exist on the same row, they must have identical monomers in all positions corresponding to row-wise reactions. The same is true for columns. I wrote a computer program that attempted to optimally place an arbitrary set of sequences in an array of this type, with complete freedom of which monomers flowed in each channel during each step and complete freedom whether each step was to be performed row-wise or column-wise. The main result was that sequences can rarely exist on the same row or column unless the sequences are very highly similar.

Other array synthesis techniques, including ink-jet synthesis, light-directed synthesis with micromirror arrays, and robotic synthesis in microtiter plates, are ideally suited to making arrays of arbitrary sequences. Furthermore, these methods are easily reconfigured, meaning that a new set of sequences does not require any equipment modification.

It turns out that one can also fabricate microfluidic synthesis devices with the same degree of flexibility as these approaches. Thorsen *et al.* [268] demonstrated an individually addressable array device, consisting of an array of chambers that could be selectively purged. Though designed such

³Though I use the word "sequence" implying the synthesis of polymers, this discussion is equally valid for more general forms of combinatorial synthesis, in which one generates products by a sequence of reactions, not necessarily adding a piece each time, nor necessarily adding new units to the same molecular site.

that chambers were filled one whole row at a time (with the same fluid), a few simple modifications could be made to the design to allow selective loading of chambers as well. The modified chip could be used for combinatorial chemistry if the chambers were open to a derivatized substrate, if the surfaces of each chamber were derivatized with appropriate starting groups, or if the chambers could trap solid support microbeads.

In this hypothetical modification of their design, the introduction of reagents would be a sequential process. First, the first row would be loaded with monomer X, and column valves would be opened in turn for each chamber requiring monomer X at the current position. Next, the second row would be loaded with monomer X and so on until every array element of the chip requiring monomer X at the current sequence position had been reacted.

An alternative design is shown in Figure 7.11, in which all rows in the entire chip can be preloaded with a particular reagent. Chambers requiring reaction with the currently loaded reagent are then opened in turn. Each chamber is individually addressable by a row and column valve. Because all rows are preloaded, each reaction cycle can be significantly faster. An additional advantage of this design is that chambers remain sealed if they are not active. Only the active chamber has its double-valves (at its entrance and exit) opened. In contrast, in the design of Thorsen *et al.*, all chambers in a column are opened when a column valve is opened. Though there is flow through only one chamber, valve release in the other chambers leads to the possibility of sample contamination or loss by diffusion or evaporation.

Small modifications to the designs can be made to allow different styles of synthesis. For example, with the inclusion of partially closing valves, synthesis can proceed on trapped solid-support beads. Alternatively, a different valve configuration could allow the double-valves at the inlet and outlet of each chamber to be independently controlled. This would allow reactions requiring solventexchange (by evaporation) and would allow accurate metering of reagent volumes by dead-end filling. Synthesized molecules can remain tethered to the substrate or solid-support beads or can be cleaved and purged from the chambers one at a time. Applications other than synthesis are possible with



Figure 7.11: Design and operation of an individually-addressable microfluidic array synthesizer. (Top) Design of the synthesizer. Only a small portion (six reaction sites) are shown for clarity. The fluid channel is shown in blue, and two control layers are shown in red and green. A multi-layer chip architecture could be used to implement this design (see Chapter 6). The blue squares represent reaction chambers. Each chamber is isolated by a double-valve at the bottom (entrance) and top (exit). Valves are indicated by "X"s. In each double-valve, one valve is controlled by a column selector valve, and one is controlled by a row selector valve. Thus the operation is like a Boolean OR-gate: the double-valve remains closed if either the row or column valve is closed (or if both valves are closed). Only if both are opened can fluids flow through the chamber to react with the molecules being "grown" on the substrate by solid-phase synthesis. (Bottom-left) Prior to a reaction step, all row and column selector valves are closed. A reagent is introduced into all fluid channel rows (dark blue). (Bottom-right) To allow the reagent to react with a particular array site, one row selector valve and one column selector valve are opened (indicated by asterisks and lighter colouring). Note the new pattern of "X"s indicating which valves are still closed. A single chamber is opened, allowing fluid to flow through to the output port. Other chambers in the same row or column are still completely isolated by valves. It is not shown in the figure, but row and column selector valves can be controlled via multiplexers as in [268] to limit the number of off-chip connections.

these microfluidic device designs. For example, one could trap biological cells in chambers and deliver different molecules (such as drug candidates) to each chamber.

7.4.4 Related work

The use of microfluidic channels to pattern substrates and to perform reactions in a combinatorial fashion is not new. However, to our knowledge, the use of microfluidics for array synthesis and the concept of virtual rotation of channel direction are.

To perform patterning of surfaces with different substances in parallel, Delamarche *et al.* [57] fabricated PDMS devices containing parallel open channels (as small as 1 μ m in size) in their surface. These devices were reversibly sealed to glass slides to flow through solutions of proteins (by capillary force), resulting in reaction with the substrate along the flow path. The authors observed that reactants were quickly lost to the walls and substrate, and thus a continuous supply of fresh reagents was necessary to ensure that the farthest end of the channel was reacted. This problem was solved in later work by fabricating devices containing fluid reservoirs at the inlets and outlets [138]. Notably, Delamarche *et al.* found the channel walls to give very sharp edges on stripes, except for the small amounts of reactants that were able to migrate between the reversible PDMS-substrate seal. With covalent bonding, one would not expect to observe this problem.

Ermantraut *et al.* [75] report the fabrication of arrays by using elastomeric masks held in place on the substrate to confine the regions of synthesis to holes in the elastomer membrane. This method allows reactions to proceed in parallel, much like light-directed synthesis. Oligonucleotides were synthesized with phosphoramidite chemistry at 99% efficiency in spot sizes as small as 1 μ m. This method offers considerable synthesis flexibility as the patterns of holes in each membrane can be designed to produce arbitrary sets of sequences. However, the need to remove the old membrane and align a new membrane prior to each synthesis step introduces the same disadvantages as our 1-layer passive microfluidic devices discussed earlier. A similar method at a larger scale was reported by Livesay *et al.* [173] in which reagents are flooded over a microtiter plate rather than a flat substrate. Synthesis occurs on solid-support beads trapped in each well. A physical mask is inserted above the microtiter plate to control which wells the reagents can enter during each step.

Another technique that has been used for the parallel synthesis of oligonucleotide arrays by phosphoramidite chemistry is PDMS stamping [297]. Patterned PDMS stamps were "inked" with coupling reagents and pressed against the substrate for each synthesis step. Similar to physical masking techniques that require masks to be exchanged or reoriented, stamping suffers the same alignment challenges and risks of contamination.

Ismagilov *et al.* [125] report the fabrication of PDMS devices consisting of two sets of channels, in different layers, crossed at right angles. At each intersection point, a small fluid-filled chamber with porous membranes on both sides is interposed between the two crossing channels. The membranes ensure that the fluid inside remains stationary and that cross-contamination between the channels is prevented. Product is generated when molecules from each channel diffuse across their respective membranes into the chamber and react. The device implements a combinatorial chemistry step by permitting all possible pairwise reactions between the reagents in the first set of channels with the reagents in the second set of channels. A few variations, such as the presence of a gel in one set of channels, were also reported. Since the fluid in the reactors remains stationary, and leftover reagents from previous steps cannot be eliminated, it is not likely this method could be used for multiple synthesis steps (e.g., oligonucleotides or peptides).

7.4.5 Advantages of microfluidic synthesis

In situ synthesis of arrays with microfluidics offers many advantages over alternative techniques. The use of physical barriers to confine reactions implies that conventional synthesis chemistry can be used. For example, there is no need for modifications to confer light-sensitivity. Conventional DNA and peptide chemistry gives higher cycle efficiencies and therefore a significantly larger fraction of molecules with the desired full-length sequence at each array position. In addition, smaller feature sizes and higher array densities should be possible since microchannel walls completely prevent diffusion and other effects that can reduce resolution. Though very tiny nanoscale channels can be fabricated, the lower practical size limit will likely be determined by such factors as the length of time needed to flow the reagents through the channels or the minimum size of synthesis sites needed to ensure a sufficient number of molecules for performing the desired assay.

In contrast with light-directed synthesis, microfluidic synthesis does not require an expensive optical setup for every chip that is being synthesized at one time. In addition, one can use some of the microfluidic chip area to perform the reagent handling that is normally performed by a bulky DNA synthesizer in many of the schemes described above. The parallelism of microfluidic synthesis provides speed advantages compared with serial methods such ink-jet and spotting methods. The fact that the microfluidic device is a sealed environment may also be helpful, for example to eliminate evaporation, especially in reactions requiring heating.

An additional advantage is the possibility that the fluidic network that was used to synthesize the array can be used afterwards to deliver the analyte directly to the tethered probes on the chip. Hybridization times in microarray experiments have been dramatically reduced by such schemes [251, 273] because the diffusion distance for a target molecule to reach a probe is reduced from an inch or more (the total array size) down to the width of a single microchannel. Furthermore, the channel structure could control the delivery of different analytes to different parts of the chip, permitting parallel, multiplexed assays [246]. Integrated microfluidic devices may also combine synthesis and analysis in other interesting ways.

7.5 DNA array synthesis

I attempted to fabricate DNA arrays with microfluidic devices to demonstrate the principles and methods outlined above. Initially I worked with PDMS devices as we had not yet begun our exploration of solvent-resistant microfluidics.

7.5.1 Early experiments

Since solvent-resistance data for PDMS suggested that PDMS was compatible with acetonitrile but not with other reagents involved in DNA synthesis (dichloromethane, tetrahydrofuran, pyridine), I initially attempted synthesis with "passive" microfluidic devices (Figure 7.8). These devices need only be compatible with the coupling reagents, consisting of phosphoramidites and activator (tetrazole) dissolved in acetonitrile; other reactions in the synthesis cycle can be performed by immersing the substrate into reagent baths. Pre-mixed standard phosphoramidite reagents were purchased from Applied Biosystems.

Three PDMS devices were fabricated, then treated with 0.12 M HCl to improve wetting and flow characteristics, and dried by baking at 120°C before use. Prior to each coupling step, the appropriate microfluidic device was aligned and sealed to the glass substrate and installed in a jig that applies mechanical pressure to help maintain adhesion. The jig was similar to that in Figure 4.4, except that fluids were not delivered through the glass. Nitrogen was then flowed through all channels (observed by bubbling through ethanol) to ensure all channels were open and had not collapsed during the clamping procedure. Filter-ferrules (Upchurch Scientific) were used in HPLC fittings delivering reagents to the jig to prevent particulate contaminants from entering the PDMS microchannels. As an additional measure, coupling reagents were diluted $5\times$ with dry acetonitrile to prevent precipitation inside microchannels that otherwise occurs due to loss of acetonitrile by evaporation or diffusion into PDMS.⁴ The jig was placed inside a glove bag containing a dry argon atmosphere.

Each cycle of DNA synthesis was carried out using a standard phosphoramidite synthesis protocol. Immediately prior to coupling, channels were flushed with dry acetonitrile for several minutes. Coupling reagents were then flowed through the channels under 5–7 psi fluid pressure, with vacuum applied at the outlets. After completely filling each channel (1–2 min), the flow was stopped for 20 min. All four nucleotides were reacted in dedicated channels in parallel. Once coupling was complete, each channel was flushed with acetonitrile then nitrogen. The device was then disassembled from the substrate, and further steps were performed in reagents baths. The substrate was immersed in mixture of Cap A and B (1:1 v/v) for 1 min followed by an acetonitrile rinse. Next, it was reacted with Oxidizer solution for 1 min, followed by another acetonitrile rinse. Finally, it

⁴Note that even this dilution represents a huge excess of coupling reagents—even much greater than in a commercial synthesizer. This is because the glass surface has far fewer reaction sites available than the collection of controlled-pore glass (CPG) beads typically found in synthesis columns.

was immersed in Deblock solution for 30 seconds, followed by a final acetonitrile rinse. I observed after synthesis that the initially hydrophobic glass slides had hydrophilic patches (observed during drying) corresponding to the paths of fluids in the microchannels, indicating that a chemical change had occurred on the surface.

Detection of products proved challenging due to the small (theoretical) quantities produced and because the molecules were permanently tethered to the substrate. Conventional methods such as HPLC and UV spectrometry were not possible [163]. Instead, I attempted to monitor the success of synthesis steps by coupling a fluorescently labeled phosphoramidite—a method used by workers at Affymetrix to measure coupling efficiences [189, 212]. Coupling solution was prepared by dissolving 5 mM Cy-3-CE Phosphoramidite (indodicarbocyanine 3-1-O-(2-cyanoethyl)-(N,N-diisopropl)phosphoramidite) (Glen Research) and 50 mM Phosphoramidite dT (Applied Biosystems) in dry acetonitrile. However, this labeled nucleotide exhibited a high degree of non-specific binding that was indistinguishable from covalent coupling. It is possible that the non-specific binding is related to contamination of the chemistry by PDMS or molecules trapped in the PDMS. Since the alignment of each device to the substrate took considerable time (up to 30 min), it is also possible that the chip absorbed a significant amount of moisture during that time, contaminating later coupling steps. Another method of monitoring synthesis reactions is radioactive labeling; however, we did not have access to the needed materials and facilities.

To continue our investigations, we proceeded first to optimize an alternative detection protocol, based on DNA hybridization. In this protocol, fluorescently labeled strands bind to complementary DNA strands tethered to the surface and can be visualized via fluorescence imaging. The protocol was first optimized using DNA manually spotted onto the substrate (rather than synthesized). We then confirmed that the detection protocol worked as expected when DNA was synthesized on the substrate inside a flow cell connected to a commercial DNA synthesizer. Finally, this protocol was used to verify the principle of stripe synthesis in millifluidic Teflon flow cells. Since the Teflon flow cell has roughly the same exposed substrate surface area as the one-layer PDMS microfluidic devices, the millifluidic principles should be readily scalable down to solvent-resistant microfluidic devices.

7.5.2 Hybridization optimization

Hybridization conditions (including prehybridization and stringency wash) were optimized by spotting presynthesized amino-modified oligos onto aldehyde slides and hybridizing with fluorescently labeled targets. Sequences are shown in Figure 7.12. All oligos were synthesized by the Caltech Biopolymer Synthesis and Analysis Resource Center. We chose to work with 10-mers as this is the size we determined to be the minimum useful size for performing gene expression analysis of simple organisms with an *n*-mer array (see Chapter 8). 6-mers would also have been suitable (to test the 6-mer synthesis chip design), but we found 6-mer hybridizations to have poor repeatability.

In typical experiments, slides were patterned with 10mer-1 on one half of the surface and 10mer-2 on the other half. Hybridizations were performed in the wells created when a PDMS gasket containing punched holes was sealed against the slide. The wells allowed different hybridization experiments to be carried out in parallel on the same array. (This was necessary because our hybridization targets were both labeled with the same dye—Cy3.) Both targets (Complement-1 and Complement-2) were hybridized to each probe at several different DNA concentrations (ranging from 0.16–100 μ M) to assess hybridization stringency.

10mer-1: Complement-1:	3'	5' CY3 -	А А Т Т	C C G G	C C G G	A C T G	АА- ТТ	- S - L 5'	3' -	
10mer-2: Complement-2:	3'	5' CY3 -	A C T G	а а т т	C C G G	C A G T	АА- ТТ	- S - L 5'	3' -	

S: SPACER L: AMINE LINKER

Figure 7.12: DNA sequences used for hybridization optimization. Sequences are shown for tethered probe molecules 10mer-1 and 10mer-2 and fluorescent hybridization targets Complement-1 and Complement-2. Probes consist purely of A and C nucleotides to minimize secondary structure formation that could interfere with hybridization. The probes differ at five nucleotide positions. Each probe sequence contains the C12 Spacer Phosphoramidite (S) and C7 Amino Modifier (L), both from Glen Research (Sterling, VA). The amino modifier results in covalent tethering when solutions containing these sequences are deposited on aldehyde slides.

Probes (10mer-1 and 10mer-2) were pipetted manually onto ArrayIt Silylated Slides (TeleChem International) in a printing solution consisting of $5 \times$ SSC, 0.001% SDS (sodium dodecyl sulfate), and 50 μ M DNA. The slides were then left to dry at room temperature for 24 h and subsequently washed and blocked (to passivate remaining aldehyde groups) according to the slide manufacturer's recommended protocol. (We modified the protocol slightly—all wash steps were extended to 5 min duration.)

Due to the low melting temperatures and the wide range of melting temperature estimates given by various algorithms for short oligos, prehybridization and hybridization conditions were optimized by exploring the parameter space and comparing hybridization signal and stringency. Published protocols for cDNA [104] and short oligonucleotide hybridizations [64] were used as a starting point. Details of the optimized protocols follow. Note that identical protocols were used in later experiments where the DNA was synthesized in place on glass substrates.

To reduce non-specific binding of fluorescently-labeled DNA and thus background fluorescence, a prehybridization step was performed prior to hybridization to passivate any reactive functional groups remaining on the surface. Prehybridization solution (5× SSC, 0.1% SDS, 10 mg/mL BSA) was prepared and heated to 40°C. A PDMS barrier was placed around the region of interest on the substrate, filled with this solution, and maintained at 40°C for 2 h. The barrier was then removed, and the slide was rinsed with deionized (18 MΩ) water and dried with nitrogen.

Hybridization solution (4× SSC, 0.05% SDS, 0.2 mg/mL BSA, 0.16 μ M DNA) was prepared for each of the two labeled targets. The solution was pre-cooled to 4°C. Hybridizations were carried out by pipetting solutions into PDMS gaskets with holes cut at the locations where hybridization reactions were desired. Cover slips were placed over the gaskets to prevent evaporation. The use of gaskets allows simultaneous hybridization of multiple probes, even if they are labeled with the same fluorophore. Hybridization was carried out at 15°C in darkness for at least 2 h. Once complete, coverslips were carefully removed and wells were emptied with a pipette to prevent carryover of solution between the wells when the PDMS gasket was removed. Stringency washing was caried out by immersion of the substrate in a series of four successive wash solutions (W1, W2, W3, W4) for 5 min each. W1 (1× SSC, 0.03% SDS, \sim 9°C); W2 (0.2× SSC, \sim 11°C); W3 (0.05× SSC, \sim 13°C); W4 (water, \sim 15°C). The ramping temperature was achieved by refrigerating plastic centrifuge tubes containing 50 mL of each wash solution to about 9°C, the performing the entire wash sequence with all tubes unrefrigerated. Washed slides were dried with nitrogen and scanned immediately on a GenePix 4000A (Axon Instruments) or ArrayWorx (Applied Precision) microarray scanner.

7.5.3 Surface derivatization

In addition to optimizing the hybridization protocol, we also optimized the surface derivatization protocol. Surface derivatization is the process whereby the substrate is functionalized with reactive groups on which synthesis can begin.

Several slide preparation protocols have been reported in the literature. Typically, glass slides are treated with a silane that presents terminal hydroxyl (or amine) groups on which synthesis can begin, for example N-(3-(triethoxysilyl)-propyl)-4-hydroxybutyramide [189, 163, 87] or 3aminopropyltriethoxysilane [31, 248]. Often a DMT-protected linker phosphoramidite (such as hexaethyleneglycol) is coupled to the entire surface in a long coupling reaction [189, 31, 87]. The additional linker length increases the efficiency of subsequent couplings by moving them away from the surface [163, 184, 241].



Figure 7.13: Silane linker for DNA synthesis. The structure of N-(3-(triethoxysilyl)-propyl)-4-hydroxybutyramide linked to a glass solid support is shown.

We observed best hybridization results (lowest background, highest specificity, most consistent) for slides derivatized with N-(3-(triethoxysilyl)-propyl)-4-hydroxybutyramide according to a protocol adapted from the above references (see Appendix A.2.6). The structure of this silane is shown in Figure 7.13.

7.5.4 Synthesis on substrates with a DNA synthesizer

To ensure hybridization could be used to detect and distinguish sequences synthesized *in situ* on glass substrates, we performed syntheses with modified commercial DNA synthesizers—an ABI 380B (Applied Biosystems) (donated by the Caltech Biopolymer Synthesis and Analysis Resource Center) and a Beckman Coulter Oligo 1000M (on loan, courtesy of Beckman Coulter). An additional synthesizer, a Gene Assembler Special (Pharmacia LKB) donated by Frances Arnold at Caltech, was not used due to lack of an available fume hood in which to operate this machine. The Oligo 1000M contains a built-in trityl monitor to compare the reaction efficiency of successive synthesis cycles. This provided useful feedback during bulk synthesis experiments; however the signal level during *in situ* synthesis on surfaces was too low to give accurate readings. The trityl alarm was disabled during such syntheses to avoid the synthesis being aborted.

Standard phosphoramidite reagents for the ABI 380B were purchased from Applied Biosystems and stored and used according to the supplier's recommended procedures. Pressure was delivered to the machine from a cylinder of dry argon. The synthesizer required extensive repairs, cleaning, and calibration prior to use. To verify correct operation, we synthesized 25-mer and 21-mer primers for λ -DNA and ran a PCR (polymerase chain reaction) assay using λ -DNA as a template. Primers were synthesized on standard CPG columns and cleaved (and deprotected) by standard ammonium hydroxide treatment. PCR reactions were performed with unpurified product; ammonia was "removed" simply by dilution. Comparison of the PCR product with a standard DNA ladder by gel electrophoresis indicated that the expected portion of the template had been amplified, thus implying successful synthesis of the two primers. Optical absorption measurements at 260 nm indicated a single stranded DNA concentration of 0.250 mM, in agreement with the expected concentration as determined by the synthesis "scale" (determined by functional group loading in the solid support column). In initial experiments with the ABI 380B, controlled-pore glass (CPG) solid support material was removed from standard columns and replaced with shards $(2\times3-4 \text{ mm})$ of derivatized glass. With these modified columns in place, standard synthesis programs were run to synthesize the desired sequence on the glass surfaces. However, it proved difficult to handle the small glass shards and to perform hybridization experiments. Results were inconclusive, perhaps due to damage of surfaces during handling.

Subsequent experiments were performed with the Oligo 1000M. (Our ABI 380B experienced frequent malfunctions due to leaky valves or electronic errors.) Experiments were performed with the standard 200 nmol "Economy" synthesis program. Reagents for the Oligo 1000M were purchased from Beckman Coulter and stored and used according to recommended procedures. A cylinder of dry helium supplied pressure to the machine.

To permit synthesis on standard glass microscope slides rather than glass shards, I fabricated the Teflon fluid delivery jig shown in Figures 7.14c and 7.15. It consists of a Teflon block clamped against a derivatized glass slide. The Teflon block contains a machined depression or channel that serves as a flow cell. This jig is connected in place of a standard column by redirecting the column input and outputs on the DNA synthesizer. Though the volume of the fluid cell was similar to the volume of the column, flow rates were slightly different and the Oligo 1000M synthesis programs required modification of flow times to ensure that the Teflon flow chambers were completely filled with reagents during each synthesis step. The Teflon block actually contained *two* separate chambers allowing two sequences to be synthesized simultaneously on a single substrate by connecting to two different synthesis columns on the machine. Initial fluid cells contained a circular chamber approximately 1 cm in diameter. This large synthesis surface area permitted PDMS gaskets with punched holes to be overlaid for performing multiple hybridizations to each synthesized region. After demonstration of successful synthesis, the size of the flow cells were scaled down to more closely approximate microfluidic synthesis. Flow cells with millifluidic channels (\sim 2 mm in width) were machined.



Figure 7.14: Beckman Coulter Oligo 1000M DNA synthesizer. (a) DNA synthesizer with lid open (tilted up sideways). (b) Synthesizer with the reagent platform rotated to show the reagent bottles behind

(deblock, activator, cap 1, cap 2, and oxider). (c) Synthesizer with the Teflon flow cell inserted in place of a standard synthesis column to perform synthesis on flat substrates.



Figure 7.15: Teflon flow cell for millifluidic solid-phase DNA synthesis. (a) Schematic of Teflon flow cell that connects in place of a synthesis column in a commercial DNA synthesizer. A flow chamber is machined in a Teflon plate. The machining process results in a raised "lip" of Teflon around the machined area that acts as a seal when a derivatized glass slide is pressed against it. Reagents are delivered from the synthesizer through HPLC fittings. The reagents flow through the flow cell, reacting with the derivatized surface of the glass and exit the other side. Typically the chamber was mounted vertically such that the inlet was at the bottom and outlet at the top to help eliminate bubbles. (b,c) Several different Teflon flow cells. Note that each actually contains two flow cells for two simultaneous syntheses. The circular cells in b are matched in volume to the volume of the standard synthesizer column cartridge. All other cells were designed to pattern lines on the surface to test synthesis at intersections of stripes.

1 cm

In typical experiments, two 15-mer sequences (see Figure 7.16) were synthesized *in situ* on different parts of a derivatized substrate. These sequences correspond to the 10-mers used for hybridization optimization but contain an additional 5-nucleotide spacer segment. After synthesis, substrates were rinsed with acetonitrile and deprotected in a 1:1 solution of ethanol and ethylene diamine for 1.5 hr at room temperature to remove side-chain protecting groups. Hybridization experiments were then performed in wells in PDMS gaskets as described above. Each of the Cy3-labeled complements was hybridized to both 15-mer sequences to verify specificity and compare hybridization quality. A fluorescence image of a successful hybridization with good specificity is shown in Figure 7.17.



(b) Partial sequences for intersection test

Figure 7.16: **DNA sequences used in millifluidic synthesis experiments**. (a) Basic sequences. The two 15-mer sequences, 15mer-1 and 15mer-2, correspond to the sequences 10mer-1 and 10mer-2 in Figure 7.12, but contain an additional five nucleotide spacer (ACACA) at the 3' end. The sequences are composed only of A and C nucleotides to prevent secondary structure formation that could interfere with hybridization. Also shown are the two complementary 10-mer sequences labelled with Cy3 for detection of the 15-mers. (b) Partial sequences. For the intersection test, stripes of half-15-mers were synthesized in perpendicular directions to produce full 15-mers at the points of crossing.



Figure 7.17: **Demonstration of hybridization specificity onto synthesized DNA stripes**. Fluorescence image (Cy3 channel) of a hybridized slide. Two stripes of DNA (15mer-1 and 15mer-2) were synthesized on a derivatized glass substrate by inserting a flow cell in place of a synthesis column on a commercial DNA synthesizer using standard phosphoramidite chemistry and reagents. After deprotection, slides were hybridized with 10-mer complements of the two sequences. Hybridization solutions were placed into small wells punched through a PDMS gasket such that four separate hybridization experiments were performed against each stripe. The hybridizations exhibit the correct specificity and have low background.

7.5.5 Millifluidic synthesis and detection

The thin millifluidic flow cell channels provided a means to synthesize nucleotides in stripes and test the principle of stripe synthesis. Stripes of DNA consisting of the first halves of two 15-mer sequences were synthesized first in parallel horizontal stripes. Next, the other halves of the two sequences were synthesized in parallel vertical stripes crossing the first ones. At the stripe intersections, nucleotides in the second synthesis couple to the strands from the first synthesis, thus extending them to full 15-mers. The intersection of two pairs of parallel stripes resulted in four different 15-mers as shown in Figure 7.18. The glass slide was then rinsed with acetonitrile and deprotected in a 1:1 solution of ethanol and ethylene diamine for 1.5 hr at room temperature. After, the slide was rinsed twice with ethanol and dried with nitrogen. Hybridization to Complement 1 showed good specificity for its complement (5'-15mer-1B-15mer-1A-3'). This successful result also indicated that full 15-mers were fabricated at intersections. Several additional control experiments were performed, each omitting critical steps (such as omission of the 8th nucleotide or omission of the deblocking step) between synthesis of the two sets of stripes.

7.5.6 From millifluidics to microfluidics

The total substrate area exposed to the channel contents is similar for both the PDMS microfluidic devices and the Teflon millifluidic channels. Though we were not able to fabricate Teflon devices with smaller channels, we have no reason to believe the chemistry would not work equally well (if not better) in narrower channels. When scaling down, the total reaction surface area would not change significantly. However, due to dramatically reduced channel depth, the total reaction volume would be decreased substantially. Reagents may have to be replenished at a faster flow velocity to maintain initial concentrations. Scaling down will also increase the time needed to fill a channel, a delay that must be taken into account if driving the synthesis via an external controller such as a commercial DNA synthesizer. In addition, as the volume discrepancy between the reaction volume of the DNA synthesizer and that of the microchannel increases, it may become necessary to alter the synthesis program, or shunt part of the reagent volume to waste rather than waiting for the full quantity to pass through the microfluidic chip.

7.6 Peptide array synthesis

In addition to DNA arrays, we attempted to fabricate peptide arrays to demonstrate microfluidic combinatorial synthesis. Peptide chemistry is less sensitive to contamination by air (moisture) and can be performed without the use of an inert atmosphere. To become familiar with the chemistry, manual synthesis on commercial solid support resin was first performed. Next, peptide synthesis reactions were performed in Teflon millifluidic devices (Figure 7.15) to verify that reactions were occurring as expected. To scale down to microfluidic devices, these same reactions were attempted in PDMS devices sealed to glass.



Figure 7.18: Demonstration of DNA extension when intersecting stripes of DNA are synthesized. (Top) Synthesis scheme. Stripes of DNA were synthesized by clamping a custom-built Teflon flow cell to a derivatized glass slide and flowing reagents from a commercial DNA synthesizer. In the first synthesis, two 8-mers were patterned in horizontal stripes. The slide was realigned in the synthesis jig and a 7-mer synthesis performed in the perpendicular direction. A total of four different 15-mers were fabricated at the intersections in this manner (written inside squares in the $5' \rightarrow 3'$ direction). After synthesis, the slide was deprotected, and a hybridization was performed against Cy3-labeled oligo Complement-1. (Bottom) The fluorescence image (Cy3 channel) shows strong fluorescence only at the location of the correct sequence, 1B1A, indicating successful synthesis of the full 15-mer as well as good specificity. There is also a reasonably large signal for intersection 1B2A due to mismatch binding. (There is only a single internal nucleotide difference compared with the perfect match.) The large background on edges of channels is due to poor flow characteristics along the edge where the Teflon meets the glass.

7.6.1 Manual synthesis

The peptide sequence N–YGAFLSF–C was synthesized manually according to standard Fmoc chemistry. Fodor *et al.* [78] reported this sequence (prepared by light-directed synthesis) to be highly labelled by the mouse monoclonal antibody 3E7.

Synthesis was performed on polystyrene Fmoc-F-resin (Applied Biosystems), which already has the first amino acid (F) attached with a substitution of 0.66 mmol/g. To perform a 0.1 mM synthesis, 152 mg of the resin was used. All reactions were performed in a 10 mL glass-fritted tube with vacuum applied to the bottom to drain reagents between steps. The resin was first swelled for 30 min in dichloromethane.

Each synthesis cycle was performed as follows:

- 1. Wash. The tube was filled with NMP, closed, stirred for 1 min on a rotator, and drained by vacuum. This step was repeated 5 times.
- 2. Deprotect. The tube was filled with 8 mL of 20% piperidine in synthesis-grade DMF and rotated for 5 min. The solution was drained by vacuum and then the tube was refilled and rotated for an additional 10 min.
- 3. Wash. The tube was washed as in step 1.
- 4. Coupling. Fresh coupling solution was prepared prior to each reaction. A molar excess of 4× was used. 0.4 mmol Fmoc-protected amino acid (Novabiochem) was dissolved in 1000 μL NMP. 0.4 mmol HBTU (Novabiochem) and 0.4 mmol HOBt (Novabiochem) were dissolved in 800 μL DMF. These solutions were mixed for 5–6 min. 0.8 mmol DIEA was added and mixed for about 1 min. The tube was filled with this coupling solution then closed and stirred for at least 30 min on the rotator.
- 5. Ninhydrin Test. After coupling, a ninhydrin test was performed to verify completeness of coupling. Failure would indicate that the coupling should be repeated. In most couplings, we estimated the loading of uncoupled amines to be 1–2 μmol/g. Compared with the original resin loading, this represents a coupling efficiency of 99.7–99.8%.

Acetylation (capping) was not performed. After the final Fmoc deprotection step, the resin was washed $5-6\times$ with DCM. The resin was then lyophilized.

Side-chain deprotection and cleavage from the resin were performed in 95% TFA in water at 4°C. 5 mL of this solution was added to the dry resin in the tube, sealed, and installed on a rotator. After a few seconds, the lid was removed to release generated gas. The tube was then rotated for 1.5 h. Separation and purification were then carried out. The tube was drained into 40 mL cold (4°C) tert butyl methyl ether. An additional 1 mL TFA was washed through the tube. The resulting solution was then centrifuged at full speed for 2–3 min, the supernatant was poured off, and additional ether was added and mixed. This step was repeated three times. On the last, the tube was filled with room temperature ethyl ether. Finally, the precipitated peptide was captured by flushing this solution through a filter and then eluted by redissolving with 60% acetonitrile in water (with 0.1% TFA). The peptide was lyophilized and then purified by collecting the HPLC peak (214 nm detector). Mass spectrometry indicated the correct peptide product at high purity.

The high coupling yields observed throughout the entire reaction suggest that this peptide sequence exhibits low sequence-specific folding (i.e., there was no interference with the synthesis).

7.6.2 Millifluidic synthesis

To investigate the synthesis of peptides on surfaces, synthesis was carried out on amine-derivatized slides using a Teflon flow cell (Figure 7.15) containing channels approximately 2 mm wide. The Teflon cell contained two channels to perform two syntheses simultaneously on two different parts of the substrate. The volume of each channel was measured to be about 75 μ L. In order to ensure complete filling, an "elemental volume" of 200 μ L was selected for all reactions. Diffusion coefficients of amino acids [299, 52] suggest that reactions should last at least 30 min to ensure sufficient time for diffusion if using a stopped-flow technique. If using continuous flow, it is possible that this time could be reduced due to the continous supply of fresh reagents near the surface at the full original concentration.

To determine coupling reagent concentrations, I estimated the number of synthesis sites. ArrayIt SuperAmine substrates (TeleChem International) have an amine loading of 5×10^{12} /mm² [123]. The approximate glass surface area in contact with fluids inside one chamber of the flow cell is 0.59 cm². Thus, approximately 1.2×10^{15} or 2.0 nmol surface amine groups should be available for reaction. Therefore a 40 μ M coupling-solution concentration would be required assuming a 4× excess of reagents and a 200 μ L volume. However, in the literature, peptide arrays are typically coupled with 5 mM solutions of activated amino acids [242]. Given that this concentration has been used successfully in many studies, I chose to use it as well. Presumably the huge excess of reagents will further improve the reaction efficiencies. For 200 μ L of coupling solution, I used 3 μ mol of reagents—a factor of 3 is built in to account for the fact that only about 1/3 of the coupling volume fits inside the reactor.

Syntheses were carried out directly on amine-derivatized surfaces. The substrate was mounted vertically and solutions flowed from bottom to top. Reagents were placed in polypropylene centrifuge tubes and were delivered to the flow cell by pressurizing the head space with a syringe. Reagents were switched by manually moving the tubing connected to the flow cell from one reagent tube to another. Each synthesis cycle consisted of steps similar to the manual synthesis protocol above:

- 1. Wash. The reactors were washed by flowing 200 μ L of NMP through the flow cell. This was repeated 5 times.
- 2. Coupling. Coupling was performed twice, each time filling the reactors with 200 μ L of solution and stopping the flow for 30 min. Fresh coupling solutions were prepared prior to each coupling reaction.
- 3. Wash. An NMP wash was performed as in Step 1.
- 4. Deprotect. Deprotection was performed 3 times, each time filling the chambers with 200 μ L of solution and stopping the flow for 5 min, 5 min, and 10 min, respectively.

Note that, unlike the manual synthesis protocol, coupling is performed first; this is because the derivatized slide is not initially Fmoc protected. After synthesis, the chambers were washed with

DCM 6 times and dried by flowing a nitrogen stream through them. Next, protecting groups were cleaved by introducing a total of 400 μ L of 95% TFA in water through the chamber during a 3 h period. Note that it was necessary to plug the outlet to avoid the deprotection solution being ejected as gas was generated. The substrate was then washed by flowing 50% TFA in water through the reaction chambers, followed by continuous flow of purified water (2000 μ L total). Nitrogen was flowed through the channels overnight to dry the substrate. Fmoc amino acids were purchased from AnaSpec; HBTU and HOBt, from Novabiochem.

Two sequences, N–PGGFL–C and N–YGGLF–C, were synthesized on a single substrate by the above method. Incubation with mouse antibody 3E7 (Abcam Ltd.) then FITC goat anti-mouse (Abcam Ltd.) did not yield the expected fluorescence pattern: 3E7 should bind to YGGFL but not to PGGFL [78].

In order to debug the synthesis, I explored other detection options that could be used to monitor each step of the chemistry rather than requiring the synthesis of a complete 5-mer. Analysis of the deprotection solution (containing Fmoc group) by mass spectrometry was inconclusive, as was analysis of short 1-mer and 2-mer peptides. Measurement of the optical absorption of Fmoc in solution after deprotection (Section 7.2.3), however, proved to be effective. The accuracy of the standard empirical formula was verified by adding deprotection reagents to a solution containing a known quantity of an Fmoc-protected amino acid. It was necessary to modify protocols slightly (notably, by reducing the volumes used) to yield a detectable signal. Deprotection solutions were collected from the flow cell output during each synthesis. Absorbance measurements were performed without dilution and were compared with a blank consisting of 20% piperidine in DMF. The quantity of Fmoc is given by [235]:

(mmol Fmoc) =
$$\frac{A_{301}}{7800} \times (\text{sample volume in mL}).$$
 (7.1)

I performed several experiments to verify successful peptide synthesis reactions. To provide more flexibility in the products that could be analyzed in solution, I made extensive use of the Fmocprotected Rink Amide Linker (RAL) (AnaSpec). This linker is reacted with the substrate in a standard coupling reaction as if it were an amino acid, but can be cleaved after synthesis of a full peptide by treatment with 20% TFA in water (we used 95% TFA). This releases the peptide into solution.

In Experiment 1, I performed coupling of RAL to the substrate in flow chamber A and omitted RAL from the coupling solution in flow chamber B. Products of the deprotection step (10 min 200 μ L, 14 min 200 μ L, 15 min 200 μ L, with 650 μ L flush) were collected and quantitated. For sample A, I measured $A_{301} = 0.018$, corresponding to 2.9 nmol Fmoc. For sample B, I measured $A_{301} = 0.005$, corresponding to 0.8 nmol Fmoc. The results are consistent with expectations: sample B contains essentially no Fmoc, and the amount of Fmoc in sample A is relatively close to the estimated amine loading of the substrate, 2.0 nmol. In Experiment 2, the first experiment was repeated with chambers reversed and coupling reactions extended (60 min instead of 30 min). Collection of deprotection solution (15 min 200 μ L, 15 min 200 μ L, 10 min 200 μ L) gave the reverse results: sample A, $A_{301} = 0.005$ (0.4 nmol); sample B, $A_{301} = 0.031$ (2.4 nmol).

Experiment 3 investigated the use of shorter coupling times—just a single 10-min reaction. Chamber A was coupled with blank coupling solution; Chamber B with RAL. Deprotection solution was collected after reaction (10 min 200 μ L, 5 min 100 μ L). Results indicated a slightly lower amount of product: sample A, $A_{301} = 0.008$ (0.31 nmol); sample B, $A_{301} = 0.050$ (1.92 nmol).

The large amount of "noise" in these experiments, leading to a significant absorbance in the solution expected to be blank, led me to discover that dyes with significant 301 nm absorbance were being leached by the ferrules in the HPLC connectors of the flow cell and components of the centrifuge tubes acting as reagent reservoirs. A ferrule soaked for 1 h in deprotection solution gave an apparent signal of 0.4 nmol Fmoc, and a piece of the lid of a centrifuge tube gave an apparent signal of 0.6 nmol Fmoc. While the soaking times were longer than the deprotection reaction times, these results could explain at least part of the observed contamination.

I next investigated the possibility of multiple couplings in Experiment 4. In Chamber A, RAL was coupled in the first step and leucine (Leu) in the second. In Chamber B, RAL was coupled in the

first step and a blank coupling solution in the second. A coupling protocol of 2×5 min was used, and deprotection consisted of 5 min incubation with 150 μ L of solution, 5 min with 150 μ L, and finally a 100 μ L flush. After the first coupling step, the following Fmoc quantities were observed, indicating RAL had been successfully coupled in both chambers: sample A, $A_{301} = 0.049$ (2.5 nmol), sample B, $A_{301} = 0.039$ (2.0 nmol). Measurements after the second coupling indicated that the second coupling in Chamber A was successful ($A_{301} = 0.041$, 2.1 nmol) and that the lack of coupling in Chamber B resulted in no Fmoc being available for release ($A_{301} = 0.007$, 0.4 nmol). From these results, we can also estimate that the cycle efficiency was 84%.

Attempts to verify cleavage of the Rink Amide Linker were not successful. In typical experiments, RAL was coupled to the solid support. Deprotection was carried out in one chamber to remove the Fmoc but not in the other. A cleavage reaction was carried out in both chambers and the product was collected for absorbance analysis. However, absorbance measurements were inconsistent, and it was not possible to interpret the results—perhaps the cleaved linker has significant absorbance at 301 nm.

7.6.3 Microfluidic devices

Several attempts were made to repeat the small scale synthesis in a PDMS microfluidic channel and measure the optical absorbance of the deprotection solution. One-layer PDMS microfluidic chips were fabricated by curing Sylgard 184 on a mold for 30 min at 80°C. Cured chips were removed from the mold, sealed to amine-derivatized substrates, and baked overnight at 80°C. PDMS was chosen as, at the time, it was the only available microfluidic device material capable of making reliable seals to amine-derivatized glass substrates. Previous experiments had shown PDMS to exhibit moderate swelling in dimethylformamide (DMF) and N-methyl-pyrrolidinone (NMP), and permanent channel damage (collapse and deterioration) after 48 hours of flow through channels. However, chips did not delaminate from the surface under these conditions. The microchannel design was the first pattern of the passive 6-mer chip design in Figure 7.8. It contains four independent channels, each of which can serve as a reaction "chamber". Each channel is 100 μ m wide and several centimeters long, having a total surface area of fluid-glass contact of about 0.21 cm²—approximately 1/3 the surface area in the Teflon fluidic device. The volume of each channel/chamber, however, is significantly smaller: about 0.2 μ L instead of 75 μ L. The "elementary volume" of solutions was therefore reduced to about 2.5 μ L rather than the 200 μ L used in the Teflon flow cell. Theoretically, the reduced dimensions should permit faster reactions as the diffusion time is dramatically reduced.

Basic synthesis experiments (e.g., Experiment 1) were performed in an identical manner as for the Teflon flow cell, using two PDMS microchannels as reaction chambers. However, optical absorbance measurements showed very inconsistent results (including occasional negative absorbance values) for repeated attempts.

Samples of PDMS were soaked in 20% piperidine/DMF to determine if prolonged exposure would leach compounds or induce other changes to alter the absorbance of the solution. No effect was observed after 1 h immersion. However, tests showed that piperidine itself has a significant optical absorbance at the same wavelength as the Fmoc group. Thus an imbalance in the amount of piperidine being collected for each sample could lead to the observed differences, for example if the PDMS selectively absorbs piperidine or DMF from the solution as it is flowed through. In one test, a 500 μ L solution flowed through PDMS for 24 h showed an absorbance of $A_{301} = 0.030$ (apparent 1.9 nmol Fmoc). An alternative detection method is needed to continue studies in PDMS. Alternatively, a preferable option would be to perform synthesis in microfluidic chips fabricated from solvent-resistant materials (Chapters 3, 4, and 5); however, it has not been possible to bond such chips to derivatized glass substrates.

7.7 On-bead array synthesis

Microfluidic synthesis can produce arrays directly on flat substrates or on trapped beads. The former offers the possibility for the highest density due to its simpler design and is more practical for large arrays. However, the latter is useful in many instances. For example, a trapped column has far greater surface area than a flat substrate and would yield much larger quantities of products. If the final products are to be cleaved and removed from the chip (or moved to another area of the chip), then on-bead synthesis is preferable. Products could be released by cleaving them from the support, or the support can be flushed out of the chip with molecules still attached. One advantage of the latter is that the final deprotection and cleavage reagents need not be compatibile with the microfluidic chip.

Another situation in which bead synthesis would be useful is if the microfluidic device cannot be adhered to an appropriately derivatized surface. In many of the most successful solvent-resistant microfluidic technologies that we have demonstrated (Chapters 4 and 5), it has been impossible to bond a push-down microfluidic device directly to (derivatized) glass. Though in principle it may be possible to modify the polymers used in these devices to present certain functional groups at the surface, such modifications have not been attempted since the structures are proprietary.

For proof of principle testing, we designed a solid-phase synthesizer chip (Figure 7.19) that operates as outlined in Figure 7.20. The chip has a main flow path that is fed by a series of individually-valved inputs for various reagents—monomers, wash solutions, and other reagents. For example, in DNA synthesis, these are used for derivatized solid-support beads, nucleotides dissolved in acetonitrile (A,C,G,T), oxider solution, activator solution, deblocking solution, acetonitrile (as a wash solvent), capping solution (2 parts), and helium or argon. The wash solvent should always be located furthest upstream from the column. Reagents can be delivered individually or can be mixed by the on-chip rotary mixer [43]. Though reagents could simply be mixed by diffusion by opening two values at once, the mixer provides a means to mix reagents in precise ratios (by loading the desired amount of each reagent into the mixer) without having to account for differences in fluidic resistance from each inlet or differences in fluid properties. Reagent switching is implemented on-chip to avoid the problem of fluid volume mismatch that exists between external fluid controllers (e.g., a commercial DNA synthesizer) and the microchannels. Note that a multiplexer is not used in this design for two reasons: (i) it complicates the washing process when switching reagents; and (ii) dedicated valves allow greater flexibility. The flow path goes to a column, consisting of resin/beads packed behind a partially closing valve [179]. The 200 μ m wide column has a square profile so that it is not completely closed when the valves at its ends are actuated. These valves close to a sufficiently small gap to trap particles such as 0.7 μ m derivatized silica microspheres, yet leave a sufficiently large gap that solutions can flow, thus acting as "frits". The column can be loaded with resin/beads of any functionality for the desired synthesis. The desired product is synthesized by programming the delivery of reagents and wash solvents to the column. Once finished, the product can be extracted by flushing the beads out and performing the cleavage reaction off-chip, or cleaving can be done on-chip and the product collected at the output. The design in Figure 7.19 is intended to synthesize only a single product, but it would be straightforward to scale up to a parallel chip. By sharing reagent inlets, the design of a parallel synthesizer would not be significantly more complex than an individual synthesizer.

In addition to being a proof of concept for a highly parallel synthesis chip, a single synthesizer on a chip offers several advantages. For example, equipment cost can be significantly reduced, as HPLC valves and fittings used in macroscopic automated synthesizers are quite expensive. The device could synthesize products on very small scales if needed—often the smallest scale of DNA or peptide synthesis is far too large, and product and reagents are wasted. It is also possible to integrate and automate additional aspects of the synthesis on the chip such as cleavage from the solid support, purification, and analysis, possibly for feedback control of the reaction. Furthermore, the chip could be integrated with other sophisticated functions such as screens for binding affinity or drug effects.

Significant steps were made in realizing this chip implementation. However, due to lack of sufficiently reliable solvent-resistant chips to fabricate a fully working device, multi-step synthesis has not been demonstrated. Chips were fabricated first from Sylgard 184 (Dow Corning) using typical methods (Chapter 2). Frit valve fabrication is detailed in Appendix A.1.4. Devices were spin-coated with CYTOP diluted 1:10 in Fluorinert FC-75 and bonded to CYTOP-coated PDMS by the methods in Section 3.5.1.2. Due to the large particle size in commercial CPG synthesis columns (typically 50–80 μ m), these beads could not be used in this device—the particle diameter is on the same order as the channel dimensions. Instead, silca microspheres (0.8 μ m and 1.5 μ m) were purchased from Bangs Laboratories, Inc. (Fishers, IN) and derivatized by a protocol modified from



Figure 7.19: Design of a solid-phase synthesis chip. (Top) CAD design. Fluid channels are shown in blue, control channels in red, and the main synthesis column (in the fluid layer) in green. Note that most valves have been labelled at their control channel inlet, rather than at the actual valve position, to avoid clutter. The main flow path is indicated by the dotted line. Reagents, beads, wash solvents, etc. are introduced via Inputs 1 to 12. Reagents may come from connections to pressurized bottles. Each has a dedicated valve to allow individual reagents or specific combinations to be injected. A rotary mixer actuated by a peristaltic pump is included in the flow path for reagent combinations that must be thoroughly mixed before entering the column (e.g., nucleotides and activator, in DNA synthesis). The rotary mixer on this chip has a serpentine shape rather than a circular shape such that the volume is sufficiently large to fill the column. The column is flanked by "frit valves" to trap solid-support beads that are flowed through as a slurry during the column-packing phase. For testing purposes, this chip was designed to have a very large column and mixer (to ensure a large quantity of product), and to have wide spacing between input ports (to address the problem of delamination near holes that was commonly observed in solvent-resistant materials). In production, this chip could be dramatically reduced in size. (Bottom) Photograph of a commercial peptide synthesizer with two columns (Applied Biosystems 433A) compared in scale with the synthesizer chip. Not shown for the microfluidic chip are the pressure source and small vials of reagents.



Figure 7.20: Details of solid-phase synthesis chip operation. (a-c) Operation of mixer flow control valves. (Note that the size of the mixer has been reduced in these diagrams for clarity.) Valves can be configured (a) to bypass the mixer; (b) to flow through the mixer for washing or for loading plugs of reagents sequentially into the mixer; or (c) to mix the loaded reagent plugs. In the latter configuration, the peristalic pump (not shown) is activated to circulate the flow through the serpentine channel. (d-e) Operation of column flow control valves. The valves at the inlet and outlet of the column are "frit valves". They close only partially to allow liquids to pass through while holding the solid-support column in place. The inlet frit valve is only needed if reverse flow is used during synthesis to agitate the beads. The outlet frit valve must remain closed permanently unless it is desired to flush the solid-support resin/beads out of the chip. The remaining three values choose between (d) a bypass configuration (e.g., when initially purging reagent inlets, when switching between reagents, or when flushing the flow path after loading each plug of fluid into the mixer) and (e) a flow-through configuration. The latter is used in many situations: column packing (a slurry of resin/beads is injected); reaction (the next reagent for synthesis is injected); washing (the wash solvent is injected). Not shown is one additional configuration in which the fully closing value at the column exit can be closed if it is desired to stop the flow to allow solutions to react for a prolonged period with the growing product on the resin. In all figures, filled red rectangles represent closed values (or frit values), while open red rectangles represent open valves.

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LeProust *et al.* [163]. First, the beads—originally shipped in water—were resuspended in ethanol in preparation for derivatization. Beads were centrifuged in a microcentrifuge tube, allowing a significant portion of the supernatant to be removed. Ethanol was then added and the tube was vortexed. This sequence of steps was repeated several times to ensure that most of the water had been eliminated. Beads were then derivatized with 3-aminopropyltriethoxysilane (see Appendix A.2.5) using ethanol as a solvent. Beads were constantly stirred during derivatization.

In partially working chips, packing of the column was successfully demonstrated. We found it important not to close valves on top of beads as the beads become embedded in the CYTOP coating and prevent subsequent valve closure. Not surprisingly, the packed column significantly reduces the flow rate through the channel, sometimes to the point of clogging. We were able to flow several reagents through the column, including dichloromethane (as required in DNA synthesis). CYTOPcoated chips did not confer sufficient resistance for long term flows, however. Eventually swelling occurs and blocks the channels—probably at inputs ports, where the chip is constantly exposed even while other reagents are being flowed through the device. Saurabh Vyawahare designed an alternative microfluidic synthesis device intended to connect to a DNA synthesizer in a similar fashion to the Teflon flow cell described earlier in this chapter. Because fluid handling is performed externally, high-swelling solvents are only in contact with the chip during the time of reaction.

7.8 Summary

Combinatorial arrays are a powerful tool combining the benefits of combinatorial chemistry and high throughput screening. Arrays can be created using a variety of technologies. For very large combinatorial arrays of compounds, deposition methods such as spotting, ink-jet printing, etc. run into practical difficulties in terms of storing and manipulating the individual compounds. *In situ* synthesis of arrays is the only reasonable solution. Array synthesis methods include light-directed synthesis, ink-jet synthesis, and microfluidic synthesis. The use of microchannel walls rather than light exposure to delineate spot boundaries allows the use of conventional DNA or peptide synthesis chemistry, which is more efficient and results in a much higher purity synthesis. Moreover, microchannels give a more distinct boundary to the reaction area, offer the potential for reduced size, and eliminate the need for costly optical components such as photomasks for each step of the reaction or a digital micromirror array and controller.

The stripe synthesis method provides a simple and elegant approach for generating combinatorial arrays of compounds such as DNA. This method is generalizable to any solid-phase synthesis chemistry. We have improved on the original concept of Southern *et al.* and devised a microfluidic synthesis device that needs to be sealed only once to the (derivatized) substrate, eliminating potential errors due to misalignment and contamination. This strategy also lends itself to further miniaturization and automation. Microfluidic devices with tens of thousands of individual fluidic elements have already been demonstrated using channel sizes of 50–100 microns [48], and channels as small as 100 nm have been demonstrated with some technologies. This suggests that arrays synthesized by microfluidics might one day contain millions or billions of different compounds such as DNA, RNA, PNA, peptides, oligosaccharides, and small molecules. It is unlikely that this potential capability can be matched by other array synthesis methods.

We have taken several steps towards the microfluidic synthesis of DNA and peptide arrays. In conjunction with the development of solvent-resistant microfluidics (Chapters 3, 4 and 5), sophisticated combinatorial chemistry applications in microfluidic chips are not far off.

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Chapter 8

Universal Gene Expression Analysis with Combinatorial Arrays

8.1 Introduction

The ability of DNA microarrays to simultaneously measure thousands of binding interactions has led to their rapid adoption in many applications: gene expression profiling [252, 183], DNA sequencing [65], genomic fingerprinting [157], and studies of DNA binding proteins [28], to name a few. Gene expression profiling, in particular, has exploded into an enormous field encompassing a wide variety of applications. For example, in the field of functional genomics, comparison of expression levels across many different experimental conditions [127, 24, 61], or between wildtype and knockout or overexpressed cells, helps to determine gene function and regulatory network structure. Differences in induced expression changes in closely related types of cancer have been used as a means for reliable diagnosis [4]. In the pharmaceutical industry, expression studies help to correlate drug response (positive or negative effects) with genetic profiles to predict the effects of the drug in new patients. Gene expression profiling is also used in the field of developmental biology to untangle the mysteries of development and aging, and in a variety of other fields to determine the biological

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response to drugs, infections, and environmental toxins. A typical experimental setup is illustrated in Figure 8.1.



Figure 8.1: Typical experimental setup for gene expression profiling. Cells are harvested from the sample of interest and the messenger RNA (mRNA) is extracted and labeled. One common labeling technique involves reverse transcription of the mRNA into complementary DNA (cDNA) in the presence of fluorescently labeled nucleotides. The labeled sample is then hybridized to a microarray consisting of many spots, each with single-stranded DNA of a particular (known) sequence tethered to the array substrate. cDNA from the sample binds to complementary sequences on the microarray and can be detected quantitatively by fluorescence imaging. The brightness of each spot reflects the amount of the corresponding mRNA present in the original sample and is thus an estimate of the level of expression of the corresponding gene. Often experiments are performed in a differential fashion to cancel out many sources of errors. In this case, two labeled samples (with different fluorophores) are hybridized simultaneously to the microarray. One sample acts as a reference and is compared to a sample prepared under a different condition. For example, one can compare cells that have and have not been exposed to a drug, or one can compare cells from normal tissue with those from cancerous tissue. The pattern of binding of each sample to the microarray is observed in a different fluorescence "channel" and an analysis is performed to determine for each gene a ratio of the expression in the experimental sample to the reference sample. The ratios are assumed to represent expression changes induced by the differences in the experimental conditions between the two samples. Note that later in this chapter, the term "gene" is used loosely to refer to the cDNA being hybridized to the array, and the term "oligo" or "n-mer" is used to refer to the probe DNA tethered to a single spot on the array.

Diverse methods for fabricating expression arrays have been developed in the past several years, some based on the deposition of cDNA libraries and/or oligonucleotides by robots [238] or inkjet printers [204], and others based on *in situ* DNA synthesis employing photolithographic [37], micromirror [203], or ink-jet technology [113]. A drawback of all of these methods is that one must carefully choose—in advance—which sequences to probe. As a result, revisions to the arrays to correct mistakes or incorporate new genomic information are costly, requiring arrays to be redesigned and manufactured. It is desirable to have a universal gene expression chip that is applicable to all organisms, ranging from bacteria to human, including those that lack complete cDNA libraries or whose genomes are not yet fully sequenced.

One way to realize universality is to synthesize a combinatorial *n*-mer array containing all 4^n possible oligos of length *n*, the key problem being to find a value of *n* that is large enough to afford sufficient specificity, yet is small enough for practical fabrication and readout. Combinatoric *n*-mer arrays can conveniently be fabricated in a small number of simple steps using conventional solid phase synthesis chemistry and arrays of parallel fluid channels in perpendicular orientations to mask the reagents. This microfluidic synthesis technique, described in detail in Chapter 7, has the potential to fabricate arrays with spot sizes as small as the tiniest microchannels that have been demonstrated—about 100 nm.

Until high-resolution, non-optical readout methods become practical, microarray densities will ultimately be constrained by the optical diffraction limit. With this lower bound of about 0.28 μ m on pixel size, *n*-mer arrays are limited to 8×10^9 distinct spots per square inch, corresponding roughly to a 16-mer array on a 1-inch-square chip. While it is possible to fabricate arrays with larger surface areas we consider here arrays whose sizes are comparable to the current state-of-the-art in order to facilitate sensitivity comparisons. We therefore address the question of whether one can extract useful gene expression information from combinatorial arrays of short (i.e., $n \leq 16$) oligonucleotides.

We first develop an analytical model to predict, for a given value of n and a particular genome, the average "ambiguity" of the resulting hybridization pattern. With this model, we argue that for a certain minimum value of n, the ambiguity is sufficiently low that individual gene expression levels can be extracted from the hybridization data.

8.2 Results and discussion

8.2.1 Basic analytical model

Hybridization of a single labeled mRNA species to an *n*-mer array will cause numerous spots to fluoresce, yielding a characteristic "fingerprint" pattern. A diverse sample of mRNA transcripts yields an equilibrium hybridization pattern which is a linear superposition of numerous overlapping fingerprints, a pattern from which gene expression levels can be deduced by inverting a huge matrix of size 4^n —the number of distinct sequences on the array (see Section 8.3.1). This calculation is impractically large, but can be avoided by taking advantage of the vast redundancy inherent in a combinatorial array. One can ignore the ambiguous oligonucleotides that bind many different transcripts, instead concentrating on the information-rich oligonucleotides that bind few transcripts. We formalize this approach by defining the "degeneracy" of an *n*-mer as the number of different mRNA transcripts it can capture, which of course depends on the transcript uniquely; however, it is more realistic to expect to find small oligonucleotide groups, each oligo of which binds only to transcripts in a small independent group. In these cases the aforementioned matrix has vastly reduced dimension, is sparse, and is in block-diagonal form, greatly simplifying its inversion. The lower the average degeneracy, the easier is the construction of the block-diagonal matrix.

We now describe an analytic model that predicts the average degeneracy of the $N_o = 4^n$ distinct oligonucleotides on an *n*-mer array when analyzing a transcriptome of N_g "genes". An individual mRNA transcript of length ℓ has $b = \ell + 1 - n \approx \ell$ subsequences¹ of length *n*, any of which can serve as a site for binding the complementary *n*-mer affixed to the array. Assuming the transcript has a *random* nucleotide sequence, the probability that a *particular n*-mer "captures" the transcript is $p = b/N_o$. This is a simple Bernoulli trial. To compute the expected number of *different* transcripts to which the *n*-mer binds (i.e., its degeneracy, *d*), it is necessary to carry out N_g Bernoulli trials—one for each transcript. The result is a binomial distribution of degeneracies, which can be approximated

¹Transcripts typically have lengths on the order of 1000 nucleotides, thus $\ell \gg n$.
by the Poisson distribution,

$$P_{\text{Binomial}}(d; N_g) = \binom{N_g}{d} p^d (1-p)^{(N_g-d)} \approx P_{\text{Poisson}}(d; \lambda) = \frac{e^{-\lambda} \lambda^d}{d!},$$
(8.1)

where $\lambda = N_g p$ is the average degeneracy.

Not all genes have exactly the same length. One can account for non-uniform transcript length by computing the degeneracy distribution as a weighted average of Poisson distributions:

$$P(d; \bar{d}) = \sum_{\ell=0}^{\infty} P_{\text{Poisson}}(d; \lambda(\ell)) f(\ell), \qquad (8.2)$$

where $f(\ell)$ is the fraction of transcripts with length ℓ . The mean value of this new distribution is:

$$\bar{d} = N_g \bar{p} = \frac{N_g \bar{b}}{N_o} \approx \frac{N_g \bar{\ell}}{N_o},\tag{8.3}$$

where $\bar{\ell}$ is the average transcript length.

The predictions of this model are compared with the true degeneracies calculated from yeast ORFs and mouse transcripts in Table 8.1 and Figure 8.2. It is well known that there are significant statistical biases in nucleotide and codon distributions [202]. Despite the fact that this model neglects these variations, its predictions agree surprisingly well with the genomic data. The reduced agreement for larger average degeneracy values can be attributed primarily to a "clipping" effect that occurs when the average degeneracy value is close to the maximum possible degeneracy value (i.e., the number of genes), a regime in which we are not interested.

8.2.2 Accounting for mismatches

In practice, hybridization is imperfect and stable duplexes can form between strands that are not perfect complements. As a first approximation, we suppose that the hybridization stringency can be tailored to prevent duplex formation when the number of mismatched positions exceeds some thresh-



Figure 8.2: Comparison of predicted and actual degeneracy histograms. Degeneracy histograms determined from actual yeast genomic sequences (square markers) are compared with predictions of the analytical model (continuous line). Each histogram shows the fraction of *n*-mers having each degeneracy value. Predicted curves were obtained by taking a weighted average of Poisson distributions as in Equation 8.2, with the weights corresponding to the distribution of transcript lengths in yeast. There are no fitted parameters. Actual histograms were generated with custom computer software that counted the degeneracy of each *n*-mer in the yeast genome. (a–d) Histograms for the case of 0 mismatches, for n = 9, n = 10, n = 11, and n = 12, respectively. (e–h) Histograms for the case of 1 mismatch for the same range of *n*-values. Similar results were obtained for the mouse genome (not shown). (Reproduced from [275] with permission. Copyright Cold Spring Harbor Laboratory Press, 2002.)

		0 mi	smatches	1 m	ismatch
Organism	n-mer size	\bar{d} (actual)	\bar{d} (predicted)	\bar{d} (actual)	\bar{d} (predicted)
yeast	7	479.3	544.2	4190	11970
yeast	8	130.2	135.9	2120	3399
yeast	9	33.42	33.96	790.0	950.9
yeast	10	8.420	8.485	245.8	263.0
yeast	11	2.110	2.120	70.29	72.07
yeast	12	0.5275	0.5295	19.39	19.59
mouse	9	130.2	134.1	3308	3754
mouse	10	32.66	33.44	976.2	1037
mouse	11	8.161	8.343	273.8	283.6
mouse	12	2.037	2.081	74.96	77.00
mouse	13	0.518	0.519	20.27	20.77
mouse	14	0.127	0.130	5.442	5.569

Table 8.1: Comparison of average degeneracy predictions with actual data. Average degeneracies are tabulated for both yeast and mouse, for various values of n and two different hybridization stringencies (0 mismatches and 1 mismatch). Predicted values were determined from the analytical model, Equation 8.3, while actual values were tabulated from actual yeast and mouse genomic sequence data.

old, m. Implementing this assumption requires one to establish hybridization and wash conditions that simultaneously provide adequate stringency for all spots on the array.

Comparing the melting curve for a perfectly matched duplex with that of a mismatched duplex indicates that a "window" of hybridization temperatures exists within which the perfect match is stable and the mismatch sufficiently unstable that the two can be distinguished. Fortunately, the width of this temperature window is largest for short oligonucleotides, due to single-nucleotide mismatches having an increasing destabilizing effect as oligo length is reduced. Numerous experiments have demonstrated that single-nucleotide mismatches can reliably be distinguished from perfect matches. This capability is exemplified by Wang *et al.* [284], who designed several huge microarrays (with 150,000–300,000 features) to detect single nucleotide polymorphisms (SNPs) in the human genome. They were able to resolve single-nucleotide central mismatches for all features on each chip simultaneously. The discrimination of end mismatches is somewhat more difficult due to the narrower range of suitable temperatures [64], but successful techniques have been demonstrated by several groups. Kutyavin *et al.* [156] employ minor-groove-binding molecules that stabilize properly formed double helices. Yershov *et al.* [301], Stomakhin *et al.* [255], and Maldonado-Rodriquez *et al.* [176] describe methods whereby duplexes with properly matched ends are stabilized by the phenomenon of "contiguous base stacking". It has also been reported that this level of discrimination can be achieved by hybridizing DNA to a PNA array, due to the higher mismatch sensitivity of DNA-PNA binding compared to DNA-DNA binding [117, 223, 291]. Of particular note for *n*-mer arrays where n is relatively short, it has also been observed that discrimination is simpler with shorter oligonucleotides due to the larger *relative* differences in binding of the perfect and non-perfect matches to the target [64].

To simultaneously achieve adequate discrimination across the whole *n*-mer array requires a means to reduce the intrinsic variation in melting temperatures (due to the variation in CG content from 0%-100\%, among other factors). This is an active area of research, and already a number of groups have demonstrated successful techniques with small arrays. For example, Sosnowski *et al.* [245] report single-nucleotide mismatch discrimination under the same hybridization and wash conditions for two different sequences differing in intrinsic melting temperature by 20°C. More recently, chips with several thousand addressable spots have been produced based on this "electronic stringency control" method [105]. Other approaches include the addition of auxiliary molecules during hybridization [224, 128], the use of modified bases, or modification of the DNA backbone [110], to homogenize melting temperatures. Despite the fact that progress in array technology may yield nearly perfect hybridizations, for practical purposes we have relaxed this requirement in the conclusions that follow. We therefore assume that sequences can bind with up to one mismatch.

Mismatches increase the probability p that a gene binds to a particular immobilized n-mer. The increase is a simple multiplicative factor,

$$c = \sum_{k=0}^{m} \binom{n}{k} 3^k,\tag{8.4}$$

reflecting the increased number of subsequences that are *sufficiently* complementary (i.e., having $\leq m$ mismatches) for binding to the *n*-mer. The factor *c* enters the equation for average degeneracy (Equation 8.3) simply as a multiplier. An alternative viewpoint is that the number of distinct oligonucleotides on the array is reduced by this factor to $N'_o = 4^n/c$. Furthermore, because the decreased number of spots corresponds to a lower effective value for the *n*-mer length:

 $n' = \log_4 (4^n/c) = n - \log_4(c)$, one can quantify the effect of mismatches. When the analytical model is modified to include mismatches, we find excellent agreement between predictions and actual calculation (Table 8.1).

8.2.3 Truncation of transcripts

The size of the *n*-mer array is not the sole degree of freedom available to reduce the average degeneracy; one can also reduce $\bar{\ell}$, the average transcript length (see Equation 8.3). With appropriate nucleases and controlled reaction conditions it should be possible to truncate the length of all transcripts before hybridization according to one of two schemes: (1) reduction in transcript length by an average length $\overline{\Delta L}$ from one end, or (2) reduction of all transcripts to the same average length \bar{L} . For example, the duration of enzymatic digestion could be tailored to remove a desired average number of nucleotides from all transcripts (scheme 1). To implement scheme 2, one could protect the transcripts along a desired length (e.g., by polymerizing a second strand for a controlled time), subsequently digesting away the remaining unprotected portion. Since truncation would occur prior to hybridization, it can be incorporated into the analytic model simply by replacing $\bar{\ell}$ everywhere with $\bar{\ell} - \overline{\Delta L}$ or with \bar{L} , depending on the truncation scheme. Figures 8.3a and 8.3b demonstrate that the model continues to yield accurate predictions with truncated transcripts in addition to mismatches.

8.2.4 Estimating n

Having validated the model over a wide range of parameter values, we can estimate useful sizes for n-mer arrays. Figure 8.3c illustrates combinations of parameter values that are predicted to yield an average degeneracy of 1, (i.e., the "ideal" case), for which gene expression levels can be trivially solved. As shown for the case of 1 mismatch, to achieve this target in yeast requires a 14-mer array if transcripts are untruncated or a 12-mer array after transcript truncation to about 80 bp. In mouse, the target degeneracy is nearly realized with a 15-mer array without truncation or a 14-mer array after truncation to 90 bp.



Figure 8.3: Comparison of predicted and actual average degeneracy. Predictions of average degeneracy are compared with calculations from actual sequence data, for the case of 1 mismatch: (a) yeast; and (b) mouse. Continuous lines represent predictions (with no fitted parameters) of average degeneracy as a function of the *n*-mer length, n, for varying degrees of transcript length truncation to a fixed length, L, computed from Equation 8.3 with modifications for mismatches and length truncation. ("Raw" designates the untruncated cases.) Discrete points represent the actual average degeneracy values tabulated from genomic sequence data. Due to the presence of many ESTs in the mouse UniGene database, the average transcript length for mouse is reported as much lower than yeast, so we have included a predicted curve for a hypothetical average gene length of 1500 bp. (c) Predicted relationship between parameter values to achieve an average degeneracy of 1 (the trivial case). (Adapted from [275] with permission. Copyright Cold Spring Harbor Laboratory Press, 2002.)

Our results so far have considered the average degeneracy of *all n*-mers on the array. However, when the degeneracy is sufficiently low, only a tiny subset of the oligos are needed for monitoring individual gene expression levels. A logical starting point is to consider, for each gene, the minimum degeneracy *n*-mer to which it can bind. Transcripts having "minimum degeneracy" equal to 1 are obvious trivial cases, as they can be monitored uniquely by a single array spot. Of the remaining transcripts, those that share their minimum degeneracy oligo with only trivial genes are also trivial by such an association. Statistically, a sufficiently large fraction of genes having a minimum degeneracy of 1 should render all genes trivial. Modifications to our purely analytic model fail to make accurate predictions for small subsets of oligonucleotides, presumably due to the underlying non-randomness of real genomes. However, beginning only with a histogram of the minimum degeneracy values for all genes in an organism (Figure 8.4), it is easy to estimate the likelihood of the above associations and predict the total fraction of genes whose expression levels can be trivially solved (see Section 8.3.4). To check these predictions, we wrote a computer program to determine exactly the fraction of trivially solvable genes based on the individual gene sequences.

A few results for the case of 1 mismatch are summarized in Table 8.2. In general, we found that nearly all genes turn out to be trivial if the fraction of genes having minimum degeneracy equal to 1 (Figures 8.5a and 8.5b) is at least about 80%. With a 10-mer array and transcript truncation to 50 bp, 98.8% of yeast transcripts are trivial. Most of the non-trivial genes are in fact unsolvable because they have identical sequences after truncation. Omitting the truncation would eliminate this problem and also simplify the experimental protocol. No truncation is needed with a 12-mer array, in which case 99.8% of transcripts are trivial. Upon close inspection, we found that most of the non-trivial genes may actually be unsolvable because they differ by only a few base pairs from one another. Similar results were obtained for mouse. With a 12-mer array and truncation to 100 bp, 97.9% of mouse transcripts are trivial; 99.6% of mouse transcripts are trivial with a 13-mer array and no truncation. Note that these required n values for both yeast and mouse are lower (by 1 or 2) than the previous predictions (Figure 8.3c), which were based on the *average* degeneracy taken



Figure 8.4: Minimum degeneracy histograms for the mouse genome, assuming 1 mismatch. Each histogram shows the fraction of transcripts having a given *minimum* degeneracy value. The minimum degeneracy of a transcript is determined by finding the degeneracy of all oligos to which it can bind and then selecting the lowest. Data for the histograms were generated by custom computer software that examined actual sequence data to find the *n*-mer with lowest degeneracy that binds to each transcript (allowing for up to 1 mismatch). As expected, increasing *n* and decreasing the transcript length both increase the proportion of genes having low minimum degeneracy. (a) 11-mers, no truncation; (b) 11-mers, truncation to 50 bp; (c) 12-mers, no truncation; (d) 12-mers, truncation to 100 bp. (Reproduced from [275] with permission. Copyright Cold Spring Harbor Laboratory Press, 2002.)

over all *n*-mers. It is likely that even smaller arrays can be used if one is willing to expend more computational effort and address also the non-trivial cases.

Organism	n	Truncation	Fractionwith $d_{min} = 1$	Fraction trivial (predicted)	Fraction trivial (actual)	Inherent redundancy
yeast	10	50 bp	0.887	0.988	0.987	10.96
yeast	12	none	0.966	1.000	0.998	54.14
mouse	12	100 bp	0.809	0.996	0.979	6.17
mouse	13	none	0.906	1.000	0.996	20.28

Table 8.2: Predicted and actual fraction of genes that can be trivially solved for several useful array sizes. All data assume single mismatches. For each set of parameters, several quantities are listed. The fraction of transcripts with a minimum degeneracy of 1 ($d_{min} = 1$) was tabulated from the raw genome sequence data based on the *n*-mer size and truncation length. The predicted and actual fractions of transcripts that can be trivially solved were determined by the methods in Section 8.3.4. It is notable that in the cases shown here (and others not shown), nearly all genes could be trivially solved even when the fraction of genes with $d_{min} = 1$ was only 80%. Inherent redundancy (i.e., the average number of "unique oligos" per transcript) is also included for reference. In most cases where a high fraction of transcripts are trivially solvable, the intrinsic redundancy was observed to be on the order of 10.

8.2.5 Redundancy

Microarrays using oligonucleotides generally require more than one probe per gene to produce reliable results. With the decreased feature sizes and shorter probe lengths of combinatorial *n*-mer arrays, the importance of redundancy is likely to be even greater. Thus, while in principle only a single oligo is needed to monitor each gene, in practice one would use multiple oligos to allow averaging over independent measurements. Redundant measurements reduce the relative impact of experimental variations in binding and readout and increase the level of confidence in the measured values [162], particularly for genes expressed at low levels [136].

An approximate measure of the inherent level of redundancy in an array is the average number of "unique oligos" per gene. This quantity can be predicted by dividing the total number of unique oligos (i.e., oligos that bind to only one gene)—determined from either the Poisson model or the actual genomic data—by the number of genes. For the four array sizes discussed in the previous section, the average redundancy is on the order of 10 unique oligos per gene (see Table 8.2).

To ensure that a high fraction of genes have *at least* 10 unique oligos per gene, computing the *average* is not sufficient: the fraction must be calculated directly from the genomic sequence



Figure 8.5: Fraction of transcripts having minimum degeneracy equal to 1. Plots show the fraction of transcripts having minimum degeneracy (d_{min}) equal to 1 (i.e., binding to an oligo that does not bind to any other transcripts) over a range of *n*-mer sizes and truncation lengths *L*, assuming 1 mismatch. ("Raw" designates untruncated cases.) (a) yeast; (b) mouse. It turns out that when at least a sufficient fraction ($\approx 80\%$) of transcripts have $d_{min} = 1$, nearly all gene expression levels can be trivially solved. (Reproduced from [275] with permission. Copyright Cold Spring Harbor Laboratory Press, 2002.)

data. We used customer computer software to do so. For yeast and a hybridization stringency of 1 mismatch, an 11-mer array with truncation to 100 bp ensures that 97.0% of genes bind to at least 10 unique oligos. A 13-mer array with truncation to 200 bp ensures that 99.6% of genes bind to at least 10 unique oligos in mouse with 1 mismatch. In Figure 8.6, actual redundancy is plotted against predicted redundancy for several sets of parameter values. These plots suggest that in order to have a large fraction of genes with the desired redundancy x, one should choose a set of parameters that *predicts* an average redundancy of about 10x.

8.2.6 Conclusions

Since the mouse genome is only slightly smaller than the human genome, the results above provide an estimate of the required size for a *universal* array, namely $n \ge 12$ for truncated transcripts or $n \ge 13$ for untruncated transcripts. To ensure a redundancy of at least 10 unique oligos per gene, the required size is $n \ge 13$. Both figures are well within the limit of practical fabrication and readout ($n \le 16$). While not universal, arrays as small as n = 10 would permit the study of microorganisms as complex as yeast.

In addition to universality, combinatorial *n*-mer arrays offer other significant advantages. For instance, since selection of *n*-mers with which to identify transcripts is performed in software, data can be reanalyzed (avoiding additional experiments) as genomic sequence data is updated. In addition, the selection criteria can easily be modified to incorporate additional constraints on parameters, such as spot quality and melting temperatures, to yield higher quality results. Besides gene expression analysis, combinatorial *n*-mer arrays have potential applications in such diverse areas as DNA sequencing by hybridization [65], the study of DNA binding proteins [28], and genomic fingerprinting [157].

As a final note, we point out that while combinatorial *n*-mer arrays can be *fabricated* without genomic knowledge, our analysis strategy does make use of known genomic sequence data as a prerequisite for *interpreting* the data. These data now exist in an essentially complete form for several bacteria, yeast, worm, fly, mouse, and human, among many other organisms. A comprehensive list



D

1000



Figure 8.6: Comparison of predicted and actual redundancy. Redundancy is defined here as the number of unique oligos that bind to a gene. Since unique oligos bind to no other genes, their hybridization signal serves as an unambiguous measure of the expression of that gene. If there are several unique oligos for a gene, then several independent measurements are made—this is what is meant by "redundancy". On the horizontal axis is the predicted redundancy, computed simply as the average number of unique oligos per gene (i.e., the total number of oligos with a degeneracy of 1 divided by the total number of genes). On the vertical axis is the actual fraction of genes having at least x unique oligos. Each plotted data point represents a particular *n*-mer size and truncation length. Some specific combinations are shown in Table 8.2. A general trend that can be observed in these plots is that in order to ensure that a large fraction of oligos have the desired redundancy x, one should choose a set of parameters that gives a *predicted* redundancy of about 10x. (a) Data for yeast (1 mismatch); (b) Data for mouse (1 mismatch).

a

Actual fraction of genes having at least 'x' unique oligos 1

0.8

0.6

0.4

0.2

0 ∔∎ 0.001 v=1

of completed and ongoing genome projects is available at http://www.genomesonline.org/ [20]. For unsequenced organisms, by performing multiple hybridization experiments, we believe that it may be possible to deduce partial gene expression information without prior genomic knowledge.

8.3 Methods

8.3.1 Mathematical analysis of gene expression

A hybridization experiment can be expressed as the matrix equation $\mathbf{S} = \mathbf{H} \cdot \mathbf{E}$, where $\mathbf{S} =$ $(S_1, S_2, ..., S_i, ..., S_{N_q})^T$ is the vector of measured signal intensities and $\mathbf{E} = (E_1, E_2, ..., E_j, ..., E_{N_q})^T$ is the vector of unknown transcript concentrations (i.e., expression levels). For a particular set of hybridization conditions, **H** is a constant matrix if the system is in chemical equilibrium and the array is not saturated. Each coefficient H_{ij} of **H** is closely related to the melting temperature (affinity) of the binding interaction between transcript j and oligo i, and can be estimated using semi-empirical formulae [27, 101] or measured by calibration experiments with known quantities of various mRNA species. Deducing transcript expression levels is reduced to the computational problem of solving the above system of equations for E. Since it is impractical to directly invert H, our approach is to find a projection \mathbf{P} , such that $\mathbf{H}' = \mathbf{P} \cdot \mathbf{H}$ is a square $N_g \times N_g$ matrix. The vast reduction in dimensionality allows one considerable freedom in choosing a projection, and choosing \mathbf{P} such that \mathbf{H}' is invertible and in block diagonal form permits trivial determination of expression levels: $\mathbf{E} = (\mathbf{P} \cdot \mathbf{H})^{-1} \cdot (\mathbf{P} \cdot \mathbf{S}) = \mathbf{H}'^{-1} \cdot \mathbf{S}'$. We simplify the problem by choosing a hybridization stringency m and setting all elements of **H** to zero for which the corresponding transcript and oligo require more than m mismatches to bind. We then search for a projection by beginning with the minimum degeneracy oligo for each gene and then selecting additional oligos until \mathbf{H}' is invertible and the desired level of redundancy is achieved. The projection is simplest to construct when many rows have mostly zero entries—that is, when many oligos have low degeneracy.

8.3.2 Source of sequence data

Genomic sequence data for degeneracy calculations were drawn from public gene sequence databases for two organisms: yeast (*Saccharomyces cerevisiae*) and mouse (*Mus musculus*). These two organisms were selected because of their availability and because they are representative of the two ends of the eukaryotic genome size spectrum.

Yeast sequence data was obtained from the Saccharomyces Genome Database at Stanford University (http://www.yeastgenome.org/). We downloaded the complete set of coding sequences from ftp://genome-ftp.stanford.edu/pub/yeast/yeast_ORFs/orf_coding.fasta.Z on December 14, 1999. For this database, $N_g = 6306$ and $\bar{\ell} \approx 1420$. Since identical gene sequences cannot be distinguished by any microarray, duplicates were removed, leaving $N_g = 6276$ unique genes.

Sequences for mouse were downloaded from the UniGene system at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/UniGene/). We downloaded the file ftp://ftp.ncbi.nlm.nih.gov/repository/UniGene/Mm.seq.uniq.Z, Build 74. Though this database does not contain the complete genome of mouse, it contains both genes and ESTs representing a substantial portion of the expressed genome. For this database, $N_g = 75963$ and $\bar{\ell} \approx 471$. Due to the many ESTs, the average transcript length is quite small. Thus we included some calculations with a longer hypothetical average gene length.

8.3.3 Degeneracy calculations

To calculate degeneracy values from actual sequence data, we wrote a computer program that scans through the sequences comprising a transcriptome, tallying the number of times each subsequence of length n (n-mer) is encountered in different transcripts. Accounting for length truncation to length \bar{L} is accomplished by examining only the first \bar{L} characters of each transcript. To deal with mismatches, each subsequence of length n within a transcript is expanded into a set of all sequences that differ by at most m nucleotides from the original subsequence. Sequences containing non-A,C,G,T characters were ignored (0% of sequence data in yeast; 1–2% in mouse). From the list of degeneracy values for each of the 4^n possible n-mers, the *average* degeneracy is easily calculated for comparison with the analytic model. In addition, the degeneracy list itself is used to generate a histogram showing the fraction of n-mers having each degeneracy value, for comparison with theoretical histograms calculated from Equation 8.2.

While counting n-mers is very simple in principle, the enormous size of the problem for large values of n (e.g., there are over a billion 15-mers) introduced several technical challenges. In early versions of our program, we observed that the memory requirements exceeded the physical RAM (random access memory) of the computer and thus caused a significant amount of swapping to disk. which slowed the program by several orders of magnitude. To avoid this problem, our counting program was coded in C++, a language that permits a high degree of control over memory usage. At the start of a "run" (for a particular organism, value of n, and number of mismatches m), the program loaded the genome sequence into memory and declared a large array with one entry to store the tally for each *n*-mer. As the genome was scanned, each encountered *n*-mer was converted to a number, determined by interpretting its DNA sequence as a base-4 number, with nucleotides representing the digits. This number served as an index into the array of tallies, allowing the proper tally to be incremented. Once all genes were scanned, the tally data was written to a file—one value per line—with implicit line numbers serving as the *n*-mer identities. For very large values of n, the entire array did not fit in memory so the program was run in several "passes". If, for example, two passes were required, the program would first count only *n*-mers occurring in the first half of the list of all possible *n*-mers, ignoring any *n*-mers from the second half that were encountered while scanning the genome. After writing the data to a file, the genome was scanned a second time, this time tallying only *n*-mers in the second half (ignoring those in the first). The resulting data was appended to the first file.

It should be noted that we settled on this strategy after trying several other options. Languages such as perl and PHP permit arrays to be created dynamically as the program runs; thus tallies only need to be stored for *n*-mers that have been encountered at least once while scanning the genome. Since most *n*-mers are not encountered at all (for large n), far fewer array elements need to be stored. However, this advantage is offset by the fact that accessing each element in a dynamic array is much slower than in a static array. Furthermore, it seemed that dynamic languages required about $100-1000 \times$ more memory to store the same amount of data, thus requiring a much larger number of passes through the genome. As a result, the C++ program ran considerably faster overall.

8.3.4 Predicting the fraction of solvable expression levels

The fraction of trivially solvable expression levels is estimated in a probabilistic fashion from a minimum-degeneracy histogram derived from actual sequence data (e.g., Figure 8.4). These histograms were generated by a computer program that makes use of the list of *n*-mer degeneracies to determine the lowest degeneracy oligo to which the transcript can bind. A minimum-degeneracy histogram indicates the fraction of genes (x_i) having each value of minimum degeneracy, *i*.

Genes having minimum degeneracy equal to 1 are clearly trivial because their expression level can be deduced unambiguously from the fluorescence of the minimum degeneracy oligo. A fraction x_1 of all transcripts fall into this category. Those genes having minimum degeneracy equal to 2 are trivial if the other gene that shares the degeneracy 2 oligo has minimum degeneracy equal to 1. Of all transcripts, a fraction $x_2 \cdot x_1$ are expected to fall into this category. Similarly, those genes having minimum degeneracy equal to 3 are trivial if both of the other (distinct) genes that share the degeneracy 3 oligo have minimum degeneracy equal to 1. Statistically, a fraction $x_3 \cdot x_1 \cdot (x_1 - 1/N_g)$ of genes should fall into this category. Continuing in this fashion, one obtains a summation that estimates the fraction of genes whose expression levels can be solved trivially.

A computer program was written to examine actual gene sequences in order to determine the exact total fraction that could be trivially solved. As above, all genes having minimum degeneracy equal to 1 are clearly trivial. Each of the remaining genes is handled in the following manner. First, all n-mers to which the gene binds are identified and sorted in increasing order of degeneracy. Then, for each n-mer in turn, the *other* genes that bind the n-mer are identified. If all of these other genes have minimum degeneracy equal to 1, then the original gene is trivial by its association. If this condition is not met for any of the n-mers to which the gene binds, then the gene is declared non-trivial.

Chapter 9

A Probabilistic Method for Determining Gene Relationships from Expression Data

9.1 Introduction

One of the ultimate goals in biology is to understand the function of all genes and the structure of the interaction networks among them. Aside from its scientific value, a complete and detailed understanding would have a profound impact on the field of medicine. For example, it would become possible to design accurate diagnostics for nearly any condition, and it would be possible to accurately predict the effectiveness and side-effects of drugs or genetic treatments [103].

Microarrays (discussed in Chapters 7 and 8) and SAGE (serial analysis of gene expression) have proven to be powerful tools in the pursuit of this goal, providing genome-wide high-throughput measurements of cellular mRNA levels ("gene expression levels") as a readout of the state of the underlying genetic network. Experiments are often designed to compare the state of the genetic network under two or more conditions. For example, one can observe how the pattern of expression of genes involved in development changes over time, or one can observe the difference in network state between healthy tissue and cancerous tissue. Alternatively, one can monitor the induced changes in expression due to a perturbation such as a structural network change (e.g., knockout or overexpressed gene) or a temporary change induced by a drug, toxin, pathogen, hormone, or other factor. By observing patterns of induced changes, it is possible to make some hypotheses about the underlying genetic network structure. For example, genes that show similar changes in expression under a variety of conditions are likely to be closely related, perhaps performing a similar function or belonging to the same regulatory pathway.¹ In a growing body of knowledge in the literature and in numerous online databases, such hypotheses are being pieced together into a unified picture that will ultimately describe the whole underlying system. Already, the individual hypotheses have suggested useful biological experiments and have helped to identify new candidate drug targets and diagnostic markers for further exploration.

With the vast amounts of data being generated by microarray and SAGE experiments, the field of bioinformatics has proliferated. Many statistical techniques have emerged to deduce relationships among genes from this wealth of data, in order to assign functions to previously unknown genes and to piece together the network of gene regulation. In this chapter, I first briefly review several such techniques and argue the advantages of non-metric techniques, such as Guilt by Association (GBA), in particular. Though this method was developed by Walker *et al.* [282] to infer the relatedness of genes based on cDNA library data, we have extended it so that expression ratio data can also be analyzed. I present a detailed description of our modifications as well as our implementation of the modified algorithm in computer software. The software uses several tricks to permit the calculations to be performed in a reasonable amount of time. Our computed estimates of gene relationships (p-values) are available in an online database for further investigation.

9.2 Analyzing expression data

In a typical microarray study,² messenger RNA (mRNA) is extracted from a sample, transcribed into complementary DNA (cDNA), and labeled with a radioactive or fluorescent marker. A microarray contains thousands of spatially-identified tethered DNA "probes". When the sample is hybridized to

¹It should be noted that a lack of correlation between expression patterns does not necessarily indicate that genes are unrelated. It may simply mean that the set of experimental conditions was not sufficiently broad to cause changes in the relevant pathway.

 $^{^{2}}$ Though I use the term "microarray" for concreteness, the discussion of experimental principles and data analysis is valid for a wide variety of high-throughput technologies that measure gene expression.

the array, these probes capture complementary cDNA molecules from the sample, and the intensity (radioactivity or fluorescence) measured at each array position can thus be read out to determine the concentration of the corresponding cDNA species in the sample. This concentration corresponds to the original mRNA level or "gene expression level".

To cancel out many uncertain sources of noise and variation, experiments are often carried out in a differential fashion and one determines an *expression ratio* for each gene, where the expression level in one sample is divided by the level in a reference sample. A differential experiment may be performed by labeling each of the two samples with a different fluorophore and hybridizing them to the same microarray, or by measuring absolute levels on two different microarrays. The resulting expression ratios are associated with the experimental differences between the two samples. A ratio greater than 1 indicates that the gene was up-regulated (expressed at a higher level) compared to the reference, and a ratio less than 1 indicates that the gene was down-regulated (expressed at a lower level) with respect to the reference.

While an individual differential experiment can provide meaningful information, studies typically compare many samples (prepared under different experimental conditions) to the same reference. The term "condition" is used in the broadest possible sense. Some examples of experiments that have been reported include: (i) comparing samples taken at different times or stages of development to a baseline sample; (ii) comparing samples from different tissue types or different types of cancers to a pool of cDNA from all samples; or (iii) comparing samples exposed to certain nutrients, drugs, toxins, or pathogens to an untreated sample. The result of performing such a series of experiments is a set of expression ratios for each gene, called an expression "vector", "profile", or "pattern". We are interested here in the analysis of such vectors.

Numerous methods have emerged for sifting through the vast quantities of published gene expression data. They are based on the simple idea that genes showing similar patterns of expression across many experiments are likely to be related in function or to play a part in the same biological pathway. One important distinction among analysis methods is how the similarity of expression patterns is comapred. "Metric" methods use a distance measure—such as Euclidean distance or Pearson correlation distance—that satisfies the axioms of a metric. Distance metrics satisfy certain mathematical properties, including the triangle inequality, which states that:

$$d_{AC} \le d_{AB} + d_{BC},\tag{9.1}$$

where d_{ij} is the distance between the expression vectors of two genes *i* and *j*. A common criticism of methods using distance metrics is that certain biological relationships cannot be accurately described due to this constraint. For example, if genes X and Y have unrelated biological functions yet are both regulated to some degree by a common transcriptional regulator Z, then one would expect the proper description to be $d_{XZ}, d_{YZ} \ll d_{XY}$, which is not compatible with the triangle inequality. Other shortcomings of distance metrics have been cited as well. For example, methods based on Euclidean distance cannot handle expression vectors with missing data points (due to the inability to properly orient an incomplete vector in expression space), nor can they properly handle important relationships such as negative correlations between genes [33]. Furthermore, distance metrics tend to be highly sensitive to measurement errors in expression data (which is inherently very noisy), and many metrics introduce biases such as assigning more significance to larger ratio values in the expression vector [32]. "Non-metric" measures of gene relatedness, such as probabilities [282] or mutual information [32], do not suffer these drawbacks.

A second important distinction among analysis methods is whether the final description of the relationships consists of gene clusters or gene networks (e.g., Bayesian [83, 131] or Relevance [32] networks). Clustering is the process of finding groups (clusters) of genes with the most closely related expression vectors. A wide variety of methods have been used [129], including k-means, Gaussian mixture models, fuzzy c-means, self-organizing maps [262], and hierarchical clustering into dendrograms [70]. Since the emergence of high-throughput platforms for measuring gene expression, clustering has been the predominant method of analysis and has led to a great many important

biological discoveries. However, clustering has many shortcomings. First, many methods do not permit genes to be members of multiple clusters, therefore preventing an accurate description of genes involved in multiple pathways or under the control of multiple regulatory factors. Some methods have difficulty describing other biologically relevant situations, such as negatively correlated genes, or genes exhibiting non-linear relationships. Another drawback is that some clustering algorithms require seemingly arbitrary quantities such as the final number of clusters or other parameters to be known in advance. Lastly, clustering methods use global correlation measures and attempt to place *all* genes into clusters, even though it is unlikely that the weakest relations are believable.

Network approaches tend to extract prominent relationships from the observed data rather than trying to fit all of it. This can be especially useful in situations where gene relationships are only apparent under a small subset of experimental conditions and would be masked in global comparisons. In the Relevance network approach of Butte and Kohane [32], a non-metric quantity called mutual information is computed for each pair of genes to indicate the probability that they are related, and then a threshold probability level is imposed, effectively converting all the probabilities into binary values: related or unrelated. Butte and Kohane permuted their data to determine the maximum mutual information that could be obtained by random chance and used this as a cutoff. The result is several disjoint networks of genes, each containing links representing only the most believable gene relationships; improbable links are simply discarded. The threshold value affects the size of these networks and the number of connections between them. In practice, one must tune the threshold probability to achieve the desired trade-off between false positive and false negative error rates. Bayesian approaches attempt to determine the most probable genetic regulatory network structure given the available data. Again, rather than just grouping genes that behave similarly, a Bayesian network precisely identifies specific links between individual genes. Bayesian networks have the advantage of being able to naturally incorporate different measurement models into the analysis (e.g., noisy or stochastic expression data rather than fixed ratios) [32] and even to combine different types of data into the analysis [83, 131]. For example, clinical and protein interaction data could be combined with expression ratio data to increase the accuracy of predictions.

To avoid the many pitfalls associated with clustering using distance metrics, the methods described in the next sections use non-metric probability calculations. These probabilities can be used to identify candidate drug targets or diagnostic markers by finding genes closely related to known targets, or can be used to construct relevance networks.

9.3 Guilt by Association

Our non-metric analysis method described in the next section is based closely on the Guilt by Association (GBA) algorithm introduced by Walker *et al.* [282], in which pairs of related genes are identified based on their "associations" in tissue libraries. A tissue library (also known as a cDNA library) is essentially a collection of the mRNA content of a sample that has been reverse transcribed into cDNA. Transcripts are identified and counted by a method such as sequencing or SAGE. GBA measures the association between genes with a non-metric probability function to avoid the disadvantages of using distance metrics.

Library data is attractive for analysis because it is often more quantitatively accurate than microarray data, especially for transcripts expressed at very low levels [213, 124]. In addition, transcript counting is far less noisy [282, 280] and can more accurately detect even very slight differences in expression levels between samples [213]; in microarrays, differences smaller than a factor of 2 are often considered insignificant and are thus ignored. Furthermore, library data is more "portable" than expression ratio data because it consists of absolute measurements of transcript abundance. SAGE measurements from different experiments can be directly compared, whereas the differences between microarray formats, reference samples, and normalization strategies make direct comparison of microarray experiments difficult [213].

Like microarray data, many cDNA libraries have been published online [13]. Data from several different libraries can be combined to construct expression vectors for each gene. If a pair of genes has similar expression vectors across a set of libraries then the genes are likely to be related.

The first step in GBA analysis is to discretize the library expression data. Walker *et al.* used binary values: 1 if the gene was present in a given library (regardless of the number of copies); and 0 if the gene was absent. Table 9.1 illustrates library data that has been discretized in this manner. Discretization is intended to simplify the analysis, as well as to reduce the impact of any quantitative differences between different libraries (e.g., if they are normalized or subtracted), and to remove the magnitudes of expression to allow the detection of relationships between genes that are not linear and monotonic.

cDNA Library:	1	2	3	4	5	6	7	8	9	10	
Gene A	1	0	0	1	1	0	1	1	0	1	
Gene B	1	1	0	1	1	0	1	0	0	1	
Gene C	0	0	1	1	0	0	0	1	1	0	
:											

Table 9.1: Example of discretized expression of genes in cDNA libraries. Each row of the table represents the expression of a particular transcript (gene or EST) in many different libraries (represented by columns). A value of "0" indicates that the transcript was not detected in the library, while a value of "1" indicates that at least one copy was found.

For each pair of genes, Walker *et al.* compute the probability (p-value) that the observed pattern of co-expression could have arisen by random chance. To do so, the discretized expression data is organized into a 2×2 contingency table (Table 9.2). This table summarizes the pattern of *co*expression of the two genes. Entries represent the number of libraries in which gene A and gene B are both present (n_{11}) , the number of libraries in which the genes are both absent (n_{00}) , and the number of libraries in which one gene is present while the other is absent $(n_{10}$ and $n_{01})$. For each row and column, "margin totals" are computed. For example, the row totals r_1 and r_0 represent the total number of libraries in which gene A was present or absent, respectively. The total of these margin totals is the total number of libraries, N. It is from this table that the p-value is computed. One makes the "null hypothesis" that the genes are independent and computes the probability that the observed pattern of co-expression could occur randomly, *assuming fixed margin totals*, then tests the validity of this hypothesis. A low p-value implies that the null hypothesis should be rejected and that the genes are likely to be related.

	Gene $B = 1$	Gene $B = 0$	Total
Gene $A = 1$	$n_{11} = 5$	$n_{10} = 1$	$r_1 = 6$
Gene $A = 0$	$n_{01} = 1$	$n_{00} = 3$	$r_0 = 4$
Total	$c_1 = 6$	$c_0 = 4$	N = 10

Table 9.2: Example of co-expression pattern of genes in cDNA libraries. This 2×2 contingency table summarizes for genes A and B the co-expression data from Table 9.1. Each of the values in the table represents a certain number of cDNA libraries. n_{11} is the number of libraries in which gene A is present and gene B is also present; n_{10} is the number of libraries in which gene A is present but gene B is absent, etc. The last row and column are "margin totals". The row total $r_1 = n_{11} + n_{10}$ is the total number of libraries in which gene A was found, and $r_0 = n_{01} + n_{00}$ is the total number of libraries from which gene A was absent. Column totals represent analogous quantities for gene B. The total of the margin totals is simply the total number of libraries, $N = r_1 + r_0 = c_1 + c_0$. The data in the contingency table are used to compute the likelihood that the pair of genes is related, by methods described in the text.

One method for evaluating the null hypothesis is to perform a chi-squared test. First, an *expected* count is computed for each table cell (row i, column j),

$$E_{ij} = \frac{r_i \times c_j}{N}.\tag{9.2}$$

The chi-squared statistic is then computed:

$$\chi^2 = \sum_{i,j} \frac{(O_{ij} - E_{ij})^2}{E_{ij}},\tag{9.3}$$

where O_{ij} is the *observed* count in each cell (from the original contingency table), and the sum is computed over all four cells. Observing that there is just one degree of freedom (e.g., n_{11}) when margin totals are fixed, one then computes the probability of the χ^2 statistic. A low probability indicates a large deviation between expected and observed counts and hence the relation between the genes is non-random.

The validity of the chi-squared test depends on having a sufficient sample size. However, for many pairs of genes—particularly those present in very few cDNA libraries—the minimum requirements for validity are not met. One commonly used validity requirement is that the total sample size must be greater than 40 or that all tables cells must have expected values of at least 5 if the total sample size is in the range 20–40. A thorough analysis of validity conditions was reported by Tejedor and Andrés [266]. When the conditions are not met, the p-value calculation must instead be performed by an alternate method such as Fisher's exact test. This involves computing the probability of observing the actual data or more "extreme" data with the same margin totals. One enumerates all possible tables with the same margin totals, computes the probability of each table, then sums all probabilities that are less than or equal to that of the observed table. The probability of one particular table (i.e., one particular co-expression pattern) is:

$$P_{2\times2} = \frac{\binom{N}{n_{11}}\binom{N-n_{11}}{n_{10}}\binom{N-n_{11}-n_{10}}{n_{01}}\binom{N-n_{11}-n_{10}-n_{01}}{n_{00}}}{\binom{N}{r_1}\binom{N-r_1}{r_0}\binom{N}{r_1}\binom{N-r_1}{r_0}} = \frac{r_1!r_0!c_1!c_0!}{N!n_{11}!n_{10}!n_{01}!n_{00}!}.$$
(9.4)

This equation can be interpreted as the number of possible arrangements of data with the observed numbers of correlations $(n_{11}, n_{10}, \text{ etc.})$ preserved, divided by the total number of possible arrangements of data with only the restriction that the observed margin totals are preserved.

For 2×2 contingency tables, it is straightforward to enumerate all other possible tables to determine which are more extreme because there is only one degree of freedom. One needs only to vary one of the cells over all possible values and compute the other cells using the margin totals, omitting any tables containing negative-valued cells. An example is shown in Figure 9.1 for the contingency table of Table 9.2.

The lower the p-value (whether computed by a chi-squared test or Fisher's exact test), the less likely is the null hypothesis, and the more likely it is that the pair of genes is related. Walker *et al.* used Guilt by Association with Fisher's exact test to determine which genes were most closely associated with known genes involved in prostate cancer [282], Parkinson's disease and schizophrenia [281], and the cell cycle [280]. In the first two studies, a set of 522 human cDNA libraries was used; in the third, 1176 libraries. It was observed that the computed p-values for known gene relations were lower (therefore more significant) than correlation coefficients [281]. Furthermore, several known relations were not detected by correlation methods but were detected by GBA [282, 280].

It should be noted that the magnitudes of the p-values are only approximate because several assumptions made by the calculations do not hold in general. For example, the cDNA libraries



Figure 9.1: Example of performing Fisher's exact test. (a) The contingency table from Table 9.2 with the margin totals shown. (b) All possible tables with the same margin totals. The value underneath each table is the probability, $P_{2\times2}$, of that particular table from Equation 9.4. The overall p-value for the observed data is then the sum of all probabilities less than or equal to 0.114: P = 0.114 + 0.071 + 0.005 = 0.190.

are not all independent—many belong to sets of experiments with only small differences between each sample. However, even with appropriate corrections, the most reliable relations remain significant [282].

Though only cDNA library data were analyzed by Walker *et al.*, presumably this method can also be applied to data from single channel oligonucleotide experiments such as those using Affymetrix GeneChips or arrays using radioactive labels.

9.4 Extension of GBA to expression ratio data

Building on earlier work by Brody and Quake (http://thebigone.stanford.edu/yeast/), we extended the Guilt by Association method to use differential expression *ratio* data rather than library data. The motivation for this was two-fold: (i) there is far more expression ratio data that is publicly available; and (ii) ratios can represent richer, more-complex relationships between genes than presence or absence in cDNA libraries. Furthermore, by combining data from multiple microarray studies it may be possible to uncover gene relationships that are not clearly apparent in any individual study. Our algorithm involves discretization of expression data prior to analysis and

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a non-metric measure of the relation between gene pairs, for the same reasons put forth by Walker et al. [282, 281, 280].

Brody and Quake analyzed yeast expression data from one set of microarray experiments by a simplified approach; here we further develop the algorithm and analyze human expression data combined from many different microarray studies.

9.4.1 Algorithm

To perform a modified Guilt by Association analysis, we first discretized expression ratios to three distinct values, +, -, or 0, representing up-regulated, down-regulated, or unchanged expression, respectively. Discretization helps to address the problem of high variability in ratio data and also helps to identify complex non-linear correlations by ignoring the magnitude of expression changes. A hypothetical set of discretized data is shown in Table 9.3. We performed the discretization simply by imposing a fixed "noise threshold". Ratios that exceeded the upper threshold, $T_{+} = 1.414$, were designated up-regulated; ratios that were smaller than the lower threshold, $T_{-} = 0.707$, were designated down-regulated; and all others were designated as unchanged. Our simple method has several shortcomings—for example, it ignores genes that undergo only small expression changes in response to perturbations. However, several more sophisticated approaches have been reported in the literature for determining whether expression changes are statistically significant. For example, one can apply statistical analysis of variance (ANOVA) [146, 66, 144], or one can design experiments to include redundant measurements and additional controls [145, 107]. At the time our work was performed, most published datasets were not properly analyzed and did not supply all the needed information (such as raw scanner data) for us to be able to correct the analysis. Furthermore, many datasets did not include sufficient experimental replicates and controls to eliminate many sources of noise and variation. The field has progressed tremendously in the past 5 years, and several recent studies now include estimates of statistical significance with each expression ratio.

Analogous to the 2×2 case, the pattern of co-expression of the discretized data for each pair of genes (Table 9.3) can be summarized in a 3×3 contingency table (see Table 9.4). This table lists the

Experiment:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Gene A	+	0	0	-	+	0	+	+	+	0	-	-	0	+	+	
Gene B	+	+	0	—	+	_	+	0	Х	Х	+	0	+	_	_	
Gene C	Χ	+	-	+	0	0	0	+	-	0	0	-	0	0	+	
:																

Table 9.3: Example of discretized gene expression ratios in expression datasets. Each row corresponds to a particular transcript (gene or EST). For each experiment (column), the ratio is expressed as + (up-regulated), - (down-regulated), or 0 (unchanged). Note that sometimes the data for a particular gene is missing from an experiment due to a defect in the array or other problem (indicated by an "X"). When comparing two genes, only experiments in which both genes have a valid data point are included in the calculation.

number of experiments in which the genes are both up-regulated (n_{++}) , both down-regulated (n_{--}) , etc. Margin totals are computed for the table and then a p-value is computed based on a chi-squared test or Fisher's exact test. With fixed margin totals, there are four degrees of freedom in a 3×3 table. The chi-squared test is valid if no more than 20% of the expected frequences are less than 5 and none is less than 1. In many cases, this condition is not met, even in datasets consisting of dozens of individual experiments. For example, highly correlated genes have a high value for n_{++} but low values for all other table cells.

	Gene $B = +$	Gene $B = 0$	Gene $B = -$	Total
Gene $A = +$	$n_{++} = 3$	$n_{+0} = 1$	$n_{+-} = 2$	$r_{+} = 6$
Gene $A = 0$	$n_{0+} = 2$	$n_{00} = 1$	$n_{0-} = 1$	$r_0 = 4$
Gene $A = -$	$n_{-+} = 1$	$n_{-0} = 1$	$n_{} = 1$	$r_{-} = 3$
Total	$c_{+} = 6$	$c_0 = 3$	$c_{-} = 4$	N = 13

Table 9.4: Example of co-expression pattern of genes from discretized ratio data. The pattern of co-expression of genes A and B in Table 9.3 are summarized in this contingency table. n_{++} is the number of microarray experiments in which both genes were up-regulated, n_{+0} is the number of experiments in which gene A was up-regulated while gene B was unchanged, etc. Margin totals are computed for each row $(r_+, r_0, \text{ and } r_-)$ and column $(c_+, c_0, \text{ and } c_-)$. From the table, the likelihood of the null hypothesis (that the genes are independent) is computed using a chi-squared or Fisher's exact test.

Using generalizations of Fisher's exact test to 3×3 tables, a p-value can be computed as follows.

The probability of a single table is given by:

$$P_{3\times3} = \frac{r_+!r_0!r_-!c_+!c_0!c_-!}{N!n_{++}!n_{+0}!n_{+-}!n_{0+}!n_{00}!n_{0-}!n_{-+}!n_{-0}!n_{--}!},$$
(9.5)

and the overall p-value is obtained by summing probabilities over all possible tables that have the same margin totals as the observed data but are more "extreme". In 3×3 tables with fixed margin totals, there are four degrees of freedom so it is much more difficult to find all the tables than in the 2×2 case. When dealing with datasets consisting of on the order of 100 experiments, we observed that calculations for some pairs of genes involved the evaluation of tens of thousands of possible tables. Brute force methods can easily identify all tables, but more efficient algorithms have also been developed [190, 191, 46].

9.4.2 Implementation

We wrote computer software in perl and C++ to create and maintain a database of gene expression ratio data (in original and discretized form) along with p-values between each gene pair computed by the GBA method. The database is available online at http://thebigone.stanford.edu/pvalue/. Raw microarray data was obtained from the Stanford Microarray Database (http://genome-www.stanford.edu/microarray/). We downloaded data from several available experiments relating to human tissue and cell lines, as indicated in Table 9.5.

		Number	Number of
Reference	Description	of Genes	Experiments
[127]	Fibroblast response to serum	8600	19
[24]	Peripheral blood mononuclear cell response	7600	182
	to bacterial infection		
[211]	Breast tumours	8100	84
[232]	Clustering of genes based on tumour type in cancer cell lines	8000	68
[4]	Distinguishing types of B-cell lymphoma by expression differences	17900 (cDNA clones)	133
[210]	Expression patterns in mammary epithelial cells and breast cancers	5000	33
[294]	Identification of cell-cycle associated genes in cancer cell lines	16300, 29600	90
[61]	Response of macrophages to a bacterial tran- scription factor required for virulence	22600 (cDNA transcripts)	53
[59]	Temporal expression profile of prostate can- cer cell line after treatment with synthetic an- drogen	18000	30

Table 9.5: Human microarray datasets used in GBA analysis.

Most microarray publications list probes used on the arrays by their GenBank Accession Number [19]. We consulted the UniGene database (build 150) [214] to determine the gene (or EST) represented by each probe. Storing the raw data based on sequence identifiers rather than genes allowed us to easily keep up to date³ with UniGene as genes were added and corrections were made with each new "build". Using the UniGene database also allowed us to aggregate data from all probes representing the same gene. For each unique gene (UniGene "cluster"), a single expression ratio for each microarray experiment was determined by taking the median of the ratios of all constituent probes. Expression ratios for all genes/clusters were then discretized and stored. The latest version of our database contains expression data for approximately 35000 unique clusters.

Though combining measurements in this manner makes theoretical sense, we noticed in several cases that probes corresponding to the same UniGene cluster had very different expression patterns and probably should not be combined. These cases may indicate errors in the UniGene database or may represent misidentification of probes in array experiments. It is expected that UniGene errors will eventually be resolved with future updates so no effort was made at this time to detect or correct these questionable cluster assignments. However, in the meantime, we did build a second database of discretized expression ratios based on individual sequences rather than clusters. This database contained approximately 80000 entries. For clarity in the subsequent discussion, only the first database is described.

For each pair of genes in the database, a p-value was computed based on the discretized ratio data and stored. Storing p-values is necessary if one wishes to search the database for the most probable gene relationships, for example. Due to the large number of gene pairs, we chose not to store *all* p-values but rather only those less than a certain threshold (10^{-2}) . Not only did this reduce the data storage requirements, but it also dramatically improved the speed at which results could be returned when querying the database. The threshold was selected somewhat arbitrarily, but a later analysis (see Figure 9.2) revealed it to be an acceptable choice because only p-values much lower than this are thought to represent significant relationships in our database.

 $^{^{3}}$ Each update requires that sequences be reassigned to clusters, expression ratios be re-aggregated and rediscretized, and p-values be re-computed for all gene pairs. To improve efficiency, one could detect which sequences and clusters had been affected by the update and re-compute only those.



Histogram of p-values computed by GBA

Figure 9.2: Analysis to determine p-value representing the threshold of significance. Due to violations of certain assumptions of the GBA algorithm, the magnitudes of the computed p-values are not reliable. To determine at what p-value gene pairs can be considered to have a significant relation, we performed a simple graphical analysis. A histogram was generated, indicating for each p-value the fraction of gene pairs having that p-value or lower. The continuous line shows the fraction that would be expected by random chance (i.e., 1/p), and the square markers indicate the fractions tabulated from our database of p-values (combined from 9 datasets using the p_{min} algorithm). One can observe that the GBA data is distinctly non-random. At a p-value of 10^{-4} , the lines cross. For lower p-values, there are more gene pairs in the GBA data than expected by random chance, suggesting that 10^{-4} represents the threshold of significance. This is only an approximation and must be tuned to achieve the desired trade-off between false positives and false negatives.

P-value calculations were implemented in C++ code, using logarithms of the relevant equations to improve computational accuracy. Three different p-values were calculated for every pair of genes in our dataset: (i) the individual table probability $P_{3\times3}$, (ii) the chi-squared probability, and (iii) the probability computed by Fisher's exact test.

Despite the apparent complexity of Equation 9.5, $P_{3\times3}$ can be computed very inexpensively by pre-computing a table of $\log(n!)$ for n = 0..N once at the start of the run. (The time needed for this pre-computation is amortized over all genes pairs.) Compared with Brody and Quake's original code, this simple modification reduced the execution time by 20%. The implementation of the chi-squared calculation [216] is also relatively inexpensive.

On the other hand, Fisher's exact test is a very expensive calculation, due to the large number of tables that may exist for a given set of margin totals. Though brute force methods can be used to find all tables and compute their probabilities, more efficient algorithms have been published [190, 191, 46]. We implemented this computation by calling an external FORTRAN 77 subroutine published by Mehta and Patel [191]. I modified the code slightly to avoid duplicating some calculations when calling the subroutine billions of times. Calculations of the Fisher's exact test p-value for some gene pairs took many seconds (on an 800 MHz AMD Athlon computer), so it was not practical to complete a full run for all gene pairs (0.6 billion pairs in one database, 3.2 billion pairs in the other). However, it was not necessary to perform the full calculation most of the time—in many cases the other calculations provide an excellent approximation.

For very small p-values, the single-table probability agrees very well with Fisher's exact test. This is not surprising—when the p-value is low, there are very few, if any, additional contingency tables that are more extreme than the observed data; therefore, there are few terms in the Fisher summation. For increasing p-values, the values rapidly diverge. It may be possible to derive a threshold p-value below which the single-table value can safely be used as an approximation to the Fisher value within some specified tolerance.

Though not accurate, the single-table p-value also has some utility for high p-values. Because it is always an *under*estimate of the Fisher's exact test p-value, it can be used as a quick screen to avoid unnecessary and expensive calculations. If the single-table p-value is greater than the database cutoff value, then we know that the gene pair will not be stored in the database because the Fisher's exact test p-value will be even higher. Thus the full Fisher's exact test computation can be skipped.

We also found that the agreement between Fisher's exact test and the chi-squared test was quite good in most cases, for both high and low p-values. The only exceptions were cases where the requirements for validity of the chi-squared test were not satisfied. For example, highly correlated genes frequently had low values in many cells of the contingency table. This suggests that the chisquared method can be used to compute most p-values to a good approximation, except in cases where Fisher's exact test must be used due to violation of the validity conditions.

It should be noted that in Brody and Quake's analysis of yeast expression data, all p-values are based on the single-table value. Thus, it is expected that the results are only accurate for the lowest (most significant) p-values. Fortunately, these are the ones that are generally the most interesting.

In addition to the software mentioned above, additional programs were written in the PHP scripting language to provide a web-based interface to the database. The database can be browsed for gene pairs having the smallest p-values (i.e., most probable relationships) or for all genes having a probable relationship with a particular gene (identified by UniGene cluster, sequence accession number, gene name, or gene description). For each pair of genes, the p-value is given along with links to view the raw or discretized data on which the calculation was based. One other quantity that is shown is the "dot product", computed by multiplying integer representations of the discretized ratios (+1, 0, -1) for the two genes in each experiment and summing over all experiments. Gene pairs that are highly correlated will have a large positive dot product, pairs that are highly anti-correlated will have a large negative dot product, and those related in more complex ways will have an intermediate value.

9.4.3 Combining datasets

Since many published microarray studies explore only a small range of experimental conditions, a large part of the cell's genetic network is not interrogated, and the relationships between many pairs of genes remain hidden. By combining expression measurements from multiple studies, however, one can compare expression vectors across a much broader range of conditions, and relationships are more likely to be revealed, if they exist. As more and more studies are published, the effectiveness of combining them will improve.

We pursued two approaches for combining sets of experiments ("datasets") from multiple studies. In the first, we simply combined all datasets into very long expression vectors for each gene, such that each vector contained ratios from all experimental conditions in all studies. However, we observed that low p-values were being computed for many pairs of genes thought to be unrelated.

This problem arises from the details of how microarray experiments are performed. In the ideal case, an experiment would compare samples consisting of a single cell type to a reference consisting of an identical cell type. The observed differences in the samples would reflect real expression changes resulting directly from the experimental conditions. However, many studies use mixed cell types, either inadvertently because micro-dissection was not used to isolate individual cells during sample preparation, or because samples were intentionally pooled. Pooling is often performed to ensure that the reference sample contains molecules representing all cDNA sequences to avoid the problem of dividing by zero in ratio calculations.

Comparing different cell types in a microarray experiment results in systematic biases in the expression ratios for the whole set of experiments. For example, when one cell type is compared to a pool of cell types, expression ratios reflect biases such as the fundamental differences in expression levels between cells of different types in addition to any real expression changes due to the experimental conditions. Such biases can lead to the appearance of false correlations (see Figure 9.3). It could also be argued that biases such as expression differences due to differences in cell type represent meaningful information that should be included in the analysis; however, in practice, it is not possible to differentiate meaningful biases from the many possible meaningless ones.

The majority of public datasets available at the time of this analysis had obvious biases such as different cell types, though a few had no obvious biases [127, 294].⁴ Instead of restricting our analysis

 $^{^{4}}$ It is likely that even these apparently bias-free studies have some sources of hidden bias such as differences between dyes or detectors in the two fluorescence channels [18].



Figure 9.3: Example of bias problem when combining expression datasets. Biases present in microarray experiments, due to the use of pools of cell types in the reference sample, for example, lead to problems when performing p-value calculations on combined datasets. (a) A hypothetical dataset where gene X (upper row) appears up-regulated in all five hypothetical experiments due to a bias, and gene Y (lower row) appears down-regulated in all experiments. The contingency table for this particular dataset is shown at the right. Because all measurements are identical, the fact that gene X and gene Y show a correlated pattern of expression is not considered significant. The p-value is very high: P = 1.000. Thus, a systematic bias does not create a problem (i.e., false positive) if the dataset is analyzed individually. (b) Another hypothetical dataset where gene X appears unchanged in all experiments, and gene Y appears up-regulated in all experiments due to a bias. Again, the contingency table and (insignificant) p-value are shown. (c) If these two datasets are combined into long expression vectors containing all experiments from both sets, the two groupings of biased measurements now falsely appear highly significant (P = 0.008). The low p-value arises because it is unlikely that independent genes would be observed to be co-expressed with $n_{+-} = 5$ and $n_{0+} = 5$ in 10 experiments. Thus, combining datasets with different biases into long expression vectors leads to very misleading p-values.

to this small fraction of studies, however, we combined expression measurements by an alternate approach.

For each gene pair, we computed p-values separately for each dataset, then combined them into a single p-value. There are several ways of accomplishing this. The simplest method is simply to take the minimum of all the p-values:

$$p_{min} = \min(p_1, p_2, \dots, p_k), \tag{9.6}$$

where k is the number of datasets and p_i is the p-value computed from the *i*th dataset. The rationale behind this approach is that gene relationships will be revealed in some sets of experiments but not in others. If at least one set shows a significant relation, then it is sufficient to assume the genes are related. Other methods for combining p-values have been reported as well [306, 292]. For example, one can compute a "Fisher statistic",

$$S = -2\sum_{i=1}^{k} \ln p_i, \tag{9.7}$$

and then compute an overall p-value by interpretting this statistic as a χ^2 value with 2k degrees of freedom. One problem with this method is that it requires *all* p-values to be stored in the database—a cutoff cannot be used. It is not clear what is the best method to combine values: the first method is vulnerable to outliers with low p-value, while the second may allow a significant result in one dataset to be "washed out" among many datasets that show no significant result. It is also unclear whether all individual p-values should be weighted equally or whether p-values derived from datasets consisting of more experiments should be given more weight.

We observed that among the 9 datasets used in our analysis, only a few p-values were available to be combined for each gene pair. Some of the missing values are due to our use of a p-value cutoff when building the database. However, the p_{min} algorithm is unaffected because the missing values can safely be assumed to be greater than any of the included values. Other missing values reflect the fact that not all microarrays contain the same set of genes, and thus the comparison of expression
patterns for a given gene pair may not be possible on some arrays. By including many more datasets in the analysis, one can increase the average number of individual p-values that are combined for each gene pair.

9.4.4 Results

In our database, many of the lowest p-values corresponded to expected relationships such as genes that code for different modules of the same protein complex (such as major histocompatibility complex (MHC) proteins and immunoglobulins) or genes that are in close proximity on the chromosome. A large fraction of highly significant relations involved at least one unknown gene. These pairs most likely represent identical genes. Indeed, a more recent version of the UniGene database (build 186) shows that many of the pairs we identified initially have now been merged into the same UniGene cluster. This suggests the method could be used to assign putative functions to unknown ESTs used as microarray probes.

Among the lowest p-values are also pairs of genes representing different enzymes in the same metabolic pathway, as well as many pairs of genes involved in the cell cycle, including many of the same relations found by Walker [280]. Our database did not contain very many of the relationships pertaining to prostate cancer, Parkison's disease, and schizophrena as found by Walker *et al.* [282, 281]. Presumably this is simply due to the fact that the microarray experiments included in our analysis did not include all of the relevant genes and that the experimental conditions in these studies were not designed to perturb the relevant pathways.

In addition to verifying several of the most significant relations, we found that many sets of significant gene pairs picked randomly from the database correspond to suspected biological relations. Since it is very tedious to perform literature and database searches for each gene in a pair to determine whether the relation makes biological sense, we instead compared our results to suspected groups of significant genes published in an extensive study by Segal *et al.* [239]. As part of that study, more than 450 biologically significant "modules" of related genes were identified. We found that most

pairs of genes with low p-value picked randomly from our database consist of genes belonging to the same module.

In spite of these comparisons, there is an overall lack of sources of "correct answers" against which to compare generated hypotheses [33]. Eventually, improved annotations and more complete databases will allow algorithms such as ours to be fully evaluated in terms of the accuracy and completeness of the set of predicted relationships.

9.5 Related Work

Earlier sections in this chapter have described the relation of our work to the GBA method of Walker et al. [282, 281, 280] who analyzed profiles of expression in cDNA libraries.

Our work also has many characteristics in common with the work of Butte and Kohane [32], in which vectors of yeast expression ratios were discretized into n subranges and compared based on their mutual information. Mutual information is a non-metric measure of the shared information between two vectors. The higher the mutual information, the less likely the vectors are randomly related to one another, and the more likely there exists a biological relationship between the genes. The authors' analysis revealed many relationships that could be validated in the literature, including pairs of identical genes, genes in the same pathway, and genes with similar functions. Butte and Kohane selected n = 10 in their analysis, which is significantly higher than our value of n = 3. While higher values of n utilize more information from the expression ratios, they increase the susceptibility to noise. Our n = 3 approach has the advantage of being compatible with statistical approaches that determine whether a gene is significantly up- or down-regulated. This is particularly helpful in the case of genes that do not exhibit wide swings in expression levels and for which small changes in expression can often be very significant. Such small expression changes are typically ignored by methods that look only at magnitudes of expression ratios.

Bowers *et al.* [26] recently reported an interesting analysis of protein "phylogenetic profiles" (as opposed to gene expression profiles) that bears some similarity to our work. For each protein, an Ndimensional profile is constructed, with ones and zeros representing whether the protein (or a close homolog) is present or not present in each of N organisms. Rather than analyzing pairs of profiles, the authors investigated protein triplets. They identified pairs of profiles, a and b, that individually were not good predictors of a third profile, c, but whose logically combined profiles described c well. Comparisons were based on a non-metric measure related to the entropy of the individual and joint profiles. Each triplet could be classified as one of eight possible "logic relationships" and could be combined together to infer the structure of the protein interaction network. The analysis of triplets can detect relations that might go unnoticed if only examining pairwise relationships. The authors suggest that the underlying principles of their analysis could be applied to other sets of genomic data including expression profiles. Perhaps the work of Butte and Kohane [32] or our modified Guilt by Association algorithm could serve as a starting point.

Other areas of related work include Bayesian networks [83, 131] and Boolean networks [165]. These approaches can model additional dimensions of relationships between genes, including temporal (causation) and spatial effects, ultimately leading to a more accurate and complete picture the genetic network in humans and other organisms. However, the data for performing such analyses remains scarce. In the meantime, methods such as those described above for predicting gene relationships based on expression data will continue to be immensely useful in deducing the functions of unknown genes and discovering new candidate drug targets and diagnostic markers.

9.6 Future Directions

At the time we created this database, it was difficult to draw meaningful conclusions, beyond the simple verification of some known biological relationships, due to the relatively small number and narrow range of published human microarray studies available, and due to the many errors and omissions in the UniGene database. Furthermore, without the raw image data it was not possible to determine for low expression ratios whether the degree of up- or down-regulation should be considered significant. It is likely that many low ratios were misclassified by our simple threshold approach.

In recent years, UniGene has been updated many times and hundreds of new human microarray studies (consisting of many thousands of individual experiments) have been published, reflecting a much more complete set of interrogation conditions. In addition, a far greater set of known relations now exists in databases and in the literature for assessing the accuracy of the results. Updating the database to include hundreds of new microarray studies and to employ a more sophisticated discretization algorithm would consume considerable computing resources but could ultimately produce a valuable data mining tool. Researchers at Peking University have implemented a public database, *GBA server* [296], that accumulates EST library data and p-value calculations based on the GBA methods of Walker *et al.* [282]. A similar implementation for the modified GBA method that we have presented here could potentially serve as a valuable resource for the online bioinformatics community. An attractive feature of GBA databases is that their effectiveness and reliability increases with time as more and more data is integrated.

An additional worthwhile direction of future work concerns the user interface. In addition to presenting the output as a list of genes with significant relations, it would be useful to explore the use of web-based graphical tools such as TouchGraph [240] to display interactive relevance networks of the relations having p-values below some threshold. A simple analysis (Figure 9.2) suggests a p-value cutoff of 10^{-4} or lower for our current database. We found 67193 pairs of genes with a p-value below this threshold (out of about 0.5 billion possible pairs). It is not unreasonable to graphically navigate a set of data with this size.

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Appendix A

Methods

A.1 Fabrication of microfluidic molds

Relief molds for microfluidic device casting were created by photolithographically patterning photoresist on silicon wafers or glass microscope slides.

A.1.1 Photomask preparation

Low resolution (3386 dpi) photomasks were printed on plastic transparencies at Mika Color Corp. (Los Angeles, CA). These masks are acceptable for simple designs, but printed features were found to "overflow" the designed boundaries by as much as 20 μ m.

Designs with critical channel widths or closely spaced features were printed at CAD/Art Services, Inc. (Poway, CA) at a resolution of either 8000 or 20000 dpi, depending on accuracy requirements.

Transparency photomasks were adhered to flat borosilicate glass plates (Chemglass Inc., Vineland, NJ) with clear tape.

A.1.2 Mold patterning

Photoresists were processed according to manufacturers' suggested protocols for the desired channel pattern thickness (channel depth). SJR 5740 (Shipley), SPR 220-7 (Shipley), and AZ 50XT (Clariant) positive resists were typically used for fluid-layer molds. Various negative resists of the SU8 family (MicroChem) were used for control-layer molds.

A.1.3 Mold rounding

It is necessary that fluid channels have a rounded profile to allow complete closing of valves at modest pressures. To cause rounding of photoresist channel features on fluid layer molds, molds were heated above the melting temperature of the resist. Molds patterned with SJR 5740 or SPR 220-7 were heated to 120°C for 20–30 min. Molds patterned with AZ 50XT were heated to 160°C for 1 h.

A.1.4 Fabrication of fluid layer molds with frit valves

Frit valves (for trapping microbeads, for example) were created by including non-rounded (square) channel segments in the fluid layer at the desired valve positions. Typically the square channels were 200 μ m wide and were crossed by valves with 200 μ m width. The fluid layer design was printed onto two different photomasks—one containing channel segments to be rounded, the other containing the square channel segments. At junctions, the rounded segments were designed to overlap the ends of the square channel segments by 30–50 μ m.

Rounded and flat channel segments on the mold were fabricated from two different photoresists typically SU8 resist for the square segments and SJR 5740, SPR 220-7, or AZ 50XT for the rounded segments. Due to its high chemical stability, SU8 segments were patterned first, according to the manufacturer's protocol using a negative photomask. Processing of the second resist was then carried out directly on top of the SU8-patterned wafer. During the exposure step, the second (positive) photomask was aligned to the SU8 features visible on the wafer. After development, the newly patterned channel features were rounded according to Secton A.1.3. The SU8 photoresist does not undergo rounding under those conditions.

Note that for rounded and square channel features of $10-12 \ \mu m$ in height, a significant jump in height (60–70 μm) can occur at junctions of the two resists. For this reason, frit valves should be positioned at least several hundred microns away from junctions.

(Tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane provides an effective mold release coating for replication molding of many polymers. The coating is applied by placing the mold in a dessicator with a small amount (200μ L) of silane in a small dish of paraffin oil. The dessicator is pumped with a vacuum pump for 2 min to vaporize the silane and then is sealed closed. Treatment requires continued exposure for about 1–2 hours in this vapour.

A.2 Glass Protocols

A.2.1 RCA glass cleaning

Standard 1 mm thick 1×3 inch and 2×3 inch glass microscope slides were cleaned as follows. First, slides were mounted in a rack and sonicated for 30 min in a solution of 2% Micro-90 soap (Cole-Parmer Instrument Company, Vernon Hills, IL) in deionized water. Slides were then rinsed 3 times with deionized water. A batch of RCA-1 cleaning solution (6 parts deionized water, 4 parts 27% ammonium hydroxide, 1 part 30% hydrogen peroxide) was prepared and heated on a hot plate until it bubbled vigorously. The slide rack was then immersed in this solution for 60 min with continued heating and stirring. Subsequently, slides were rinsed 3 times with deionized water and then stored underwater in sealed containers to prevent contamination before use.

A variety of alternative glass cleaning methods are reviewed and compared in [51].

A.2.2 Glass microchannel etching

Microchannels were etched in glass using a protocol adapted from Stjernström and Roeraade [254]. Glass microchannel layers were fabricated from RCA-cleaned (Appendix A.2.1) microscope slides. SJR 5740 photoresist (Shipley) was used as an etch mask. Resist was spin-coated at 1500 RPM for 60 s. After soft-baking for 2.5 min at 105°C, the resist was exposed through the desired positive photomask (channels dark) for 1.5 min on a Karl Suss mask aligner (UV lamp intensity 175 W). The resist was developed by immersing for approximately 2 min in Microposit 2401 developer diluted 1:4 in deionized water. A uniform layer of resist was spun and soft-baked on the other side of the glass to prevent etching from the back side. Alternatively, clear tape could be used as an etch barrier. Channels were etched by immersing the slide with the channel surface facing down in a solution of equal parts of 1N HCl, deionized water, and buffered oxide etchant (6:1) at 25°C. Channels were etched until the desired channel depth was reached (etch rate approximately 0.8 μ m/min). Slides were then rinsed 3 times with deionized water and then washed in acetone and isopropanol to remove the photoresist. Slides were washed again in water and then dried before use.

A.2.3 Drilling holes in glass slides

Solvents must sometimes be delivered to microfluidic fluid channels through the glass substrate—for example when only the bottom surface of the device is compatible with the solvents.

Holes in glass were drilled using a 0.75 mm diameter diamond-tipped Triple Ripple drill bit (C. R. Laurence Company Inc., Los Angeles, CA). The desired locations of the centers of the holes were first marked on the glass with a water-insoluble marker. With the slide immersed in water, the drill bit (spinning at a high speed setting) was brought down slowly to the glass surface. After drilling to a depth of 100–200 μ m, the drill was raised to allow water to enter the hole. This process was repeated until the hole was drilled completely through the glass. To prevent flaking on the back side, it was necessary to firmly push the slide against a second glass slide or other rigid support as the drill bit broke through the back surface.

A.2.4 PDMS/glass oxygen plasma bonding

Oxygen plasma treatment can be used to covalently bond PDMS to itself or to glass. The mechanism is thought to be related to the breaking of bonds on each surface during treatment followed by the formation of Si–O–Si bonds when the two surfaces are brought into contact.

Details of the process are poorly understood, but numerous groups have reported processing parameters that work, and a few have published systematic optimizations of bonding [21]. In general, clean PDMS surfaces (for example, using HCl treatment), low plasma powers, and short treatment times lead to the highest bond strengths. One set of optimized parameters is 75 W power at 75 mTorr pressure for 10 s duration [137]. Typically, surfaces must be placed into contact within about 1 min of treatment unless oligomers are extracted from the cured PDMS [160]. Drops of non-swelling solvents such as methanol or ethanol can be placed between treated surfaces to act as a lubricant during alignment and to prolong the working time. Assembled devices are heated to evaporate the solvent and complete the bonding process within several minutes.

Typically, I treated surfaces at a pressure of 90–100 mTorr for 15 seconds. The plasma power was not critical: powers from 25 W to 75 W resulted in successful bonding. 1–2 drops of methanol were placed between surfaces during alignment. Capillary action helps to pull the bonding surfaces together as the methanol evaporates during the subsequent bake at 80°C for several hours.

A.2.5 Amino-derivatization of glass slides

RCA-cleaned (Appendix A.2.1) glass slides were derivatized with amino groups by treatment with 3aminopropyltriethoxysilane (Sigma-Aldrich). Slides were rinsed twice with acetone to remove water and then immersed in a solution of 2 vol% APTES in acetone for 30 min at room temperature with stirring. Slides were then rinsed twice with acetone to remove unreacted silane and twice with deonized water. They were then dried and baked at 120°C for 30–60 min to anneal the silane coating and finally stored dry until use. Toluene or 95% ethanol were also found to be suitable solvents.

A.2.6 Derivatization of glass for DNA synthesis

A silanization solution of 1 vol% silane in 95% ethanol was prepared and stirred for 1 h. The silane, N-(3-(triethoxysilyl)-propyl)-4-hydroxybutyramide, was purchased from Gelest, Inc., Morrisville, PA. Prior to treatment, RCA-cleaned (Appendix A.2.1) slides were immersed in 10% sodium hydroxide for 3 min at 70°C, rinsed with deionized water, immersed in 1% HCl for 1 min, washed twice with water, and dried with nitrogen and transferred to a dry slide rack. Dried slides were wetted by immersion in ethanol and then transferred to the silanization solution for 1 hour with stirring. After treatment, slides were washed three times with ethanol, dried with nitrogen, baked for 2 h at 115°C, and stored dry until use. Attempts to react this silane from the vapour phase resulted in a poor density of functional groups. Oligonucleotides synthesized on the treated surface should be deprotected by immersion in ethylene diamine (EDA) and ethanol (1:1, v/v) at room temperature rather than by ammonia treatment.

Other silanization procedures suitable for DNA synthesis can be found in the literature. For example, Maskos and Southern described the preparation of a linker that is stable under final ammonia deprotection [184]. Glass is first reacted with a silane (3-glycidoxypropyltrimethoxysilane) to which a hexaethylene glycol linker is attached.

During optimization of derivatization protocols (Section 7.5.3), I prepared several slides by this method. 15 mL silane was added to 50 mL xylene with a trace of diisopropylethylamine and mixed in a staining jar. Slides were placed in this jar, which was heated on a hot plate to a solution temperature of 80°C overnight. Slides were then washed twice in methanol, once in methanol with ether, once again in methanol, and then dried with nitrogen. Next, a PDMS gasket was placed between two silanized slides, resulting in a sealed chamber between them. A solution of 1.5 mL hexaethylene glycol with a trace of sulphuric acid was injected into the enclosed space. The assembly was baked at 80°C overnight.

A.3 Polymer film protocols

A.3.1 Measurement of polymer film thickness

Polymer film thicknesses were measured with an Alpha-step 500 profilometer (KLA-Tencor). This instrument consists of a stylus that is dragged across the surface while maintaining a constant vertical contact force. The stylus thus follows the topography of the sample, and the height profile is recorded.

To measure the film thickness on a silicon wafer mold, a small part of the film was cut or scraped away creating a step edge from the polymer down to the wafer. A profile of length 3–10 mm was taken across the edge from the polymer (high) to the silicon (low). (If profiles were taken in the reverse direction, the stylus often caught on the polymer edge and peeled it from the surface.) When measuring soft polymers, it was necessary to use a small stylus force (e.g., 1–2 mg). The film thickness was read directly from the profile after leveling. Generally the profile of the polymer was not flat near the step edge due to air gaps between the polymer and wafer created during cutting. It was thus necessary to observe the profile height at a point several millimeters from the edge.

A.3.2 Calibrating spin curve for a new polymer

Fabricating elastomeric microfluidic devices with functional microvalves requires that the thickness of the thin layer be well controlled. For new polymers, it was thus necessary to calibrate the spincoating procedure.

Several brand new glass slides or silicon wafers were prepared by treating their surfaces with the mold-release agent appropriate for the polymer being investigated. Substrates were then spin-coated with prepolymer at a variety of spin speeds (with duration and ramp speed kept constant). Samples were then cured or polymerized and measured by profilometry according to Appendix A.3.1. The layer thickness was plotted as a function of spin speed, allowing the spin speed for the desired layer thickness to be interpolated from the graph.

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