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 deter-

mine 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.

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 controlled-pore 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.

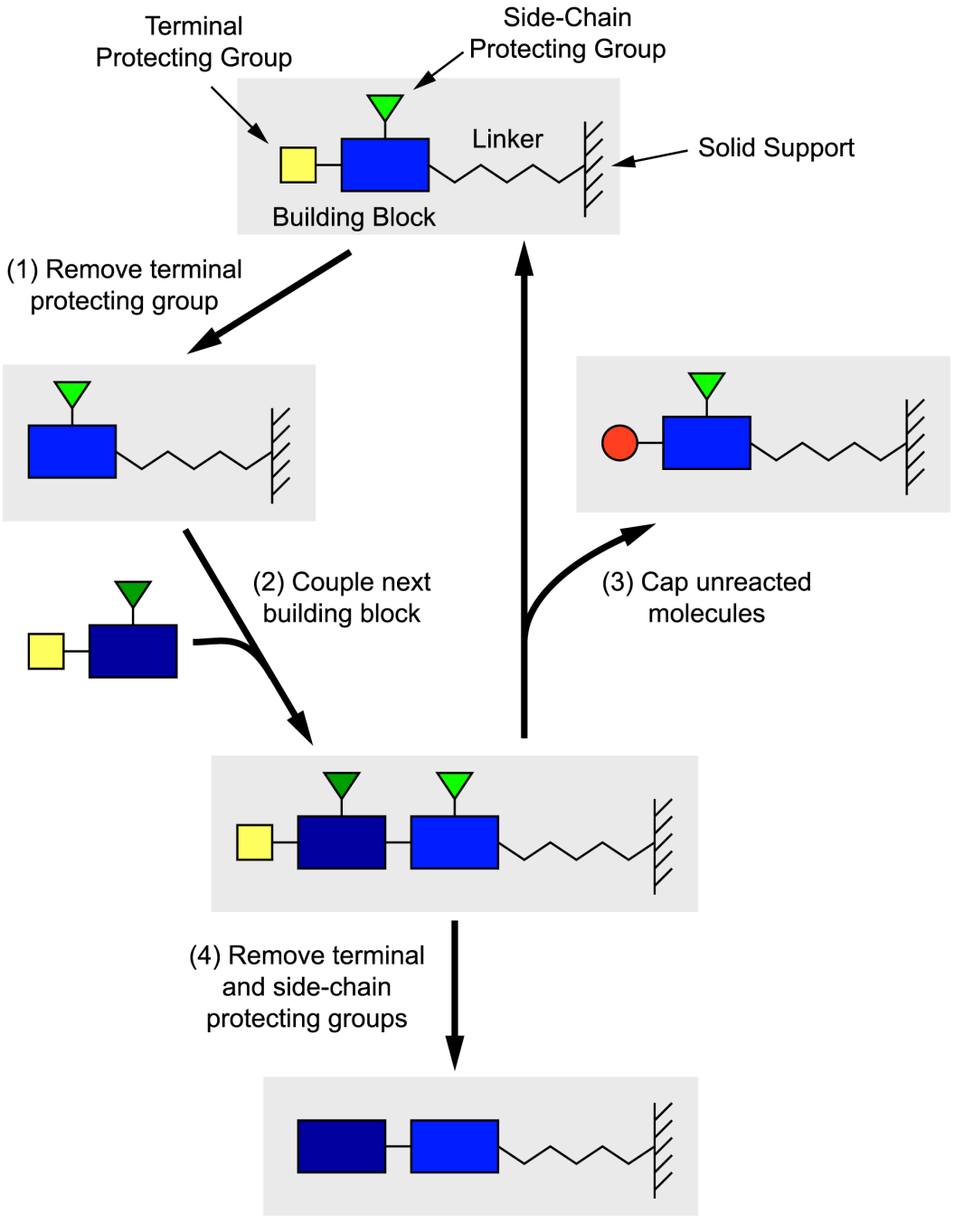


Figure 7.1: **Schematic of solid-phase synthesis**. The desired molecules are built up one building block at a time. Each building block contains a terminal protecting group that ensures only a single building block can be attached in a given step. Building blocks also contain side-chain protecting groups 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 $n-1$ and 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.

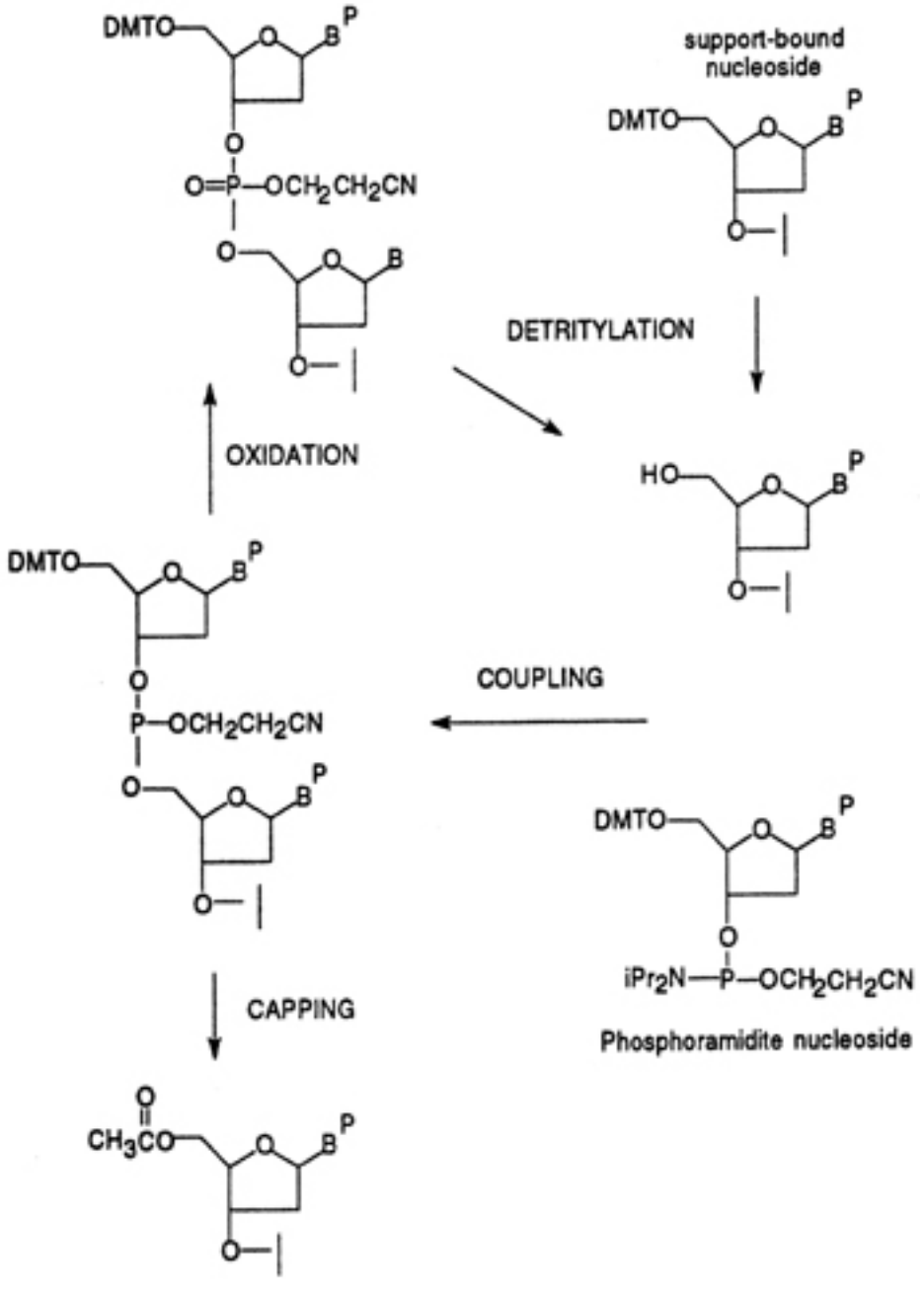


Figure 7.2: **Chemistry of DNA synthesis**. A DNA synthesis cycle begins by removing the dimethyoxytrityl (DMT) protecting group on the previous nucleoside in the molecule being synthesized, leaving an active hydroxyl (OH) group at the 5′ position. A DMT-protected activated phosphoramidite nucleoside 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 commcercial 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

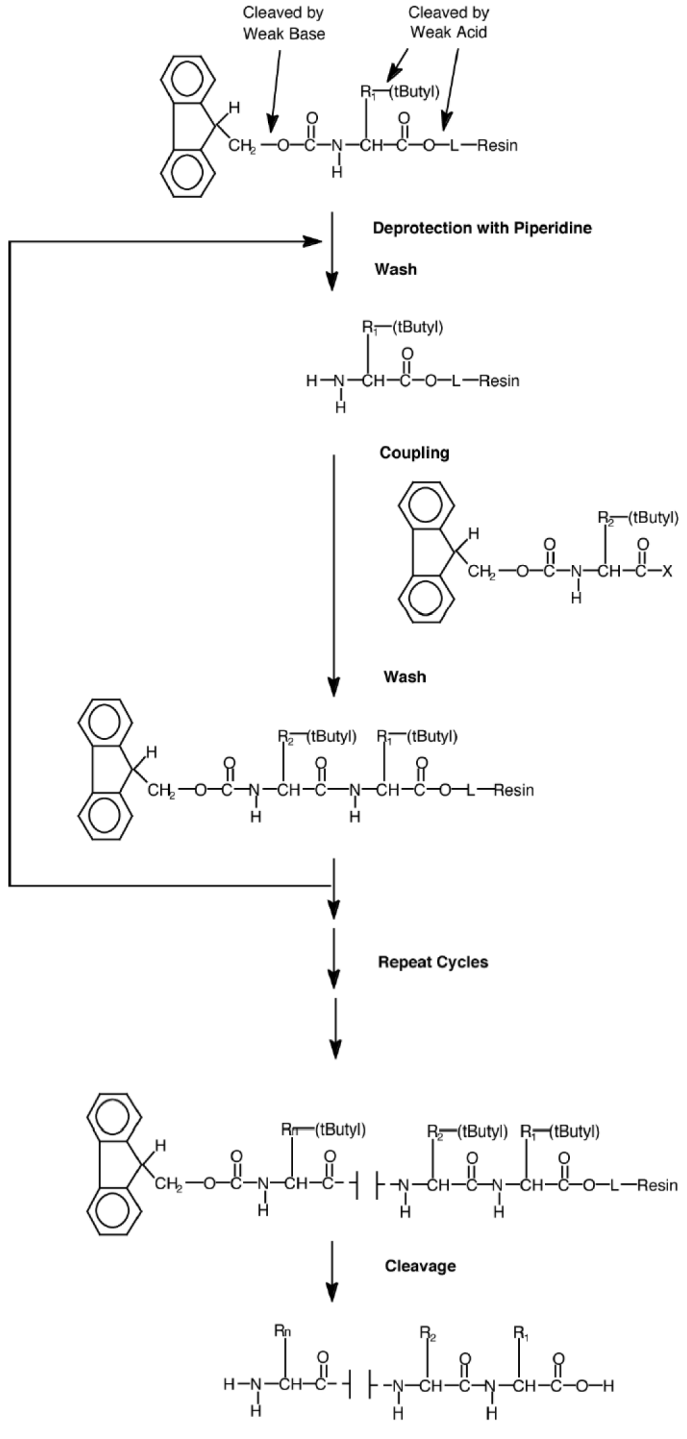


Figure 7.3: **Fmoc peptide synthesis chemistry**. In each synthesis cycle, the terminal Fmoc protecting group is removed from the growing peptide chain by piperidine, and an Fmoc-protected, activated amino acid is then coupled to the newly exposed amine. This cycle is repeated to build the desired peptide. 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.)

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 $m = 20$ for 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 na-

ture. 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 μm or greater, though 50–75 μm 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 μm thick steel foil from both surfaces (see Figure 7.4). At the tip, the pens were approximately 100 μm wide by 13 μm thick, containing a trench about 30 μm wide by 7 μm deep. We demonstrated printing of spots as small as 20×40 μm (corresponding to densities up to 25000 spots/cm^2) 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).

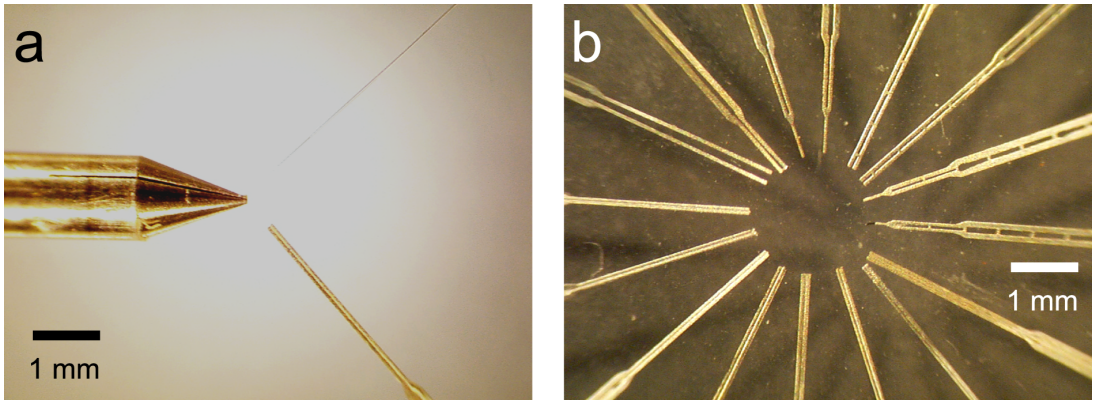


Figure 7.4: **Microfabricated stainless steel trench pens**. (a) Comparison of commercial slot pen (left) machined by conventional methods with our microfabricated trench pen (right). The tip sizes are similar; however, the trench pens are capable of smaller spot sizes. This is presumably due in part 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 μm wide by 7 μm 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 μm, 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

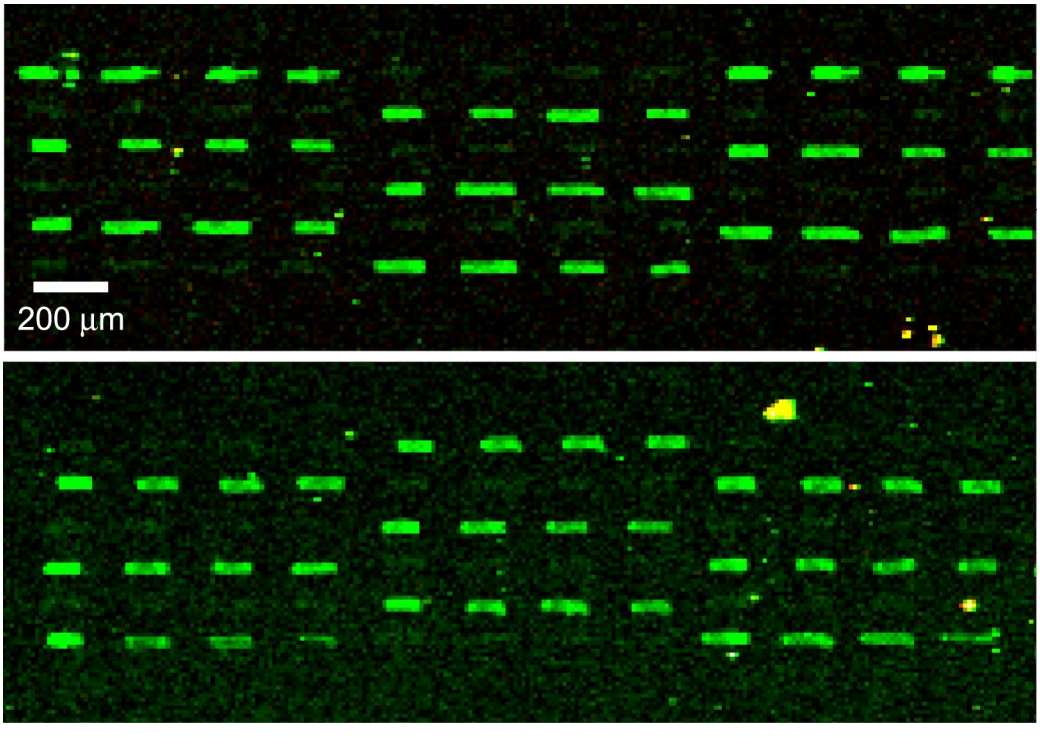


Figure 7.5: **Hybridization to an array printed by stainless steel trench pens**. Two 10-mer probe sequences were spotted onto a glass substrate with our microfabricated trench pen in an alternating fashion. (Top) Complement 1 hybridizes selectively to probe 10mer-1 and does not hybridize to probe 10mer-2 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 μm 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 disk-shaped 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 cm^2 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 solid-support 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 μm were demonstrated with cycle efficiencies of 85–95%. Arrays with features as small as 18 μm have been reported in the literature [169]; however, the technology is thought to be capable of printing arrays with 10 μm features, corresponding to a density of 10^6 probes/cm^2 [11]. Illumination through a chrome photomask deblocks only selected areas of the substrate. Up to $20n$ 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 μm separated by a 3 μm 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 10×10 μm 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 μm have been reported using photomasks [283], while feature sizes in arrays fabricated with digital micromirror arrays have reached 14 μm [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).

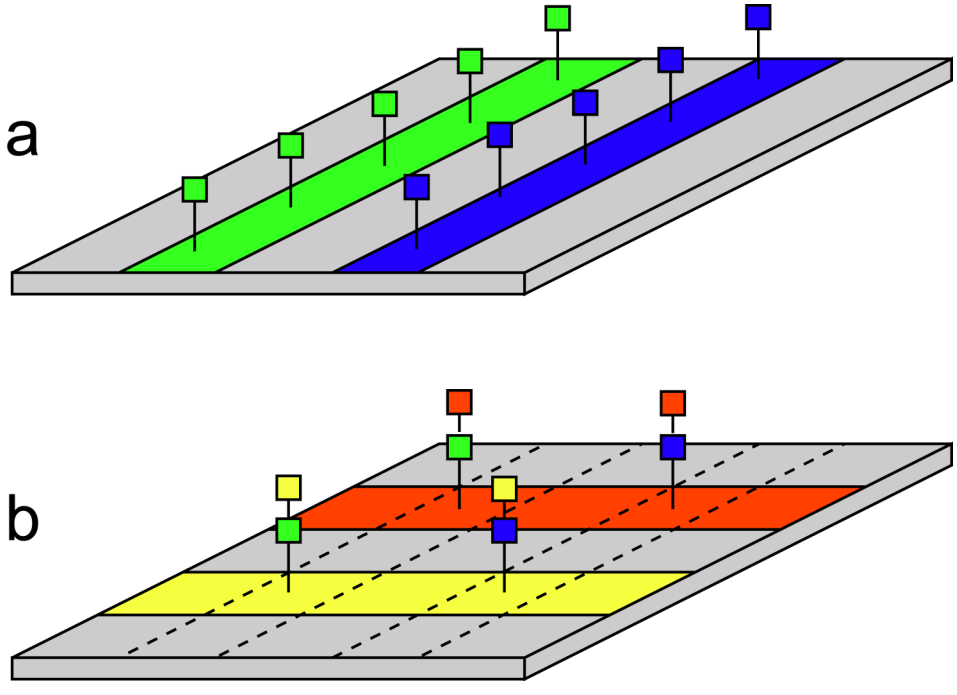


Figure 7.6: **Principle of *in situ* solid-phase synthesis by surface striping**. Using microchannels or other means, one can confine reagents to flow in a thin stripe along the substrate surface. By flowing the appropriate reagents to perform coupling of a monomer (e.g., nucleotide, amino acid, etc.), 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.

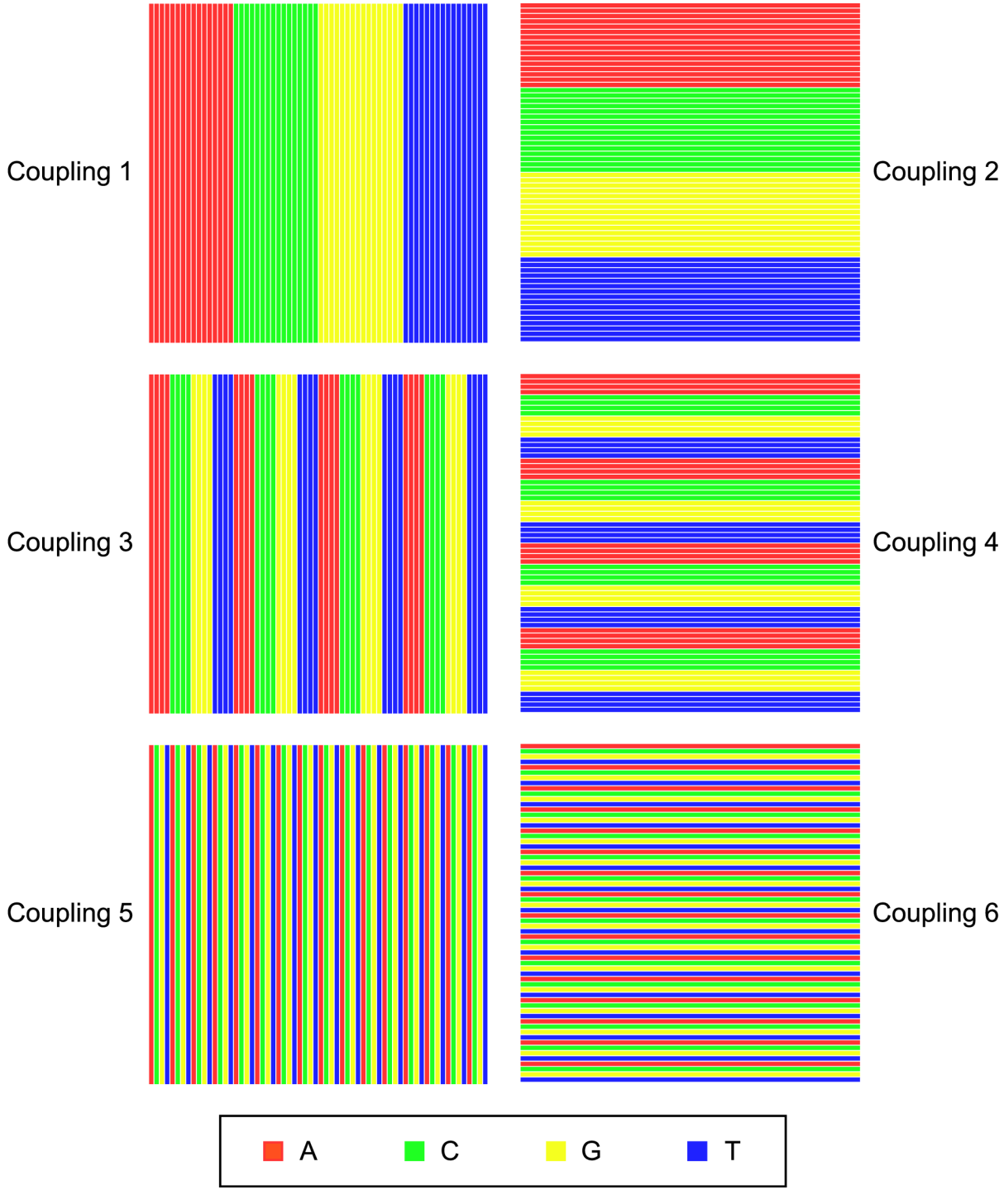


Figure 7.7: **Pattern of nucleotide coupling steps to build all DNA 6-mers in 6 steps**. There are 4 "monomers" from which DNA is synthesized: A, C, G, and T. To make all possible 6-mers by the stripe synthesis method, one requires an array with $4^6 = 4096$ spots, or 64 rows by 64 columns. In the first 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 inital 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.

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 diamond-shaped 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.

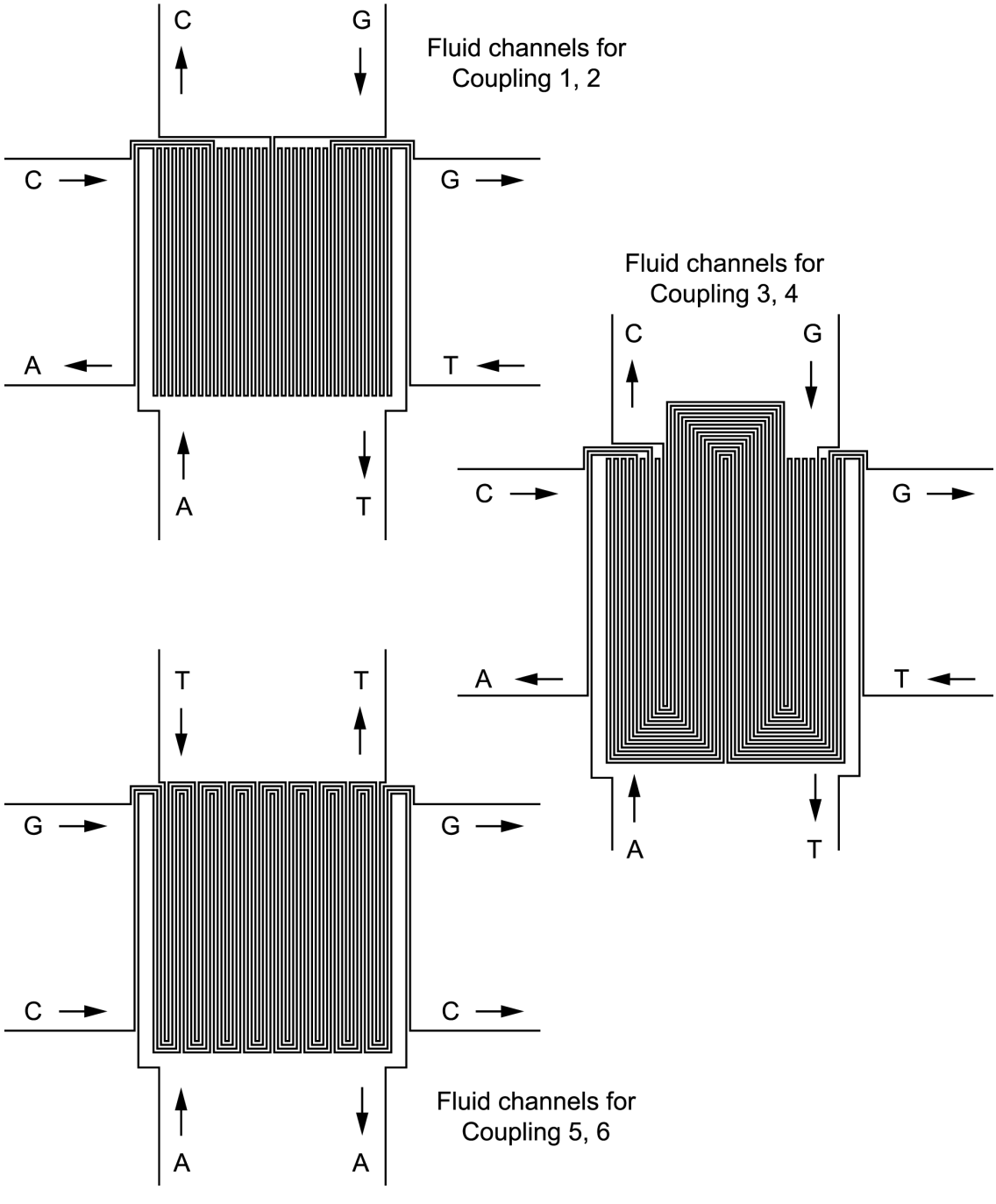


Figure 7.8: **Scheme for synthesizing all DNA 6-mers with passive microfluidic devices**. Essentially the passive device consists of 64 parallel channels. To reduce the number of chip inlets, these channels are tied together such that all channels carrying nucleotide A are joined to a single inlet, etc. 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.

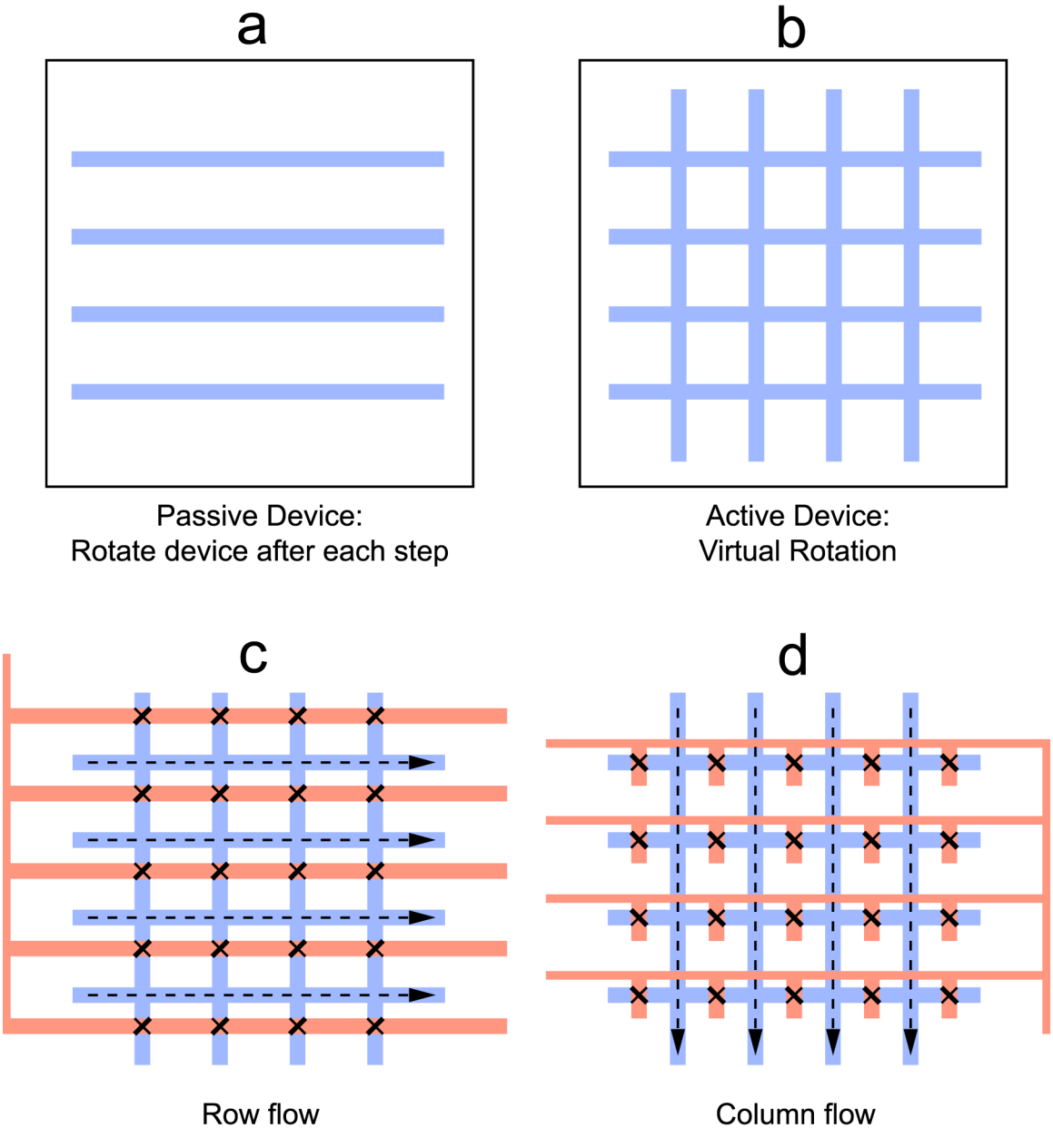


Figure 7.9: **Switching the flow direction (row or column) in a grid of microchannels**. (a) Design of a *passive* microfluidic device. Fluid channels are shown in light blue. Reagents are flowed in rows for one step of the synthesis, then rows are flushed and dried. The device must then be physically 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.

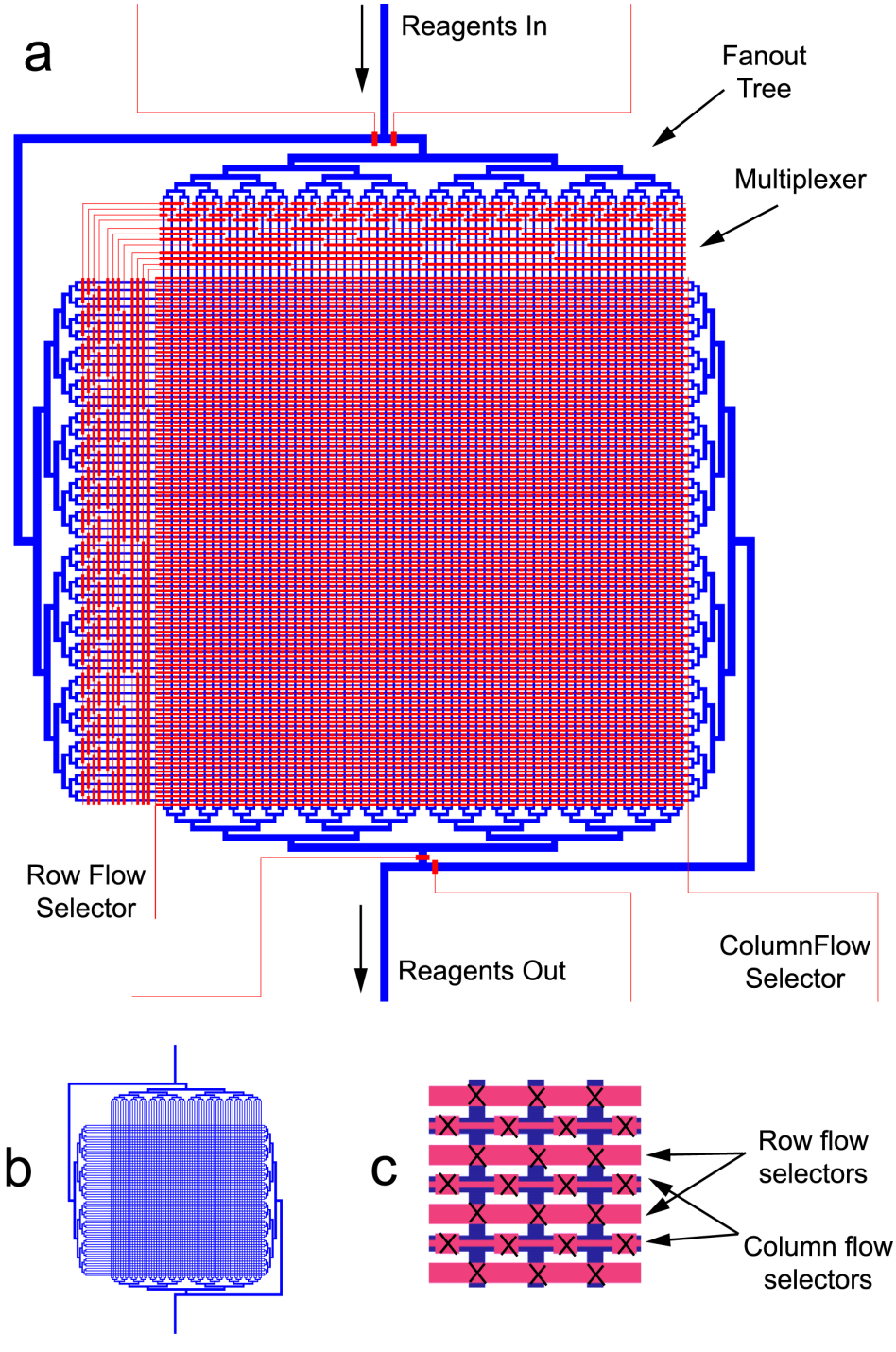


Figure 7.10: **Design of an active DNA 6-mer synthesis device**. (a) Unlike the passive design of Figure 7.8, in which three different fluidic devices are needed, the active approach requires only a *single* device containing a fluid layer (blue) and a control layer (red). Rotation of flow direction 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.

Partially closed valves allow fluid flow but prevent the escape of the beads. In a typical step of synthesis, valves preventing flow in one direction (row or column) would be completely closed. The other valve bank would remain partially closed to hold the beads while reagents were flowed through the columns or rows. (Alternatively, dedicated frit valves 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

[3]Though 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 solvent-exchange (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

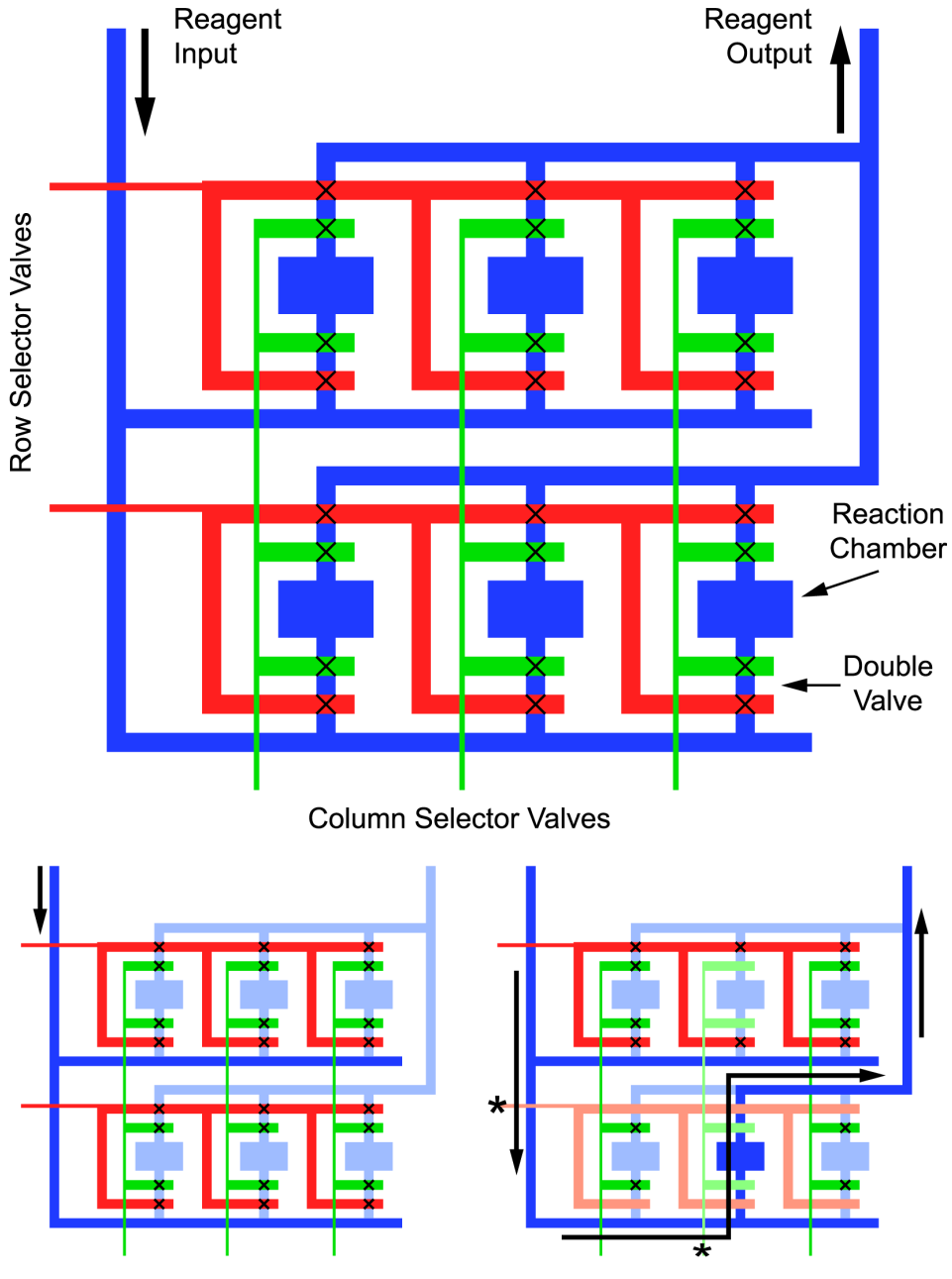


Figure 7.11: **Design and operation of an individually-addressable microfluidic array synthesizer**. (Top) Design of the synthesizer. Only a small portion (six reaction sites) are shown for clarity. The fluid channel is shown in blue, and two control layers are shown in red and green. A multi-layer chip 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 μm 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 μm. 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× 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

[4]Note 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 μM) to assess hybridization stringency.

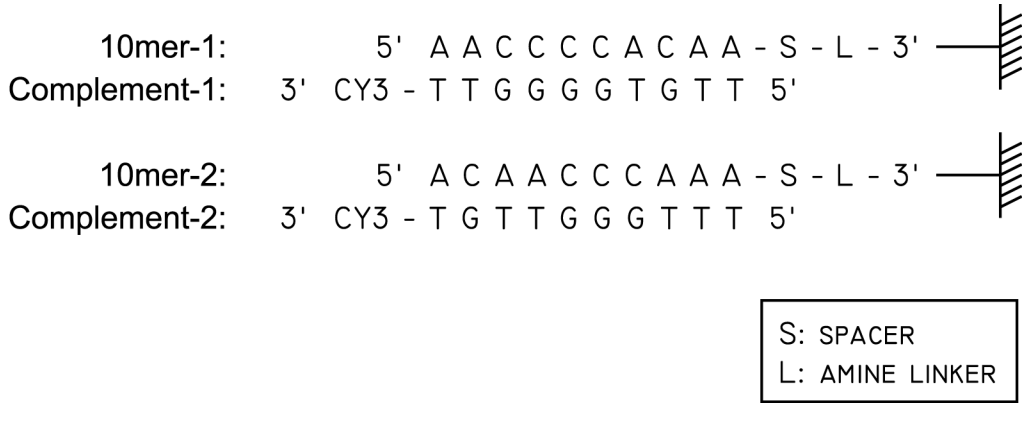


Figure 7.12: **DNA sequences used for hybridization optimization**. Sequences are shown for tethered probe molecules 10mer-1 and 10mer-2 and fluorescent hybridization targets Complement-1 and Complement-2. Probes consist purely of A and C nucleotides to minimize secondary structure formation that could 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× SSC, 0.001% SDS (sodium dodecyl sulfate), and 50 μM 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 μM 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, ∼9°C); W2 (0.2× SSC, ∼11°C); W3 (0.05× SSC, ∼13°C); W4 (water, ∼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 3-aminopropyltriethoxysilane [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].

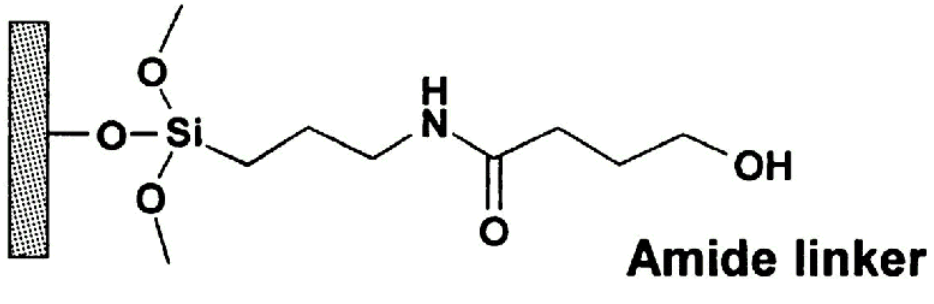


Figure 7.13: **Silane linker for DNA synthesis**. The structure of N-(3-(triethoxysilyl)-propyl)-4-hydroxybutyramide linked to a glass solid support is shown.

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 (2×3–4 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 (∼2 mm in width) were machined.

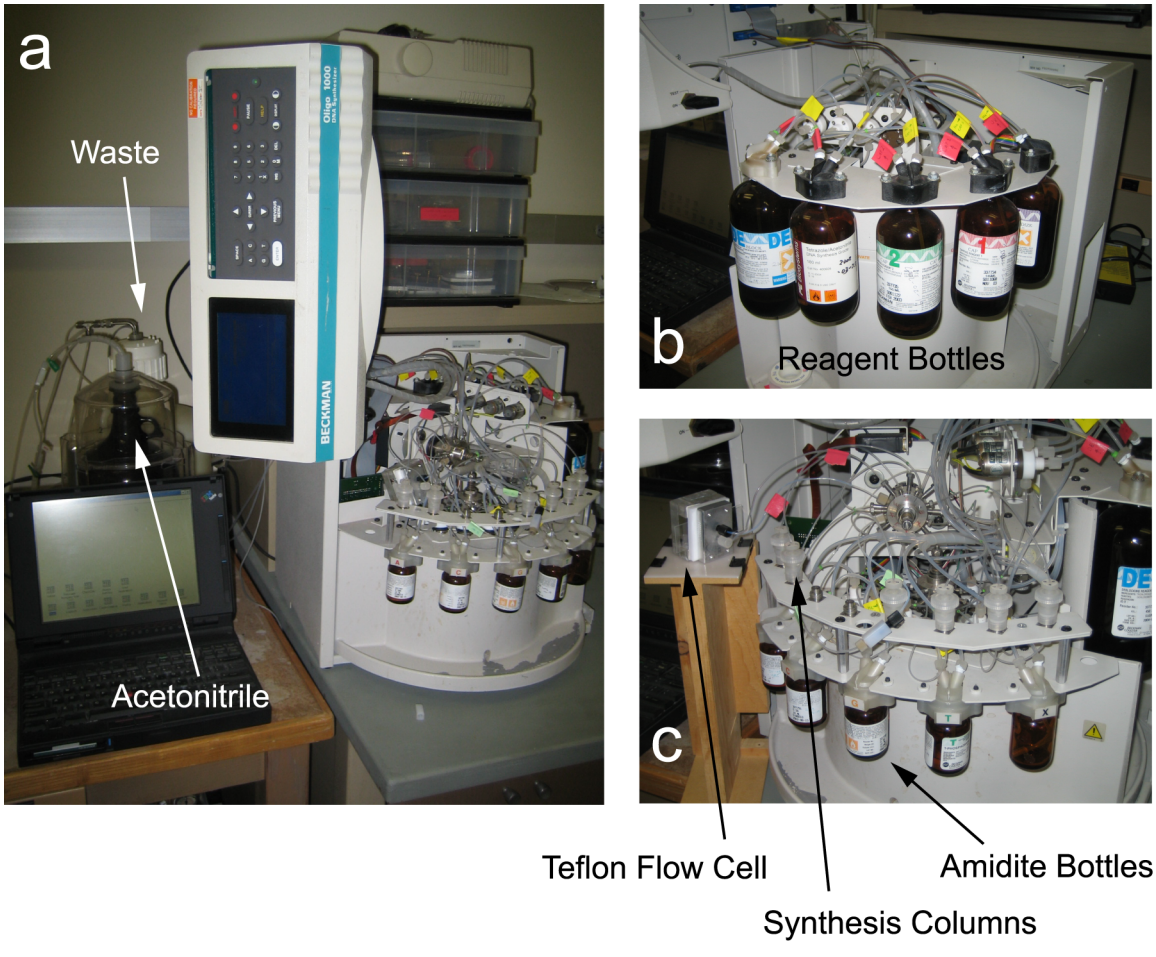


Figure 7.14: **Beckman Coulter Oligo 1000M DNA synthesizer**. (a) DNA synthesizer with lid open (tilted up sideways). (b) Synthesizer with the reagent platform rotated to show the reagent bottles behind (deblock, activator, cap 1, cap 2, and oxider). (c) Synthesizer with the Teflon flow cell inserted in place of a standard synthesis column to perform synthesis on flat substrates.

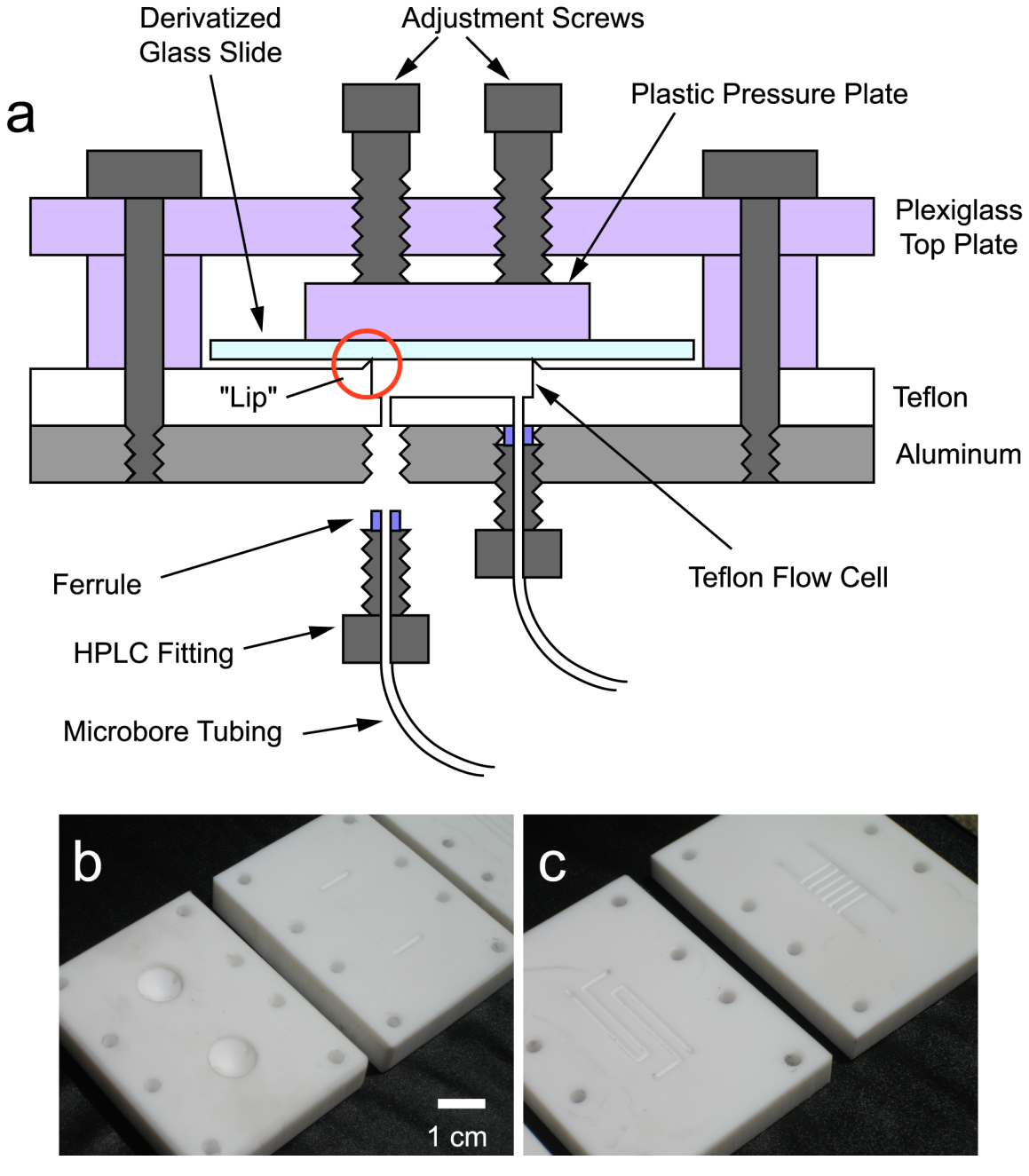
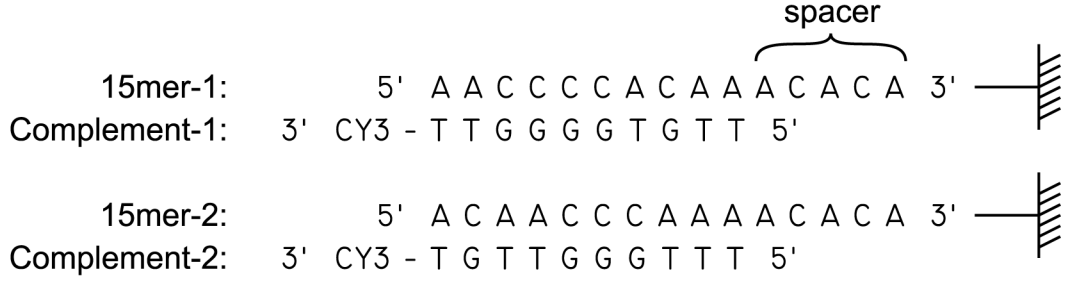
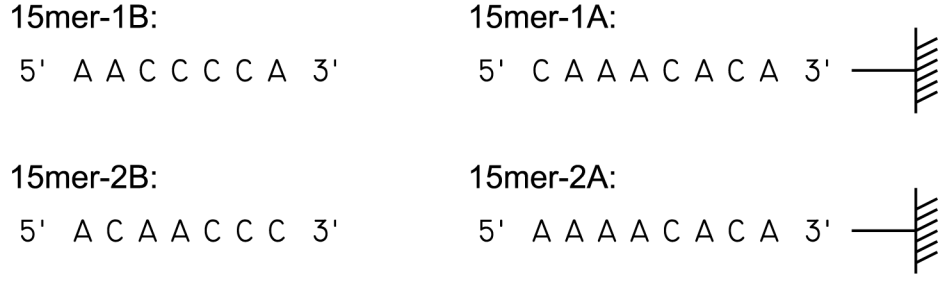


Figure 7.15: **Teflon flow cell for millifluidic solid-phase DNA synthesis**. (a) Schematic of Teflon flow cell that connects in place of a synthesis column in a commercial DNA synthesizer. A flow chamber is machined in a Teflon plate. The machining process results in a raised "lip" of Teflon around 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.

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.



(a) Basic sequences



(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.

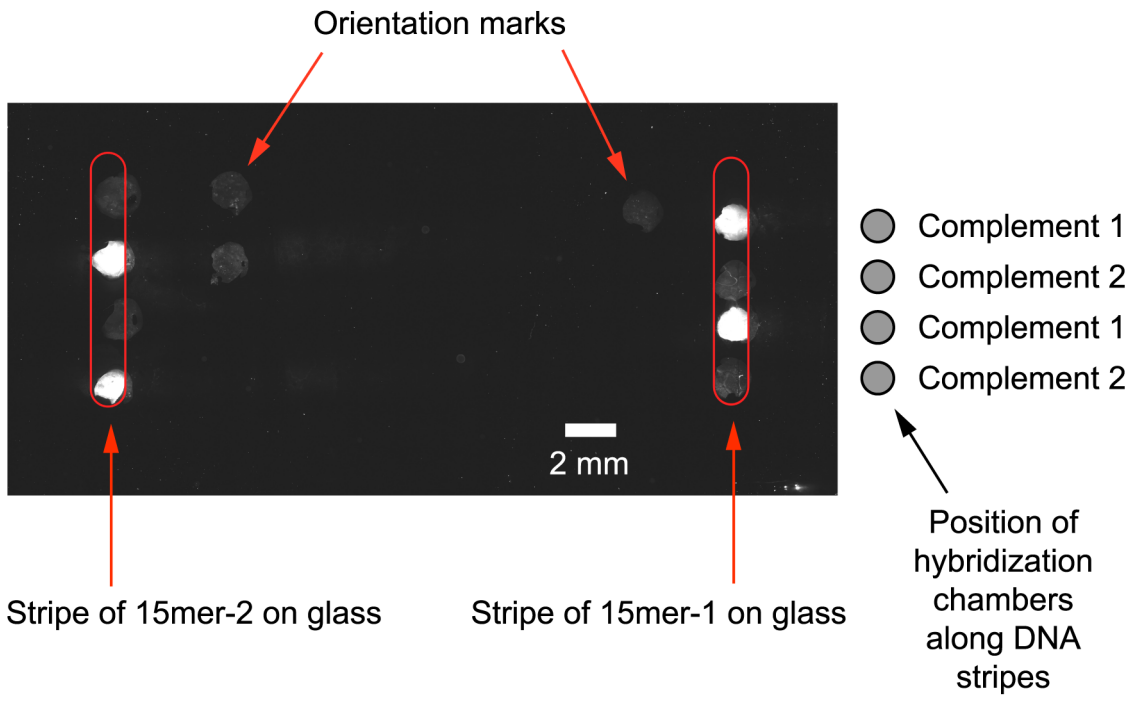


Figure 7.17: **Demonstration of hybridization specificity onto synthesized DNA stripes**. Fluorescence image (Cy3 channel) of a hybridized slide. Two stripes of DNA (15mer-1 and 15mer-2) were synthesized on a derivatized glass substrate by inserting a flow cell in place of a synthesis column on a commercial 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.

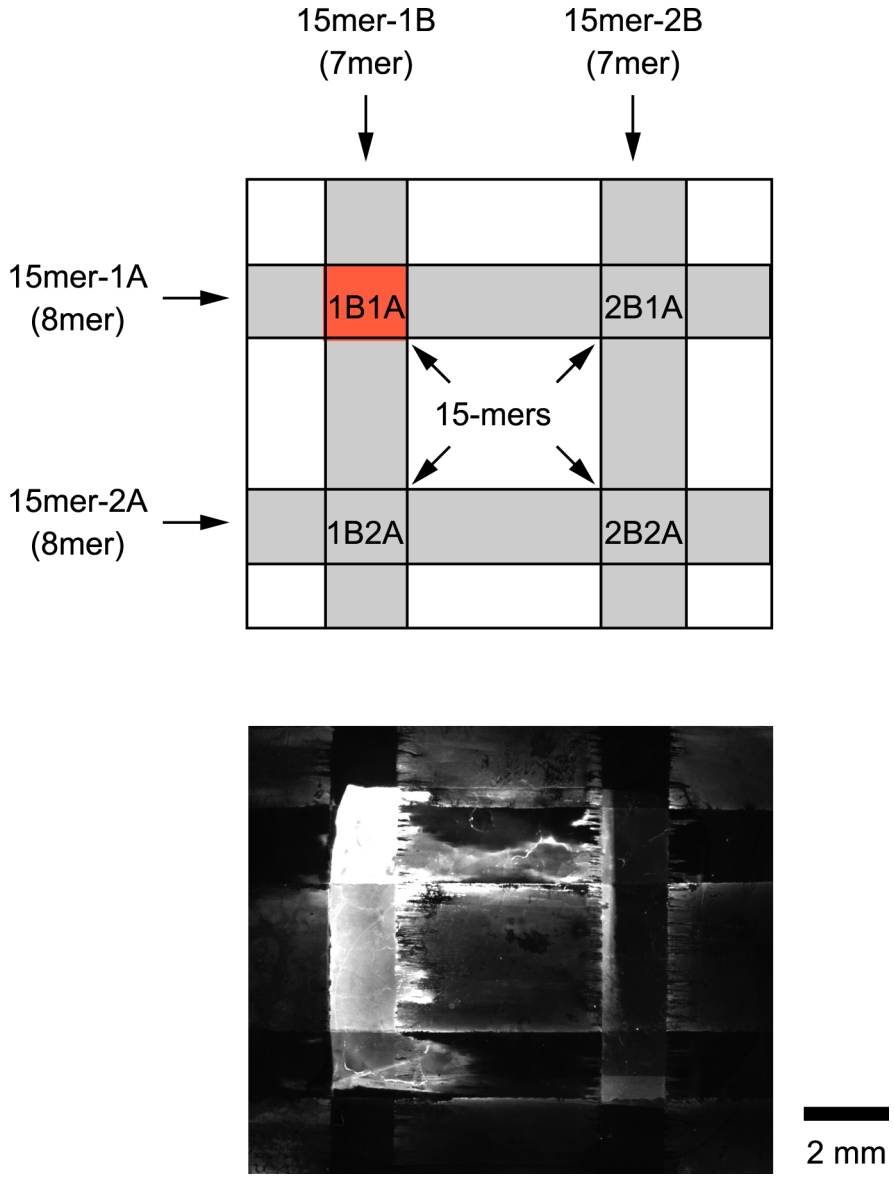


Figure 7.18: **Demonstration of DNA extension when intersecting stripes of DNA are synthesized**. (Top) Synthesis scheme. Stripes of DNA were synthesized by clamping a custom-built Teflon flow cell to a derivatized glass slide and flowing reagents from a commercial DNA synthesizer. In the first synthesis, 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× 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 μL. In order to ensure complete filling, an "elemental volume" of 200 μL 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 $5 \times 10^{12}/\text{mm}^2$ [123]. The approximate glass surface area in contact with fluids inside one chamber of the flow cell is 0.59 cm^2. Thus, approximately 1.2×10^{15} or 2.0 nmol surface amine groups should be available for reaction. Therefore a 40 μM coupling-solution concentration would be required assuming a 4× excess of reagents and a 200 μL 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 μL of coupling solution, I used 3 μmol 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 μL of NMP through the flow cell. This was repeated 5 times.

2. Coupling. Coupling was performed twice, each time filling the reactors with 200 μL 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 μL 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 μL 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 μL 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]:

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

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 Fmoc-

protected 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 μL, 14 min 200 μL, 15 min 200 μL, with 650 μL 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 μL, 15 min 200 μL, 10 min 200 μL) 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 μL, 5 min 100 μL). 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 μL of solution, 5 min with 150 μL, and finally a 100 μL 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 μm wide and several centimeters long, having

a total surface area of fluid-glass contact of about 0.21 cm^2—approximately 1/3 the surface area in the Teflon fluidic device. The volume of each channel/chamber, however, is significantly smaller: about 0.2 μL instead of 75 μL. The "elementary volume" of solutions was therefore reduced to about 2.5 μL rather than the 200 μL 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 μL 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 valves 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 μm 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 μm 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 μm), 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 μm and 1.5 μm) were purchased from Bangs Laboratories, Inc. (Fishers, IN) and derivatized by a protocol modified from

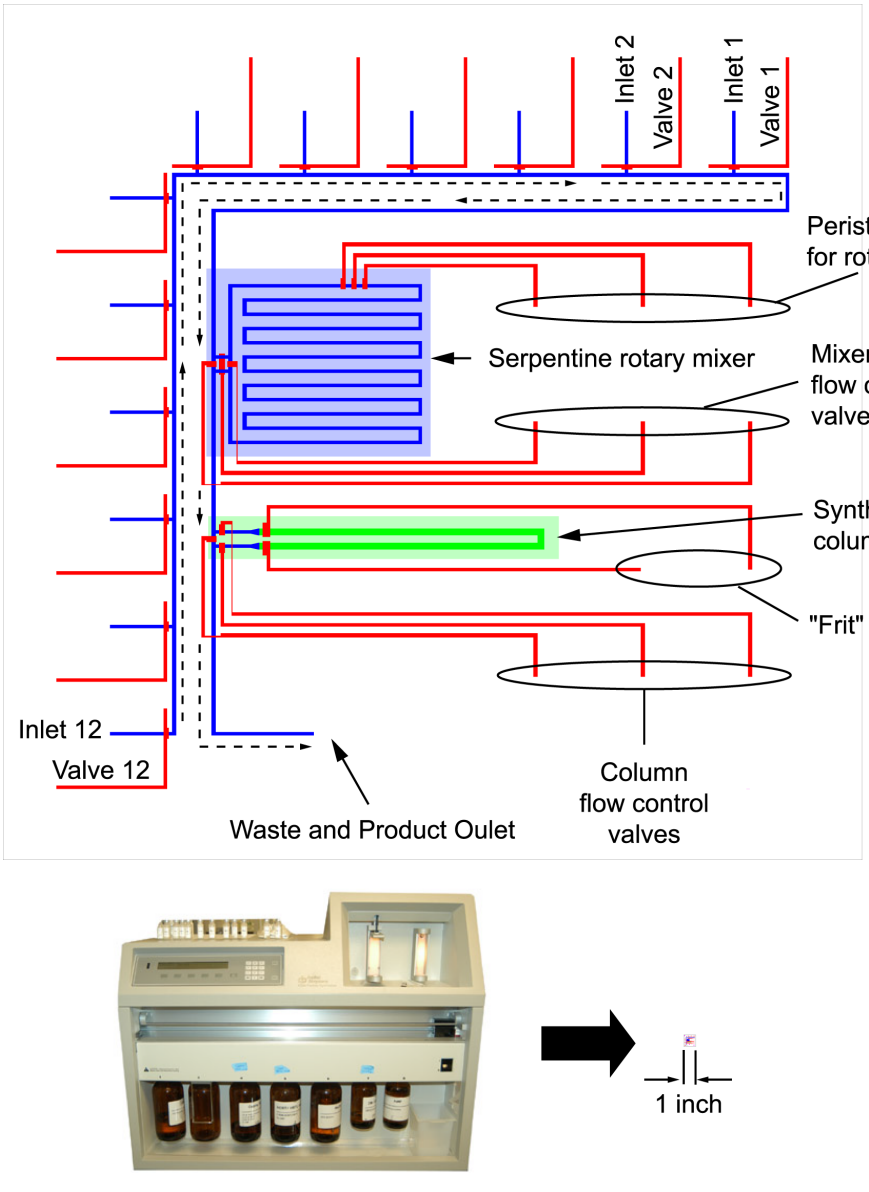


Figure 7.19: **Design of a solid-phase synthesis chip**. (Top) CAD design. Fluid channels are shown in blue, control channels in red, and the main synthesis column (in the fluid layer) in green. Note that most valves have been labelled at their control channel inlet, rather than at the actual valve position, 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.

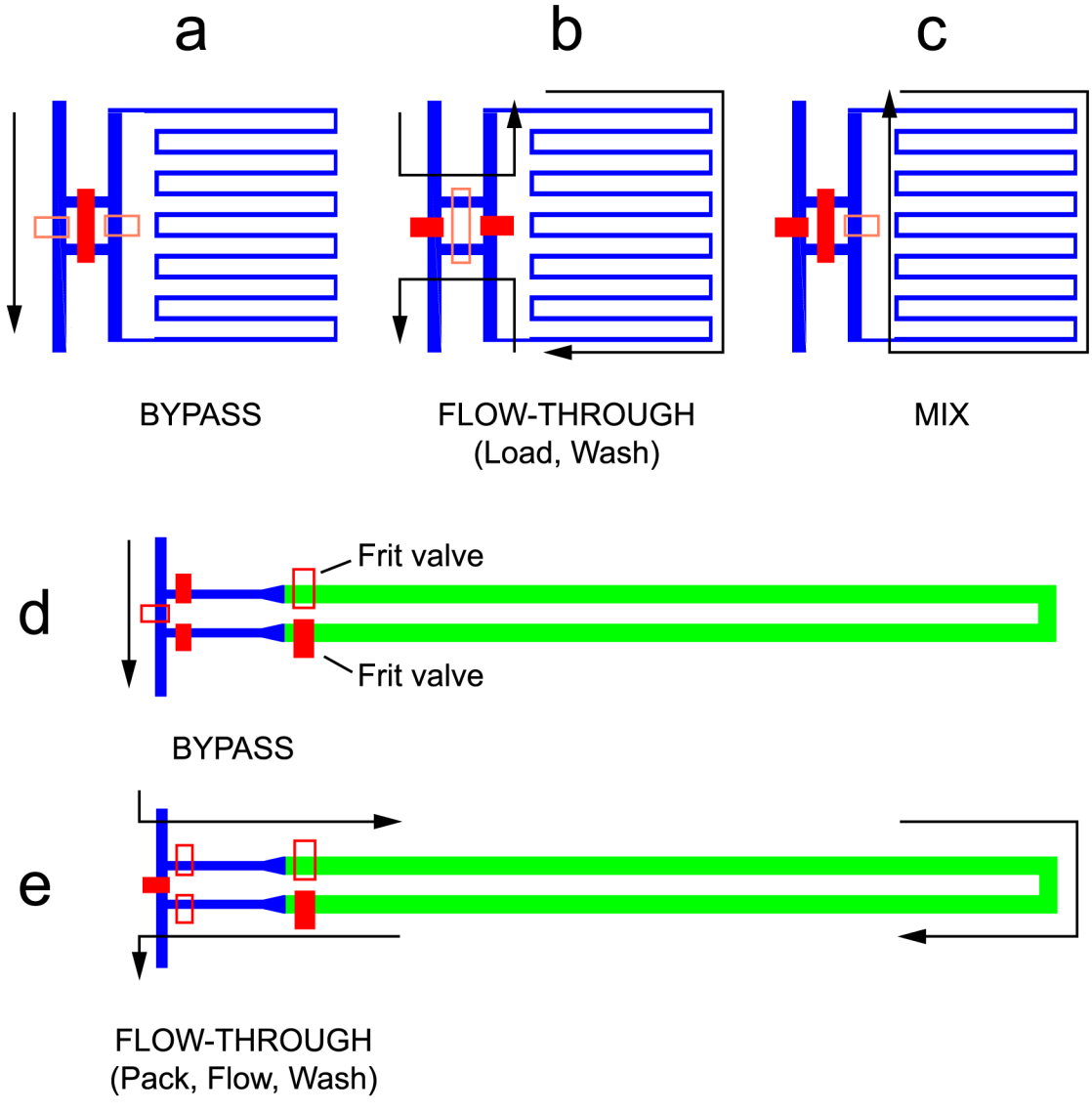


Figure 7.20: **Details of solid-phase synthesis chip operation**. (a-c) Operation of mixer flow control valves. (Note that the size of the mixer has been reduced in these diagrams for clarity.) Valves can be configured (a) to bypass the mixer; (b) to flow through the mixer for washing or for loading 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 valves 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 valve 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 valves (or frit valves), while open red rectangles represent open valves.

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). CYTOP-coated 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.

Acknowledgment

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