

# Microfabricated Devices for Genetic Diagnostics

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## Invited Paper

*This paper presents a review of microfabricated devices for genetic diagnostics. Genetic diagnostics are powerful technology drivers and excellent candidate applications for miniaturization technologies because the demand for inexpensive genetic information is essentially unlimited, and the cost and time for the diagnostic decreases with sample volume. Genetic information is stored in long DNA molecules in solution. This information is processed and extracted using a series of enzymatic and other chemical reactions well known in molecular biology. Processing of DNA molecules in the microscale hence requires the implementation of microfluidic devices capable of handling, mixing, thermal cycling, separating, and detecting nano- and picoliter liquid samples. This paper discusses some of the fundamental macroscale protocols used for genetic analyses and how these processes scale down to microscopic volumes. The construction and performance of microfluidic devices for DNA amplification, separation, hybridization, and detection are discussed, showing that so far, no fundamental impediments exist for genetic diagnostics based on microelectromechanical systems. Some of the unresolved storage and packaging issues and future challenges for the practical implementation of these devices are also presented.*

**Keywords**—Biological sensors, microfluidics.

## I. INTRODUCTION

A fundamental requirement for the commercial success of any microfabrication technology is an application with a very large demand. These applications are essential technology drivers that provide sufficient economic pull for the adequate recovery of facility costs that sustain continued research into new and improved devices at very low unit cost. This paper discusses a new type of application for microelectromechanical systems (MEMS) that not only satisfies this requirement but also promises enormous potential for growth.

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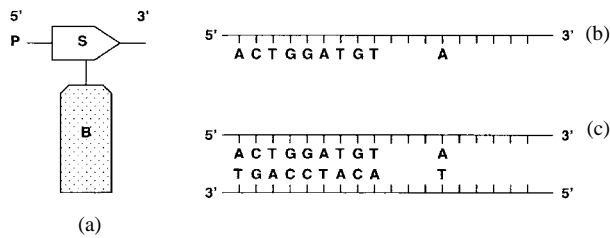
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Genetic tests (or assays) have an enormous scope of applications in biotechnology and medicine, ranging from agriculture and farming [1] to the detection of pathogens in foods [2]–[4] to genetic diagnostics on human subjects [5]–[14]. Currently, about 400 diseases are diagnosable by molecular analysis of nucleic acids, and this number is increasing daily. Many of these assays were recently developed as part of a major thrust initiated by the National Institutes of Health aimed at making medicine a more quantitative science [15]. Further, many more assays will undoubtedly follow in the near future as more genetic information is discovered by major research undertakings such as the Human Genome Project [16]–[19]. Humans have approximately 100 000 genes that could be potentially tested for defects or the propensity for diseases. Essentially with the same procedure, the contents of every gene on any form of life could be examined. Such a broad base application may prove to be the ultimate technology driver of all time.

Recently, there has been much interest in the implementation of microfluidic devices for genetic assays. These devices are excellent candidates for miniaturization because 1) the demand for genetic information is essentially unlimited, and hence determined only by the cost of information retrieval, 2) the performance and costs of genetic assays can be improved in the microscale, 3) the same microfabricated part can be used for many different assays by changing the nature of its reagents, not the device construction, and 4) genetic assays can benefit from the automation and control provided by miniature electronic devices.

The implementation of these devices presents new and interesting technological challenges. Genetic information is contained in a long polymer of nucleic acid, typically in solution in a weakly saline water-based buffer. The extraction of genetic information involves a series of chemical manipulations of the sample requiring mixing with reagents, thermal cycling, labeling, and fragmentation analysis using conventional molecular biology protocols. A miniaturized device for genetic assays is hence a chemical reactor capable of performing some or all of these functions in microscale volumes, including the detection of the assay outcome.



**Fig. 1.** (a) DNA nucleotide, (b) single-stranded DNA chain, and (c) double-stranded DNA with complementary base pairs. The two strands are joined by hydrogen bonds.

This paper begins with a basic discussion of the nature of genetic information, followed by a summary of the most important procedures used for testing and reading this information. This is followed by some scaling arguments that favor the use of miniaturization. Next, a review of microscale protocols and their demonstration in microfabricated devices is presented, followed by a discussion of important problems that must be solved for the implementation of practical engineering devices. This paper concludes with some projections on future research directions.

## II. THE NATURE OF GENETIC INFORMATION

This section very briefly discusses the basic principles and terminology in molecular biology that are necessary for the engineering understanding of the task at hand. Genetic information in humans is stored in the cell chromosomes. Each chromosome consists of long, compactly packed, supercoiled linear polymer strands of deoxyribonucleic acid (or DNA) [20]. The chromosome information is stored as a long string of DNA fragments grouped as genes, each expressing an identifiable function or characteristic of the organism. In humans, for example, each of the 46 chromosomes is  $50 - 400 \times 10^6$  units long, while the single chromosome in the *E. coli* bacteria is  $4 \times 10^6$  units long.

The units of a single DNA strand are called nucleotides. Each nucleotide consists of a base (B), a sugar linkage (S), and a phosphate bridge (P), as shown in Fig. 1(a). The sugar linkage gives the nucleotide directionality with two distinct ends labeled 5' and 3'. There are four types of nucleotides corresponding to four different bases: adenine, guanine, cytosine, and thymine, commonly labeled A, G, C, and T. Nucleotides can only be linked in a specific direction forming single strands of DNA, as shown in Fig. 1(b). Individual bases are hydrophobic, but strands of DNA are quite soluble in water due to the polar backbone. Single-stranded DNA tends to attach (or hybridize) through weak hydrogen bonds to another strand of complementary base pairs (G-C and A-T), forming a double strand (or duplex), as shown in Fig. 1(c). Double-stranded DNA is more stable in water because the hydrophobic bases are hidden by hydrophilic backbones.

All information relevant to cell growth and regulation is stored in this form. To perform an analysis, first the DNA must be extracted from the cell nucleus and purified. This procedure involves the disruption of cell membranes by

chemical exposure to a detergent [21]. This is typically followed by purification using centrifugation or other methods for removal of cell debris, proteins, and enzymes, leaving DNA in solution.

DNA assays take two general forms. In diagnostic applications, the assay detects the presence of a specific base pair (bp) fragment in a fingerprint pattern matching fashion. In sequencing applications, the assay yields the actual base-pair order. Sequencing assays inherently provide much more information than fingerprinting assays since test patterns are often not unique due to the presence of mutations. Both assays are performed using a set of well-known molecular machinery described below.

## III. DNA ANALYSIS METHODS

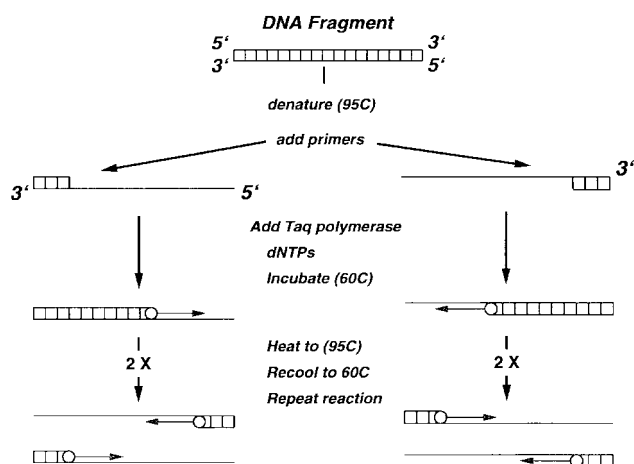
Conventional genetic assay protocols use five fundamental chemical procedures for the analysis of DNA [22]. These procedures are the workhorses of molecular biology and must be reproduced in the microscale.

### A. Chemical Amplification

DNA samples are often present at concentration levels that are too low for any direct test. Therefore, chemical amplification is used to increase the concentration of the sample. The amplification basically consists of a set of reactions that allow a DNA molecule or fragment to duplicate. Further amplification is hence obtained by repeating the procedure. Amplification factors as large as  $2^{30}$  are common, which permit detection based on single DNA molecules.

Several schemes can be used for amplification, but all of these use powerful enzymes. Enzymes are "miracle worker" protein catalyst molecules that can manipulate and modify DNA strands present in every living organism. In particular, polymerase enzymes assemble complementary strands of DNA from a single-strand fragment. This enzyme scans single strands of DNA and, starting from a specific location, captures matching nucleotides from solution and connects them to the single strand, assembling the complementary strand one base at a time like a zipper.

Molecular replication takes place when a double-stranded fragment is first thermally separated (or denatured) into two strands followed by the polymerase action. This procedure is known as the polymerase chain reaction (PCR) [11], [23]–[27], shown in Fig. 2. A typical amplification uses the high-temperature resistant Taq polymerase enzyme extracted from a heat-resistant microorganism (*Thermus aquaticus*) mixed with the unknown DNA sample (or template), an adequate supply of nucleotides [deoxyribonucleose triphosphates (dNTP's)], and primers that determine the starting point of the replication. The length of the amplified fragment is controlled by cleaving the DNA ahead of time or by auxiliary primers. These primers are short synthetic chains of matching nucleotides (or oligonucleotides) that bind to the single-stranded DNA at specific locations, thus flanking the region of DNA that gets amplified.



**Fig. 2.** The polymerase chain reaction. The double-stranded fragment is first separated (denatured) into two strands by heating the sample to 92°C. The primers next attach to the 3' ends of DNA. Starting from the primer, the enzyme scans the single strands and reconstructs the complementary strand at 60–70°C, producing two double-stranded fragments. The cycle can then be repeated [22].

The amplification begins by denaturing of DNA at 95°C, yielding single strands of DNA. The temperature is next decreased, permitting the attachment of the matching primers to the single strands and the enzyme action. At the end of this cycle, two double strands of DNA are formed. The cycle can then be repeated 20 or 30 times to provide a very large amplification factor. The success of the reaction depends on the correct composition of components, precise temperature control, particularly during the denaturing and annealing phases, the nature of the reactor walls, and the presence of contamination, which can inhibit the enzymatic action.

After the completion of  $n$  cycles, the concentration of template increases by a factor

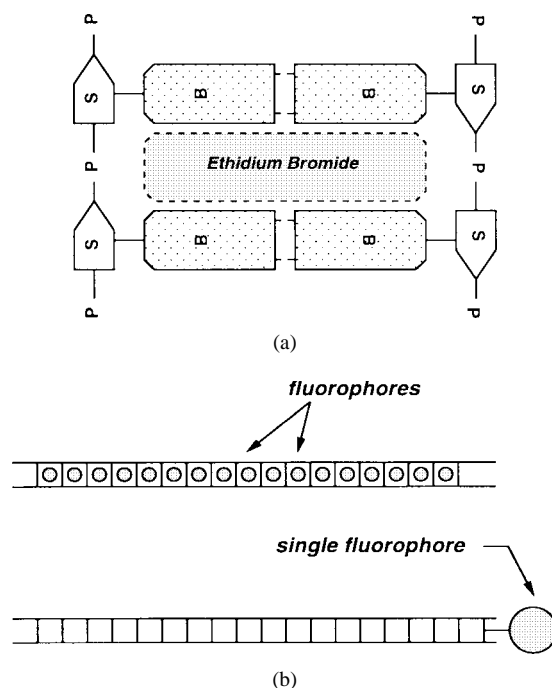
$$F = (1 + E(n))^n \quad (1)$$

where  $E$  is an efficiency factor, a function of  $n$ . For  $n < 20$ ,  $E \approx 1$ , but for  $n > 20$ , the efficiency drops. PCR techniques are often used for qualitative assays since the duplications yield exponentially growing uncertainty. Some quantitative analysis is nevertheless possible when adequate controls are added to the sample [24], [28]–[30].

The polymerase reaction is not the only amplifying mechanism. Other types of reactions [24], [31], [32], such as the strand displacement mechanism (SDA) [33], [34], can provide large amplification factors with the added benefit of isothermal operation. Conventional PCR amplifications are made in macroscopic thermal cyclers accommodating multiple reaction vials. Macroscopic cyclers require roughly 90 min to complete the amplification; therefore, faster thermal cyclers are desirable.

### B. Fluorescence and Staining Visualization

The presence of DNA fragments is commonly detected by introducing in the mixture a suitable label molecule that binds to the fragment. Early labeling methods used



**Fig. 3.** Different type of DNA fluorescent dyes. (a) Intercalating dyes fit between two base pairs. (b) Single fluorophores can also bind to the end of the fragment.

radioactive  $^{32}\text{P}$  incorporated in the fragment nucleotides. Most modern labeling methods use fluorophore dyes that emit light when bound to DNA under external excitation [8], [35]. Light-emitting labels are extremely sensitive, permitting the detection of individual molecules [36]–[38] in femtoliter samples. Hence, these dyes are almost universally used for the visualization of DNA fragments. Both the type of bonding and excitation mechanism for these dyes varies.

Intercalating dyes such as ethidium bromide (EtBr) fluoresce when excited by ultraviolet (UV) light only when bound between two nucleotides in double-stranded DNA, as shown in Fig. 3(a). Since a fragment can accommodate one intercalating label per base pair, a single DNA molecule can contain hundreds of fluorophores and emit a strong signal. Intercalating dyes affect somewhat the migration of fragments during separations; therefore, for best accuracy, sometimes a single fluorophore is attached at the end of the molecule.

Light emission can be excited in several ways. Conventional fluorescent labels require the excitation from UV light; hence, the emission signal must be separated from the excitation using filters and dichroic mirrors. Light emission can also be excited chemically [39], [40] and by electrochemical reactions [41], [42]. Electrochemiluminescence (ECL) tagging methods use a large bright  $\text{Ru}(\text{bpy})_3^{2+}$  end label that emits light in the presence of an electrochemical reaction. This technique is becoming increasingly important as it is regarded as the most sensitive [43], [44] tagging method.

DNA fragments with light-emitting tags can be observed using epi-fluorescence microscopes [45] with in-

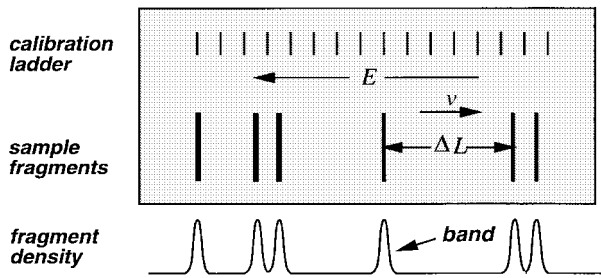


Fig. 4. DNA fragments are separated into bands due to their different drift velocities. The calibration ladder serves as a reference.

tensified photomultiplier tube or cooled charge-coupled device (CCD) cameras [46]–[48]. Chemiluminescent and electro-chemiluminescent tags can be easily observed using conventional and cooled CCD's [46], [49], [50].

### C. Restriction Digestion

The analysis of replicated DNA often involves fragmentation (or digestion) of the molecule. Restriction endonuclease enzymes split double-stranded DNA at specific locations. Restriction enzymes are essential for analyzing chromosome structure and sequencing very long DNA molecules.

Most enzymes recognize short DNA sequences of four to eight base pairs, cleaving the fragment at this site at a point of symmetry. More than 90 enzymes have been characterized and are available for these operations [51], [52]. Restriction enzymes are used to cleave DNA molecules into specific fragments that are more readily analyzed. The size distribution of such fragments can serve as a fingerprint for a DNA molecule.

### D. Electrophoretic Separations

Electrophoresis is a technique used for separating DNA fragments of different sizes from a mixture [53], [54]. DNA fragments in solution are negatively charged; therefore, they drift under the presence of an applied field  $E$  with velocity

$$v = \mu_i(N_i)E \quad (2)$$

where  $\mu$  is the fragment mobility. The mobility depends on the type of mobile phase and the fragment size  $N_i$ . Therefore, if the mixture is introduced as a single band at a starting point in a mobile phase, the fragments are separated into bands composed of different sizes of DNA as they drift in a “race track” fashion as shown in Fig. 4. The fragment separation is  $\Delta L = \Delta\mu E t$ , where  $t$  is the drift time. The fragment position along the track and the band pattern are indicative of the fragment size and the number of fragments present in the mixture.

DNA fragments hold uniform charge to mass ratio, making  $\mu$  independent of size in liquid phases; therefore, an auxiliary molecular sieve is needed for the separations. The sieving medium is typically an entangled polymer matrix in the form of a gel. The mobility of DNA fragments in gels is roughly inversely proportional to the logarithm of the fragment size [54]–[60], and the type of sieving matrix

depends on the fragment size range. Both linear and cross-linked polymers are used. Agarose, for example, is used for large fragments ( $>3$  kb), while a denser matrix like polyacrylamide is used for shorter fragments. Both double- and single-stranded DNA fragments can be separated with gels. The resolution of single-stranded fragments is higher than that of double strands. Denaturing and heated gels (also known as sequencing gels) are hence often used for optimum results.

The resolution of the separation is measured by the number of theoretical plates  $\mathcal{N}$ , which is equal to the square of the number of distinguishable bands that fit within the length of the gel. When the bandwidth is determined by diffusional spreading with diffusion coefficient  $D$ , then [54]

$$\mathcal{N} = \frac{\mu V}{2D}. \quad (3)$$

Therefore, high-resolution separations require a high voltage  $V$ . This relationship is independent of the gel length but only holds for uniform fields. Typical macroscopic gels use voltages as high as 2 kV over distances of 20–100 cm. The maximum field is limited by both heating effects and the development of fragment comigration. Conventional macroscopic gels are cast in the form of thin multilane slabs. Due to Joule heating, electric fields in slabs are limited to 5–40 V/cm; hence, good separations require many hours. Further, the slab gel preparation is labor intensive. Scaling of the slab is therefore greatly advantageous.

DNA fragments can also be separated in capillaries 10–300  $\mu\text{m}$  in diameter [61]. This technique greatly benefits from the increased surface-to-volume ratio of the gel that permits the use of much higher fields and faster separations. The resolution of bands is further enhanced through the use of highly focused laser-induced fluorescence [62]. Typically, these capillaries are 50 cm long, yielding high-resolution separations in under one hour [61]. State-of-the-art capillary electrophoresis (CE) systems use fields of up to 1200 V/cm achieving separation of 250 bands in 10 min [56], [63]. In CE systems, the capillary walls affect the sieving matrix polymerization and the separation quality; therefore, for best results, noncross-linked gels are often used [55], [58], [60], [63]–[66].

### E. The Sanger Sequencing Scheme

Replication techniques are combined with electrophoretic separations to devise a mechanism for reading the base-pair sequence of a DNA fragment [22], [67]. Like PCR, the duplication of the single-stranded DNA starts at the primer location, but it is randomly terminated at locations where a specific base is present. The random termination is caused by the introduction of a small concentration of dideoxy nucleotides (ddNTP's) of one type (ddA, ddC, ddG, or ddT) in the initial mixture. These special nucleotides terminate the polymerase duplication when captured. The reaction thus generates complementary strand fragments terminated at all possible positions of the matching dideoxide.

In the Sanger scheme [67], four of these reactions are carried out with each termination base, as shown in Fig. 5.

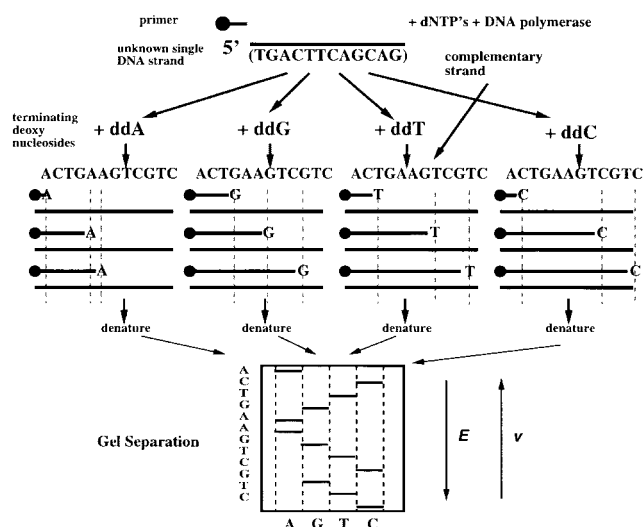


Fig. 5. Sanger method for sequencing.

When these fragments are separated in a gel in four separate lanes, the relative positions of the fragments indicate the location in the sequence where a specific base is present; hence the sequence is read out directly. In this method, the sequence accuracy and the maximum readable fragment length depend on the gel uniformity and resolution. State-of-the-art Sanger sequencing systems can read fragments about 1000 bases long using denaturing gels.

#### F. Hybridization Techniques

Hybridization is the term used for the hydrogen bonding of two complementary single strands of DNA, thus forming a duplex. This renaturation process occurs at specific temperature and salinity conditions. In hybridization-based analyses [8], [68], [69], one of the strands is known (a DNA probe) and the other unknown. The hybridization bond is specific since it occurs only when there is a match of complementary strands. The presence of a double strand in the mixture (detected by fluorescence) is indicative of a match; hence hybridization serves as a sequence detection mechanism.

There are many different kinds of hybridization, but the most relevant to this paper uses DNA probes that are immobilized. These probes are attached to a rigid surface using a linker molecule [70], as shown in Fig. 6. The DNA probes can be either synthetic oligonucleotides or longer DNA fragments typically arranged in array form [71]–[73].

The application of these type of devices is wide and will probably extend further due to the massive parallelism present in large arrays. Several groups have developed theoretical methods for sequencing DNA on these devices [74], [75], but only sequencing of very short fragments has been achieved successfully [76]. Nevertheless, the technique seems to hold some promise for longer fragments [77].

There are several difficulties associated with array hybridization techniques. Hybridization recognition is never perfect, especially for short duplexes, and single base

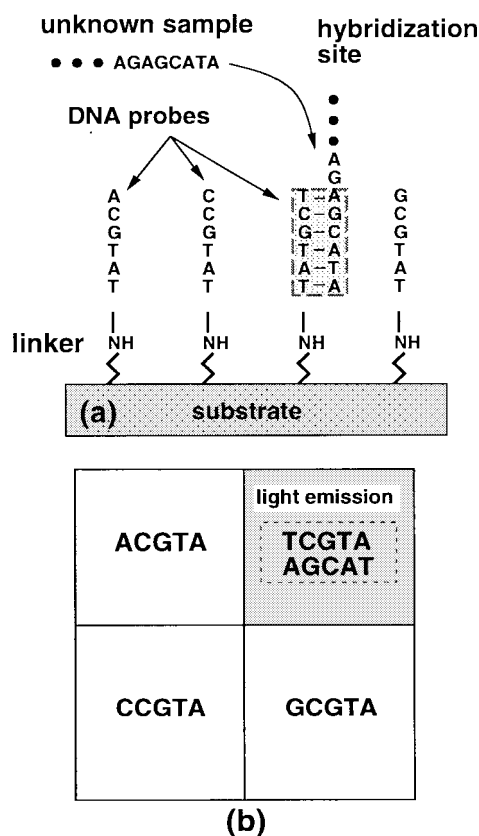


Fig. 6. (a) Example immobilized oligonucleotide probes showing hybridization of unknown to specific probe. (b) Probes are arranged as planar arrays. The hybridized regions can be detected by the fluorescence of the duplex.

mismatches can occur. Oligomer probes that are self-complementary can fold on themselves or bind with neighbors. Also, short duplexes are stable under nonstringent conditions that favor the internal pairing of the sample [78]. An obvious difficulty lies on the interpretation of the data. Most arrays have probes of short length (12–20 mer), but a typical fragment size distribution for a digested unknown does not necessarily match that for the probes. Therefore, smaller fragments can hybridize to many probes, and the fluorescence pattern for different fragment sequences is not unique.

Despite these pitfalls, the number of possible patterns can be very large; therefore, excellent pattern matching can be achieved for fingerprinting applications. To illustrate this point, let a single strand of DNA be divided into  $m$  equal fragments each  $p$  bases long. Say for  $p = 8$  and  $m = 20$  under ideal hybridization conditions, in a complete,  $4^p = 65,536$  oligomer array, the number of distinct sequences that yield the same fluorescence pattern is

$$N_s = m! \approx 2.4 \times 10^{18} \quad (4)$$

and hardly unique (as required in sequencing). On the other hand, the recognition error rate

$$\epsilon = \frac{m!}{4^{p \cdot m}} \approx 10^{-78} \quad (5)$$

is extremely low. For longer or undigested targets, the hybridization bond can occur at any location where there is a match with the probes and the nonuniqueness is very much reduced [75] due to overlaps.

Hybridization arrays have been constructed on polypropylene [73], glass [72], [79], [80], and silicon [81]. Reading of these arrays is typically done using confocal epi-fluorescence microscopes [82], [83] with cooled and intensified CCD's. Detection of hybrids is especially difficult as the size of the array pixels continues to shrink. Conventional CCD's have been used with radioactively labeled samples [84]. Recently, gold particles chemically bonded to the duplex sites have been used as a means of hybridization detection [85].

A typical DNA assay may involve a combination of the above techniques. For example, the process may include the extraction of nucleic material from a cell followed by amplification, staining, restriction digestion, and electrophoretic separation or hybridization. The implementation of these protocols in the microscale hence requires microdevices capable of handling liquid samples, mixing, thermal cycling, and detection.

#### IV. BENEFITS OF MINIATURIZATION AND SCALING

In macroscopic protocols, relatively large microliter volumes of DNA are handled in vials and manually loaded into desktop thermal cyclers and gels separators. These macroscopic systems are slow and expensive. For example, a typical sequencing protocol requires 6–8 h to complete at a cost of several hundred dollars. Further, the high cost of these systems restricts these tests to laboratories.

Alternatively, smaller sample volumes can be analyzed with miniaturized equipment. There is ample justification for the miniaturization of these systems in both clinical and research settings [72], [86], [87]. Scaling down the assays results in an increase in throughput due to reduced analysis times, reagent cost reductions due to minuscule reaction volumes, and much lower system costs due to introduction of batch fabrication techniques. Fig. 7 shows the effects of scaling on the assay parameters for a cubic sample of linear dimension  $L/S$  with  $S$  as a scaling parameter. The volume of sample and cost of reagents scale by  $S^{-3}$ . The same scaling factor affects the thermal cycle time, while separation time scales by  $S^{-1}$  (a much celebrated argument [88]). Ultimately, the minimum assay time is limited by the speed of the enzyme itself (30–100 bp/s [11]). Microsystem cost scales by  $S^{-2}$ , but it is limited by the package cost.

The benefits of scaling come at the expense of stretching detection limits. For a fixed concentration, the number of molecules in the sample scales by  $S^{-3}$ . If the detector area is fixed, the signal-to-noise ratio (S/N) is severely degraded by  $S^{-3}$ . The S/N reduction is not as severe if the detector area scales with the sample ( $S/N \propto S^{-1}$ ). This favors the use of miniaturized detectors placed close to the sample.

Scaling also increases the surface-to-volume ratio (S/V) of the sample, accentuating the influence of surface phe-

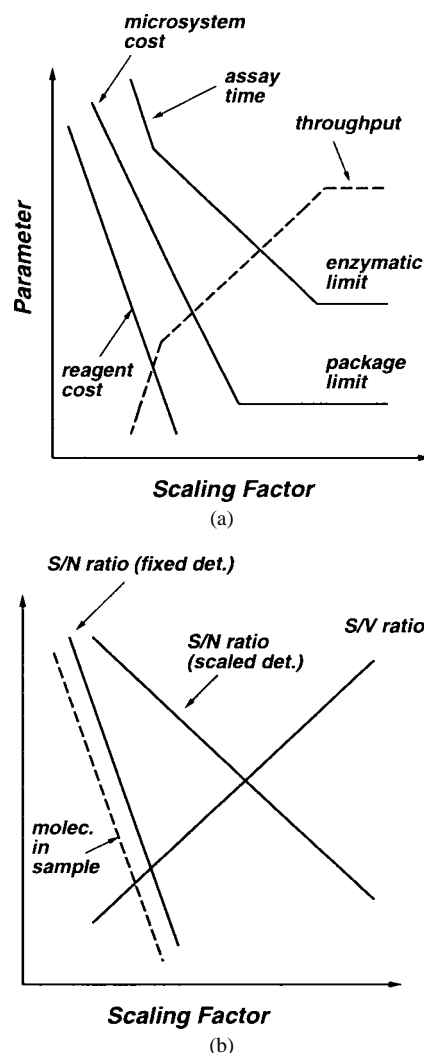


Fig. 7. Scaling of assay parameters. (a) Costs and time are reduced. (b) The detector signal-to-noise ratio is degraded by scaling.

nomena such as enzyme-wall adsorption and sample evaporation that may affect the microsystem performance. Further, scaling causes hydrodynamic problems. The resistance of capillaries scales by  $S$ ; hence, sample transfer requires high pressures. Capillary forces are also scaled by  $S$ , making sample localization very difficult and the control of surface properties essential.

It seems reasonable to assume that the scaling factor is determined by detection noise. At a scale of  $10 \mu\text{m}$ , the number of DNA molecules (100–1000) may reach the practical detection limits. It is conceivable that scaling factors of 100 or larger are possible when handling picoliter samples. This results in 100-fold increases in throughput and similar decreases in cost.

In recent years, several devices have been constructed that demonstrate the benefits of scaling. Some of these devices are rudimentary in construction, but they provide very convincing evidence that further miniaturization and systems integration should be pursued for this application.

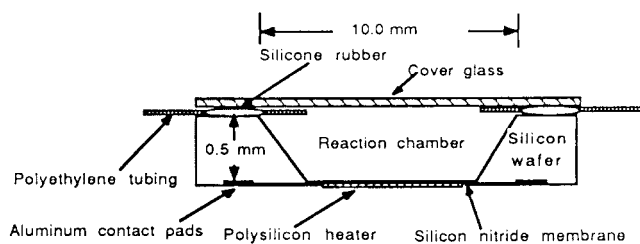


Fig. 8. Silicon microwell for PCR reaction [89].

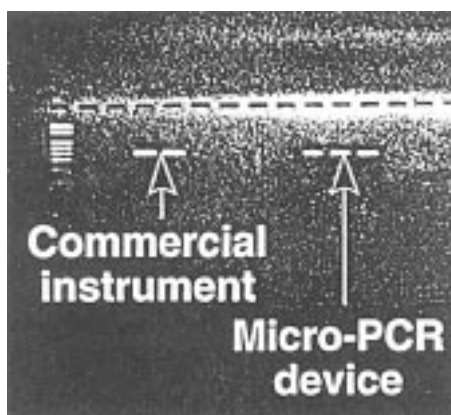


Fig. 9. Comparison of DNA fragments from conventional and microwell PCR [89].

## V. MICROFABRICATED DEVICES

Over the past decade, the miniaturization of DNA assays has been investigated by several groups using a broad range of fabrication technologies and materials. While the construction for many of these devices is often rudimentary, these simple devices serve well as microscale protocol demonstrators

### A. Microfabricated Amplification Devices

A number of micromachined devices [89], [90] have been developed to accomplish faster amplification cycles by basically reducing or eliminating the large thermal mass present in macroscopic systems.

The first of these devices was developed by Northrup [89], [91] at Lawrence Livermore National Laboratories (Figs. 8 and 9). This device consists of a microwell cavity structure formed in a silicon substrate by anisotropic etching. The well bottom is a thin silicon nitride membrane with polysilicon heaters on the underside. This type of structure is essentially the same used for many bulk micromachined pressure sensors; hence, it can be fabricated cheaply. The well lead is a glass slide bonded to the top. Due to its small thermal mass, this structure can be heated at rates of  $15^{\circ}\text{C/s}$  and cycle times of about 1 min. A 20-cycle amplification in a  $50\text{-}\mu\text{L}$  microwell was carried out roughly four times faster than in a conventional cyler with a much lower power budget.

The microwell approach is suitable for array detection of multiple pathogens. Arrays of 8 and 24 wells with and

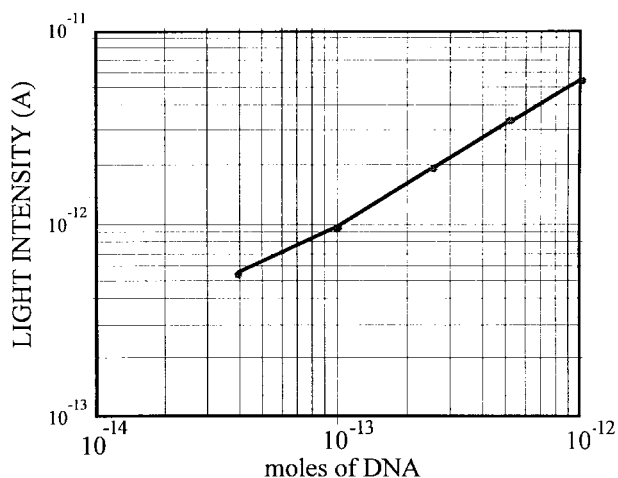


Fig. 10. Recorded light intensity versus moles of single-stranded DNA in micromachined ECL cell [95].

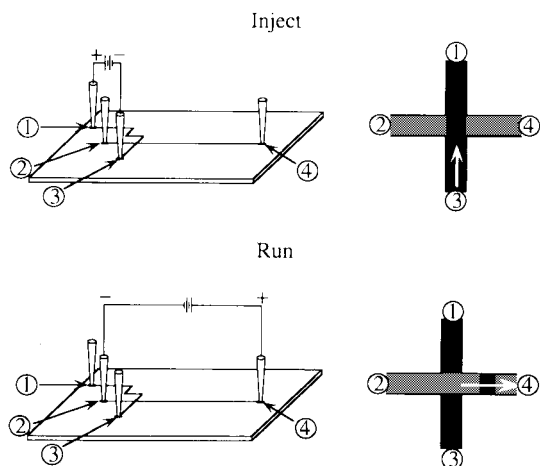
without heaters have been fabricated on silicon anodically bonded to glass [92], [93]. Reagents are loaded into each well using inkjet-type methods to provide capability of *in situ* fluorescence. One of the major problems with the silicon-based array devices is their cost. Simpler arrays of polypropylene microwells have been fabricated using conventional moulding and stamping methods with well depths of  $250\ \mu\text{m}$ . These passive devices require an external cooled CCD camera readout; therefore, they must be used with an expensive reader.

Recently, diode detectors have been used in silicon microwell structures to provide quantification of PCR products by electrochemiluminescence [94], [95] (Fig. 10). These devices show a detection limit of 40 fmoles of DNA, which is substantially less than that achievable in commercial macroscopic devices.

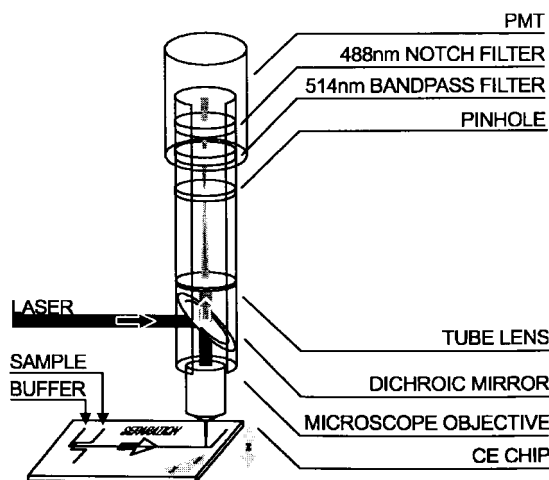
### B. Electrophoresis Microdevices

In CE systems, band resolution is not only determined by the separation voltage but also the length of the sample injection plug. High-resolution separations with millimeter-sized plugs require long capillaries ( $\approx 1\ \text{m}$ ) and separation times. Faster separation times could be achieved with shorter, microfabricated channels if used along with microinjection and more sensitive band detection schemes.

The feasibility, properties, and performance of electrophoresis devices microfabricated on planar glass substrates have been studied by many analytical chemists [86]–[88], [96]–[109]. These devices consist of two crossing perpendicular channels. The first channel defines the sample injection plug and the second separates the sample. These channels are made by wet etching two  $10\text{-}\mu\text{m}$ -deep crossing grooves on Pyrex glass wafers [88]. Platinum electrodes are next deposited and patterned, and channels are next sealed with a top glass wafer with access holes bonded to the substrate, thus forming two crossing capillaries. Due to the presence of the metal steps, hermetic bonding can be accomplished using cement or by thermally fusing the



**Fig. 11.** Simple cross-channel capillaries etched into glass are capable of fast, high-resolution DNA separations. (Reprinted with permission from [65], ©1995 American Chemical Society.)

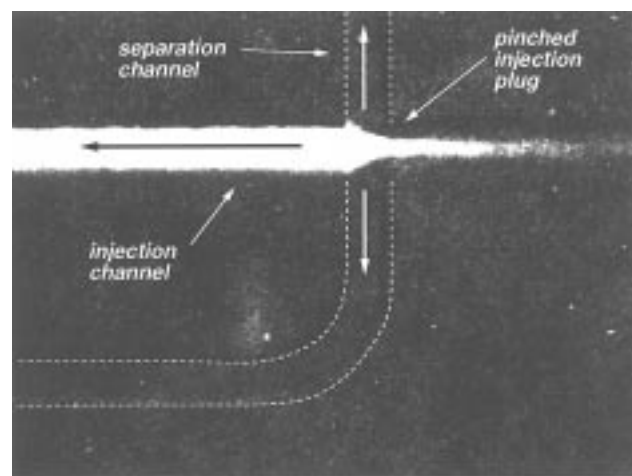


**Fig. 12.** Confocal epi-fluorescence readout for CE chips [112].

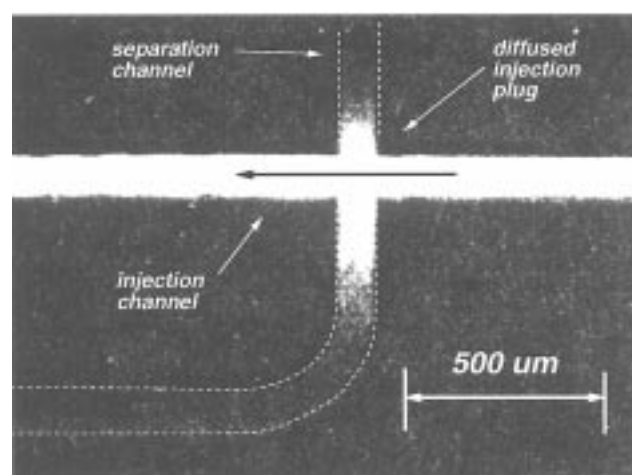
glass pieces at 660°C. Other methods of fabrication for these devices have been recently developed [110], [111].

Fig. 11 shows a typical etch pattern configuration. The most important part of the device is the channel intersection, which determines the size of the injection plug. After introduction of the mobile phase, the sample is placed in the lower reservoir. A low voltage is next applied across the vertical capillary, forming a long plug that fills the capillary with sample (with no separation). Next, the vertical capillary voltage is turned off, and a high voltage is applied across the horizontal capillary, moving the plug of sample at the intersection forward and resulting in a high-resolution separation. Typically, the migrating bands are recorded using a confocal fluorescence microscope focused at a specific spot [97], [112], as shown in Fig. 12.

In the above scheme, the injection plug length is enlarged due to lateral diffusion and convection eddies at the intersection [104], [113]. The cross-channel voltage configuration can be changed to form a sharper plug. Fig. 13 shows the injection plugs under two different schemes.



(a)



(b)

**Fig. 13.** Comparison of injection plugs under two different bias configurations. In (b), the vertical channel is unbiased; hence, the plug is free to diffuse. In (a), counter currents are injected in the separation channel, resulting in a sharper plug of constant volume. The arrows indicate the direction of the currents (opposite to the DNA motion). (Adapted from [104] with permission, ©1994 American Chemical Society.)

In the top photograph, counter currents are injected into the separation channel to prevent the plug diffusion. The resulting plug volume is constant, yielding higher resolution separations.

The performance of planar crossing channels for separations of DNA was demonstrated by Woolley [65]. Fig. 14 shows separations of DNA fragments on a short,  $8 \times 50 \mu\text{m}^2$ , 3.5-cm-long capillaries. About 433 fragments were distinguishable in about 10 min separation time using fields of 200 V/cm. The chip separation is about 3–5 times faster than conventional CE and 50 times faster than slab gels. Direct sequencing has also been carried out in these structures using multicolor dyes.

The separation quality also depends on the type of separating medium. For DNA fragments, the polymerization uniformity of conventional slab gel matrix materials in capillaries such as polyacrylamide is difficult to control.

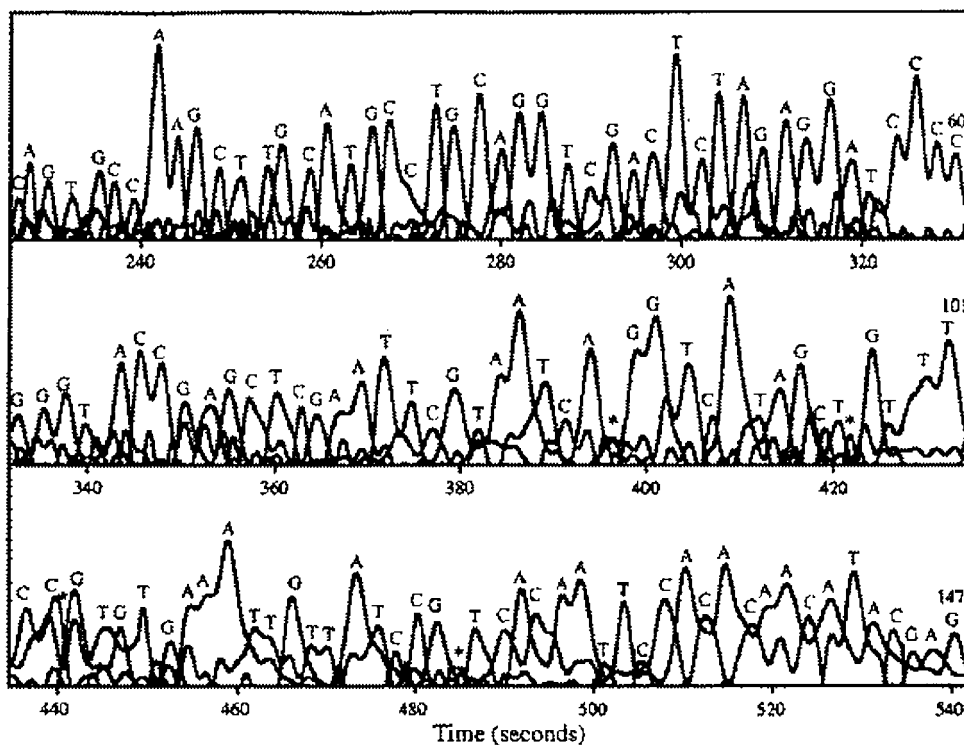


Fig. 14. Separation of DNA fragments in planar glass chips using multicolor dyes and confocal microscopy detection. (Reprinted with permission from [65], ©1995 American Chemical Society.)

For this reason, unpolymerized liquids such as linear acrylamide and hydroxyethylcellulose (HEC) are often used [61]. Unlike cross-linked polymers, these sieve materials can be pumped out of the channel, allowing the reuse of the chip. In all of these devices, the presence of DNA bands was observed using fluorescence microscopy techniques. Therefore, these devices must be used in a laboratory setting.

Recently, separation channels have been fabricated on silicon substrates with on-chip detectors and filters [114]. DNA separations are possible on silicon substrates because high voltage drops, which may break down the isolation dielectrics [115], are generally not required and when needed can be properly handled using very thick dielectrics. The presence of individual bands has been observed with fairly high S/N's (of about 100), as shown in Fig. 15. The fabrication of these devices is discussed in Section VI. On-chip detectors eliminate the need of expensive readout optics and open the road to low-cost disposable devices.

### C. Micromachined Hybridization Arrays

Hybridization array devices can be micromachined using lithographic techniques [80], [116], [117]. Fodor's technique [116] permits the photo-induced solid synthesis of oligonucleotides, as shown in Fig. 16. First, a glass substrate is coated with a linker molecule that has a photochemical removable protecting group [118], [119]. Upon the illumination of UV light, the protecting group is removed at selected areas. Next, the first nucleoside

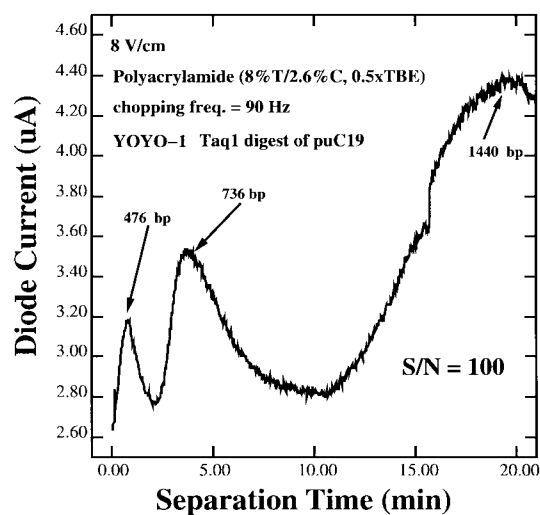
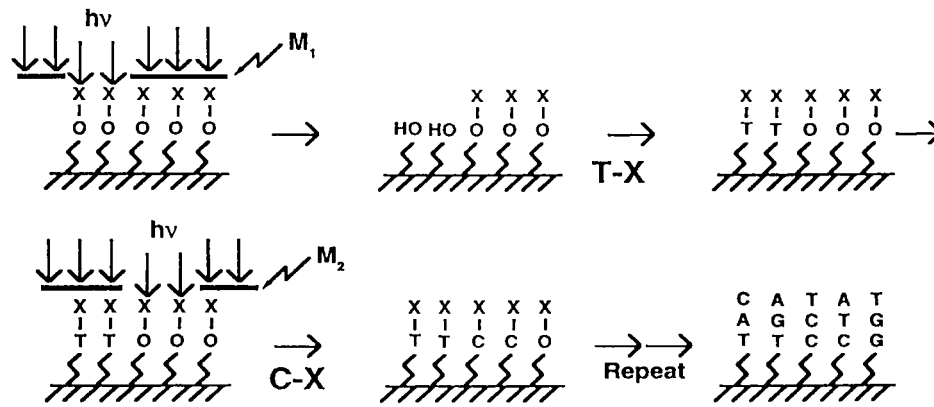


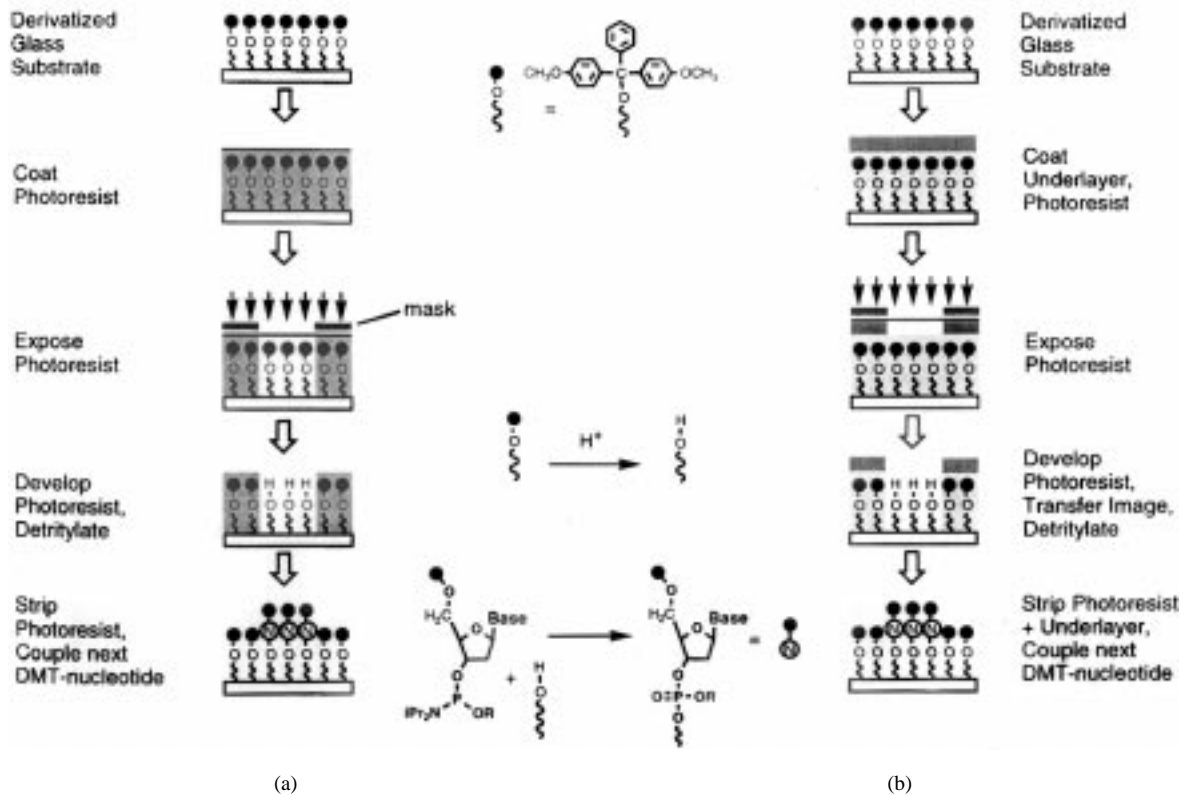
Fig. 15. Low-resolution separation of DNA fragments recorded with on-chip detector diodes, thus eliminating the need for expensive readout optics [114].

with a photo-labile attached to its 5' end is placed on the substrate, bonding only to the deprotected linkers. The cycle is repeated to build oligonucleotides of arbitrary length one base at a time.

A similar technique that uses protecting photoresist has been reported to produce smaller, 8  $\mu\text{m}$  pixels [80]. The two-layer process shown in Fig. 17(b) is necessary to protect the oligonucleotide chemistry from the basic ( $\text{OH}^- > 0.1 \text{ M}$ ) resist developer. The polyimide (XU-218, CIBA-



**Fig. 16.** Assembly of oligonucleotide probes using photolithographic solid synthesis. Four lithographies are required for a base; therefore, oligonucleotides with 16 bases require 64 lithographies. (Reprinted from [118] with permission, ©1996 National Academy of Sciences, USA.)



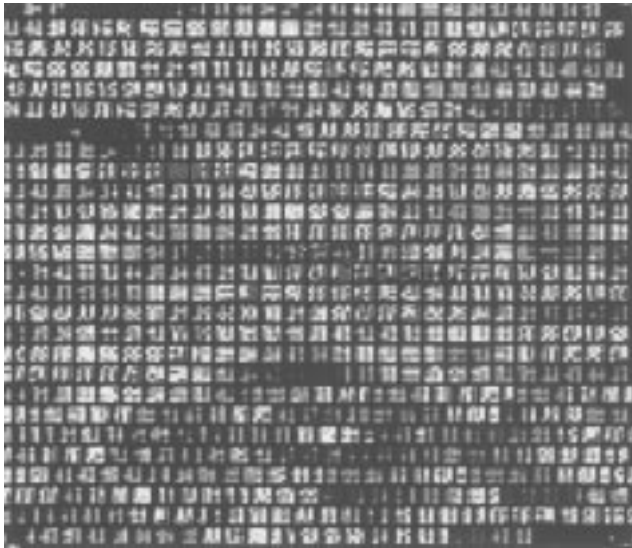
**Fig. 17.** Oligonucleotide array fabrication using photoresist as a mask. In (a), the resist blocks the deprotected regions. In (b), a two-layer polyimide/resist is used to protect the oligonucleotides from the resist developer. The polyimide layer can be etched using compounds that do not hydrolize the oligonucleotide protection groups. (Reprinted from [80] with permission, ©1996 National Academy of Sciences, USA.)

Geigy) can be patterned after the resist development with a solvent.

The array probe patches range from 50–200  $\mu\text{m}$  each; therefore, 1  $\text{cm}^2$  can contain 10–40 000 different probes. The number of possible oligonucleotides is much larger—yielding, for example, for a 15-mer probe  $4^{15} \approx 10^9$  combinations. Dense arrays with 20 000–96 600 probe patches have been reported [120]. Other lithographic methods are reported in [117].

Finished arrays are mounted in flow-cell plastic packages. Fig. 18 shows the example fluorescent array signature of a test for the HIV virus. These type of devices are now widely used in genetic research [83]. Currently, the fluorescent signal for these devices is read with a confocal fluorescence microscope, as shown in Fig. 19.

One of the important factors in these devices is the detection time. The hybridization of the sample to the probes is a diffusion-controlled phenomena, and hence

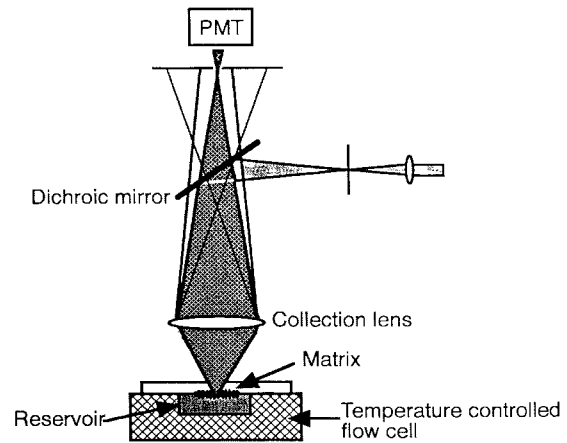


**Fig. 18.** Fluorescent signature of hybridization array for HIV virus assay. (Reprinted from [72] with permission, ©1995 BioTechniques.)

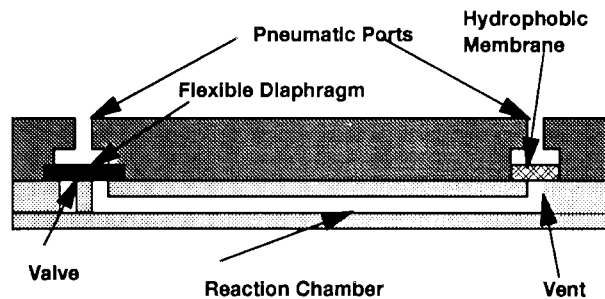
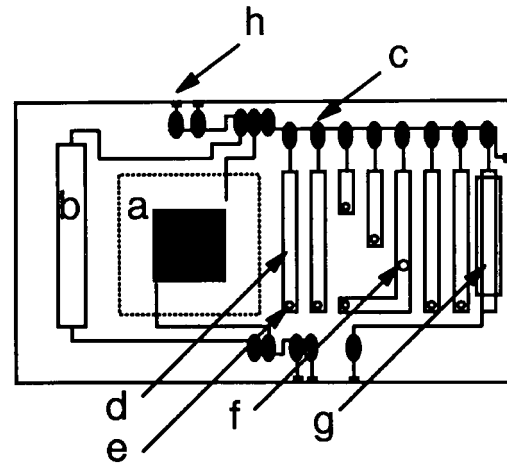
slow. After the sample is poured on the array, it is typically necessary to wait 90 min for the hybridization to complete. Recently, the hybridization speed was enhanced by the use of electric fields that direct the sample to the probes. Sosnowski [81] fabricated a platinum electrode array device on a silicon substrate passivated with silicon nitride and a permeable agarose gel. Through the application of an electric field between the electrode and the sample, the hybridization time was reduced 25-fold. The reduced time is attributed to the enhanced transport of charged molecules to selected microlocations and the resulting localized increase in the sample concentration.

A seemingly useful property of field-directed oligonucleotide arrays is the capability for selective denaturing. This is important, as hybridization can still occur even in the presence of single base-pair mismatches (SBPM's), which makes the interpretation of the data difficult. By reversing the direction of the field and scrutinizing the field magnitude, it is possible to remove the incorrectly hybridized strand over perfect matching ones. Sosnowski [81] achieved electric-field-induced SBPM denaturing in a few seconds at a rate four times faster than denaturing of exact matches. In these devices, a multiplexed electrode plane is hence required; therefore, high-density, electrically addressable arrays will require a multiplexing circuit plane. A 128 microelectrode complementary metal-oxide-semiconductor (CMOS) chip has been reported by Calliat [121]. In this device, the actual probe immobilization was directed to specific electrodes using an oligo-pyrrole scheme [122], [123].

Further advances in this area will require improvements in inexpensive fluorescence detection. Some attempts have been made for simpler, less expensive detection techniques. Recently, hybridization arrays with radioactive labels have been imaged by placing the array in close proximity to a conventional CCD chip, eliminating the expensive readout optics [84]. A more practical implementation of these



**Fig. 19.** Confocal fluorescence microscopy readout for hybridization arrays. (Reprinted from [72] with permission, ©1995 BioTechniques.)

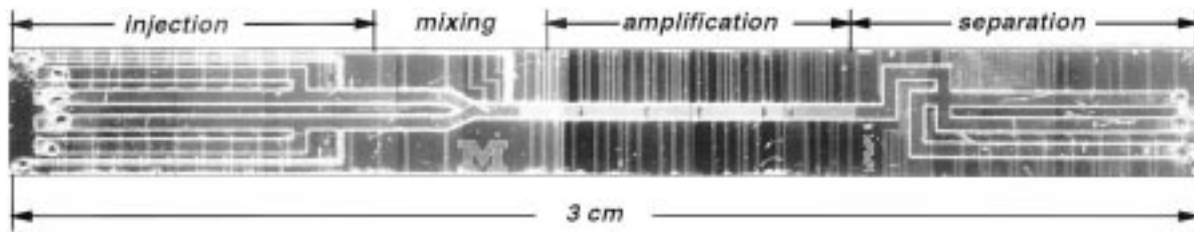


**Fig. 20.** The Affymetrix fluid processor is constructed using micromachining of flexible plastics. The system is driven pneumatically [133]. The substrate holds a) an oligonucleotide array, b) an auxiliary hybridization chamber, c) diaphragm valves, d) reagent chambers, e) hydrophobic vents, f) a linking chamber, g) an extraction outlet, and h) fluidic ports. The processor measures 40×70 mm (reprinted with permission from [133]).

ideas is likely to be based on electrochemiluminescent labeling, which lacks the adverse effects of radioactive sample handling.

#### D. Fluid Processors

The devices above generally require sample preparation protocols involving dosing of reagents, mixing of reagents



**Fig. 21.** Example integrated glass device with injectors, mixers, amplification chamber, separation, and detection [136].

with the sample, and injection of the mixture into the device. These functions are performed by a fluid processor.

There is a substantial amount of literature on the miniaturization of these systems [124]. Most of these are made by complicated bonding techniques and many planes of functionality [125]–[132] for valves, gaskets, and pumping devices; therefore, simpler and less expensive alternatives are desirable. Valves are a major source of difficulties in these systems. Micromachined mechanical valves are difficult to implement and require complex fabrication processes and large operating power.

To date, there have been only two implementations successfully applied to genetic assay systems. The first implementation uses a valveless approach. In this system, sample and reagents are transported in a liquid medium using electroosmotic flow controlled by external voltages. Therefore, the sample flow is controlled in much the same way as current in an electrical circuit. This technique was applied on glass chips for mixing, separation, and labeling applications. This scheme, however, is not without pitfalls, since PCR DNA amplification cannot be accomplished.

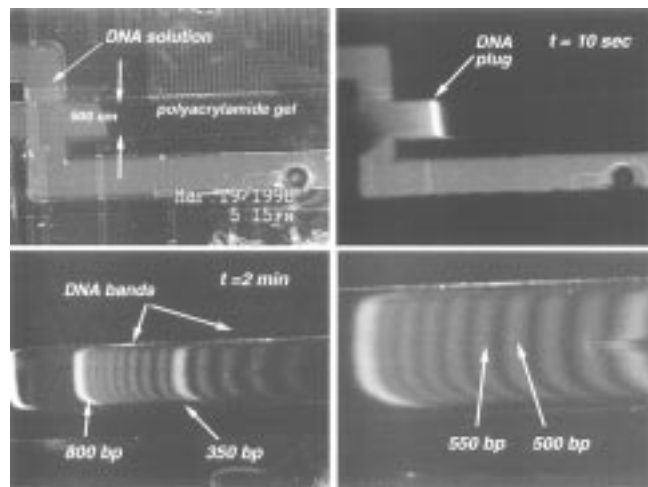
In a second approach, Anderson [133] (Fig. 20) uses pneumatic actuation for moving the sample and driving a set of passive valves. The device was micromachined on a polycarbonate plastic substrate. Plastic materials are beneficial for these devices because the diaphragm valves do not require special gasket materials.

The plastic processor consists of a set of chambers of different volumes interconnected by fluidic busses. Sensorless positioning and venting of excess gas is achieved using porous hydrophobic membranes.

## VI. INTEGRATED TECHNOLOGIES

The devices discussed above are good demonstration vehicles for feasibility of molecular assays in microscale environments, but their practical implementation for low-cost applications presents more problems.

Many of these devices are crude in construction and not manufacturable in their present forms. Further, DNA assays require a combination of these devices, but there is no unifying platform that supports them [134]. In addition, most of these devices require external optical readers that restrict their use to laboratory environments, increasing the assay cost substantially. A solution to these problems is an inexpensive integrated fabrication technology that ac-

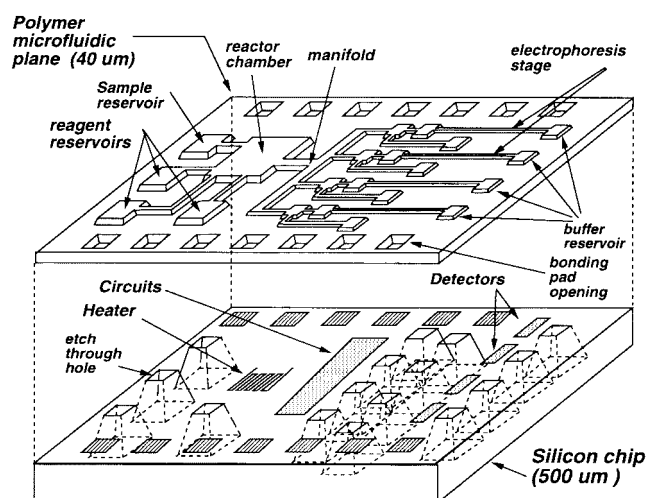


**Fig. 22.** Injection and separation of DNA fragments on integrated device. The channel is  $500 \times 50 \mu\text{m}^2$  (50 bp ladder,  $0.13 \mu\text{g}/\mu\text{L}$ , SYBR Green, 8 V/cm, 10%T:2.6%C polyacrylamide) [136].

commodates all the necessary fluid, thermal, and detection functions under a common substrate [135].

Simple integrated devices have been fabricated by Anderson [133], Woolley [134], and Burns [136]. Woolley's device combined a vertical PCR microwell with a planar glass chip CE stage to perform DNA amplification and separation functions in less than 45 min. Anderson's device incorporated an entire assay in a micromachined cartridge. In this device, DNA extraction, PCR, *in vitro* transcription, fragmentation, hybridization, and washing were all carried out under external computer control.

Burns' device incorporated simple injectors, mixer, heating chamber, and separation channel with detectors (Figs. 21 and 22). Diode detectors were constructed in a silicon substrate with a thick optical filter deposited on the surface that blocks UV excitation. Heaters and electrodes are next defined on the surface and passivated with silicon dioxide and p-xylylene. A network of capillaries and chambers is next formed above the surface by bonding an etched glass wafer on top of the silicon substrate. In this device, sample and reagents can be driven through the system pneumatically or by heated gas trapped in pockets behind the sample [137], and the sample spread is controlled through a series of hydrophobic stops [138]. This device demonstrated the motion, amplification, separation, and detection (Fig. 15) of DNA samples integrated in a single part.

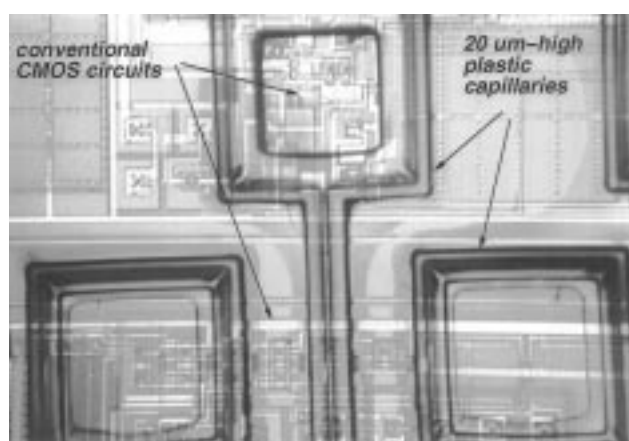


**Fig. 23.** The polymer fluid channels and reservoirs are constructed by deposition of plastics on top of a silicon substrate. Etch-through holes are formed in the silicon substrate to serve as funnel-like inlets and reservoirs for the sample, reagents, and buffer solutions [139].

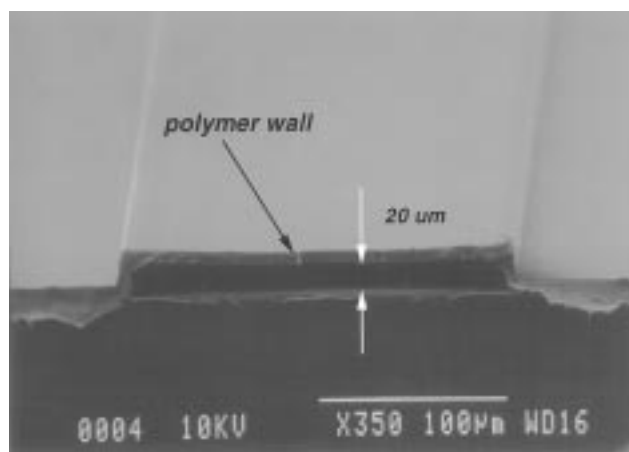
Integrated bulk glass devices are not manufactured easily because the surface of the detector substrate is not flat; hence, hermetic bonding can only be achieved using epoxies. Further, heating protocols are very inefficient because of the high thermal conductivity of silicon.

Alternatively, these devices can be fabricated using flexible plastics [139]–[146]. These materials can be bonded or deposited on most substrates relatively easily [147]. Further, many plastic materials are biocompatible, transparent, and provide a high degree of chemical and thermal isolation. Thick layers of plastic can also provide the necessary dielectric isolation between the detector and CE electrodes. Plastic-on-silicon devices have been fabricated by several groups [139]–[141], [144], [145]. These microfluidic systems are partitioned into two stacked levels of functionality, as shown in Fig. 23. These devices are fabricated using a simple three-mask, low-temperature, integrated-circuit-compatible process that allows the integration of microfluidic and circuit elements. The capillary structures are constructed using a thick layer of sacrificial photoresist covered with a vapor-deposited plastic, p-xylylene [147], or combinations of p-xylylene with thick polyimides. After etching holes through the walls, the sacrificial resist is removed by immersion in acetone. These fluidic elements are conformal to the substrate, thus overcoming the planarization required by bonded structures. The capillaries are also optically transparent and can range from 0.5 to 50  $\mu\text{m}$  in height. Fig. 24(a) shows a top photograph of plastic polyimide/p-xylylene capillaries constructed on top of a regular CMOS circuit substrate. Fig. 24(b) shows the cross section of p-xylylene channels constructed on top of a silicon substrate. The top wall is 10–20  $\mu\text{m}$  thick.

The resulting channels have fairly large volumes, are optically transparent, are conformal to the substrate, and are perfectly hermetic. One of the major virtues of the plastic fabrication process is that it only uses weak chemicals and



(a)



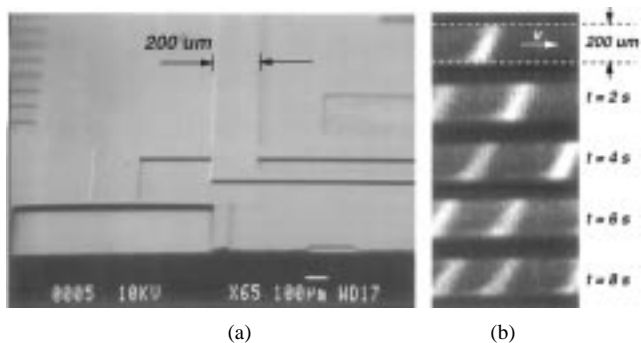
(b)

**Fig. 24.** (a) Top view of polyimide/p-xylylene channels constructed on top of a CMOS circuit. The low-temperature process does not degrade any of the circuit performance. (b) Example cross section of a p-xylylene polymer channel [139]–[141].

low temperatures ( $<150^\circ\text{C}$ ); thus, the fluidic plane can be constructed on top of any conventional circuit substrate without chemical attack or thermal degradation of the underlying circuits. Several microfluidic devices have been fabricated using this technology. Webster [140] fabricated CE channels with integrated detectors [136], as shown in Fig. 25.

In integrated devices, a droplet of DNA sample must be mixed with reagents and transported throughout the system; therefore, a suitable mechanism for the injection and propulsion of droplets is needed. In the Affymetrix processor [133], these functions are performed using a set of externally driven pneumatic valves; therefore, this system requires an external pump or compressed air source. The internal generation of sample propulsion mechanisms is difficult due to the need for valves that require moving parts. At the same time, the small dimensions of the capillaries make the pressure drops for inducing motion fairly large.

Recently, the motion of samples within these devices has been achieved, taking advantage of the large capillary



**Fig. 25.** Capillary electrophoresis device fabricated using plastics on silicon. (a) The channel cross section is  $200 \times 20 \mu\text{m}^2$ . (b) Photograph showing separation of fluorescently labeled DNA fragments in plastic CE channel at elapsed times (HEC, 0.5%, 100 V/cm) [140].

pressures present in these capillaries. Handique [138] used hydrophobic patches to stop the wetting of the solution and a thermally expanding bubble to cut and propel individual sample drops. Man [141] developed capillary stops and injectors using a variant of the plastic process. The device uses a sharp neck in the channel that creates a surface-induced pressure barrier that stops the flow. The injector was driven by the pressure of an electrolytically generated oxygen bubble formed just behind the neck region. Electrolytic bubbles hold two important virtues. The bubble volume can be precisely metered since the integrated current is representative of the moles of gas generated. In addition, the power dissipation required for the bubble generation and drop motion is three orders of magnitude smaller than for the thermal drive. The injector shown Fig. 26 only requires  $100 \mu\text{W}$  of power to drive the sample forward.

Unlike glass devices, plastic devices are more susceptible to the rapid evaporation of buffer and gels due to the larger solid-phase diffusion coefficients. Typically, a combination of plastic capillary walls with glass coatings is necessary to eliminate these adverse effects.

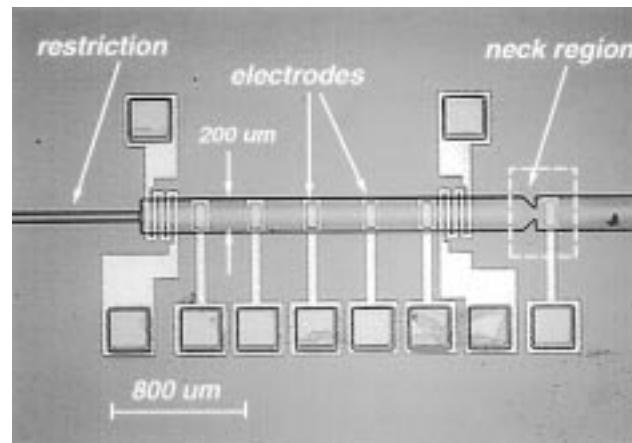
## VII. PACKAGING, INTERFACING, AND STORAGE CHALLENGES

While much progress has been achieved toward the miniaturization of genetic assay devices, there are still fundamental challenges that must be resolved before the practical realization of these systems. In this section, we discuss areas of research that are important but have been neglected and propose several practical solutions.

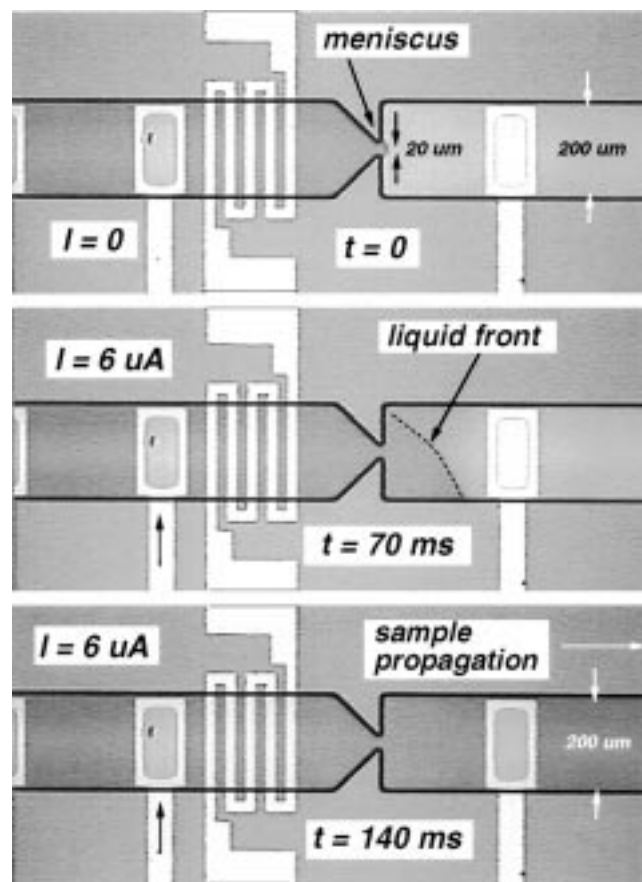
Two important research areas that have not been addressed are 1) chip packaging and interfacing and 2) reagent and chip storage. These important topics affect the operation of the device and the cost of testing apparatus and assay.

### A. Packaging and Chip Interface

These microscopic devices require both electrical and fluidic connections. The package must satisfy both of these functions. Electrical connections typically take the



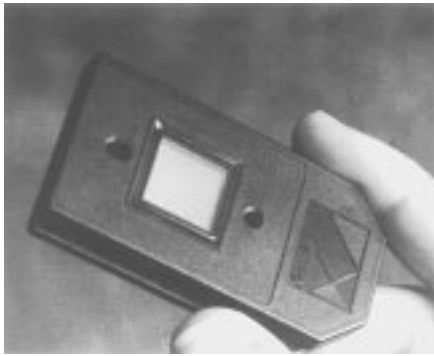
(a)



(b)

**Fig. 26.** (a) Example stop valve/injector implemented using plastics on silicon. The sharp lateral constriction forms a pressure barrier that stops the flow. (b) The injector is activated by the formation of an oxygen bubble that overcomes the barrier reestablishing flow [141].

form of wire bonds, while fluid connections require a reservoir or pocket for the introduction of the sample. From the packaging point of view, it makes sense to isolate these intrinsically different signal paths for several reasons. Electrical connections are fragile and do not need to be exposed; hence, they can be protected effectively using epoxies. On the other hand, the fluid interface must be open



**Fig. 27.** The Affymetrix package houses a flow cell containing the oligonucleotide array, a window, and rubber sealable membranes for the injection of the sample. (Reprinted from [72] with permission, ©1995 BioTechniques.)

for the introduction of liquid samples. A possible approach may use the backside of the chip for the fluid inlets while the front is used for the bonding wires.

The fluidic interface is equally important and introduces additional problems. Sample loading in these devices is in general a difficult operation. A representative sample of 1  $\mu\text{L}$  drop of DNA in solution requires a reservoir volume of 1  $\text{mm}^3$ . This volume is fairly large compared to the total chip inner volume. Even at this larger volume, the introduction of sample in these small reservoirs requires good alignment and a very small dispensing device. These problems can be eliminated if an intermediate part that connects the macroscopic to microscopic scales is constructed.

Based on the above, two alternative interfaces could be feasible. A large reservoir with a sealable membrane could be incorporated within the package. This approach could permit the injection of relatively large 5–10  $\mu\text{L}$  samples with a syringe, as shown in the example of Fig. 27. A second alternative could use direct wicking of the sample into the system. To date, there is no literature available on practical packaging and interfacing schemes for electrophoretic devices.

### B. Chip and Reagent Storage

Unlike microelectronic devices, DNA assay devices use perishable reagents and enzymes. Therefore, these devices can be operated in several modes that accommodate this constraint. In a possible scheme, the reagents are loaded after fabrication and preserved chemically or by physical means (dehydration, cooling, or freeze-drying). This scheme was reported by Albin [92] for PCR microwell arrays. Reagents, enzymes, and *in situ* fluorescence reporters were dehydrated on each well after their introduction using an inkjet-type device.

Alternatively, the reagents could be loaded just prior to the assay. Since the volume of the microfluidic part is very small, it seems that the first scheme may be difficult to achieve due to the gradual evaporation of the sample, especially when gel materials are involved. Larger reagent volumes could also be stored within a chip package, externally loaded by the testing apparatus. The latter option

is unlikely since it would require relatively large volumes of reagents and enzymes. To date, practical ways to store these reagents have not been well developed. These are important topics of research as they affect the cost of the assay and reading equipment.

## VIII. SUMMARY

The demand for genetic information is essentially unlimited, but conventional laboratory genetic assays are slow and expensive. Assay cost and time can be reduced by several orders of magnitude if the size of sample and analysis apparatus are reduced to microscale dimensions. The virtues and feasibility of micromachined devices for genetic assays have been recently demonstrated by many research groups.

The enormous number of commercial applications has fueled very rapid technological advances in these devices spanning the fields of molecular biology, chemistry, and microfabrication. This paper provides a tutorial of the basic molecular biology protocols that must be reproduced at the microscale, reviews current microfabrication technologies and devices used for genetic diagnostics, and discusses important technological and manufacturing challenges that must be addressed for the practical implementation of these devices.

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