On-Chip DNA Band Detection in Microfabricated Separation Systems

Sundaresh N. Brahmasandra\textsuperscript{a}, Brian N. Johnson\textsuperscript{a}, James R. Webster\textsuperscript{c}, David T. Burke\textsuperscript{b}, Carlos H. Mastrangelo\textsuperscript{c}, Mark A. Burns\textsuperscript{a}

\textsuperscript{a}Dept. of Chemical Engineering
\textsuperscript{b}Dept. of Human Genetics
\textsuperscript{c}Dept. of Electrical Engineering and Computer Science
The University of Michigan, 2300, Hayward Street, Ann Arbor, MI 48109

ABSTRACT

In recent years, there has been tremendous interest in developing a complete, high-volume, DNA analysis system using microfabrication techniques. Key to the success of such systems, is the development of a high-resolution separation and detection system for analyzing DNA reaction products. Over the past decade, many researchers have demonstrated that micromachined fluidic devices are capable of performing many of the required functions of such a device. However, all of these devices rely on expensive external fluorescence imaging systems that may limit the realization of a low cost, miniature DNA analysis chip. We have developed an on-chip fluorescent detection system and used it to detect individual DNA bands migrating in a microfabricated electrophoretic device. Fluorescence-based detection of DNA bands is achieved by incorporating a highly sensitive photodiode beneath the electrophoresis channel along with a thin film optical filter deposited above the diode. A miniaturized detection system, with sensitivity comparable to macroscale detection systems, could accelerate the realization of integrated ‘lab-on-a-chip’ systems.

Keywords: DNA electrophoresis, microfabrication, photodetector, fluorescence, on-chip detection, interference filter.

INTRODUCTION

Over the last two decades, few areas have witnessed changes of the magnitude observed in molecular biology in general and DNA/RNA analysis in particular. The biological and biomedical sciences, from agriculture to forensic science have enthusiastically incorporated DNA/RNA analysis into their experimental procedures.\textsuperscript{1} Extraction of the wealth of genetic information has become routine and accessible worldwide, even for laboratories with limited resources. Rather than satisfying the demand, the initial burst of information has stimulated appetites for even greater breadth and depth of scientific data\textsuperscript{2}. In 1990, the National Institutes of Health and Department of Energy initiated an ambitious, global project known as the Human Genome Project. The goals of this project are to characterize all the human genomic material by improving existing genetic maps, constructing physical maps of entire chromosomes and ultimately to sequence the entire genome\textsuperscript{3}. Also in the field of medicine, analytical demand continues to increase, as new knowledge is gained by scientists. Since clinical science is concerned with what is wrong in a particular individual, knowledge of individual variation is essential to clinic-level diagnosis. Consequently, human genetic analysis is rapidly turning out to be a study of populations\textsuperscript{4}.

A major challenge of the Human Genome Initiative and any future large-scale DNA analysis demand is the development of rapid and accurate analysis technology with minimized processing cost\textsuperscript{5}. The present state of the art for determining the

\textsuperscript{1} Contact information:-
(Mark A. Burns) Phone: (313) 764 4315 : Fax: (313) 763 0459 : Email: maburns@umich.edu
DNA sequence is defined by the 'Sanger dideoxy' method. These have been designed for experimental flexibility and not for economy of repetitive tasks. A major limitation of conventional procedures is the large number of operator interactions, long separation times, short read lengths, significant post-processing time and high cost of reagents. Thus, future analytical devices must balance cost and accuracy with the rapidly increasing need for genetic information.

One approach to developing an alternate analysis system has been to miniaturize individual components of the entire process of DNA analysis. This reduction in size will not only cut reagent costs, but also offer improved speeds of separation and more efficient reactions due to reduction in transport distances. While the use of very small diameter capillary systems for electrophoretic separations has been well established since the early 1980's, recently silicon microfabrication technology has been used to microfabricate electrophoretic systems.

**MICROFABRICATED ELECTROPHORESIS SYSTEMS**

A wide range of mechanical and electrical devices can be machined on silicon. Over the past 30 years, the fabrication technologies for this material have developed at a rapid pace, resulting in today's sophisticated microelectronic circuits. Silicon structures are processed using batch fabrication and lithographic techniques. Today, silicon fabrication techniques are available to simultaneously construct micrometer and sub-micrometer structures on large area wafers (400 cm²) yielding millions of devices per individual wafer.

Although this technology was initially applied to the fabrication of microelectronic devices, the same techniques can be used for the fabrication of microscopic mechanical systems. As a mechanical building material, silicon has well known fabrication procedures, and it is available in inexpensive, ultra-pure crystalline form. Over the past decade, a number of microfluidic devices have been developed using silicon microfabrication technology that allows the construction of miniaturized "chemical reactors". Miniaturized capillary electrophoresis (CE) chips were pioneered by Harrison and Manz and used to separate fluorescent dyes and fluorescently labeled amino acids. More recently DNA restriction fragments and oligonucleotides have been separated using these CE chips. Integrated analysis systems capable of performing PCR amplification followed by subsequent product sizing, DNA restriction digestion followed by size-fractionation and capillary electrophoresis with integrated electrochemical detection have also been achieved.

The devices discussed above demonstrate the feasibility of molecular assays in microscale environments, but their practical implementation for low-cost application presents significant additional effort. For example, most of these devices require external optical readers that restrict their use to laboratory environments, thereby increasing the cost substantially. A possible format is an inexpensive integrated fabrication technology that accommodates all the necessary fluid, thermal, and detection functions under a common substrate. We have previously demonstrated the integration of fluidic handling and reaction components of a microfabricated DNA analysis device. However, integration of miniaturized detectors for electrophoretic analyses has so far not been attempted vigorously. While laser induced fluorescence provides detection of even single molecules, having a detection system orders of magnitude larger than the separation system significantly diminishes the benefits of miniaturization.

We have constructed a miniaturized fluorescence detector that is capable of detecting migrating DNA "bands". We have chosen to use standard gel electrophoresis and fluorescent DNA labeling methods in our devices. To date, these methods have scaled to the micron-size level with few problems. Our electrophoretic DNA separations were performed in an integrated nanoliter device, approximately 500 μm wide by 50 μm high (Figure 1). Photodetectors were constructed in a silicon substrate, with a thick optical filter that blocks UV radiation deposited on the surface. A passivation layer of p-xyylene (Parylene-C) is used to isolate the electronic components from the fluidic components. The gel matrices we have used include agarose, cross-linked polyacrylamide, modified polyacrylamide, and hydroxyethylcellulose in solution. Electrophoresis buffers and voltage conditions are standard (1X/0.5X TBE, 6-8 V/cm). The separation device has been tested extensively; both as a stand-alone electrophoresis stage and as part of an integrated system.
The electrophoresis system has a simple crossed-channel configuration to allow electrokinetic loading of DNA samples from the intersection area. The pre-formed separation gel and integral detectors are in the channel to the immediate right. The sample enters the loading region from the top channel and, if necessary, overflows into the bottom channel. The left leg of the intersection is also pre-loaded with gel to contain the liquid sample in the intersection and to prevent convective flow down this leg. Platinum electrodes are fabricated on top of the plastic passivation layer at the ends of the left and right channel, and generate the necessary electric field. Electrochemically drilled holes through the glass cap allow the addition or removal of electrophoresis running buffer. Buffer wells can be formed with silicon sealer around these holes to compensate for the evaporative loss of the buffer contacting the electrodes, if necessary.

**FIGURE 1.** Enlarged photograph of the integrated electrophoresis device showing the crossed-channel injection area, microfabricated detectors and electrodes.

**DEVICE FABRICATION**

Device construction begins with fabrication of the photodetector within a crystalline silicon substrate (**Figure 2a**). The photodetectors are positioned at multiple locations across the device. Diodes are constructed by implanting the entire surface of a 200 Ω-cm, <100>, float zone, boron-doped, p-type silicon wafer with phosphorus. Diffused layers of boron (1 x 10^{15}/cm^2) and phosphorus (5 x 10^{14}/cm^2) are then ion-implanted onto the wafer in defined regions to form the substrate contact and main lead for the diode, respectively. An interference filter (Z C & R Coatings, California) is then deposited above the diodes to allow the transmission of fluorescent-emission light (515 nm, in the existing design) from dye excited with short wavelength excitation light (<500 nm). The filter is ~6μm thick and consists of alternating layers of SiO_2 and TiO_2 thin films. A passivation layer of Parylene C (p-xylylene) is then deposited over the filter to isolate the fluidic components from the electronic components. Platinum metal is placed at defined locations for use as electrophoresis electrodes, using a ‘lift-off’ process.

Channels are fabricated (**Figure 2b**) by depositing 0.4 μm metallic layer of gold on the surface of a glass (500 μm thick) (Dow Corning 7740). A 0.06 μm layer of chromium is used as the adhesion layer. Photoresist was applied and patterned using a channel mask and developed. The metal layers were etched in a commercial gold etchant (Gold Etchant TFA, Transene Co.) and chromium etchant (CR-14, Cyantek Inc.). The accessible glass was then etched in a freshly prepared solution of hydrofluoric and nitric acid (7:3 v/v). The rate of etching was approximately 5 μm/min and the etch-depth was measured using a surface profilometer. The metal layers were removed and the wafer was rinsed in DI water, air dried and oven-dried at 100 °C for 20 minutes.

The glass side is then aligned on top of the silicon side and bonded using optical adhesive (SK-9 Lens Bond, Summers Laboratories, Fort Washington, PA). The bond was cured under a UV light source (365 nm) for 24 hours. It is wired to a printed circuit board and fitted with silicone electrophoresis buffer wells.

The left and right channels are loaded with a 10% polyacrylamide gel solution and polymerized in situ. Sample is loaded onto the top channel and injected at the cross intersection using 6-8 V/cm for 10-15 seconds. Injection is confirmed by the
appearance of a bright green band under fluorescent illumination. Excess sample is then removed, and the channel is flushed and the solution is replaced with running buffer. The compacted sample is then electrophoresed at the same electric field strength.

FIGURE 2. Simplified process flow diagram for fabrication of the silicon and glass components of the integrated electrophoresis device.

RESULTS AND DISCUSSION

1. Electrophoretic Separation

The spatial DNA resolution obtained in the electrophoresis system is comparable to systems orders of magnitude larger. **Figure 3** shows the results of a 100bp DNA ladder (Gibco BRL) run in the electrophoresis section (8 V/cm) after less than 5 millimeters of electrophoretic migration. This resolution has been reproduced many times in our devices using both 100bp and 50bp DNA ladders. The crossed intersection used to electrokinetically load sample onto the gel is robust and allows stacking of the DNA in solution at the gel boundary. Sample loads typically require 10-30 seconds and the subsequent runs last on the order of minutes. In all our runs, clearly observable DNA separation was achieved in a distance of 0.5 to 3 mm. Other non cross-linked separation media can also be used in the device and have the advantage of being replaceable. Although we have achieved similar resolution with these media, cross-linked acrylamide allows simpler loading of discrete samples on the gel. We have also performed restriction digestion separation and analysis on our micro gels with similar successful results.

We found that the operation of these gels was very similar to the macroscale counterparts. The polymerization is occasionally variable but usually results in a usable gel. Over half of the gels polymerized in situ were able to give good electrophoresis separation. The wicking of the acrylamide monomer solution into the channel allows easy loading of the matrix and monitoring of the polymerization process.
FIGURE 3. Separation of a 100bp ladder DNA achieved in a microfabricated gel electrophoresis device. Separation is achieved in ~2mm and in ~15 minutes.

The shape of the DNA bands migrating in the gel is governed by the channel shape, matrix uniformity, temperature uniformity, and by the conditions at the buffer-to-gel interface. A uniform, perfectly flat interface within a uniform, clean channel produced the sharpest bands. We have observed electrophoresis runs in this system which exhibit “smiling” and others that exhibit “frowning”. We believe that in some cases the acrylamide along the channel edges did not polymerize completely (resulting in more rapid migration along the edge, or frowning). For instances where the gel shows “smiling” the edges may simply be cooler than the bulk gel, although this has not been examined in detail. Currently, we are able to achieve flat interfaces by merely allowing the gel solution to wick into the channel, polymerize in the presence of buffer and removing excess solution upon polymerization. We are actively experimenting with hydrophobic patches to aid in generating a flat gel interface formation.

Along with the proper matrix composition and gel interface shape, an important consideration remains the application of the liquid sample to the solid gel interface. On the molecular scale, the initial application of the electric field rapidly draws the free DNA in solution to the gel interface (electrokinetic injection). At the interface, the DNA is held up by the matrix material and gradually enters the gel itself. The longer the liquid sample remains in contact with surface in the presence of an electric field, the more DNA is transferred to the gel resulting in a greater eventual signal at the detector. Unfortunately, the longer the loading process continues, the more widely distributed the DNA population is in the gel resulting in more diffuse bands and lower resolution. To date, we have used the “cross” intersection in combination with rapid stacking and sample washout to produce relatively intense, sharply defined bands.

2. Fluorescence Detector

Any DNA analysis system is ultimately limited by its ability to detect low levels of labeled DNA. Several of the most important advances in genetic analysis have been associated directly with improved levels of detection. The ability to observe DNA in real-time has had significant impact and is the basis for virtually all of the human genomic sequencing performed to date. Improvements on the basic detection system, such as new fluorescent dyes, continue to influence the overall efficiency of DNA sequencing.

Recent innovations in miniaturized electrophoresis systems have continued to rely on macroscale, external optical readout arrangements thus minimizing the benefits of microfabrication. A miniaturized detection system, with sensitivity comparable to macroscale detection systems, will aid in the realization of integrated ‘lab-on-a-chip’ systems. With this perspective, our group has developed two diode detection systems - one that can detect sample radiation and one that can detect fluorescence. They are both constructed using ion-implants into a high resistivity silicon substrate.
Although a radiation detector is simpler to construct, a fluorescence-based detector is clearly preferable, both for sample handling convenience and for detection sensitivity. The fluorescent detector we constructed begins in the same manner as the radiation detector, using ion implants to form a diode. Then, to prevent the excitation light from saturating the detector, a thin film, high-pass, interference filter is deposited over the detector surface. The filter blocks the excitation wavelength light but allows the fluorescent emission wavelengths to pass through. This filter is constructed by alternating thin SiO₂ and TiO₂ layers, and provides a very sharp spectral cutoff at 500 nm (Figure 4). The filter layers are then covered with a ~4 μm layer of p-xylene that protects the active photodetector from the ions present in the buffered DNA samples. The substrate holding the detector is next bonded to a glass cap with the etched channel network.

![Filter Performance](image)

**FIGURE 4.** Transmittance characteristics of a multi-layer optical filter on silicon detector wafer indicate negligible transmittance of UV excitation light and almost complete transmittance of (green) emission wavelengths.

![Dye Concentration](image)

**FIGURE 5.** The detection limits of the interference filter/photodiode integral detector.
We found that this detector was remarkably sensitive relative to its simple construction. Figure 5 shows the diode response as a function of DNA concentration in solution. The series of measurements was taken using one of our standard channel formats (glass channel cap, 500 μm x 50μm cross section) and a solution of 4 kb plasmid stained with SYBR Green dye. Concentrations down to approximately 2 ng/μl still gave a detectable signal over background. Based on the size of the detector (10 μm x 500 μm) and the volume of solution above it (50 μm high channel), this corresponds to a detection limit of approximately 10,000 molecules. The close positioning of the detector to the sample (a distance of ~6 μm) results in efficient collection of the fluorescent signal.

3. On-chip Detection of Migrating DNA Bands

We have used this detector with standard UV illumination source to perform separation with on-chip detection of electrophoretic migrating DNA bands. The experimental set-up for this experiment is shown in Figure 6. The setup consisted of a UV excitation source passing though an optical chopper. The chopper is controlled at the desired frequency (288 Hz) by a Lock-in amplifier. The lock-in amplifier also collects the diode response at the same frequency. This is done to minimize extraneous signal and to keep the signal to noise ratio high. The electrophoresis channel is simultaneously observed under us a fluorescence stereocope (Olympus SZX-12). Note that a simple light source such as a flashing blue light-emitting diode (LED) can also be used in place of the stereocope-chopper combination. The advantage of the fluorescence stereocope system is that the bands could be visually monitored passing over the detector while the detector signal was being recorded in real time.

![Figure 6](image)

**FIGURE 6** Experimental set up used for on-chip detection of migrating bands of DNA. Excitation (UV) can be provided by either a fluorescence stereocope passing through an optical or a blue LED flashing at a specified frequency. A lock-in amplifier is used in both cases to control the excitation source.

The response of the detector (leakage current) to UV excitation is monitored continuously throughout the electrophoretic run. As fluorescently labeled bands of DNA migrate over the detector, they are excited by the incident UV light. As a result they emit fluorescent light (green) which passes through the transparent gel and the optical filter and reaches the detector. The detector essentially sees an increase in the intensity of light and responds by a proportional increase in leakage current (Figure 7).
We have used this photodetector arrangement and detected the migration and separation of several DNA samples. Figure 8 shows separation/detection of the first seven bands of a standard 50 bp ladder DNA (Gibco BRL), labeled with the intercalating dye YOYO-1 (Molecular Probes). The separation was carried out in a native polyacrylamide gel (10%T:2.6%C). Gel and sample were loaded as previously described and the separation was carried out at 8 V/cm. The migrating DNA fragments fluoresce in the vicinity of the detector, and this results in increased leakage current of the photodetector.

The light source used for this experiment was that from a standard fluorescence stereoscope. The incident light beam was highly unstable and hence this leads to an increased noise level in the excitation signal. As a result, most of the noise observed in Figure 8 can be attributed to the disturbances in the excitation light source. Similar experiments have been performed with a “flashing” blue LED serving as the light source and the signals obtained have been significantly more stable.
CONCLUSIONS

We have developed an integrated separation and detection system using the techniques of the silicon microfabrication industry. The addition of the integral detectors does not significantly increase the cost as the detectors are also produced using the techniques of photolithography. The detector present in our existing device can easily detect fragments of double stranded DNA with concentrations of <0.1 μg/μl within each electrophoresis migrating band. This is roughly the typical concentration of DNA used in gel separations such as restriction digestion or simple amplification. Also, our current gel separation system can reproducibly resolve a 100bp or 50bp "ladder" DNA standard well into the 1000 bp range. The individual bands are clearly observed and appear essentially identical to a macro-scale electrophoresis run.

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REFERENCES


