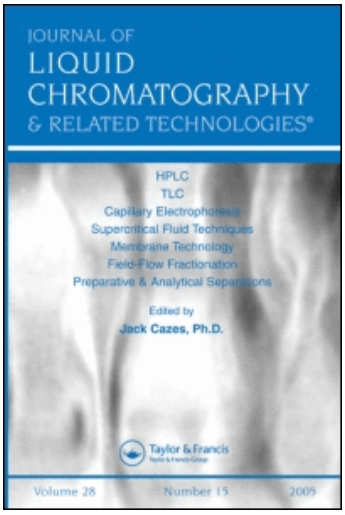

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DNA SEPARATIONS FROM NANOVIALS

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DNA SEPARATIONS FROM NANOVIALS

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ABSTRACT

Integration of 1-nL sampling vials with a capillary sample introduction ultrathin slab gel electrophoresis system is described here. The low-volume sampling capabilities of capillary electrophoresis (CE) along with the small structural size make it an ideal sampling technique for these vials. A DNA restriction enzyme digest has been sampled and separated from these vials. A comparison of the nanovial DNA separation and a separation from a larger vial are virtually identical.

INTRODUCTION

The development of faster DNA amplification methods with an emphasis on reduced reagent volumes along with integration of the sample preparation and separation systems is critical to the completion of the Human Genome Project. Many groups have been focusing on enhancing the speed and parallel processing capabilities of the separation systems through methods such as capillary array electrophoresis,[1-4] ultra-thin slab gel separations[5-9] and chip technology[10-12] to accommodate the massive number of separations needed to completely sequence a single strand of human DNA; however, aspects of the preparation steps such as speed and reagent volume have only recently been considered.

Although the polymerase chain reaction has enhanced DNA amplification technology dramatically by accurately amplifying extremely small amounts of DNA, cycling times for standard PCR volumes (200 μL) are rather long. In addition, the PCR reaction vials for sequencing 1 strand of human DNA could fill an entire room. Reduction in size of the sample chambers would assist with both problems, although manipulation of low volume samples is non-trivial. One recent demonstration of this was by Woolley et. al. whereby 20-μL reaction chambers were coupled with a chip electrophoresis system.[12]

More recently, Soper et al. reported a technique to carry out solid-phase cycle sequencing in 64-nL capillaries coupled to capillary electrophoresis.[13] We believe that this reduction in sample well size can be pushed even further.

In our laboratory, ultra-small vials ranging in size from 1 pL to 1 nL, have been micromachined in silicon[14,15] and by pressure imprinting of polystyrene[16] using standard photolithographic techniques. Numerous other fabrication methods have been used to make subnanoliter reservoirs on chips.[17-23] We have previously used these ultrasmall vials to localize small amounts of material for analysis in time-of-flight secondary ion mass spectrometry with high spatial resolution,[15] and to perform voltammetry of ferocenecarboxylic acid solutions.[16]

Manipulation of solutions with such small volumes is accomplished through the use of fused silica capillaries with inner diameters of 2- to 50-μm. Since these are the same capillaries routinely used for capillary electrophoresis, using capillary electrophoresis to sample and separate analytes from these nanovials has also been demonstrated through the analysis of catecholamines by capillary electrophoresis with electrochemical detection from 100 pL vials.[15]

In addition, 100-pL derivatization reactions of amine containing compounds have been carried out in nanovials followed by capillary electrophoresis with laser-induced fluorescence detection.[14] Analyzing DNA from nanovials can be accomplished by using a gel or sieving-buffer filled capillary; however, only one sample can be analyzed at a time.

We have recently published a method of transferring sample plugs of DNA through a single capillary onto an ultra-thin slab gel.[9,24,25] As with standard capillaries this method is amenable to sampling from very small environments such as the nanovials.

This paper describes the development of methods for transferring and handling solutions in the 9-pL to 1-nL range for subsequent separation. In particular, it focuses on the analysis of DNA samples through integration of nanovials with a capillary sample introduction ultra-thin slab gel separation system.

EXPERIMENTAL

Nanovial Fabrication

The fabrication of the silicon nanovials has been described previously.[16] In brief, a 4-in photomask was created using standard photolithographic techniques. This photomask was used repeatedly to transfer the pattern to 3-in single-crystal <100> silicon wafers. Alignment of the mask with the etch planes of the silicon crystal controlled the shape of the vials created. Through a combination of chemical and wet etching, and due to the well-defined silicon etch planes, arrays of square pyramidal nanovials were formed.

A CHA RAP-600 thermal evaporator was used to deposit a chromium adhesion layer (75 Å) followed by a gold or platinum conduction layer (1000 Å) on the wafers. For electrophoretic injection, an electrical connection was made to the metallic coating using a wire epoxied to the surface of the wafer. Sizes of the vials were determined by scanning electron microscopy (Joel Model JSM 5400, Peabody, MA) and surface profilometry (Tencor Alphastep 200).

Separation System

The capillary sample introduction ultrathin slab gel system has been described in detail.[5,24,25] A single sulfonic acid coated capillary was used to electrophoretically transfer DNA plugs to the entrance of an ultrathin slab gel (57-μm). Using buffer as a spacer between subsequent samples and translating the elution end of the capillary along the gel entrance, several distinct samples were separated in parallel with this system.

HaeIII-digested ΦX174 DNA was obtained from Sigma Chemical Co. and 15% glycerol by volume was added to retard evaporation. This solution was pressure injected into the nanovials as previously described.[14,16]

To manipulate the capillary tip into a nanovial, the injection end of the capillary was etched in hydrofluoric acid after removing the polyimide in a manner previously described.[26] The resulting capillary tip was conical in shape with a tip outer diameter between 100 and 120 μm. For analysis, the injection end of the transferring capillary was manipulated into the filled nanovial, and the injection potential was applied to the entire wafer of vials.

The capillary was subsequently returned to the separation buffer reservoir and the transferring potential was applied. Each vial was marked immediately after being filled to prevent further use and cross contamination.

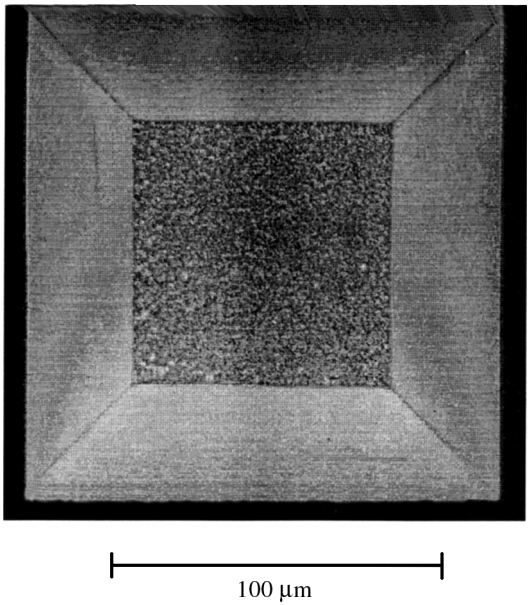

Figure 1. Scanning electron micrograph of a 1-nL flat-bottomed nanovial. The top of the vial is 172 μm and the bottom is 95 μm.

RESULTS AND DISCUSSION

Arrays of 2500 inverse square pyramidal nanovials with flat bottoms have been fabricated on 3-inch-single-crystal <100> silicon wafers using standard photolithographic and etching techniques. Scanning electron micrographs (SEM) such as the one shown in Figure 1 are used to check for variations in etching across the wafer. The ease of fabrication of the nanovials and their reproducibility makes them favorable for handling ultrasmall samples, and the precise spacing of 1 mm between the centers of the nanovials in each array facilitates automation.

One of the largest difficulties in dealing with liquid samples of this size is rapid evaporation. The relative rate of evaporation is proportional to the surface area to volume ratio of the sample; hence, evaporation is much faster with smaller samples. An aqueous sample evaporates from a 46-pL nanovial in less than 7 s under ambient conditions. There have been several methods proposed for dealing with this in larger (nL to L) containers. Evaporation from 118-nL vials has been controlled by using a thin membrane as a cover for the vials.[17] A water-saturated nitrogen stream over the sample has also been employed to reduce evaporation from microliter volumes on electrodes,[27] and continuous

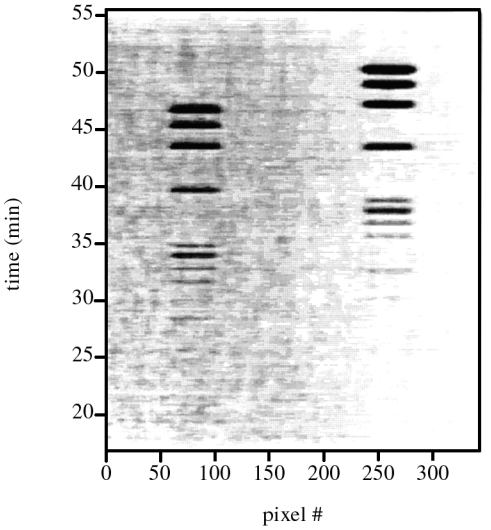

Figure 2. Top view electropherogram of *Hae*III-digested ΦX174 DNA from a 1 nL nanovial (left) and a 200 µL PCR reaction vial (right). Capillary conditions: 50 µm i.d.; 51.7 cm long; nanovial injection end, 120 µm; gel entrance end, 100 µm; Injection conditions: 10 s at 10 kV; capillary potential, 10 kV. Separation conditions: 6% polyacrylamide gel; 57 µm thick; 16 cm long; 10.5 cm to detector; separation potential, 1.6 kV.

rehydration with an additional microinjector has also been demonstrated.[28] Evaporation of samples in fast-atom-bombardment and matrix-assisted laser desorption/ionization mass spectrometry has been controlled through the use of glycerol in the sample matrix.[29,30] By placing a small amount (4 to 15% by volume) of glycerol into the solutions injected into the nanovials, the rapid evaporation rate for aqueous samples is slowed to manageable levels.

The concept of performing small scale DNA reactions has been considered. The ability to isolate DNA into small vials, and perform reactions on the nL to µL scale has significant implications for future genome research. As a first step in this direction, DNA solutions containing double stranded DNA restriction fragments and 15% glycerol have been placed in individual 1-nL nanovials. A single capillary has been used to transfer DNA sample plugs from the vials to a gel-based separation system. For comparison, an identical injection from a large vial containing 2 µL of the same DNA:glycerol solution has been made 5 min later. A top view of the separations obtained is shown in Figure 2, and individual electopherograms of the 2 injections are shown in Figure 3.

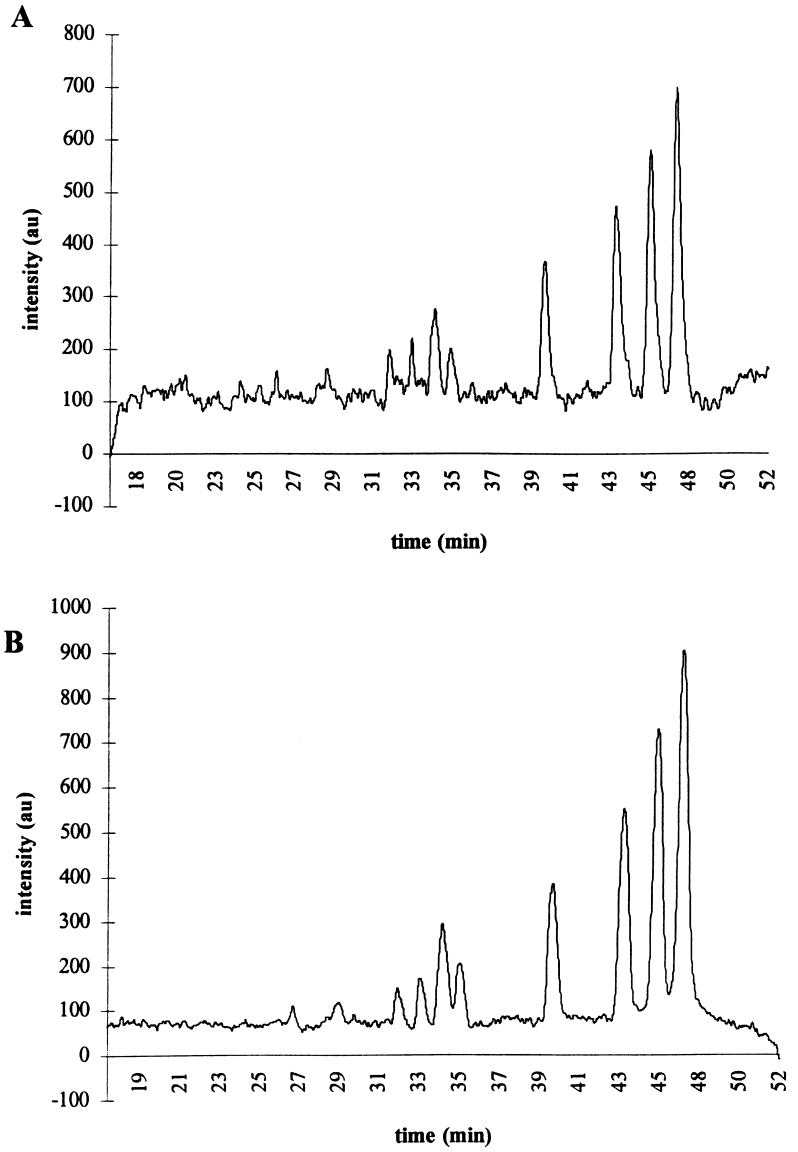

Figure 3. Single pixel electropherograms of *Hae*III-digested ΦX174 DNA injected from A. 1 nL nanovial; B. PCR reaction vial. The fragment sizes are 72, 118, 194, 234, 271/281, 310, 603, 872, 1078, 1353 bp. The time axis represents the time the DNA plugs were introduced into the capillary.

Separation of *Hae*III-digested ΦX174 DNA sampled from a 1 nL nanovial shows 10 peaks that are baseline resolved in a 6% polyacylamide gel. These separations are virtually identical demonstrating that DNA samples from 1-nL vials can be acquired and separated without difficulty.

The concept of performing reactions in multiple nanovials simultaneously, coupled with the DNA separation system might eventually lead to the high sample throughput needed for sequencing applications. Using an entire wafer of nanovials as reaction chambers for Sanger sequencing reactions or other PCR based methods would allow simultaneous amplification of 2500 samples.

This number would be a considerable improvement compared to the 96-well thermal cyclers currently on the market. In theory, 30 PCR cycles on 30 attograms of DNA performed in a 1-nL microvial would result in a DNA concentration easily detected with the current ultrathin slab gel system.[31]

The sulfonic acid coated capillaries used with the capillary sample introduction ultrathin slab gel system offer another advantage to the coupling of this system with nanovials. Upon combining the two systems, the presence of a slight electroosmotic flow in the capillary becomes apparent. As long as there is some solution in the vial initially, this EOF has the effect of rehydrating the samples in the nanovials throughout the course of DNA injections.

Nevertheless, this does not appear to affect the migration of DNA into the capillary. As the injection potential is applied, the nanovial begins to overflow; however, the negatively charged DNA migrates into the capillary without hindrance.

This seems to indicate that prevention of evaporation is not the deciding factor in using these vials for multiple separations. The presence of glycerol in the nanovials ensures enough liquid to make an electrical connection for the injection potential.

Of course techniques for rapidly filling the microvials and regulating the temperature across the entire wafer are non-trivial; however, a recent demonstration by Northrup and Mathies on a 20 μL scale indicates that such problems are not insurmountable.[12] In addition, their work suggests that removal of the salts found in most PCR reactions prior to separation is not essential for good separation resolution.

Although simultaneous amplification of multiple samples will help with the sample preparatory stage of high speed separations, it is the combination of the sample preparatory and separation stages which offers the most promise for increasing DNA sample throughput.

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