Microfluidic device reads up to four consecutive base pairs in DNA sequencing-by-synthesis

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ABSTRACT

We have developed the first fully integrated microfluidic system for DNA sequencing-by-synthesis. Using this chip and fluorescence detection, we have reliably sequenced up to 4 consecutive bps. The described sequencer can be integrated with other microfluidic components on the same chip to produce true lab-on-a-chip technology. The surface chemistry that was designed to anchor the DNA to elastomeric microchannels is useful in a broad range of studies and applications.

INTRODUCTION

The Sanger method of DNA sequencing (1) and its subsequent capillary array automation (2) has revolutionized biology and has led to the sequencing of the consensus human genome (3,4). However, this state-of-the-art technology has inherent limitations, especially in cost and read length, which make it impractical in moving to the next logical step: massive comparative genomics studies (3), aggressive disease-gene discovery and ubiquitous personalized medicine (5,6). In terms of speed and cost, sequencing only five persons took over 9 months, while the total budget of the project was over 3 billion dollars (http://www.ornl.gov/sci/techresources/Human_Genome/project/whydoe.shtmlbudget).

The limitations of the electrophoretic approach have prompted researchers to work on alternative methods: mass spectrometry (7–10), base addition with deprotection steps (11), pyrosequencing (12–16), sequencing by hybridization (17), massively parallel sequencing with stepwise enzymatic cleavage and ligation (18), polymerase colonies (19,20), sequencing using nanopores (21–24) and massively parallel single-molecule sequencing (25). While some of these methods are promising, none has yet bested electrophoresis separation. However, they may find important niche applications in areas such as SNP determination, mini-sequencing and separation. However, they may find important niche applications in areas such as SNP determination, mini-sequencing and gene expression analysis.

Here we describe a sequencing-by-synthesis method that uses microfluidic plumbing (µSBS). Briefly, the scheme involves exposing a primed DNA template to a mixture of a known type of standard nucleotide, its fluorescently tagged analog, and DNA polymerase. If the tagged nucleotide is complementary to the template base next to the primer’s end, the polymerase extends the primer with it and fluorescence signal is detected after a washing step. Iteration with each type of nucleotide reveals the DNA sequence. We implemented this scheme in a PDMS (polydimethylsiloxane) microfluidic chip and devised a novel surface chemistry to anchor the DNA to the PDMS microchannel to prevent DNA loss during feeds and flushes. The average read length was 3 bp, which constitutes proof of principle for the system as well as demonstrating the enabling technology—a heterogeneous assay fully integrated in a microfluidic system, combining active plumbing, specific surface chemistry and parallelism. This approach to DNA sequencing has advantages in economy of material and integrability under the lab-on-a-chip paradigm, while the microfluidic and surface chemistry technologies by themselves are immediately applicable to many other systems involving DNA studies, pyrosequencing being one example.

MATERIALS AND METHODS

Set-up and detection system

In each experiment, the microfluidic chip was housed in a custom-built aluminum holder, which was itself placed in a machined attachment to the translation stage of an inverted Olympus IX50 microscope. 23-Gauge steel tubes from New England Small Tube Corp. (Litchfield, NH 03052) were plugged into the chip’s control channel ports. Their other ends were connected through tygon tubing (Cole-Parmer, Vernon Hills, IL) to Lee-valve arrays (Fluidigm Corp., South San Francisco, CA) operated by LabView software on a PC computer. The same types of steel tubes and tygon plumbing were used to supply reagents to the chip’s flow channel ports.

The microscope was equipped with a mercury lamp (HBO 103 W/2 Osram), an Olympus Plan 10× objective (NA 0.25), an Olympus PlanApo 60× objective (NA 1.4) and a cooled CCD camera (SBIG ST-7I, Santa Barbara Instrument Group). Fluorescence detection was conducted using the following filter sets: Alexa Fluor 555 (ex D470/40, 500 DCLP, em D535/50), TAMRA, Lissamine and Cy3 (ex D540/25, dichroic 565 DCLP, em D605/55), both from Chroma Technology Corp., Brattleboro, VT.

Reagents

Chip fabrication. HMDS (hexamethyldisilazane) is from ShinEtsuMicroSi, Phoenix, AZ. Photoresist 5740 is from MicroChem Corp., Newton, MA. TMCS (tetramethylchlorosilane) is from Aldrich. PDMS (polydimethylsiloxane) is

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Sylgard 184 from Dow Corning, K.R. Anderson, Santa Clara, CA.

Surface chemistry. DAPEG is diacrylated polyethylene glycol SR610 from Sartomer, Exton, PA. The Pt catalyst is hydrogen hexachloroplatinate from Aldrich (26,258-7). The used polyelectrolytes are polyethyleneimine (PEI) (Sigma P-3143) and polyacrylic acid (PAc) (Aldrich 41,604-5). Streptavidin Plus comes from Prozyme (San Leandro, CA). Trisb is a buffer: Tris 10 mM (NaCl 10 mM) pH 8. TrisMg is a buffer: Tris 10 mM (NaCl 10 mM, MgCl2 100 mM) pH 8. The dUTP-Cy3 from Amersham Biosciences (Piscataway, NJ) is a dUTP nucleotide tagged with the Cy3 dye.

Sequencing. DNA1 is an 89mer biotinylated DNA template (Biotin-5'-tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcag tagctcagaCAGGAGGTCTA-3') annealed to a 14mer primer tagged with the Cy3 fluorescent dye (Cy3-5'-TAGAACCTCCGTGTTCT-3'). DNA2 is a 99mer biotinylated DNA template (biotin-5'-gccctgttctgccctgttctgccctgttctgccctgttgcttctttacacggaggttcta) annealed to the same type of primer. All DNA is from Operon Co., Alameda, CA. The sequencing feeds contained: A (10 mM dATP-Lis, 2 mM dATP, polymerase), C (10 mM ddCTP-TAMRA, 0.2 mM ddCTP, polymerase), G (10 mM ddGTP-TAMRA, 3.3 mM ddGTP, polymerase), U (8 mM ddUTP-TAMRA, 28 mM ddTTP, polymerase), all in 1× Sequenase reaction buffer with 15 mM DTT. All tagged nucleotides are from PerkinElmer (Boston, MA). All standard nucleotides are from Boehringer Mannheim (Germany). In all cases, the polymerase used is Sequenase Version 2.0 DNA Polymerase from USB Corp. (Cleveland, OH).

Microfluidic chip fabrication

PDMS microfluidic chips with integrated micromechanical valves were built using soft lithography as described previously (26) with the following modifications. Silicon wafers were exposed to HMDS vapors for 3 min. Photosist 5740 was spun at 2500 rpm for 60 s on a Model WS-400A-6NPP/LITE spinner from Laurel Technologies Corp. The wafers were baked at 105 °C for 90 s on a hotplate. UV exposure through black-and-white transparency masks was done at 180 mW/cm² for 25 s on a mask aligner (Karl Suss America Inc., Waterbury, VT). The molds were then developed for 3 min in a solution of 5:1 = deionized water:2401 MicroChem developer. Flow layer molds were baked at 100°C for 30 min on a hotplate to melt the 5740 and round the flow channels. Molds were characterized on Alpha-Step 500 (KLA-Tencor, Mountain View, CA). Channel height was between 9 and 11 μm, while main flow channel width was between 95 and 105 μm. Control channel profile was oblong, while flow channel profile was parabolic. Except for the height measurements and the flow channel rounding, the mold fabrication was conducted in a class-10 000 clean room.

Molds were exposed to TMCS vapors for 3 min. PDMS in 5:1 and 20:1 ratios were mixed and degassed using HM-501 hybrid mixer and cups from Keyence Corp. (Long Beach, CA). Then 35 g of the 5:1 was poured onto the control mold in a plastic Petri dish wrapped with aluminum foil. Five grams of the 20:1 was spun over the flow mold at 2500 rpm for 60 s on Spincoater P6700 (Specialty Coating Systems, Indianapolis, IN). Both were baked in an 80°C oven for 30 min. The control layer was taken off its mold and cut into respective chip pieces. Control line ports were punched using a 20-gauge luer-stub adapter (Beckton-Dickinson, Franklin Lakes, NJ). Control layer pieces were washed with ethanol, blown dry and aligned on top of the flow layer under a stereooscope. The result was baked in an 80°C oven for 1 h. Chip pieces were then cut out and peeled off the flow layer mold. Flow line ports were punched with the same 20-gauge luer-stub adapter. Meanwhile, 5.1 Sylgard was spun at 5000 rpm for 60 s over RCA-cleaned 22 mm #1 coverslips (27). The coverslips were then baked in an 80°C oven for 30 min. Chip pieces were washed in ethanol and blown dry before binding to the PDMS layer on the coverslips. The now assembled chips underwent final bake in an 80°C oven for 2 h.

Surface chemistry

The flow channels of the PDMS chip are filled with a mixture of DAPEG and the Pt catalyst at the volumetric ratio of 200:1 = DAPEG:catalyst. Then, the chip is baked in an oven at 80°C for 30 min. The DAPEG mixture is flushed out of the microchannels with high purity water. Alternating layers of PEI and PAc are built using 5 min feeds of 20 mg/ml solutions at pH 8, by analogy with previous work at the macro scale (28). Next, the surface is biotinylated using a kit from Pierce (28). This is followed by deposition of Streptavidin Plus at 1 mg/ml in Tris and biotinylated DNA at 7 μM in TrisMg. This completed the standard procedure for surface chemistry build up.

RESULTS

In this sequencing-by-synthesis scheme, the template to be sequenced is annealed to a shorter primer and exposed to one type of nucleotide and its fluorescently tagged analog in the presence of DNA polymerase. If the tagged nucleotide is the complement of the template base next to the 3’ end of the primer, the DNA polymerase would extend the primer with that nucleotide. Fluorescence signal is then observed after a washing step, as the tag would then be attached to the DNA template. Since the type of tagged nucleotide is known in advance, the presence of signal reveals the template base. On the other hand, if the supplied tagged nucleotide is not the correct one, the primer is not extended and no signal is observed after the washing step. Next, another type of nucleotide is fed in the same way. Once one base is determined, the scheme proceeds in exactly the same fashion to read out the next base in the template, and so on. Thus, the promise of this scheme lies in the fact that so long as the polymerase can keep extending the primer in this fashion, there is no inherent ceiling in read length, as with gels. Background signal build up and complications from FRET and fluorescence quenching can be eliminated with occasional steps of fluorescence bleaching.

In general, the dye molecules attached to the nucleotides can hinder the polymerase activity due to steric effects, such that the extension yield drops below 10% after the first tagged base (25). Thus, the feeds contain a mixture of a nucleotide and its tagged analog instead of just the tagged analog. Under suitably chosen feed concentrations, only a fraction of the
DNA population extends with tagged nucleotides, while most of the DNA builds up with a standard nucleotide and thus remains available for further extension without steric hindrance. Hence, the readout can proceed to further bases at the expense of a small portion of the DNA population per successful incorporation.

Using microfluidic technology (26) as plumbing for this scheme has a number of advantages. At the microscale, diffusion happens within seconds, which facilitates reagent exchange times. Speed is important since the sequential nature of the scheme demands that the duration of each step be kept as short as possible, so that the overall duration of a sequencing run is comparable with or better than the one of the currently available technology. Microfluidics also provides parallelism and promises significant reduction in costs by economy of scale of reagents.

The sequencing chip is shown in Figure 1. The derivatization tree supplies reagents to form surface chemistry in all five sequencing chambers at once. Lane controls direct flow of sequencing reagents from the sequencing tree into any chamber of choice. Valves are formed only where the flow segments cross over flow segments. Arrows indicate the flow direction during operation—derivatization (green) and sequencing (purple). This microfluidic architecture is applicable in any situation combining parallel derivatization of an array with individual addressability of its elements.

DNA application, the long bake that seals the coverslip to PDMS also fouls the hydroxyl groups on the glass surface, which are crucial for the PEM deposition. Shorter bakes allow PEM deposition but produce unreliable sealing. We introduced an additional featureless PDMS layer as a floor for the flow channels. That produced reliable sealing but required adapting the DNA anchorage chemistry to PDMS surfaces. PDMS polymerizes as SiH groups react with vinyl groups in the presence of a Pt catalyst. This process leaves unreacted SiH groups on the surface, which can be used as functionalization targets for vinyl groups during surface chemistry build-up. On the other hand, the polar nature of polyethylene glycol (PEG) made it a suitable substrate for PEM deposition. Thus, we selected diacrylated PEG (DAPEG) as the link between PDMS and the proven PEM surface chemistry. While UV-based PEG and biotin grafts have been shown before (29,30), our surface chemistry is the first demonstrated non-UV-based, robust, specific and tunable anchoring of DNA to PDMS where the DNA remains sterically available for enzymatic biochemical reactions and fluorescence background from non-specific binding is suppressed through surface chemistry.

We built negatively terminated PEM on top of DAPEG-treated microchannel surfaces in a PDMS chip like the one shown in Figure 1. Next, 1 μM dUTP-Cy3 in Trisb was fed through the microchannel for a few minutes. After flushing with Trisb, pictures were taken and the average counts per pixel in the microchannel were obtained. One layer of PEI was built up to make the surface positively charged. Then, the dUTP-Cy3 feed, the flushing and the detection were repeated. Since the tagged nucleotide is negatively charged, positively terminated surfaces attached 267 times more nucleotide than their negatively terminated counterparts, while control...
experiments showed PEM would not assemble onto PDMS in the absence of DAPEG. This shows the shielding quality of our PDMS-DAPEG-PEM surface chemistry.

Further experiments showed that increasing the number of PEM layers increases the surface charge density, which improves the shielding. Four alternating layers are sufficient to prevent most of the non-specific attachment, while best signal-to-noise is reached at 12 layers.

To test biotinylation, the DAPEG-PEM surface chemistry was built with eight PEM layers in a PDMS microfluidic chip of essentially the same design as Figure 1. Then the biotinylation mixture (28) was fed at 5 mM for 1, 2, 4, 8 and 16 min into lanes 1 through to 5, respectively, followed in each case by a 2 min flushing of MES 10 mM buffer. Next, pictures of all chambers were taken as background signals before the fluorophore feed, using the rhodamine fluorescence filter set. Streptavidin Alexa Fluor 555 from Molecular Probes was fed at 1 mg/ml in Trisb for 2.5 min from the derivatization tree into all chambers simultaneously by keeping the derivatization valve array closed. All unattached streptavidin was washed away with Trisb buffer and pictures were taken again with the same filter set. The net signal was extracted and converted into streptavidin surface density using a simple bulk calibration by volume fluorescence signal from a known probe concentration (Fig. 2A). The same calibration method was used henceforth.

Since all available biotin sites were saturated by streptavidin by the time of signal detection, the observed linear dependence maps the biotinylation progress. This linearity provides a tool for tuning the DNA surface density in the PDMS microchannel. The mapped range is the one of relevance to fluorescence studies as further increase in density might bring the probes together close enough for fluorescence quenching to become an issue.

To test surface chemistry stability with continuous flow, we anchored DNA1 in a device of the same layout as the one in Figure 1, using 16 PEM layers. Then we continuously flushed Trisb through one of the sequencing chambers, while simultaneously taking data every 5 min (Fig. 2B). The ‘squares’ curve shows the surface density of emitting fluorophores versus real time of continuous flushing. The ‘circles’ curve shows the expected signal, based on prior photobleaching experiments conducted without flushing. Since the measured signal is consistent with the prediction, virtually all loss of signal must be due to bleaching of the Cy3 tag on the DNA rather than loss of DNA off the surface due to anchorage failure. Hence, the surface chemistry is stable over at least 1.5 h of continuous flushing.

To test surface chemistry stability over time, we anchored DNA2 in a device of the same layout as the one in Figure 1, using 12 PEM layers. Next, from time to time, we flushed Trisb for 1 min through all chambers simultaneously and then took pictures of them using the optical system described above. The emitting fluorophores surface densities are plotted in Figure 2C and D, where each curve shows the results from a respective chamber. The consistency of these curves demonstrates the uniformity of conditions in the array, as well as the reproducibility of the results. The signal is consistent with the bleaching prediction (empty diamonds in Fig. 2C). Thus the falloff across exposures is not due to loss of material off...
the surface but due to fluorescence bleaching. The real-time pattern (Fig. 2D) shows the anchorage is stable over at least 14 h at room temperature.

To test sequencing reagents stability over time, we anchored DNA1 in a device of the same layout as the one in Figure 1, using 14 PEM layers. After fluorescence detection confirmed the successful attachment of DNA, the Cy3 tags in one of the chambers were bleached. Next, a feed containing polymerase, a nucleotide, and its tagged analog, was followed by a Trisb flush and fluorescence detection. This process was iterated with different feeds in the same chamber, to collect the sequencing data. The net increase in the fluorescent signal after each feed was converted into a corresponding change in fluorophore surface density based on individual reagent calibrations. Then, the same procedure was repeated with the next chamber, and so on. The results are shown in Figure 3A. Comparing the polymerase and non-polymerase cases for each chamber shows that everywhere the polymerase successfully extended the primer with the tagged nucleotide, while the non-specific attachment of the dye was minuscule in comparison. As the whole experiment was done with a single load of reagents, comparing the results across chambers shows that the polymerase and nucleotide remained active over at least 2 h at room temperature with no visible loss in activity.

Taken together, these experiments showed that the device and reagents are stable and capable of performing nucleotide incorporation over many hours. Hence, we could proceed to sequencing.

DNA1 was anchored in a device identical to the one in Figure 1, using 16 PEM layers. After fluorescence detection confirmed the successful attachment of DNA, the Cy3 tags in one of the chambers were bleached. Next, a feed containing polymerase, a nucleotide, and its tagged analog, was followed by a Trisb flush and fluorescence detection. This process was iterated with different feeds in the same chamber, to collect the sequencing data. The similarly extracted data showed the level of non-specific attachment and was subtracted from the previous data to produce the final results for this experiment (Fig. 3B). The measured sequence, GAUG, exactly corresponds to the beginning of the known template sequence. Thus, we have shown successful sequencing of 4 consecutive bp in a single microfluidic chamber.

We pooled the data from nine such sequencing experiments with small variations in reagents, templates and feed schemes. The resulting histogram showed the most likely read length to be 3 bp from a single chamber.

**DISCUSSION**

It is clear from the sequencing data that the signal-to-noise deteriorates so as to limit the read length in the chamber to 4 bp. We believe three factors conspire to produce this result. First, while the surface chemistry shielding works very well, it is not perfect, so there is some dye-dependent attachment (~50 dyes/μm²). Since it is non-specific, its magnitude varies enough to make it difficult to subtract as a DC-offset. Further improvements in the chemistry may eliminate this factor, but its overall impact is small next to the other two.

The second factor is that currently available polymerases have trouble incorporating even an unmodified nucleotide behind a tagged deoxynucleotide (typically 10% yield or less). Hence, at present, it makes no real difference if we use tagged deoxynucleotides or tagged terminators—in both cases, the primer does not extend any further. Every incorporation exponentially decreases the amount of DNA available for further incorporation, and so, the net signal drops with every sequenced base pair. The read length cut-off is where the net signal becomes comparable with the noise from other sources. Increasing DNA surface density would push the cut-off further along the base pair axis, but the surface density is limited on the upper end by quenching considerations and steric limitations. A better solution is to evolve polymerases whose
incorporation yield is not affected by tagged nucleotides. Cleavable nucleotides (31,32) promise to accomplish the same in a different way.

The third factor is misincorporation—in the absence of competition from other types of bases in the solution, the polymerase can make a mistake and put in the wrong base. This results in loss of synchronicity, or dephasing, among the DNA strands in the sample, and thus the signal shrinks while the background increases. This can be addressed by evolving stricter polymerases and/or by use of kinetics decay molecules to create competition among substrates.

When these techniques slow down the signal-to-noise falloff, read length will increase, while identification of repeats in the template sequence will become trivial through the multiplicity of the fluorescent signal.

Even at the current read length, µSBS is immediately applicable where the goal is to sequence only a few base pairs. We used 500 nl of reagents per feed—considerably less than is practical with conventional methods. The fundamental lower bound is the volume of the sequencing chamber, which is currently <500 pl; so, the potential for improvement is another three orders of magnitude. Although some fixed initial investment is always necessary to interface between the macro and micro worlds, it would ultimately be distributed over thousands of chambers on the chip (33).

The current readout length is also sufficient for single-nucleotide polymorphism (SNP) analysis. This field has received tremendous attention recently, with commercial platforms available from companies such as Pyrosequencing, ParAllele, Illumina, Orchid and Lynx, most of which are based around similar DNA polymerase extension reactions. The µSBS method is not yet as highly parallel as the industrial approaches, but in principle it can be extended to a similar scale. Microfluidic SNP analysis offers some advantages in fluid functionality, reagent consumption and integrability that may ultimately complement or improve the commercial platforms.

The scale of the µSBS devices couples with the detection system to require a very small amount of DNA for successful information extraction. The sequencing chamber contained <1 fmol of DNA, compared with 2 pmol in pyrosequencing (13), and at least tens of pmol in other schemes. The automated capillary method (2) reported a detection limit of 1 fmol per band, which introduces a factor of 600 for the total DNA amount necessary. The mass spectrometry method reported 5 fmol per fragment (8), or more (9), and so, suffers from the same factoring.

Such parsimonious requirements enable a further advantage of µSBS, namely, its integrability with a number of devices based on the same PDMS microfluidic technology but addressing different applications (34–36). The elimination of the need to switch back and forth between the macro and micro scales just for purposes of DNA sequencing, opens the way to a number of exciting applications, e.g. closed-loop evolution and analysis microfluidic systems.

The plumbing and surface chemistry described here are general and can serve as the basis platform for other sequencing schemes, such as pyrosequencing. Ronaghi et al. (13) used a four-enzyme system for incorporation, detection and waste elimination, and showed successful sequencing. However, the system has limitations (14), some of which are due to its solution-based format—each new extension reaction dilutes the sample and interferes with enzyme kinetics, while intermediate product accumulation limits read length. The system also loses accuracy in homopolymeric regions, because the primer extension takes longer, and the enzymatic nucleotide elimination activates too soon. The microfluidics and surface chemistry promise to eliminate all these problems. Anchoring of the template means intermediate products and nucleotides are removed fluidically without loss of DNA. This does away with enzymatic nucleotide elimination, and so, helps in homopolymeric regions. Each new feed is at the same optimal reagent concentration, which avoids dilution kinetics problems. In addition, the amount of DNA yielding the sequencing result in our system was 1 fmol versus the pyrosequencing amount of 2 (13) and 5 pmol (15). This parsimony would be important if the device is to be an integral part of a larger lab-on-chip system.

CONCLUSIONS

We have combined fluorescence, surface chemistry and microfluidic methods into a single PDMS device with broad applications to fluorescence studies of DNA. The system benefits from the standard microfluidic advantages of economy, speed and control, while our architecture combines parallel processing and individual addressability, and is thus significant to array applications. On the other hand, the novel surface chemistry described here ensures the DNA retention in the device and is the first demonstrated specific, robust and tunable grafting of DNA into PDMS microfluidic devices. This makes it an enabling technology for the related studies and applications, e.g. DNA pyrosequencing. Herein, we have proven the longevity and flow stability of the anchorage, as well as the longevity of our reagent prep. We have used the whole integrated system to demonstrate successful 3 bp DNA sequencing, which illustrates the developed technology and forms a major step towards microfluidic bulk-fluorescence DNA sequencing of longer read lengths, while the system remains fully integrable in PDMS lab-on-a-chip systems.

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