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PAPER

Thermoelectric method for sequencing DNA†

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This study describes a novel, thermoelectric method for DNA sequencing in a microfluidic device. The method measures the heat released when DNA polymerase inserts a deoxyribonucleoside triphosphate into a primed DNA template. The study describes the principle of operation of a laminar flow microfluidic chip with a reaction zone that contains DNA template/primer complex immobilized to the inner surface of the device's lower channel wall. A thin-film thermopile attached to the external surface of the lower channel wall measures the dynamic change in temperature that results when Klenow polymerase inserts a deoxyribonucleoside triphosphate into the DNA template. The intrinsic rejection of common-mode thermal signals by the thermopile in combination with hydrodynamic focused flow allows for the measurement of temperature changes on the order of 10^{-4} K without control of ambient temperature. To demonstrate the method, we report the sequencing of a model oligonucleotide containing 12 bases. Results demonstrate that it is feasible to sequence DNA by measuring the heat released during nucleotide incorporation. This thermoelectric method for sequencing DNA may offer a novel new method of DNA sequencing for personalized medicine applications.

Introduction

The genetic variations that cause a number of different diseases are now known.[1,2] Knowledge of how these genetic variations relate to disease will lead to the development of new therapeutics.[3] The comprehension of genetic variations in the overall population and the identification of specific genes of individuals through DNA sequencing will improve preventive medicine.[4] Despite improvements in sequencing technology, the expense of whole genome sequencing of an entire population is currently prohibitive. The identification of specific genes, in individuals, however, can be economically feasible and is a prerequisite for improvements in preventive medicine through the realization of personalized medicine. To achieve the promise of personalized medicine, new sequencing methods that are less expensive with the potential for more widespread application are needed in addition to those currently available for whole genome sequencing. One approach is the development of highly integrated devices that take advantage of miniaturization and simplified sequencing chemistry.[5]

Existing sequencing methodologies often provide more information than is needed for applications in personalized medicine like SNP detection.[6] Existing methods also suffer from limited read length, chemical cross-talk between neighbor wells, washing issues, false signal error due to chemical residue from prior reads, have inadequate accuracy, and are too expensive for widespread use.[4,7] Overall, low cost, high speed and good availability for physicians, patients, and researchers are important requirements for a new sequencing approach.[4] Therefore, there is a need for an inexpensive, easy to use technology that is specifically designed for these applications rather than for whole genome sequencing.

The Sanger method is the most accurate method of sequencing DNA. The Sanger DNA sequencing technology is based on DNA synthesis with incorporation of normal deoxyribonucleoside triphosphates (dNTPs) as well as dideoxyribonucleoside triphosphates (ddNTPs) also known as chain terminators that cause DNA polymerization of the growing DNA chain to cease. Terminated DNA chains are separated by size and the DNA sequence is deduced by identifying the ddNTP that terminated the synthesis of the DNA chain using fluorescently-labeled ddNTPs.[8] Other commercially available technologies are based on the real-time synthesis of DNA.[9] In methods based on the real time synthesis of DNA, DNA polymerase is used to replicate a template and the identity of each base is noted after its complimentary base is incorporated into the growing strand.[10,11,12,13,14,15]

By way of example, Pyrosequencing[15,16] is a real-time, DNA synthesis method, incorporating DNA polymerase, ATP sulfurylase, firefly luciferase, adenosine 5′ phosphosulfate (APS) and apyrase in a solution that contains a primed DNA template.

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A nucleotide incorporation event causes the release of pyrophosphate in a quantity proportional to the number of incorporated nucleotides. Pyrophosphate release triggers the following cascade of enzyme reactions that emit light, the intensity of which is proportional to the number of incorporated nucleotides and the amount of DNA.

$$(DNA)_n + dNTP \xrightarrow{\text{DNA polymerase}} (DNA)_{n+1} + PP_i \quad (1)$$

$$PP_i + APS \xrightarrow{\text{ATP sulfurylase}} ATP + SO_4^{-2} \quad (2)$$

$$ATP + \text{luciferin} + O_2 \xrightarrow{\text{luciferase}} AMP + PP_i + \text{oxyluciferin} + CO_2 + hv \quad (3)$$

$$ATP \xrightarrow{\text{apyrase}} ADP + P_i \xrightarrow{\text{apyrase}} AMP + P_i \quad (4)$$

$$dNTP \xrightarrow{\text{apyrase}} dNDP + P_i \xrightarrow{\text{apyrase}} dNMP + P_i \quad (5)$$

In reactions (1) through (5), $(DNA)_n$ and $(DNA)_{n+1}$ are DNA molecules with n and $n + 1$ base pairs, respectively, dNTP represents one of the [three natural] deoxyribonucleoside triphosphates (dCTP, dGTP or dTTP) or dATPαS which is substituted for dATP, PP_i is pyrophosphate, APS is adenosine 5′-phosphosulfate, ATP is adenosine triphosphate, SO_4^{-2} is sulfate ion, O_2 is oxygen, AMP is adenosine monophosphate, P_i is inorganic phosphate, dNDP is deoxyribonucleoside diphosphate, dNMP is deoxyribonucleoside monophosphate and hv is light. Apyrase is added to the reaction mixture to degrade unincorporated nucleotides. To expand read length and simplify primer design, ssDNA-binding protein is included.[17] Unfortunately, the read length in Pyrosequencing is limited by the stability of the enzyme system, misincorporation of nucleotides, dilution of the reaction volume, enzyme contaminants, inefficient nucleotide degradation, and difficulty in determining the number of incorporated nucleotides in homopolymeric regions due to the nonlinear light response following the incorporation of more than 5–6 identical nucleotides.[18]

In this paper, we present a novel, thermoelectric sequencing method utilizing a microfluidic platform that allows measurement of the heat released when DNA polymerase inserts a deoxyribonucleoside triphosphate into a primed DNA template without stringent control of the ambient temperature or thermopile reference junction temperature that requires only picomol quantities of DNA.

Overview of the proposed thermoelectric method for sequencing DNA

The thermoelectric DNA sequencing method presented in this paper measures the heat generated in reaction (1) above and eliminates the need for reactions (2) through (5). Like other sequencing by incorporation methods, single-strand DNA of unknown sequence serves as a template for the production of a complementary nucleic acid polymer by a polymerase enzyme. The single-strand DNA template is hybridized to an appropriate complimentary oligonucleotide primer. The resulting DNA template/primer is attached to a segment of a microfluidic device channel wall in close proximity to the measuring junctions of a thin-film thermopile to form a DNA template/primer/support complex. This complex is then exposed to a laminar flow stream of buffer solution introduced into the microfluidic device at an inlet (Inlet 1) that is proximal to the location of the DNA thereby filling the channel of the microfluidic device with a continuous flow of buffer. A second buffer solution containing, DNA polymerase, and one of the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP) is introduced into the channel through a second inlet (Inlet 2) at a location downstream from Inlet 1 and undergoes hydrodynamic focusing[19] such that it flows only over the region of the channel where the DNA template is immobilized over the measuring junctions of the thermopile. If the nucleoside that is added *via* Inlet 2 is complementary to the next base in the DNA template, polymerization occurs lengthening the complementary polymer and releasing thermal energy. In homopolymeric regions where more than one nucleotide is incorporated (*e.g.* A, A, A, …), the amount of thermal energy released is directly proportional to the number of nucleotides that are incorporated. The released thermal energy increases the temperature of the DNA template/primer/support complex causing a transfer of thermal energy from the complex to the fluid flowing in laminar flow over the complex and to the channel wall to which the DNA template is attached. A thin-film thermopile detects the temperature difference of the channel wall between the hydrodynamically focused portion of the laminar flow stream that is near to or in contact with the DNA template/primer/support complex and the portion of the laminar flow stream that is not hydrodynamically focused and remote from the double-strand DNA template/primer/support complex. The change in thermopile emf resulting from the increased temperature difference is measured with a null voltmeter. Theoretically, if the introduced dNTP is not complimentary to the next unpaired base, no change in channel wall temperature (thermopile emf) is measured Once the temperature of the channel wall has returned to baseline, the next base is added. By repeatedly introducing dNTPs sequentially, the entire unknown sequence of the DNA molecule (template) is determined. The amount of heat produced following the incorporation of a nucleotide may be amplified by including pyrophosphatase to hydrolyze the released pyrophosphate. The total amount of heat generated is dependent upon the amount of DNA reacting.

Because very small quantities of DNA are used in DNA sequencing, the measurement of the small amount of heat that is released during a nucleoside insertion event is challenging and usually performed in a temperature controlled calorimeter.[20] In this study, we avoided the need for stringent temperature control by exploiting the high common mode rejection ratio of thin-film thermopiles (Fig. S2†).[21]

The heat generated during a nucleotide incorporation event

The energetics of DNA polymerization strongly favors the addition of deoxyribonucleosides to the growing DNA strand because the high-energy bond between the γ phosphate and the β phosphate of dNTP is broken and the nucleotide is transferred to the growing DNA with a lower-energy phosphodiester bond between nucleotides. The equation for the introduction of each

nucleotide unit may be written as in eqn (6) below which includes the heat of the reaction, ΔH_r^{DNA}. In the presence of pyrophosphatase the pyrophosphate formed undergoes subsequent enzymatic hydrolysis as shown in eqn (7) releasing an additional amount of heat, $\Delta H^{PP_i}{}_r$.

$$(DNA)_n + dNTP \xrightarrow{\text{DNA polymerase}} (DNA)_{n+1} + PP_i + \Delta H_r^{DNA} \quad (6)$$

$$PP_i + H_2O \xrightarrow{\text{pyrophosphatase}} 2P_i + \Delta H_r^{PP_i} \quad (7)$$

The pyrophosphate hydrolysis reaction, eqn (7), has a large negative standard free energy change of at least −7.3 kcal mol^{-1} [22] and potentially as high as −10.9 kcal mol^{-1}.[20] If pyrophosphatase is present, the equilibrium for reaction (6) is driven further toward chain elongation and two high-energy phosphate bonds are cleaved to provide the energy needed to make each internucleotide DNA linkage.

Exothermic heats between −9.8 and −16.0 kcal mol^{-1} base-pair^{-1} have been measured for template-directed DNA polymerization using stopped-flow calorimetry.[20] The generated heat is the end result of a number of events including dNTP to dNMP hydrolysis, phosphodiester bond formation, hydrogen bond formation, and enzyme conformational changes. It is also dependent upon base identity.[20] For example, the enthalpy of dTTP incorporation (−12.3 kcal mol^{-1} base-pair^{-1}) is less than the enthalpy of dATP incorporation (−15.1 kcal mol^{-1} base-pair^{-1}).[20] Reported values for the standard molar enthalpy change for the hydrolysis of pyrophosphate, reaction (7), range from −37.0 kJ mol^{-1} (equivalent to −8.38 kcal mol^{-1}) to −12.2 kJ mol^{-1} (equivalent to −2.91 kcal mol^{-1}) depending upon the buffer used or the experimental method used to measure the enthalpy change.[23–25] The maximum and minimum total enthalpy for the two reactions is −4.38 kcal mol^{-1} base-pair^{-1} (−102.1 kJ mol^{-1} base-pair^{-1}) and −12.71 kcal mol^{-1} base-pair^{1} (−53.2 kJ mol^{-1} base-pair^{-1}), respectively, for each nucleotide incorporation event. The negative sign indicates that the overall process is exothermic and that heat is released to the surroundings.

Materials and methods

Experimental set-up

We performed thermoelectric DNA sequencing using the microfluidic device with integrated thin-film thermopile that is shown in Fig. 1. The side view (not to scale) shows the assembled device layers. Features in the top and side views of the microfluidic device include two, 1/16 inch OD, inlet ports (Upchurch Scientific, Oak Harbor, WA) attached over holes drilled into a glass microscope slide (Electron Microscopy Sciences, Hatfield, PA); a flow channel; a rectangular region where the DNA template/primer/complex is immobilized to the inner surface of a streptavidin coated glass coverslip (Arrayit, Corporation, Sunnyvale, CA); an antimony/bismuth, thin-film thermopile that is attached to the external surface of the coverslip; and a channel outlet port (Upchurch Scientific, Oak Harbor). The 100 micron deep flow channel is formed using xurography, a polymer/glass fabrication technique employing polyimide tape coated on each side with silicone adhesive (KaptonTape.com).[26] During operation, buffer solution is

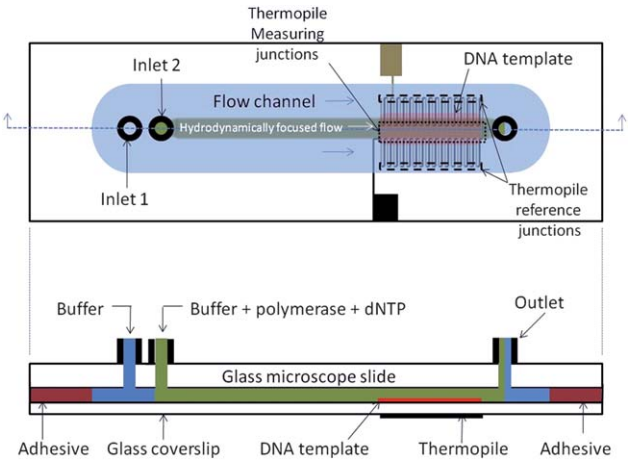

Fig. 1 Microfluidic thermoelectric sequencing device. The buffer is injected through inlet 1 while the dNTP sample is supplied through Inlet 2. The buffer carrying the sample through Inlet 2 undergoes hydrodynamic focusing and flows over the DNA template that is immobilized over the measuring junction of the thermopile.

independently introduced into the two inlet ports. The flow rates through the two inlets are adjusted such that the flow through Inlet 1 hydrodynamically focuses the fluid entering the device *via* Inlet 2. By adjusting the ratio of the flows through the two inlets, the fluid introduced through Inlet 2 flows down the centerline of the device over the immobilized DNA template and the measuring junctions of the thermopile. The fluid introduced through Inlet 1 flows only over the reference junctions of the thermopile. Laminar flow prevents the two fluid streams from mixing.[27,19] Thermal events common to both the reference and measuring junctions are rejected by the thermopile. This eliminates the need for careful control of the ambient and reference junction temperatures. To initiate sequencing, a small sample containing buffer, Klenow polymerase, and one of the dNTPs is introduced as a bolus into the Inlet 2 flow stream. When the sample reaches the template, heat is released if polymerase inserts the dNTP into the template and the temperature of the coverslip under the DNA template increases relative to the temperature of the coverslip not coated with DNA. The temperature change is detected by the measuring junctions of the thermopile but not by the reference junctions. If the dNTP is not inserted, no temperature change is detected.

Thermoelectric sequencing system. A schematic of the experimental DNA sequencing system is shown in Fig. S1.† Two Harvard Apparatus, Model '11' Plus syringe pumps (Harvard Apparatus, Holliston, MA) provide for the independent injection of buffer solutions through 0.01 inch internal diameter Teflon (ETFE) tubing (Upchurch Scientific, Oak Harbor, WA) into the inlet ports of the microfluidic sequencing device. A 0.005 inch internal diameter sample loop of known volume (Upchurch Scientific, Oak Harbor, WA) is loaded with polymerase and one of the four nucleosides using a microlitre glass syringe (Hamilton, Reno NV). The sample is injected into the buffer stream being supplied to Inlet 2 using a 6-Port Injection valve, (Model V-451, Upchurch Scientific, Oak Harbor, WA). The thermopile voltage is measured with an Agilent, Model 34420A nano voltmeter

(Agilent, Inc., Loveland, CO). The output of the nano voltmeter is recorded and processed using LabView SignalExpress (National Instruments Corporation, Austin, TX) executed on a digital computer (www.dell.com).

Thermopile manufacturing. Antimony/bismuth thermopiles with 60 thermocouple junction pairs were fabricated on 100 μm polyimide supports using a Denton model DV-502B metal evaporation system (Denton Vacuum, Moorestown, NJ). Custom designed metal shadow masks containing the patterns for creating the thermopile's thin metal lines were manufactured to our specifications by Town Technologies Inc. (Town Technologies Inc., Somerville, NJ). A rectangular piece of 100 micron thick polyimide (Kapton®, www.kaptonfilm.com) was placed behind the shadow mask designed to create the bismuth line pattern and suspended above the evaporator heat source. Bismuth metal (bismuth shot, tear-shaped, −4+30 mesh, 99.9%, Sigma-Aldrich Chemicals, www.sigmaaldrich.com) was heated until vaporized, and the vapors were allowed to condense on the support. The shadow mask containing the antimony line pattern was carefully aligned to overlap with the bismuth lines at the thermocouple junctions. The metal evaporation process was repeated using antimony metal (antimony shot, 1–2 mm, 99.999%, Sigma-Aldrich Chemicals, www.sigmaaldrich.com). Following deposition of the antimony, the thermopiles were removed from the chamber, tested for electrical continuity, and protected from physical damage using thin polyimide tape. The thermopiles were attached to the streptavidin-coated coverslip using a small quantity of cyanoacrylate adhesive (Scotch Single-Use Super Glue, 3M, St. Paul, MN).

Sequencing reagents. A biotinylated primer (5′GGA CTA TAA AGA TAC CAG GCG TT) and the oligonucleotide (5′TTA ACC GGT ACG AAC GCC TGG TAT CTT TAT AGT CCA TC) which have been designed for pyrosequencing system quality control assessment[28] were synthesized and HPLC purified by IDT (Coralville, IA). The oligonucleotides were mixed with STE annealing buffer (Sigma-Aldrich, St. Louis, MO) to obtain a final concentration of 10 pmol μL^{-1} (100 μM). An annealing procedure was performed by heating the oligonucleotides at 80 °C for 4 min and then cooling to room temperature for 60 min. The annealing of the oligonucleotides was verified using Hi-Res melting analysis performed on a LightScanner 32 instrument (Idaho Technology Inc., Salt lake City, UT). The hybridized oligonucleotides were stored at −20 °C in small aliquots.

Primed DNA oligonucleotide was concentrated in an Eppendorff vacuum concentrator, suspended in 1xSSC buffer, and immobilized on the streptavidin covered coverslip *via* biotin–streptavidin interaction (Arrayit Corporation, Sunnyvale, CA). The DNA was immobilized on a rectangular area of the coverslip over the measuring junctions of the thermopile. The coverslip was incubated in a humidity chamber (37 °C, 90% humidity) for 15 min and washed using 1× SSC buffer for 2 min followed by deionized water wash for 5 min. The dNTPs (Promega Corporation, Madison, WI), Klenow polymerase, exonuclease minus (Promega Corporation, Madison, WI) and inorganic pyrophosphatase (New England Biolabs, Ipswich, MA) were mixed with 1x Klenow buffer (50 mM Tris-HCl, 10 mM $MgSO_4$, 0.1 mM DTT).

Microfluidic device manufacture. The microfluidic device had two inlets and one outlet and a single laminar flow channel. The device was manufactured using Xurography. A cutting plotter (Graphtec America Inc., Santa Ana, CA) was used to form the microfluidic channel out of double sided Kapton® tape. The shape of the channel was designed using Adobe Illustrator (Adobe, San Jose, CA). The channel width was 12 mm and the channel length was 66 mm. The Kapton® tape was sandwiched between a 25 × 75 mm plain glass microscope slide and a 25 × 75 mm streptavidin coated coverslip that contained the immobilized primed DNA template.

Data processing. Voltmeter measurements were recorded every second using LabView SignalExpess 2009 software (National Instruments, Austin, TX). The raw data results were exported to Microsoft Excel 2007 and the baseline drift was corrected using MatLab 7.5.0 (The MatLab Inc.). After removing the baseline drift, the area under the curve (AUC, μVs) was calculated for each injection by integrating the area under the voltage *versus* time profile using the trapezoid rule.

Results

The feasibility of this novel thermoelectric method for DNA sequencing was confirmed in a series of experiments that were designed to optimize the DNA sequencing chemistry and to study the effects of several operating parameters on the thermopile response to nucleotide incorporation. The parameters investigated included the location of the immobilized oligonucleotide/primer template, the injection sample volume, the dNTP concentration, the effect of pyrophosphatase, the effect of buffer salt content (KCl), and changes in the channel height.

DNA template location

In two separate experiments, the oligonucleotide/primer template was immobilized either in a centrally located, 4 mm wide strip along the entire length of the microfluidic channel or over the 4 mm × 8 mm area defined by the measuring junctions of the thermopile. A total of 15 pmols of template were immobilized along the length of the microfluidic device, and three pmols were immobilized over the measuring junctions of the thermopile. In these experiments, the injection sample volume was 5 μL, the flow rates were 70 $\mu L\ min^{-1}$ for Inlet 1 and 25 $\mu L\ min^{-1}$ for Inlet 2, and the channel height of the microfluidic device was 100 μm. The total amounts of dNTPs and Klenow fragment injected were 67.5 nmol and one unit, respectively. One unit of Klenow Polymerase is defined as the amount of enzyme required to catalyze the incorporation of 10 nmol of deoxynucleotide into acid-precipitable material in 30 min at 37 °C. The calculated areas under the curve (AUC, μVs) for each nucleotide injection, and the response of the thermopile following the injection of dCTP nucleotide for each of these experiments are shown in Fig. 2. Immobilizing the template over the measuring junctions of the thermopile decreased the average duration of the signal by 150 s and increased the height of signal response peak by 800 nV (Fig. 2b and 2c) while increasing the total amount of heat that was detected by the thermopile (Fig. 2a).

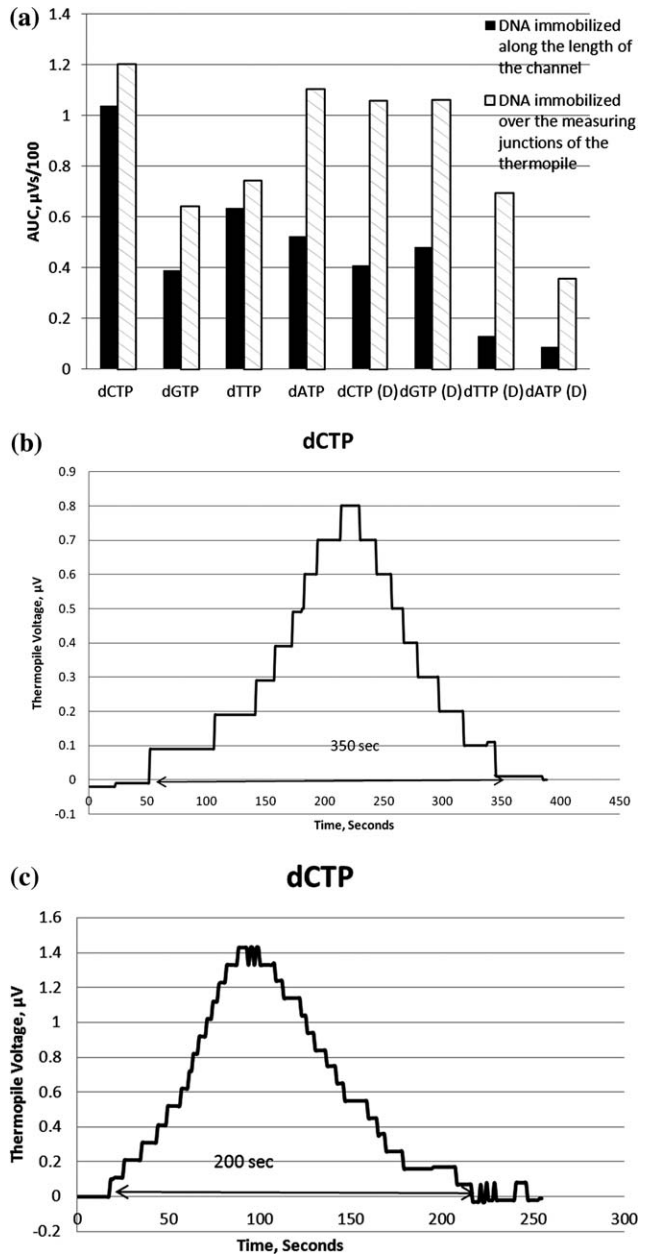

Fig. 2 Effect of DNA template location on the thermopile response. (a) Peak areas for each dNTP injection. dNTP (D) represents the total heat generated after incorporation of a sequence of two nucleotides in a homopolymeric region. (b) Thermoelectrogram of the thermopile response after dCTP injection. Template is immobilized along the length of the device, 5 μL injection volume. (c) Thermoelectrogram of the thermopile response after dCTP injection. Template is immobilized over the measuring junctions of the thermopile, 5 μL injection volume.

Injected sample volume

Sequencing experiments were also performed to evaluate the effect of injected sample volume on the DNA sequencing reaction. In these experiments, the sample volume was increased to 13 μL and the DNA was immobilized only over the area of the coverslip directly above the measuring junctions of the thermopile. The wash buffer flow rate (Inlet 1) was kept at 70 μL min^{-1} and the dNTP/Klenow polymerase mixture flow rate (Inlet 2) was kept at 25 μL min^{-1}. The channel height was 100 μm. The total amounts of dNTPs and Klenow injected were increased to 182.25 nmol and 2.7 units, respectively. Fig. 3 shows the calculated areas under the curve for each nucleotide injection and the thermopile response after the injection of dCTP using

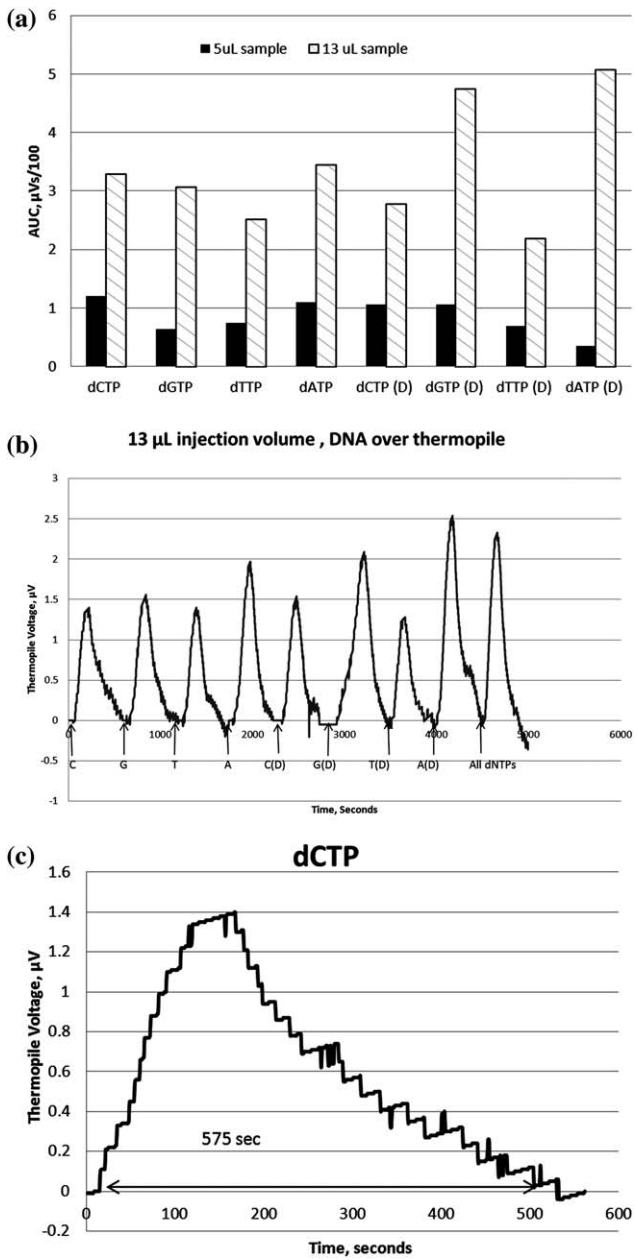

Fig. 3 Effect of sample size on the thermopile response. (a) Peak areas for each dNTP injection. dNTP (D) represents the total heat generated after incorporation of sequence of two nucleotides in a homopolymeric region. (b) Thermoelectrogram for sequencing of the whole template. 13 μL injection sample volume, 70 μL min^{-1} Inlet 1 flow rate, 25 μL min^{-1} inlet two flow rate, 182.25 nmol dNTP and 2.7 units Klenow polymerase. (c) Thermoelectrogram of the thermopile response after dCTP injection when the template is immobilized over the measuring junctions of the thermopile and the injection sample volume is 13 μL.

these parameters. Increasing the sample volume increased the total amount of heat that was detected after each injection (Fig. 3a) as well as the duration of the response (Fig. 3b).

dNTP concentration, pyrophosphatase and 1x polymerase buffer (250 mM KCL)

To increase the rate of nucleotide diffusion and DNA polymerization and to increase the magnitude of the thermopile signal, the concentration of dNTPs was increased and pyrophosphatase was added to the injection sample mixture. The salt content of the 1× polymerase buffer was also increased by adding 250 mM KCL to promote more efficient binding between Klenow polymerase and the DNA template.[29] The flow rates used for these experiments were 50 μL min^{-1} and 25 μL min^{-1} for Inlet 1 and inlet 2, respectively. The channel height was 100 μm and the sample injection volume was 13 μL. The sample injection mixture contained 540 nmol of the complimentary dNTP and 0.07 units of inorganic pyrophosphatase. Klenow fragment was injected separately and allowed to bind to the DNA template prior to the injection of the nucleotides. To assure that the maximum number of binding sites reacted, each nucleotide was injected twice and the total areas under the curve for each injection were summed. Fig. 4 shows the summed areas under the curve for each base in the sequenced template and the thermopile response after the polymerization of dCTP. Increasing the dNTP concentration and including pyrophosphatatse to the reaction mix increased the total amount of heat that was detected after each injection (Fig. 4a) as well as the height of the peak response (Fig. 4b).

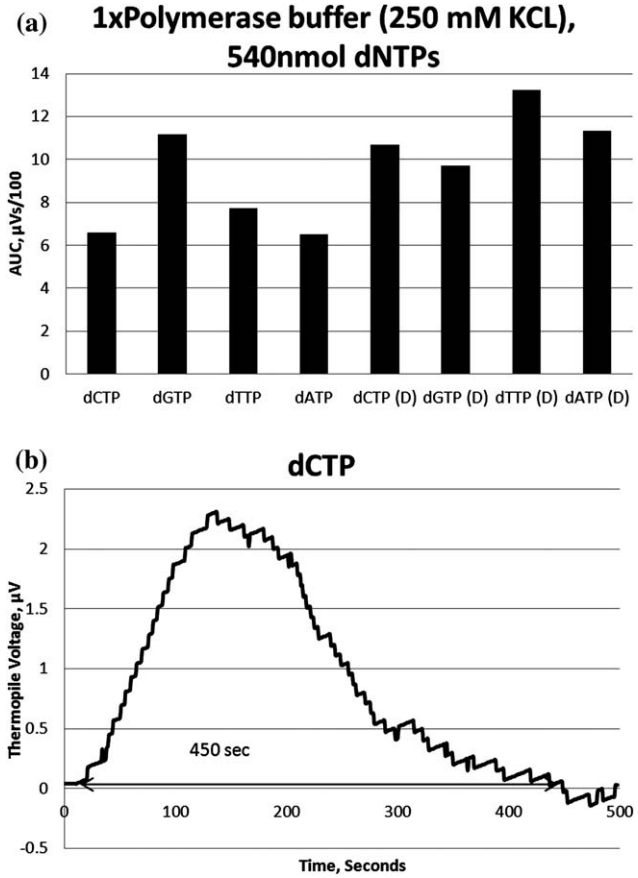

Fig. 4 Effects of adding pyrophosphatase, increased dNTP concentration, and 1× polymerase buffer salt (250 mM KCl) on the thermopile response. (a) Peak areas for each dNTP injections. dNTP (D) represents the total heat generated after incorporation of sequence of two nucleotides in a homopolymeric region. (b) Thermoelectrogram of the thermopile response after dCTP injection.

Channel height

To test the effect of the channel height on the signal response during a polymerization event, two experiments were performed using microfluidic devices with channel heights of 100 μm and 25 μm, respectively. The flow rates were 50 μL min^{-1} and 25 μL min^{-1} for Inlet 1 and Inlet 2, respectively. The amounts of dNTPs and pyrophosphatase that were injected were 540 nmol and 0.07 units, respectively. During both experiments, 3.65 units of Klenow polymerase were injected prior to the injection of the nucleotide mix. Fig. 5 compares the heat generated (AUC) and the thermopile response following the injection of the first complimentary nucleotide dCTP for each channel height. Reducing the channel height significantly increased the total amount of heat that was generated in a single injection (Fig. 5a) as well as the peak height and the duration of the response (Fig. 5b).

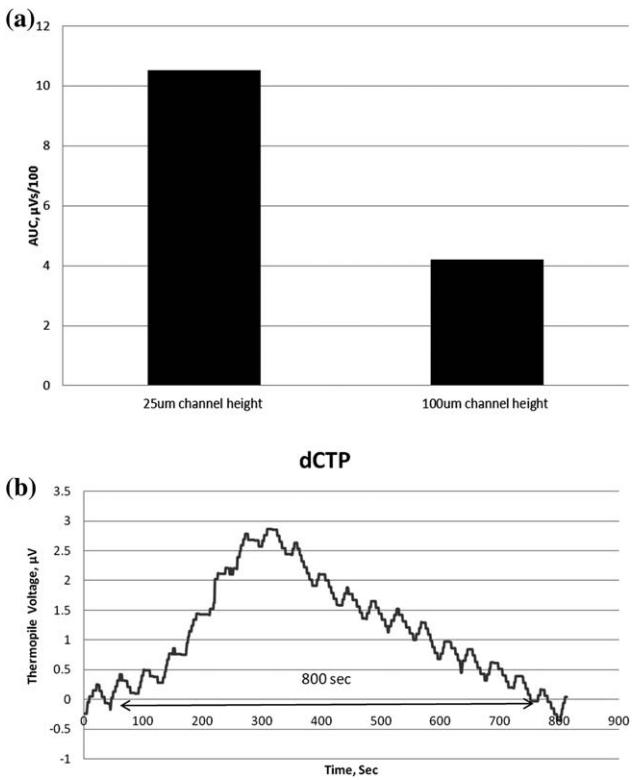

Fig. 5 Effect of channel height on the thermopile response. (a) Peak areas for each dCTP injection. (b) Thermoelectrogram of the thermopile response after dCTP injection using 13 μL injection sample volume and microfluidic device with 25 μm channel height.

Discussion

This novel method for thermoelectric DNA sequencing has been used to sequence 12 base pairs of primed oligonucleotide having the following structure:

Biotin-5'- gga cta taa aga tac cag gcg tt-3′

3'- cta cct gat att tct atg gtc cgc aa G CAT GGC CAA TT-5'

A single incorporation of each nucleotide (dCTP, dGTP, dTTP, dATP) was followed by incorporation of a sequence of two identical nucleotides in a homopolymeric region (dCTP(D), dGTP(D), dTTP(D), dATP(D). The results presented in Fig. 4a confirm that the total heat that was generated after polymerization of dNTP in a homopolymeric region is larger than the heat that is released after a single injection of dNTP. Based on our results, it should be possible to use the technology to sequence a larger number of base pairs and homopolymeric regions.

We investigated several factors that can affect the quality of the thermoelectric sequencing results. Location of the sequenced template is an important factor in obtaining good sequencing data. Immobilizing the template over the measuring junctions of the thermopile generated a more rapid thermopile response and increased the magnitude of the peak height of the resulting thermopile signal (Fig. 2a, 2b and 2c). A faster thermopile response during a polymerization event and more rapid heat dissipation following the event increases the number of base pairs that can be sequenced per unit time. Localizing the reaction over a small area above the thermopile measuring junctions eliminates the need to dissipate heat along the entire length of the channel proximal to the thermopile.

Increasing the sample injection volume increases the total amount of heat generated, the duration of the thermopile response and the magnitude of the thermopile response peak height (Fig. 3a, 3b and 3c) because there is more time for the reactants to diffuse to the polymerase/DNA template complex resulting in more polymerization. Because more heat is generated, however, it takes longer for the generated heat to dissipate and as a result the duration of the thermopile response increases from an average of 200 s to an average of 600 s (Fig. 3c). The rate of heat dissipation can be increased by increasing the flow rates but at the expense of signal strength.

Adding pyrophosphatase to the reaction mixture, increasing the salt concentration (250 mM KCl) of the 1× polymerase buffer, and increasing the concentration of dNTPs significantly increased the magnitude of the thermopile response (Fig. 4a and 4b). This is caused by several factors. Klenow polymerase binds more tightly to the DNA template when the buffer contains 250 mM KCl.[29] In addition, when Klenow polymerase is injected separately from the nucleotides prior to the beginning of a sequencing experiment it does not dissociate between each dNTP injection. Because polymerase is a large protein, if it is injected simultaneously with the dNTPs and allowed to co-diffuse excess amounts of it can interfere with the diffusion of the dNTPs and hinder the polymerization reaction. This effect may account for the smaller magnitude of the thermopile response that was measured in the experiments in which the polymerase and dNTPs were combined in the injected sample mixture. Higher dNTP concentration increased the rate of diffusion of dNTP and hence the rate of the diffusion limited polymerization reaction.

The summed areas under the curve for experiments in which each nucleotide was injected twice show that the total heat generated during double injections of the same nucleotide is more than the heat generated following a single nucleotide injection (Fig. 4a). We only observed one exception to this, the incorporation of dGTP, and in this case we observed that the magnitude of the signal was reduced by the presence of a bubble in the microfluidic device. Bubble formation in the device affects the thermopile response by altering the width of the hydrodynamically focused stream that passes over the thermopile. We did not observe twice the amount of heat generation following double-nucleotide incorporation relative to single incorporations of the same nucleotide as was theoretically expected. The non-linear response that was detected in homopolymeric regions is likely caused by the fact that not all sites available for polymerization were sequenced during a single nucleotide injection. Incomplete extension of the available sites is most likely caused by limited diffusion of the dNTPs, DNA fragmentation, and fluctuation of the flow rates. Incomplete polymerization of all available sites during a single nucleotide injection causes some of the template strands to get out of sequence. Decreasing the channel height to 25 μm decreased the dNTP diffusion distance, enhanced mass transport of the dNTPs to the sequencing template, and increased the magnitude of the thermopile signal (Fig. 5a).

The same factors account for the discrepancies in the total heat of the reaction that was measured after dTTP and dATP polymerization. Theoretically, the enthalpy of dATP incorporation is larger than the enthalpy of dTTP insertion.[22] Occasionally, we recorded heats of polymerization for dATP that were smaller than dTTP (Fig. 2a, 3a and 4a). This discrepancy is likely due to incomplete polymerization of all available sites and flow rate changes following dNTP sample injection.

The reproducibility of the measurements depends on several other factors. These factors include: the amount of DNA immobilized to the surface of the microfluidic device, the location of the DNA template relative to the measurement junctions of the thermopile, the efficiency of the annealing procedure, and the Seebeck coefficient of the thermopile.

To test the performance of the system when a non-complimentary nucleotide was introduced, either dTTP, dGTP or dATP was injected separately at the beginning of the experiment instead of the complimentary dCTP. No signal or a small response was detected by the thermopile (Fig. S4†) after these injections. The total heat measured when a response was detected was less than the response when the complimentary nucleotide was inserted. We cannot explain why a small signal was measured in some cases without performing additional experiments. The signal could be caused by degradation of the primer or dNTP misincorporation by Klenow Polymerase.[18] Further investigations are required to better understand the underlying causes of this issue.

Future improvement to the thermoelectric method for DNA sequencing will include adding single-stranded DNA binding protein (SSB) to the reaction mixture prior to sequencing and more careful control of flow rate. In previous reports, SSB has been successfully used to decrease the loss of signal that is caused

by DNA fragmentation.[18] Careful observations of the flow rates following injection of the sample showed that the flow rate can fluctuate as a result of pressure changes caused by the injection valve. The flow rate is affected by the back pressure that is created by the sample loop after the injection of the sample through the injection valve. These flow rate variations influence the hydrodynamic focusing of the two buffers and can affect the size of the reaction zone within the microfluidic device. This can cause different amounts of DNA to undergo polymerization during each injection and cause different DNA templates to be out of sequence. Further improvements of the system will require the application of back pressure regulators to correct for the fluctuation in the flow rates.

Small variations in pressure within the microfluidic device caused by the syringe pump and bubble formation can produce fluctuations in the thermopile signal.[30] The noise level when the channel height was 100 μm was approximately 10 nV. The noise caused by the syringe pump is observed as small 400 nV peaks of 50 s duration along the baseline. This noise was more prominent when the channel height was decreased to 25 μm (Fig. 5b).

Another factor that can potentially affect DNA sequencing results is the self-looping of unannealed primer. The primer used for this set of experiments does not form self-loops (Fig. S3†).[31,32]

Future modification of the microfluidic device design could include further reducing the channel height, increasing the sample volume, increasing flow rates, and adding pressure regulators to eliminate flow rate changes following nucleotide injection.

The thermoelectric DNA sequencing system was designed with the potential for application for SNP detection and genotyping of short DNA segments. The advantages of this system when compared to the current technologies available for DNA sequencing are simplicity of the system, low cost of reagents and simplified sequencing chemistry. The number of base pairs that can be sequenced per unit time depends whether or not the temperature increase of the system following one or more incorporation events can be dissipated quickly enough for applications in personalized medicine. The time required to dissipate the heat is a function of the thermal properties of the reaction zone and the rate of flow though the microfluidic device. Increasing flow will reduce the time required to dissipate the generated heat but decreases the magnitude of the temperature change detected by the thermopile. This can be overcome by temporarily increasing the flow rate following the incorporation event and then decreasing it prior to the next nucleotide injection. Because the proposed application of the thermoelectric sequencing technology is not primarily directed toward whole genome sequencing, sequencing speed is less important than in technologies designed for whole genome sequencing such as Sanger method or pyrosequencing.

The pyrosequencing method has been adapted for high throughput whole genome analysis by 454 Life Sciences, Branford, Conn. (www.454.com). Although The 454 sequencing technology can sequence larger number of base pair per unit time than the thermoelectric method for DNA sequencing, pyrosequencing suffers from chemical cross-talk, complex chemistry related to the need to produce chemiluminescent light, and excessive cost.[7] The thermoelectric method for DNA sequencing overcomes these issues by simplifying the chemistry and enzyme reaction, reducing the cost by miniaturization of the reagents and replacing expensive photon detectors with inexpensive voltage meters.

Williams *et al.* were the first to propose a calorimetric sequencing by synthesis method with the advantage that the complex chemistry associated with pyrosequencing is eliminated.[33] The Williams' method uses traditional calorimetry with integrated thermopiles to measure the temperature change of a reacting mixture contained within a chamber of finite volume when a nucleotide is inserted into a DNA template by polymerase. The disadvantage of this method is that, the detection of the small temperature change resulting from nucleotide insertion is only possible with stringent control of the ambient temperature and the thermopile reference temperature. Recently, Esfandyapour *et al.*, investigated the feasibility of thermosequencing by monitoring the heat generated in DNA polymerization using a isothermal titration calorimeter with a 1.4 mL volume and in a separate method with an infrared microscope.[7] Like the Williams method, however, thermal noise is eliminated by stringently controlling the ambient temperature and thermopile reference junction temperature. The major advantage of the proposed thermoelectric method for DNA sequencing over other calorimetric methods is the fact that it does not require control of the thermopile reference junction or the ambient temperature. In both the Williams's method and Esfandyapour's thermosequencing technology, a large reaction volume causes longer measurement time and excessively large amounts of DNA are needed to generate a measureable temperature. The novel method for DNA sequencing presented in this report overcomes these disadvantages by utilizing only three pmol of nucleic acid template to generate a detectable signal.

Conclusions

We have designed and fabricated a microfluidic device with an integrated thermopile that can be used for DNA sequencing applications. A DNA sequence consisting of 12 base pairs has been successfully sequenced. The results in this work confirm the feasibility of the thermoelectric method for DNA sequencing.

A major advantage of the proposed thermoelectric method for DNA sequencing is its simplicity. The method simplifies the sequencing chemistry by eliminating the pyrosequencing reactions that produce light. This decreases the cost of reagents and replaces expensive photon counters with inexpensive voltage detectors. The thermoelectric method might increase the potential for miniaturization and parallel processing. It promises longer read lengths because most of the causes of limited read length in current synthesis by incorporation methods arise from the complex chemistry associated with the generation of light. Additionally, the sequencing of homopolymeric regions of DNA should be possible if diffusion limitations are overcome because theoretically the total heat released is linearly related to the number of nucleotides incorporated.

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