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Recent Improvement in Miniaturization and Integration of A DNA Analysis System for Rapid Forensic Analysis (MiDAS)

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Abstract

Integration, portability, and parallel multi-sample processing are some of the most desirable features for advanced forensic DNA analytical systems. Integration leads to "sample-to-answer" systems, which can provide rapid, sensitive, and reproducible DNA analyses. Portability brings the systems directly to the scene of forensic investigations and a capability of parallel multi-sample processing allows an enhanced analysis throughput. In order to achieve the above goals, in 2010 we developed the first version of the integrated microfluidic system for rapid forensic DNA analysis, or MiDAS, followed by the second version in 2012 with improvements on the control systems, microfluidic chip designs, and amplification chemistry. In this short communication, we convey our recent progress toward further integration and miniaturization of microfluidic components. This approach will provide more flexible capacity in analytical throughput as it follows: 1) Monolithic integration of a "sample collector module" within the entire analysis workflow, 2) A multi-layer 3D cartridge design which offers capability of parallel multi-sample processing, increases portability, and reduces footprint, 3) Integration of duckbill valves into the 3D cartridge for fluidic controls among the layers and for elimination of manual sealing procedure after on-cartridge reagents loading, and 4) Improvement of polymer valving and microfluidic circuitry around PCR module for minimizing interactions of the assay reagents and reaction products with the polymer materials to increase the yield and quality of the PCR product.

Abbreviations

MiDAS: miniaturized integrated DNA analysis system; PCR: polymerase chain reaction; CE: capillary electrophoresis; DBV: duckbill valve; STR: short tandem repeat; PIDF: proportional–integrative–derivative–forward; TEC: thermo-electric controller; PCB: printed circuit board

MiDAS (Miniaturized Integrated DNA Analysis System)

Short tandem repeat (STR) DNA profiles have been widely used in forensic DNA analysis and represent individual "fingerprints" [1]. In order to acquire the STR profile, numerous procedures including DNA extraction from a biological sample, purification of extracted DNA, amplification of purified DNA, and detection of amplified DNA [2] have to be precisely performed in a specific sequence. Due to the complex nature of the process, the analysis has been performed by highly trained technicians at laboratories equipped with several bench-top instruments. The lengthy process, which can be very costly and take up to 72 hours [3], has been a major hindrance in the widespread distribution of forensic analytical systems at the scene of investigations. This, in turn, leads to difficulties in obtaining timely investigations, which are very important in forensic application, as suspects can be released from custody during the analysis [4]. Due to the current situation, various solutions have been explored to achieve rapid STR DNA profiling [3,5-7] in an integrated format [8-11].

Among them, our group has been working on developing integrated microfluidic systems for rapid forensic DNA analysis. During the development, complete integration has been our utmost focus as it provides seamless interconnection among functional modules in a "swab-to-profile" system. Our ultimate goal is to develop a rapid, sensitive, portable, and reliable DNA analysis instrument with minimum user interventions.

With this motivation, we developed the first version of the integrated microfluidic system for rapid forensic DNA analysis, MiDAS (Miniaturized Integrated DNA Analysis System) [12]. This version of instrument represented one of few advanced integrated systems [13-15] for the analysis of multiplex STR DNA profiles with buccal swab reference samples. The MiDAS was composed of a DNA processing microfluidic cartridge made of disposable polycarbonate plastics, a printed circuit board (PCB) embedded with resistive heaters and control circuitry, power supplies for running polymerase chain reaction (PCR) and capillary electrophoresis (CE), a glass (or COC plastic) CE microchip, optical equipment for fluorescent DNA detection, and a computer to run a user-interface protocol. The cartridge contained NaCl-based electrochemical pumps and polymer-based valves which were electronically controlled by the system [16]. The on-cartridge pumps and valves transferred liquid sample and reagents for buccal DNA purification, amplification of the purified DNA by multiplexed PCR, denaturation of the amplified DNA, and delivery of the denatured DNA to the attached CE microchip. The amplified STR fragments, labeled with fluorescent dyes, were separated and detected with on-chip micro-capillary electrophoresis

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for DNA profile generation.

After the first version of MiDAS in 2010, we further optimized the multiplexed PCR performance for the second version in 2012 [17]. Refinements to the proportional–integrative–derivative–forward (PIDF) temperature control in the Peltier controller (TEC) resulted in faster and more reliable performance of PCR temperature cycling. The dual-stage valve actuators, which maintained positive pressure from the heated air cavity while the molten polymer was allowed to cool in the channel, resulted in a better seal of the PCR chamber during amplification. Increasing the mechanical clamping force to the cartridge, applied by the Peltier device assemblies, reduced delamination of the double sided pressure sensitive adhesive (ARcare 90106, Adhesives Research) around the PCR chamber, resulting in less loss of the PCR product between the cartridge layers. The addition of a dedicated release channel at the bottom of the PCR chamber allowed the distance between the valves and the heated (Peltiers) area to be sufficiently apart so that more reliable valve actuation could be obtained. All these changes increased the quality of the PCR amplification and recovery of the PCR product. The encapsulation of PCR reagents into a solid phase hydrogel bead format enabled the on-cartridge pre-package of PCR reagents and simplified integration of DNA purification and PCR processes. It also provided more efficient reagents release than lyophilized powder approaches and less imprecision and complexity than mixing liquid reagents.

Integrated DNA Processing Microfluidic Cartridge

On the first and second version of MiDAS, a sample swab had to be lysed and an aliquot of a sample lysate had to be introduced into the cartridge. To eliminate the initial off-system sample processing step, we designed the further integrated DNA processing cartridge for a complete swab-to-profile system, which integrates a "swab sample lysis module" [18] where a buccal sample collection swab can be simply dropped into its chamber as shown in Figure 1(a) and processed in the following protocol as shown in Figure 1(b). Once a buccal swab is loaded into a buccal swab processing chamber, 1 mL of lysis buffer is added into the chamber. 150 µL of lysate is metered and transferred to a lysate input chamber while rest of the lysate is stored in a lysate archive chamber. The lysate then, meets with a binding buffer and magnetic beads (CS11200, Life Technologies). After going through mixing chambers, DNA in the lysate binds to the magnetic beads in an incubation chamber. Washing buffer is flowed into a washing/elution chamber while the magnetic beads are captured with a permanent magnet in the MiDAS. Then, elution buffer elutes DNA from the magnetic beads and transfers the eluted DNA to a PCR chamber. Only 10 µL of the DNA solution is metered in the PCR chamber while rest is stored in a DNA archive chamber. After a PCR is processed with the protocol in table1, the amplified DNA fragments get transferred to a denaturation chamber and denatured in presence of formamide and internal lane standard (DG1521, Promega). Denatured nucleic acids are transferred to an integrated electrophoresis microchip and separated with the protocol

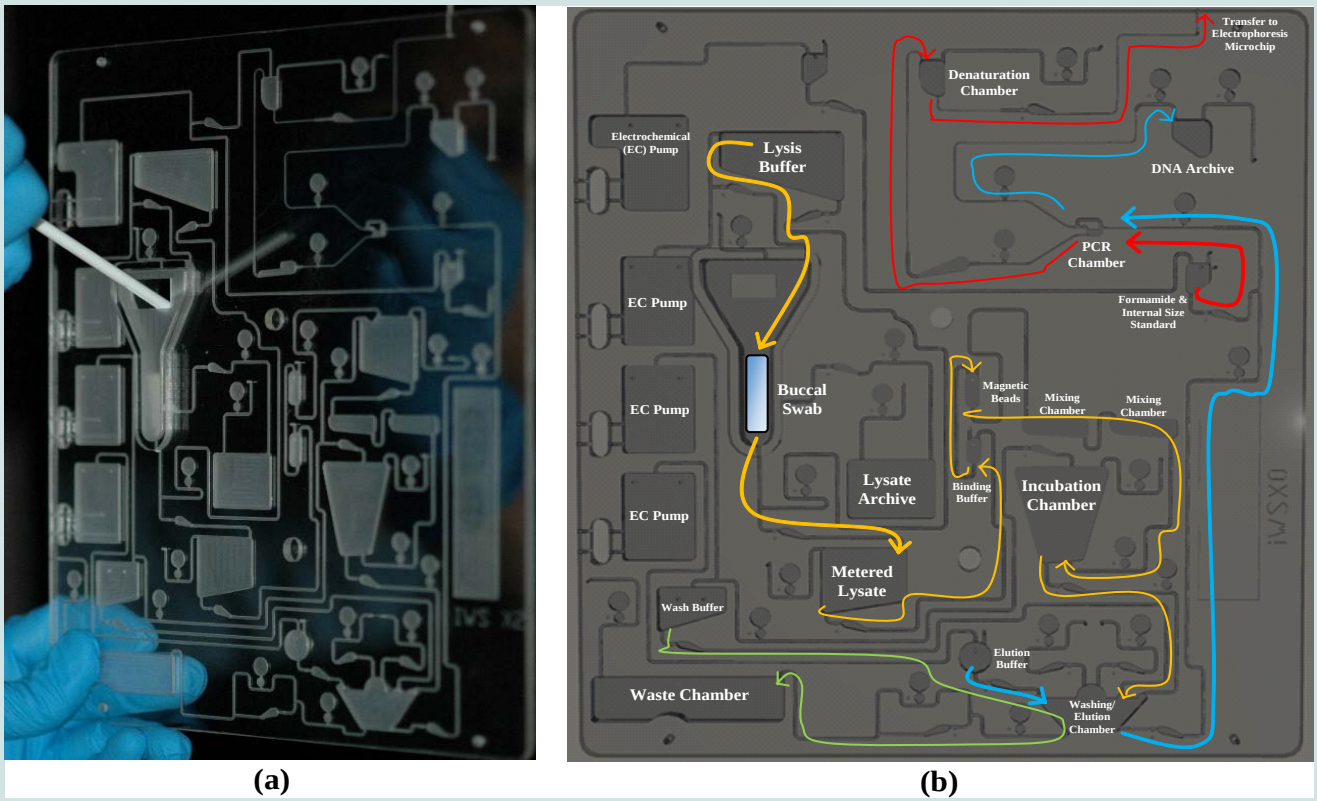

Figure 1: A fully-integrated "swab to profile" microfluidic cartridge for forensic analysis of a buccal swab sample. The cartridge integrates swab lysis, DNA extraction, multiplex PCR, and transfer of the PCR product to an electrophoresis microchip. (a) Picture of the cartridge including the buccal swab chamber where a buccal swab can be simply dropped and processed and (b) Schematic of the sample transport paths and analytical steps performed in the integrated cartridge. Swab lysis, DNA purification with magnetic beads, DNA elution, PCR, denaturation, and transfer of denatured nuclei acids to an integrated electrophoresis microchip. This protocol is indicated with yellow arrow, green arrow, blue arrow, and red arrow in order.

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in table 1 and 17 short tandem repeat (STR) loci are detected. Table 1 summarizes the specifications and subsequent characterization of the operating parameters for each functional module of the MiDAS for validating the system performance in obtaining a typical electropherogram compatible with expert system software compliant with CODIS or other databases.

By integrating the swab chamber and related microfluidic channels into the cartridge, automatic process flow could be performed from a sample swab insertion to DNA STR analysis in the MiDAS. Figure 2 shows a representative electropherogram obtained from an integrated run using the MiDAS. Although the rapid prototyping still yields a relatively rough CNC-machined channel surface, which could allow an easy entrapment of DNA molecules and consequently some sample loss, it validates that the fully integrated processing of a reference sample, from buccal swab-in to STR profile-out, could be accomplished on MiDAS.

3D Multi-layer DNA Processing Cartridge

Beyond the integration for a rapid "swab-to-profile" system, portability is another important characteristic that the MiDAS should have in order to become a major tool for on-site investigations, ultimately. For improved portability and increased throughput, we have developed a multi-layer 3D cartridge with almost a fifth of the previous footprint shrinkage or shrinking size to 43 mm width and 220 mm length while still containing all the functional modules from the 210 mm wide and 230 mm long planar cartridge. As shown in Figure 3(a), the 3D cartridge is composed of six layers. The first layer is placed in contact with heaters on a printed circuit board (PCB) and seals the second layer which contains a PCR chamber and chambers and polymer valves requiring heat to process swab sample lysis and DNA elution. The third layer contains parts of the swab chamber and a mixing chamber for binding DNA from the swab sample to magnetic beads. The fourth layer has reagent storage chambers for the lysis buffer, the magnetic beads, the wash buffer, as well as archive chambers for the sample lysate and the purified DNA. The fifth layer contains a metering chamber to accurately extract the necessary amount of swab lysate for the following processing. The first and the

Swab Lysis	Reagents		Reagent Volume				Protocol		**Efficiency**
	ChargeSwitchgNDA Normalized Buccal Cell Kit (CS11020, Life Technologies)		1mL Lysis buffer 10 µL Proteinase K				60˚C for 15 min with bubbling-based agitation		**86.2% of manual in- tube controls**
DNA Extraction	ChargeSwitch (CS)gNDA Normalized Buccal Cell Kit (CS11020, Life Technologies)								**Yield**
	CS Magnetic beads (25 µL, 1.87 mg/mL)	CS Purification buffer (30 µL)		CS Wash buffer (200 µL)			CS Elution buffer (150 µL)		**0.86 ± 0.41 ng/µL**
DNA Amplification	Reagent	Reagent Volume	DNA Mass	27 Cycle Rapid PCR Protocol					**Profile**
	Beads with PowerPlex ESI 17 (DC7781, Promega)by Q Chip	3 µL in 2 beads	1 ng@10 µL reaction volume	Activate @95.5˚C for 2 min Denature @95.7˚C for 20 sec (27×) Anneal/Extend @58.2˚C for 135 sec (27×) Final Extend@59˚C for 30 min					**Full STR Profile**
	Average of intra-color balance ratio in 4 dyes (FL, JOE, TMR, CXR)		**0.62 ± 0.08**	**Average of peak height ratio in 13 heterozygous loci**					**0.87 ± 0.08**
DNA STR Detection	Sieving and coating matrix	Loading Protocol	CE Protocol						**Resolution in base**
			Well	Sample	Sample waste	Buffer	Buffer waste	Time	
	3.5% w/v PVP/HEC with 20/80 ratio in 1×ABI 310 running buffer	50˚C for 25 min	Load	GND	800 V	GND	600 V	40 sec	**1.2 base for 100~200 base domain**
			Inject	GND	400 V	350 V	2,000 V	2 sec	
			Separate	GND	400 V	GND	2,700 V	1,000 sec	

Table 1: Specification and optimization of performance parameters for each functional cartridge module.The table summarized the results from swab lysis module (n=5), DNA extraction module (n=27), DNA amplification module (n=3at DNA template concentration of 1ng per reaction), and DNA STR detection module (n=5).

For the swab lysis, the sampleswab heads were transferred into the on-cartridge swab chamber. Lysis on the cells from the swab heads were initiated by flowing 1 mL of lysis buffer from the on-cartridge chamber with the on-cartridge pump into the swab chamber and directly lysed with the above protocol. The on-cartridge swab lysis efficiency was calculated as percentage by comparing the DNA concentration obtained from the on-cartridge lysis to the DNA concentration from the manual in-tube lysis [18].

The DNA extraction was performed by pumping 150 µL of the lysate into the chamber containing 30 µL purification buffers and the mixture was pumped into the chamber containing 25 µL of magnetic beads. The DNA bound to the magnetic beads were captured by a magnet on MiDAS and washedby flushing 200 µL of wash buffer followed by flowing 150 µL of elution buffer to release the extracted DNA from the beadsby incubating at 60 °C for 3 min [12].

In order to characterize the PCR module, the commercial DNA (360486, Life Technologies) was used as our benchmark standard. A range of DNA concentrations, from 0.25 ng up to 2 ng DNA template concentration was tested on chip (n=12) in 10 l PCR reaction. Although a full STR profile was achievable at low DNA concentration (e.g. 0.25 ng per reaction), the optimal template DNA concentration at the low end, which gave reproducible, best-balanced, and comparable peak height to benchtop control, was 1 ng per 10 l PCR reaction (Table 1). However,as for in the integrated "swab to profile" cartridge runs, 6 µL of DNA eluent would be metered into the PCR chamber where two hydrogel beads containing the 5-dye PowerPlex ESI 17 PCR amplification multimix were pre-loaded. Although the DNA concentration metered into the PCR chamber was estimated ranging from 2.7 ng to 7.6 ng, the STR profile peak height and balance was comparable to those obtained with the control DNA at 1~2 ng, due to the difference in purity and quantification methods of the template DNA.The PCR chamber was then sealed with paraffin valves and the multiplex amplification wasperformed with pre-programmed thermal cycling protocol as outlined in Table 1 [17].

The amplified DNA fragments were separated by on-chip CE separation.Polymer, 3.5% w/v PVP/HEC in a 20/80 ratioin 1 × ABI 310 running buffer (Applied Biosystems) was loaded for sieving and coating matrix and 25 µLof 1 × ABI 310 running buffer was added to each of the wells ofthe CE chip. Then, the chip was mounted into the MiDAS by clipping adapter lids in the MiDAS into the CE chip. The denatured PCR producton the cartridge was delivered into the CE chip sample reservoirand injected intothe separation channel under the above CE protocol and thelabeled fragments were detected at a point 110 mm from injection at 50 °C with laser-induced fluorescence [12,16].

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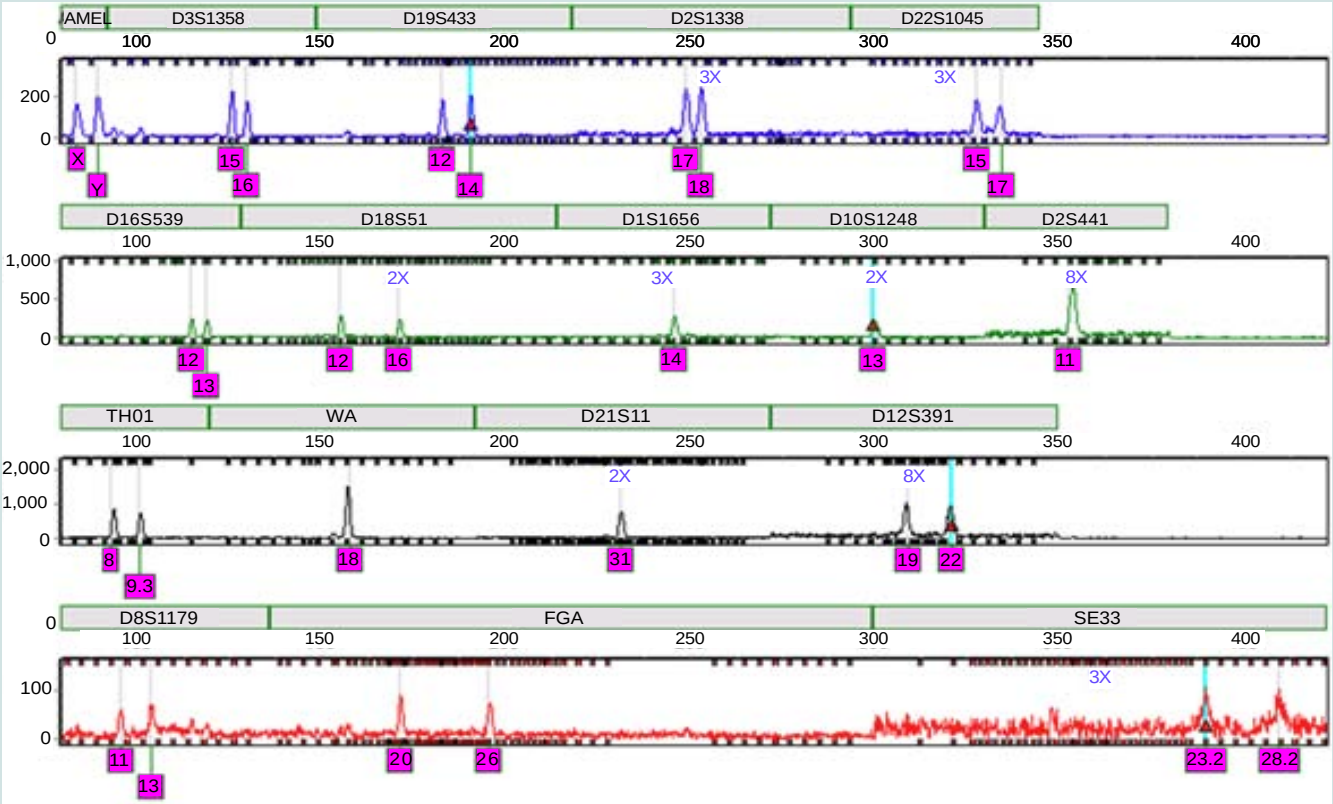

Figure 2: A preliminary electropherogram generated from the fully-integrated "swab to profile" microfluidic cartridge for forensic analysis of a buccal swab sample. The cartridge integrates swab lysis, DNA extraction, multiplex PCR, and transfer of the PCR product to the CE microchip.

sixth sealing layers are fabricated from 0.5 mm thick polycarbonate while other four layers are 3 mm thick polycarbonate with structures milled into both sides. Figure 3(b) shows the fabricated multi-layer 3D cartridge that is assembled with a prototype PCB containing resistive heaters to control on-cartridge valves and to process heat-related functions.

In addition to portability, parallel multi-sample processing is another desire for forensic analysis to achieve enhanced throughput. This can accelerate forensic analysis, which usually cannot afford the high amount of requested samples for examination due to the lengthy and labor-intensive process. In an effort to meet the requirement, the 3D cartridge has been designed to process four samples simultaneously with the same footprint of the single layer cartridge as shown in Figure 3(c) and (d).

Hybrid Valves for the 3D Cartridge

Although we can use polymer-based valves in some layer of the cartridge for planar fluidic control, we introduced some hybrid configuration for the 3D cartridge. Often, the planar microfluidic circuitry can limit the scale of cartridge shrinkage because resistive heaters and associated control circuits for the valves require sufficient space and cannot be reduced to the same size of the cartridge, leading to a bigger PCB. This eventually restricts the number of cartridges (or throughput) that can be fit into a portable system. Except the second layer of the 3D cartridge which is near the resistive heaters, fluidics on the third, fourth, and fifth layers cannot be controlled using the polymer valves as the layers are far from the resistive heaters on the PCB. Therefore, another fluidic control valve had to be sought. A new hybrid approach was selected using a duckbill valve, which is fabricated from rubber with a shape of a duck's beak at one end, allowing a unidirectional flow with prevention of backflow. The duckbill valve component used for the 3D cartridge (DU 027.002-154.01, Minivalve) was opened from 0.6 psi with an average water flow rate of 1.5 mL/min at 0.6 psi with a high linearity of forward liquid flow rate upon applied pneumatic pressure as shown in Figure 4(a). The duckbill valve showed no water leakage at 3.5 psi back pressure (The product data sheet indicates a maximum back pressure of 85 psi.) so that flow can be controlled in the 3D cartridge by placing the duckbill valves with desired flow directions as shown in Figure 4(b).

In earlier versions of the MiDAS, users had to manually seal inlet holes after introducing reagents into the cartridge, which required another user intervention. Versatility of the duckbill valves allowed easy introduction of reagents without requiring sealing the inlets with tape after the introduction as a dispenser tip on a syringe opens the duckbill valve and the valve automatically closes itself when the dispenser tip is removed from the valve as shown in Figure 4(c).

One of the most important functional modules in the MiDAS is PCR. The first version of MiDAS had variability in polymer valve operation due to a close proximity to a high temperature and high

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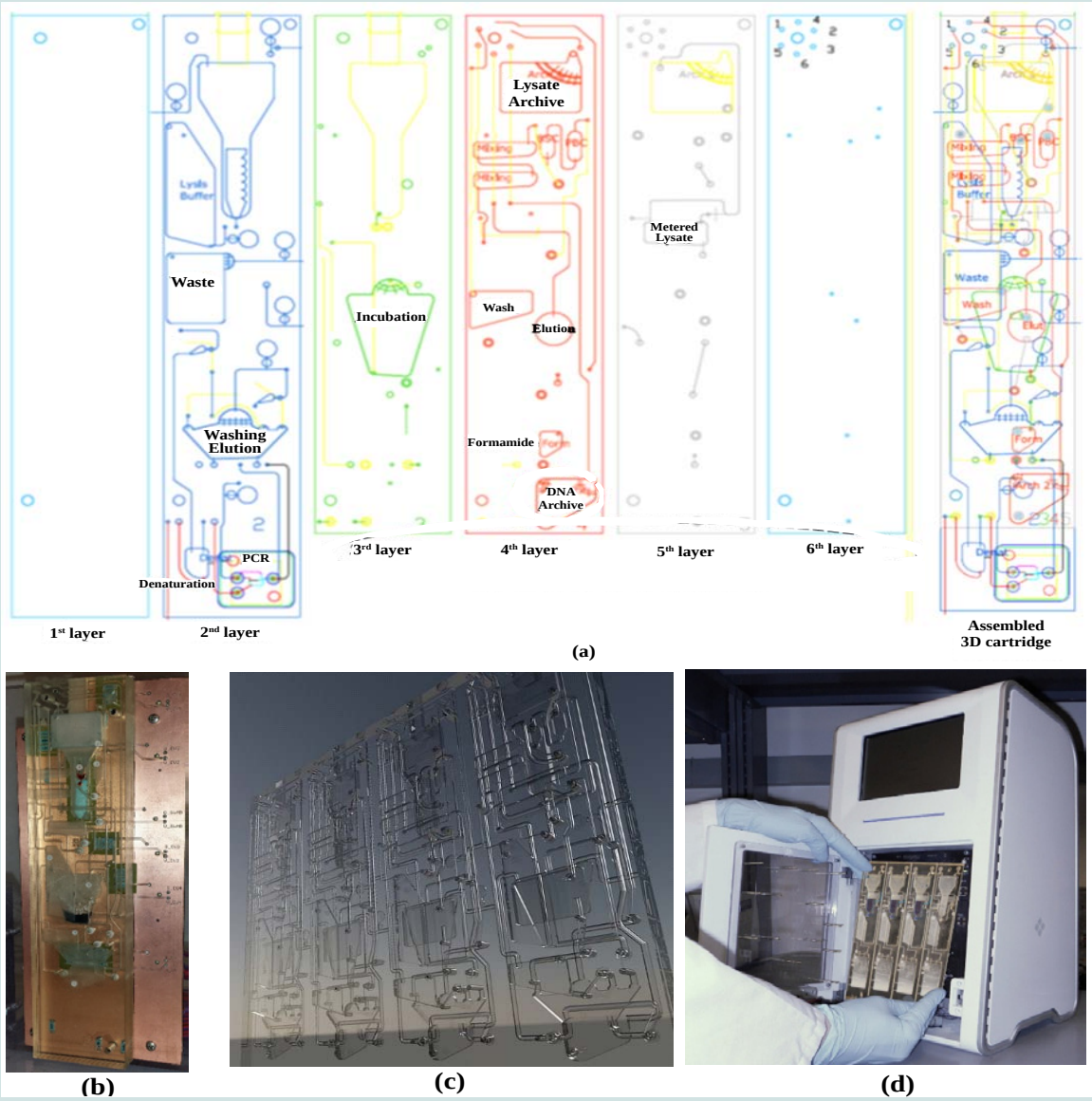

Figure 3: The novel 3D multi-layer cartridge.(a) Exploded view of the 3D cartridge comprising 6 layers for a portable forensic analysis through a miniaturization of the cartridge footprint; (b)The 3D cartridge with a prototype PCB for a portable system; (c)4-channel parallel processing cartridge for enhanced throughput in the same footprint of the single layer cartridge; and (d) A prototype analyzer that could be adapted to perform parallel multi-sample processing with the 4-channel cartridge.

pressure PCR region. Even after the re-design of the valve actuation scheme and the microfluidic circuitry for PCR [17], the mixing of PCR product with molten polymer from the valve still limited mixing ratios between the PCR product and formamide, which could lead to reduction of sensitivity and reproducibility of the DNA profiles. To address the issue, a new opening valve was designed by using only 0.6 µL of polymer (e.g. paraffin-wax), which is less than one seventh of the previous polymer volume, in order to minimize the interference with PCR product. To further minimize sample loss by condensation during the PCR, the small amount of polymer was located at the position nearest to the PCR chamber while ensuring no melting of the polymer during the PCR as shown in Figure 5(a). The small volume of polymer demonstrated a tight seal during the PCR protocol without any deterioration in multiple cartridges as shown in Figure 5(b) and there was a high sample recovery, with upto a 43% increase from the previously reported recovery volume when tested with dyed water. The valve could be then successfully opened after PCR by applying heat and air pressure from the side channel in order to flow the PCR product into a denaturation chamber.

Concluding Remarks

In this short communication, we present some of our latest developments and approaches toward further improvement and alternative configurations of the MiDAS platform. Beyond the previously published work demonstrating the successful capacity of the MiDAS integrated system for the rapid DNA analysis of forensic reference samples, we report some recent progress in the system

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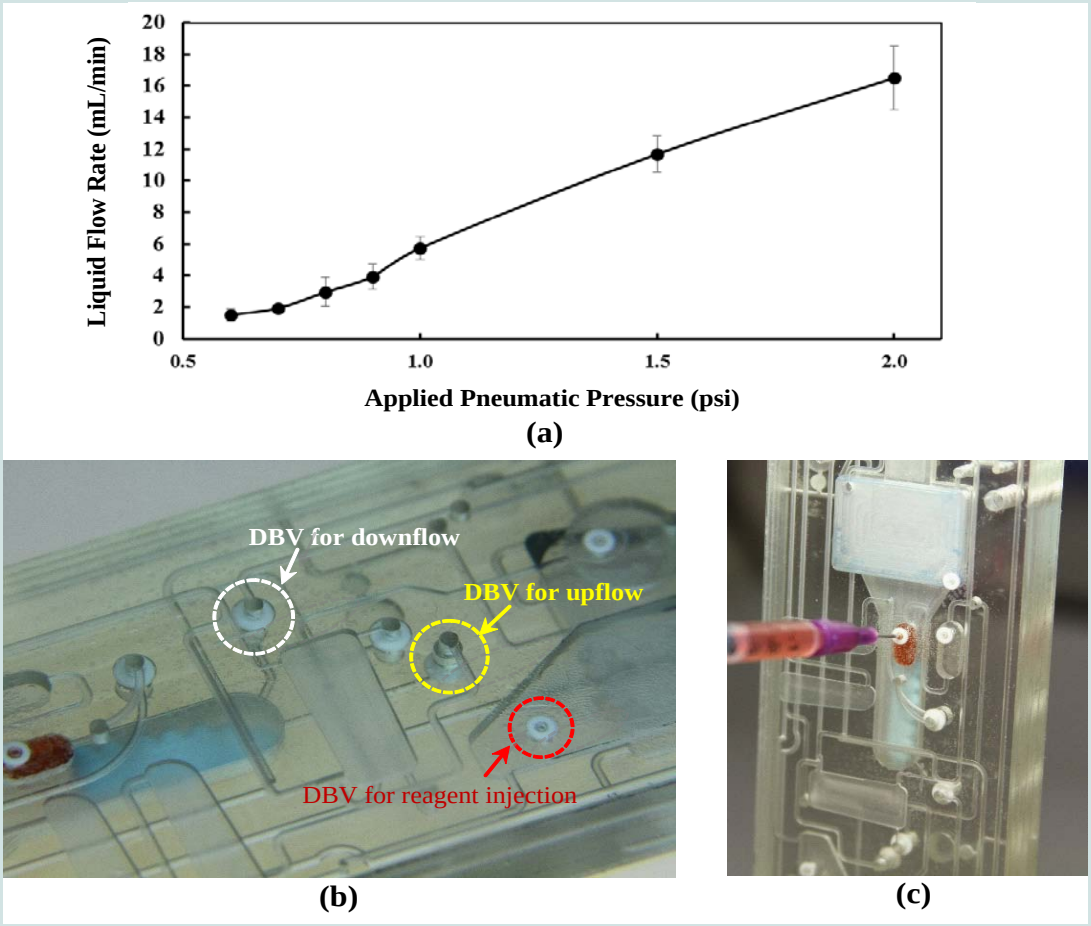

Figure 4: Hybrid duckbill valve (DBV) for the 3D multi-layer cartridge. (a) Linearity of forward liquid flow rate upon applied pneumatic pressure once a DBV is opened; (b) Integration of DBVfor fluidic control among the layers and user-friendly reagent injection; and (c) Injection of reagents through the DBV that removes the necessity to seal inlets with tape after introduction.

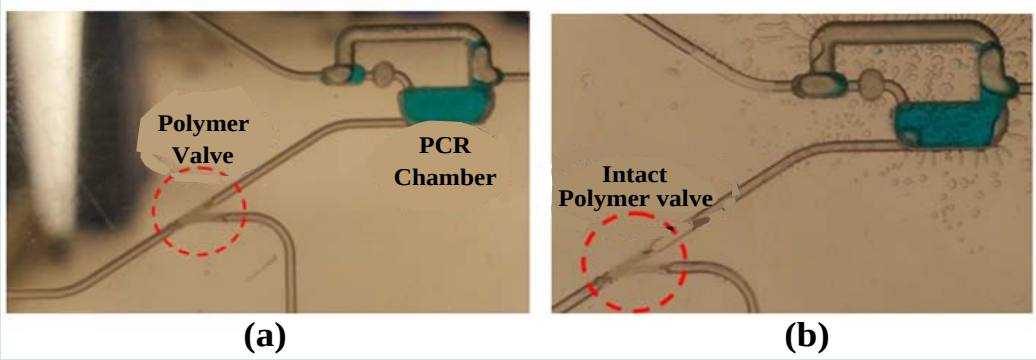

Figure 5: PCR opening valve with the minimum polymer volume to reduce PCR product loss from mixing between molten polymer and the PCR product. (a) Before PCR and (b) After PCR.

design by integrating the swab sample lysis module directly into the microfluidic workflow cartridge to fully automate the sample preparation.

The configuration of the single layer cartridge has been miniaturized into the new 3D multi-layer DNA processing cartridge which renders the MiDAS with a better portability and higher analytical throughput.

The use of a versatile hybrid combination of polymer-based and/or duckbill valves enabled not only the inter-layer fluidic controls on the 3D cartridge but also tape-free sealing after on-cartridge reagent introduction, which eliminates another manual step for the DNA analysis from the MiDAS. The new opening valve with a reduced

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polymer amount for the PCR control showed superior results with tight sealing and high recovery of PCR product to improve the sensitivity and reproducibility of the rapid DNA analysis.

Although there are still needs for optimizing designs and operating conditions, these new self-contained microfluidic cartridges are amenable for integrating a diversity of assay chemistries. Further miniaturization could reduce fabrication costs for large scale manufacturing of the MiDAS, advancing cost-effective rapid-DNA analysis for the forensics community.

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