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Development of a microfluidic chip for early and rapid detection of multiple dengue serotypes

Yu Hao Foo[1], Leon CZ Chan[2,#],

1 Smart Microfluidics (SMF), Singapore Institute of Manufacturing Technology, Nanos, 31 Biopolis Way, #04-10, S(138669), Singapore
2 Printed Intelligent Device (PID), Singapore Institute of Manufacturing Technology, 73 Nanyang Dr, S(637662), Singapore
Corresponding Author / Email: czchan@SIMTech.a-star.edu.sg, TEL: +65-6419-7450, FAX: +65-6250-3659

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It is important to distinguish between the 4 dengue virus serotypes, DEN 1, DEN 2, DEN 3 and DEN 4, when one is infected with a dengue fever episode due to lack of cross-protective neutralizing antibodies for each serotype. Secondary infections also put individuals at higher risks for severe dengue illness than those who have not been previously infected. Current gold-standard assays include reverse transcription-PCR (RT-PCR) and other laboratory and specialized labor-intensive assays like nucleotide sequencing and serotype-specific ELISA. To enable on-field diagnosis of dengue serotypes, the detection process would need to be simplified and made at least semi-automated. Polymer-based microfluidics manufacturing methods had been employed for the fabrication of such point-of-care diagnostic devices, particularly on sample loading, cell lysis, RNA extraction and RT-PCR. A downstream detection module was conceptualized and fabricated to detect the amplified DNA from provided PCR mix (product) in that sample. Further, to ensure accuracy, each serotype assay necessitates a positive control and a negative control, which constitutes 3 separate channels for diagnosis of just 1 serotype. In this study, a 6-channel bi-assay microfluidic chip was designed with pre-loaded diluent (ultrapure water) and cyanine dye, sample chamber for loading, sequential fluidic sample mixing, and integrated membranes for simultaneous (6-channel) fluidic manipulation from a single actuation source. Positive samples turned the dye from blue to violet while the negative controls remained blue. The integrated membranes provided color contrast and facilitated the manipulation of the samples to the same line of sight for simultaneous analysis, paving the way for automated color analysis via smartphone.

1. Introduction

Dengue virus is currently a widespread issue in the urban or semi-urban settings where Aedes mosquitoes tend to be omnipresent in tropical and sub-tropical countries. It is estimated that there are around 390 million cases of dengue virus infection [1] and 3.9 billion people in the world are at risk of infection with the dengue virus [2], causing around 20,000 deaths annually [3]. Infection with the dengue virus could result in dengue fever (DF), dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [4]. Typical symptoms of a dengue virus infection commonly include fever, rashes, hemorrhagic symptoms, headache, ocular pain, swollen glands, nausea and vomiting [1]–[3]. Even with adequate access to healthcare, patients may develop severe life-threatening symptoms such as internal bleeding, organ damage and severe dips in blood pressure level, causing shock that can result in fatality [5].

At present, there are multiple challenges in providing a quick and accurate diagnostic assay for dengue virus detection. Conventional diagnostic methods would include the reverse-transcription polymerase chain reaction (RT-PCR) assay which produces a highly sensitive and accurate diagnosis [6]. However, the limitations of the RT-PCR assay would include expensive reagents, equipment and skilled personnel for running the laboratory protocols [7]. Recently, optical detection methods involving aggregation of gold nanoparticles based on PNA/DNA hybridization [8], [9] had been developed, but which still requires trained laboratory personnel to perform the assay.

Microfluidics enable the development of portable diagnostics tools for point-of-care applications. These highly miniaturized devices consume minimal quantities of reagent and samples, saving costs associated with expensive reagents. It also allows for multiple analytes to be processed simultaneously with parallel circuits on a single device, which greatly increases throughput and efficiency [10].

This study reports a microfluidic chip designed with pre-loaded diluent (ultrapure water) and color-changing cyanine dye, sample chamber for loading, sequential fluidic sample mixing, and integrated membranes for simultaneous 6-channel fluidic manipulation from a single actuation source. This unidirectional actuation is part of the microfluidic chip design which enables a facile, stepwise and effective mixing of reagents, to change the cyanine dye from blue to violet for positive controls and samples, and remained blue for negative controls.

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In this work, poly(methyl methacrylate) (PMMA) was selected as the material of choice as it is low cost, have excellent optical transparency, ease of fabrication and modification, as well as its biocompatibility [11]. Thermal (fusion) bonding was selected to seal separate layers of the chip together after the micro-milling process as it is a cost effective, high-throughput method to mold thermoplastics with minimum stress developed in the network of microchannels incorporated within the chip layers. The parameters of the hot embossing process are optimized to prevent microfluidic channel deformation and clogging. Applied pressure through clamping via an applied torque, process time and operating temperature must be critically controlled to manufacture robust microfluidic chips with minimal deformation and maximum optical transparency and uniformity. Accordingly, we achieved optimum bonding when the tightening torque, holding duration and temperature were set to 1.5 Nm, 125°C, and 1.5h respectively.

2. Materials and Methods

2.1 6-channel Chip Design

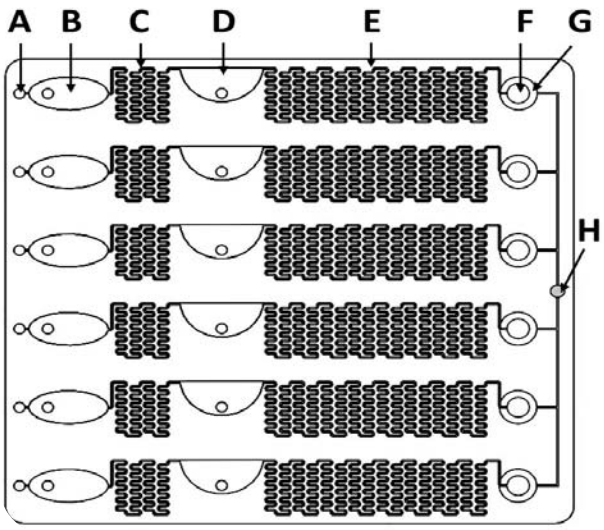

Fig. 1 Top-view of microfluidic chip with integrated membrane – A: sample loading port, B: MilliQ (ultrapure) water storage chamber, C: mixing channel for PCR mix, D: cyanine dye storage chamber, E: mixing channel for PCR product, MilliQ water and cyanine dye, F: observation chamber, G: hydrophobic membrane, H: actuation port

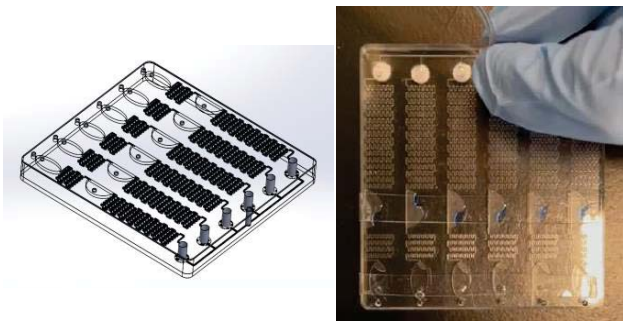

Fig. 2 (Left) Isometric view and (Right) picture of microfluidic chip (with membrane and actuation source connected to a tubing positioned at the top of the picture with the

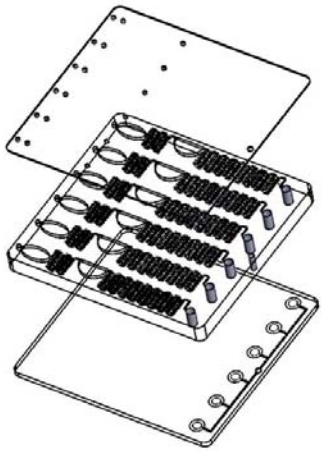

Fig. 3 Exploded view of microfluidic chip

2.1.2 Chip Fabrication and Assembly

The microfluidic chip was fabricated by employing a combination of micro-milling of the separate layers (top, center, and bottom layers) and thermal bonding afterwards to integrate all layers together along with the hydrophobic membrane, which was placed in below the observation chamber (F) in Figure 1.

PMMA sheets of 0.5mm, 1.5mm and 5mm thicknesses were micro-milled, with the center layer, illustrated in Figure 2, consisting of a serpentine structure and storage chambers. Fluidic inlets and actuation port for sample and chambers were micro-milled onto the top layer. An exit flow path connecting all 6 observation chambers with individual integrated membranes placed on the bottom layer chamber (F) leading to the actuation port (H) was micro-milled on the bottom layer.

After the micro-milling process, separate layers of the micro-milled chips undergo a washing step in an ultrasonicator to remove all machining debris resulting from the micro-milling procedure that may potentially obstruct the microfluidic channels. After the ultrasonic washing, the separate layers were wiped down with a cloth containing isopropyl alcohol (IPA) in a laminar flow cabinet to minimize dust accumulation which may affect the optical quality of the bonded assembled layers, and later wiped and disinfected with 70% ethanol. After the sterilization process, the chip was assembled, and membrane integrated within the chip. The assembled microfluidic chip was placed in a vacuum degassing oven for at least 4 hours at 80ºC. Finally, the assembled microfluidic chip was thermally bonded together with a tightening torque of 1.5 Nm on a customized bonding jig in an oven at 125ºC for 1.5 hours.

2.1.2 Experimental Design and Protocol

To minimize false positives, specific proprietary DNA sequence were identified and provided by our collaborator to represent the dengue serotypes (DEN) 2 and 3. Synthetic (commercial) DNA oligomers

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with the specified DNA sequences for DEN 2 and DEN 3 were utilized to represent the actual viral sequence for the benchtop and on-chip tests.

For the on-chip experimental runs, 16 µL of MilliQ water was pre-loaded into MilliQ (ultrapure) water storage chamber (B) and 5 µL of cyanine dye into the cyanine dye storage chamber (D) respectively, as depicted in Figure 1. In the actual application, the dengue detection chip user would then manually load 2X 4 µL samples (sample PCR product) each into sample loading ports 1 and 4 (A). Sample ports 2 and 5 were loaded with positive controls (PCR product controls spiked with specific serotype DNA), while sample ports 3 and 6 were loaded with negative controls (PCR product controls that would definitively not contain any serotype DNA).

Consequently, this 6-channel microfluidic chip design would be able to provide diagnosis for 2 different dengue serotypes (a set of 3 ports consisting of a positive control, negative control and sample constitutes 1 dengue serotype diagnosis assay). After all the PCR products are loaded into their respective sample loading ports (A), actuation can be initiated from the actuation port (H) with a syringe pump, or even manually with an adjustable pipette. The PCR product will then flow through the (serpentine) mixing channel (C) to undergo homogenous fluidic mixing with the ultrapure water, and then into the cyanine dye storage chamber (D) and incubated for 3 mins. The resulting mixture would then flow through mixing channel (E) to perform mixing with cyanine dye into the observation chambers (F) for analysis.

The microfluidic chip design was modified and optimized based on the color contrast between positive control (PC) (violet) and negative control (NC) (blue), and ease of user operation, suitable for portable dengue fever serotype differentiation and/or diagnosis.

As a comparison with a laboratory-performed benchtop control setup, 4 µL of DNA-spiked PCR product was diluted with 16 µL of MilliQ water in a 0.5 mL Eppendorf® tube, after which 5µL of cyanine dye was mixed with the diluted PCR product to represent the positive control (PC). The negative control (NC) was prepared in a separate Eppendorf tube with 20 µL of MilliQ (ultrapure) water mixed with 5 µL of cyanine dye. The benchtop setup provided a benchmark for colour contrast comparison to determine the efficacy of the on-chip setup.

2.1.3 Results and Discussion

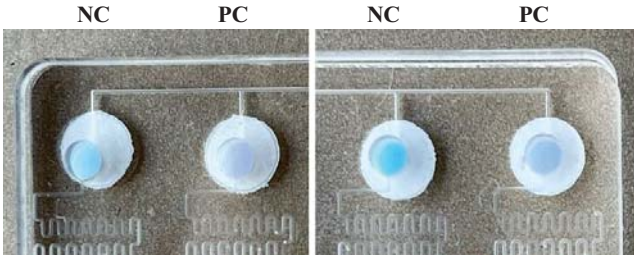
Results for benchtop setup

Commercial DEN 2 **Commercial DEN 3**

Fig. 4: Two separate sets of commercial PCR products consisting of negative control (NC) and positive control (PC) performed in 0.5 mL Eppendorf tubes. Photo taken with natural illumination of the sun in the laboratory

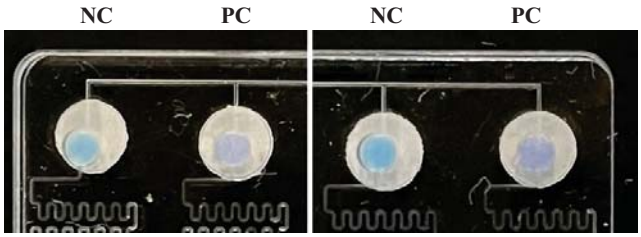
Violet coloration (change) was observed for both positive control (PC) samples while negative control (NC) samples remained blue when viewed with the naked eye. However, colour contrast between the positive control (violet) and negative control (blue) was difficult to capture with the camera.

Results for On-chip Experiments



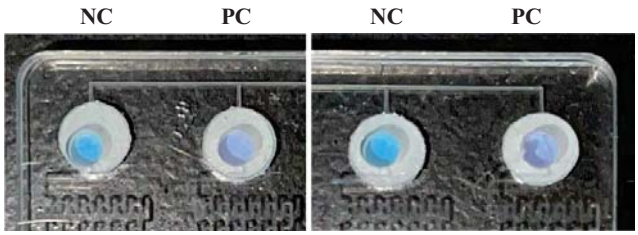
Commercial DEN 2 **Commercial DEN 3**

Fig. 5: Comparison of color contrast for NC and PC samples of two separate commercial PCR products performed on chip. Photo was taken with natural illumination of the sun in the laboratory



Commercial DEN 2 **Commercial DEN 3**

Fig. 6: Comparison of color contrast for NC and PC samples of two separate commercial PCR products performed on chip. Photo taken with calibrated D50 lighting.



Commercial DEN 2 **Commercial DEN 3**

Fig. 7: Comparison of color contrast for NC and PC samples of two separate commercial PCR products performed on chip. Photo taken with camera flash.

Color contrast for both negative control (NC) and positive control (PC) samples were noticeable with naked eye when observed under 3 different lighting conditions – natural daylight (Figure 5), calibrated D50 lighting (Figure 6) and camera flash (Figure 7). NC for both commercial DEN 2 and commercial DEN 3 samples yielded an azure blue coloration while PC samples for both commercial DEN 2 and DEN 3 synthetic DNA (oligomer) samples produced a violet coloration. On-chip color contrast was well-perceived with both the naked eye and with the camera.

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The incorporation of the integrated membrane not only assisted in increasing the color contrasts between the positive (violet) and negative (blue) samples, but it also allowed for the different fluidic plugs, flowing at slightly different flowrates due to minute pressure differences across the microchannels, to be confined above the membrane in observation chamber (F) (Figure 1) and allowed the 6 microfluidic channels to be analyzed simultaneously. This reduced the need for 6 separate actuation sources to be controlled independently, reducing the complexity required to run the dengue detection on-chip assay.

3. Conclusions

In this study, the efficacy of a downstream detection module in the form of a microfluidic chip to obtain the PCR (mix) products and detect the amplified DNA samples via colorimetric analysis was demonstrated. Positive samples turned the cyanine dye from blue to violet while negative controls remained blue. The incorporation of a meandering (serpentine) shaped liquid mixing channel allowed for simultaneous mixing and incubation of the final mixture. This resulted in quicker analyses due to reduced reaction times. The integrated (hydrophobic) membranes enhanced the color contrast of the blue and violet coloration which were better observed on-chip as compared to the benchtop setup. Several (6) samples could be tested simultaneously in a facile and replicable manner with just a single actuation source. Accuracy was ensured and chances for erroneous results were eliminated by having both positive and negative controls for each serotype assay, which constitutes 3 separate channels for diagnosis of 1 serotype.

Hence, it is demonstrated that this microfluidic chip design can successfully perform sequential fluidic samples to yield color contrast that can be discerned with the naked eye or potentially with the help of a smartphone for automated color analysis. The development of this microfluidic chip has the potential to be applied to other fields requiring efficient mixing and prolonged incubation such as PCR mix (product) preparation and detection of nucleic acid with other colorimetric assays.

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