Biotechnology Notes 4 (2023) 77–82

Contents lists available at ScienceDirect

Biotechnology Notes

journal homepage: www.keaipublishing.com/en/journals/biotechnology-notes/

A facile microfluidic chip design for DNA detection using dengue serotypes as a proof-of-concept case study

Y.H. Foo [a], Norhidayah Abu [b,c], Rafidah Hanim Shueb [b], Tuan Nur Akmalina Mat Jusoh [b], Zuhana Ahmad Zubir [c], Nur Ellina Azmi [c], Hamidah Sidek [c], Leon C.Z. Chan [a,*]

[a] Singapore Institute of Manufacturing Technology (SIMTech), Agency for Science, Technology and Research (A*STAR), 2 Fusionopolis Way, Innovis #08-04, Singapore, 138634, Republic of Singapore
[b] Department of Medical Microbiology and Parasitology, School of Medical Science, Universiti Sains Malaysia, 16150, Kubang Kerian, Kelantan, Malaysia
[c] SIRIM Berhad, Persiaran Dato' Menteri, P.O. Box 7035, Section 2, 40700, Shah Alam, Selangor, Malaysia

ARTICLE INFO

Keywords:
Microfluidics
Chip design
Dengue serotypes

ABSTRACT

Dengue fever is caused by any of the four serotypes of dengue viruses, DENV-1, DENV-2, DENV-3 and DENV-4 spread by mosquito bites and is important to distinguish between them due to lack of cross-protective neutralizing antibodies for each serotype. Secondary infections also put individuals at higher risk for severe dengue illness than those who have not been previously infected. Current preferred assays include reverse transcription-PCR (RT-PCR) and ELISA. To enable on-field diagnosis of dengue serotypes, the detection process would need to be simplified or at least semi-automated. A downstream detection module was conceptualized and fabricated to detect the amplified DNA from the provided PCR mix (product) of previously developed modular microfluidic chips involving sample loading, cell lysis, RNA extraction and RT-PCR. Further, to ensure accuracy, each serotype assay necessitates a positive control, negative control and test sample, which constitutes 3 separate channels for the diagnosis of just 1 serotype. In this study, a 6-channel bi-assay microfluidic chip was designed with pre-loaded diluent 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 will turn the dye from blue to violet while the negative controls will remain 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) or severe dengue.[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] During the COVID-19 pandemic in 2020, there was a shift of the predominant circulating strain from DENV-2 to DENV-3, leading to the largest dengue outbreak in Singapore. Up until Q4 2022, DENV-2 followed by the DENV-3 dengue serotypes were still the predominant circulating strain6.

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.[7] However, the RT-PCR assay needs other tests such as gel electrophoresis to analyze the final product which includes specific software for sample visualization and the process is time-consuming.[8] Recently, optical detection methods involving aggregation of gold nanoparticles based on PNA/DNA hybridization[9,10] had been developed, but which still require trained laboratory personnel to perform the assay.

* Corresponding author.
E-mail address: czchan@SIMTech.a-star.edu.sg (L.C.Z. Chan).

https://doi.org/10.1016/j.biotno.2023.11.001
Received 5 November 2023; Accepted 5 November 2023
Available online 9 November 2023

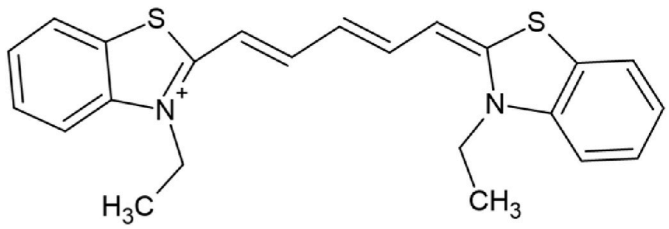

Fig. 1. Chemical structure of the dye DiSc2(5), 3,3′-diethylthiadicarbocyanine.

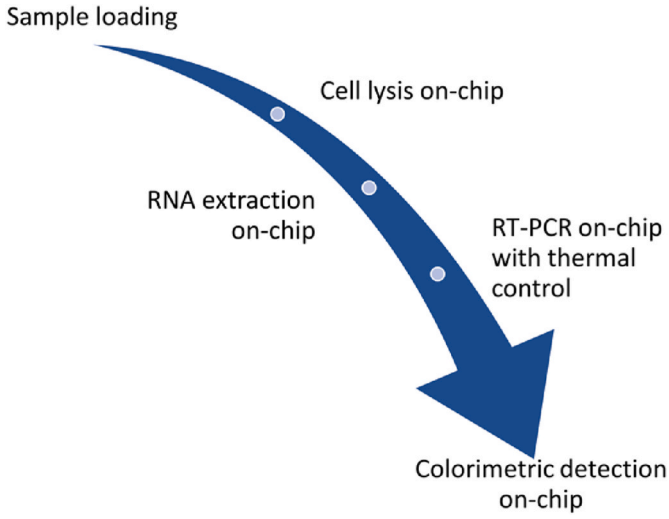

Fig. 2. Flowchart of the overarching study to incorporate the whole dengue serotype differentiation into a single microfluidic system from sample loading, cell lysis on-chip, RNA extraction on-chip, RT-PCR on-chip followed by colorimetric detection on-chip with on-chip stored cyanine dye.

Microfluidics enables the development of portable diagnostics tools for point-of-care applications. In the development of point-of-care testing, WHO has introduced the ASSURED criteria stand for Affordable, Sensitive, Specific, User-friendly, Rapid, Robust, Equipment-free, and Delivered to the end users. Therefore, the microfluidic chip as a miniaturized platform with incorporated chambers has met the criteria depending on the design and application. The microfluidic chip has been designed user-friendly, provide rapid results, and be portable for various settings to end-users without compromising its sensitivity and specificity. It allows for multiple analytes to be processed simultaneously with parallel circuits on a single device, which greatly increases throughput and efficiency.[11]

In this work, rigid poly(methyl methacrylate) (PMMA) was selected as the material of choice as it is low cost, has excellent optical transparency, ease of fabrication and modification, as well as its biocompatibility.[12] 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 with maximum transparency and optical consistency.

To determine the applicability of the fabricated microfluidic chip for the detection of dengue serotypes, we assessed the on-chip detection with DENV-2 and DENV-3. To achieve colorimetric readout on-chip, the dye 3,3′-diethylthiadicarbocyanine [$DiSc_2(5)$] (Fig. 1), which is typically utilized for DNA-PNA duplex testing[13,14] and selective protein detection in aqueous solution,[15] was employed to achieve DNA detection on-chip from PCR mix that was fed from upstream microfluidic chip modules. The cyanine dye is stored in chamber D (Fig. 3) and changes color from blue to violet upon binding to double-stranded DNA (dsDNA) (see Fig. 2 for summarized flowchart of the modular microfluidic chip development).

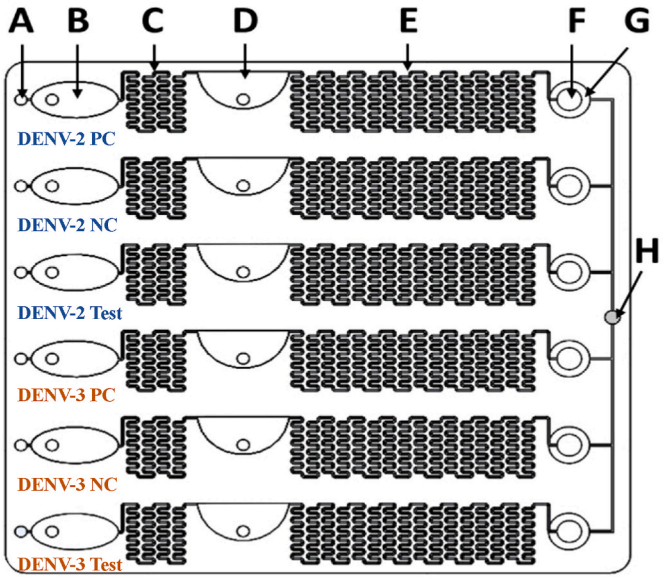

Fig. 3. Top-view of microfluidic chip for DENV-2 & DENV-3 detection – A: sample loading port, B: MilliQ (ultrapure) water storage chamber, C: mixing channel for PCR mix, D: cyanine dye storage chamber, E: mixing channel for PCR product, MilliQ water and cyanine dye, F: observation chamber, G: hydrophobic membrane, H: actuation port.

2. Materials and methods

This study with on-chip colorimetric detection on-chip is part of an overarching plan involving a facile detection method for dengue serotypes DENV-2 and DENV-3. The proof-of-concept for the detection of these 2 current predominant dengue strains would be a stepping stone for incorporating other dengue serotypes into the microfluidic system involving a series of modular microfluidic chips including on-chip sample loading, cell lysis, RNA extraction, RT-PCR with thermal control and colorimetric detection.

2.1. 6-Channel microfluidic chip design

Fig. 3 illustrates the microfluidic chip design and comprises of 6 microchannels, 3 of which form a set of positive control (PC), negative control (NC) and test samples from the upstream PCR mix. In this chip, 18 μL of sample diluent, MilliQ (ultrapure) water and 5 μL of $DiSc_2(5)$ cyanine dye were aliquoted and stored in chambers B and D respectively before use. The two sets of 4 μL PC, NC and test samples for PCR sample mixes of the DENV-2 and DENV-3 assays were added in inlets A, diluted in chamber B and then mixed in the serpentine microchannel C via vacuum actuation at port H with syringe pump withdrawal setting of 2.5 mL/min. Due to the shape and chamber capacity of D, the stored dye does not move towards chamber F until the diluted PCR mixes merge with the stored dye in chamber D. After another round of fluidic mixing in the serpentine microchannels E, the fluid flow terminates at the hydrophobic membrane in chamber F.

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

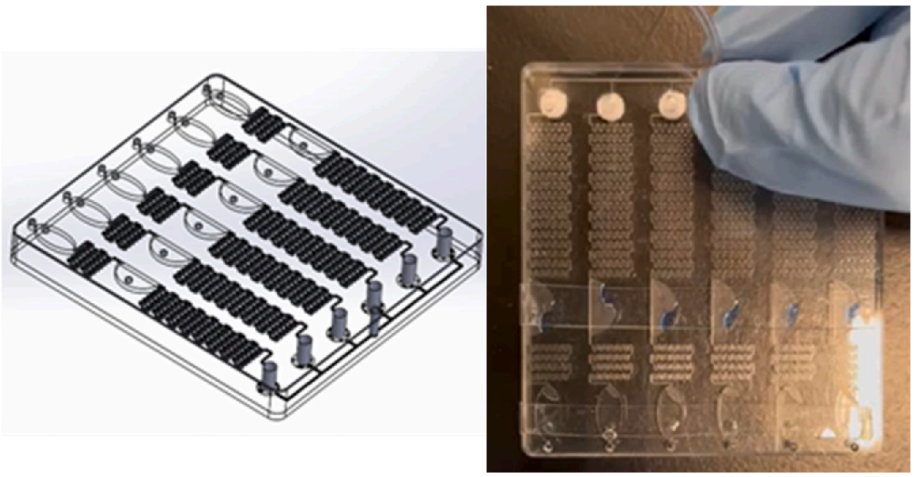

Fig. 4. (Left) Isometric view and (Right) picture of microfluidic chip (with membrane and actuation source connected to a tubing positioned at the top of the picture with the glove).

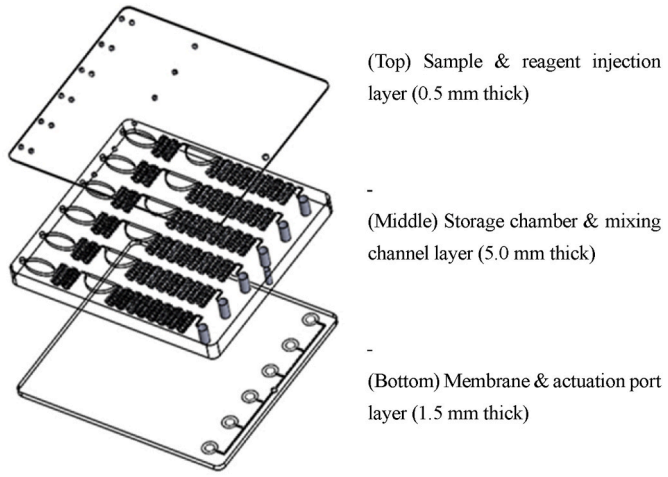

Fig. 5. Exploded view of microfluidic chip.

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).

If vacuum was applied at actuation port H without the membrane in chamber F, any minute pressure differences within the microfluidic chip due to surface roughness, presence of air bubbles or channel deformations in the range of micrometers would cause only 1 or some of the fluids in the microchannels to flow to their respective optical interrogation chambers above the membrane in chamber F. Accordingly, both DENV-2 and DENV-3 dengue serotype PC, NC and test samples can be simultaneously detected and analyzed by smartphone or by the naked eye in their respective observation chambers F.

In other words, although 6 separate fluidic inlets are required for sample introduction via chamber A, only 1 vacuum actuation port via tubing (Fig. 4) was required to perform the dilution, mixing and blue – violet color change (or lack thereof) observation on-chip.

For ease of features alignment and chip bonding, the chip design involved only 3 different chip layers - the top sample and reagent injection layer, the middle storage chamber and mixing channel layer, and the bottom membrane and actuation (vacuum) port layer, as shown in Fig. 5.

2.2. 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 below the observation chamber (F) in Fig. 3.

PMMA sheets of 0.5 mm, 5.0 mm and 1.5 mm thicknesses were micro-milled, with the center layer, illustrated in Fig. 5 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. A 0.15 μm pore size SEFAR hydrophobic membrane was laser-cut and utilized to keep liquids in chamber F.

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. The chips were fully submerged in DI water at 50 °C for 50 min, the chips rinsed in fresh DI water, and then replaced and ultrasonicated for an additional 15 min. 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 degassed and then thermally bonded together above its glass transition temperature, $T_g \approx 108$ °C. Depending on how the PMMA material was prepared, additives added, or microfluidic chip size, microchannel feature density and minimum feature size, the T_g and hence the ideal bonding parameters would be different.

2.3. Experimental design and protocol

Cyanine dye is widely used in the drug development area, detection of proteins, microarrays, flowcytometry and quantification of nucleic acid. In this study, cyanine dye was selected due to the interaction ability with DNA and RNA via the different binding modes which are intercalation between adjacent base pairs, minor groove binding, and electrostatic interaction of positively charged dye molecules with the phosphate backbone.[16] Interaction of cyanine dye with nucleic acids is accompanied by intense changes of their optical properties which help in development of the dengue microfluidic chip.

Table 1
Sequences, target gene & size (bp) of DNA primers used in this study.

Primer	Sequences (5' → 3')	Target gene & size
DENV-2	Forward- GTT CCT CTG CAA ACA CTC CA Reverse- GTG TTA TTT TGA TTT CCT TG	Envelope protein E (230 bp)
DENV-3	Forward- GGT CTC ACT TCC AGA GCA Reverse- TGA AGG CAT GTA GTC CAG	RNA-dependent RNA polymerase NS5 (102 bp)

To minimize false positives, specific proprietary DNA sequences were identified and provided by our collaborators to represent the dengue serotypes DENV-2 and DENV-3. The sequences of the DNA primers used in this study were designed by SIRIM Berhad and their expected sizes are shown in Table 1. Each primer pair was sensitive and specific as it showed high sensitivity towards positive clinical serum samples and no cross-reactivity tested by RT-PCR against culture supernatant of DENV 1–4, chikungunya virus (CHIKV), Japanese encephalitis virus (JEV), and positive clinical serum samples. The RT-PCR products of DENV-2 and DENV-3 tested on microfluidic chips were supplied by SIRIM and the sequences were provided by HUSM.

For the on-chip experimental runs, 16 μL of MilliQ water was preloaded into MilliQ (ultrapure) water storage chamber (B) and 5 μL of cyanine dye into the cyanine dye storage chamber (D) respectively, as depicted in Fig. 3.

Prior to microfluidic chip test, the viral RNA from cell culture supernatants of DENV-2 and DENV-3 were extracted and transcripted using QIAamp viral RNA mini kit and Qiagen One step RT-PCR according to manufacturer's protocol, respectively. These reverse transcription polymerase chain reaction (RT-PCR) products were used for the optimization of the concentration of the cyanine and the volume of RT-PCR products needed for benchtop test and analyzed by gel electrophoresis assay. For the specificity test, 2 μL of RT-PCR products of chikungunya virus (CHIKV) and Japanese encephalitis virus (JEV) were mixed with 18 μL RNase-free water and 5 μL cyanine dye in eppendorf tube. For the sensitivity test, the RT-PCR products from DENV-2 and DENV-3 were prepared in a few concentrations ranging from 1000 to 10000 ng/μL and mixed with 5 μL cyanine. All the reactions were incubated at room temperature for 10 min and the color changes were observed after the end of incubation time.

3. Results and discussion

In this study, we developed a downstream detection module in the form of a microfluidic chip to determine the dengue virus serotypes via colorimetric analysis based on the binding of cyanine dye with DNA from an RT-PCR product. The positive samples turned the cyanine dye from blue to violet while the color remained blue for negative samples. The specificity and sensitivity of each primer pair were determined to minimize the risk of primer dimer formation during the RT-PCR amplification process. The optimization of the RT-PCR process was also performed; however, the details of both primer and RT-PCR optimization are not included in this paper.

3.1. Results for benchtop setup

Violet coloration (change) was observed, as shown in Fig. 6 for both (positive control) DENV-2 and DENV-3, 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.

Based on the specificity test for the RT-PCR primers of DENV-2 and DENV-3 in a mix of DENV 1–4, CHIKV and JEV, the eppendorf tube test (Fig. 7a and b) and corresponding gel electrophoresis analysis in Fig. 7c and d respectively resulted in violet color changes only for DENV-2 and

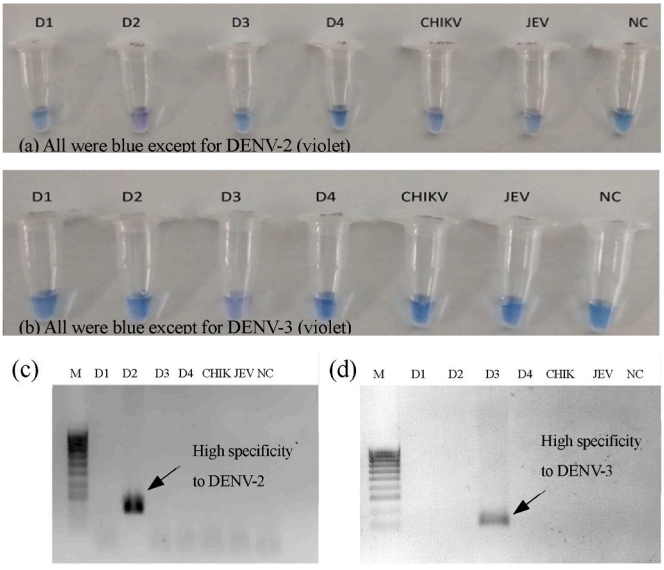

Fig. 7. Specificity tests (turns violet) of the cyanine dye for (a) DENV-2, (b) DENV-3; Gel electrophoresis analysis – RT-PCR products of (c) DENV-2 and (d) DENV-3.

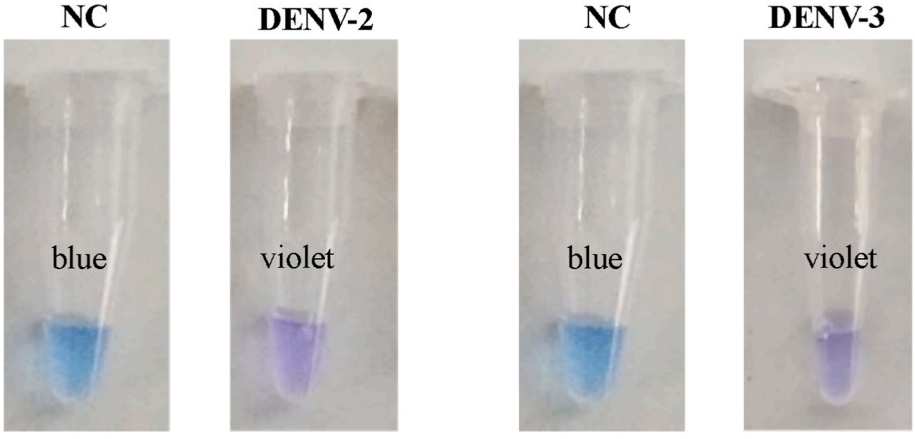

Fig. 6. Two separate sets of RT-PCR products consisting of negative control (NC) and positive control (PC) performed in 0.5 mL eppendorf tubes. Photo taken with natural illumination of the sun in the laboratory.

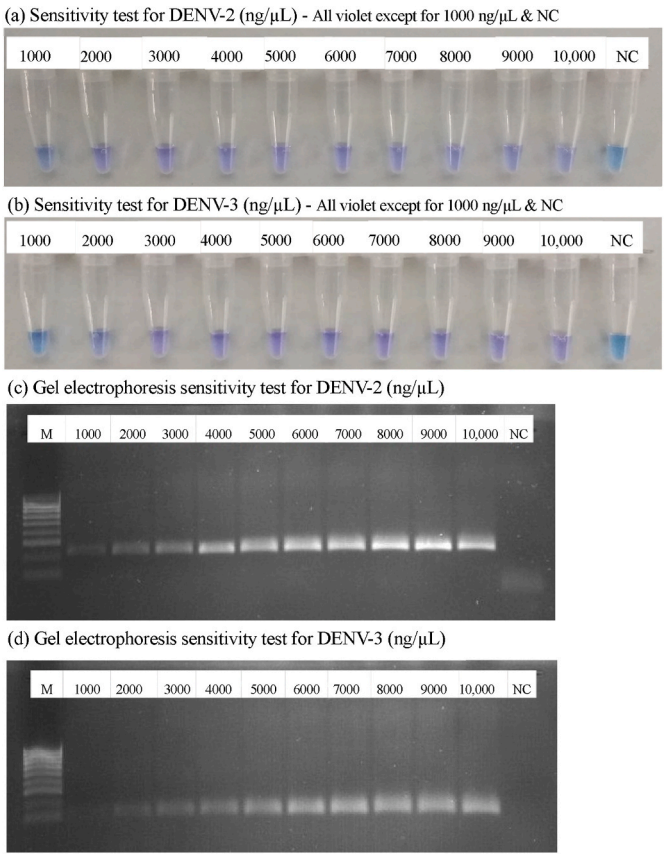

Fig. 8. Cyanine dye concentration sensitivity tests for (a) DENV-2 and (b) DENV-3; Gel-electrophoresis sensitivity tests for (c) DENV-2 and (d) DENV-3 from 1000 ng/uL to 10,000 ng/uL in steps of 1000 ng/uL.

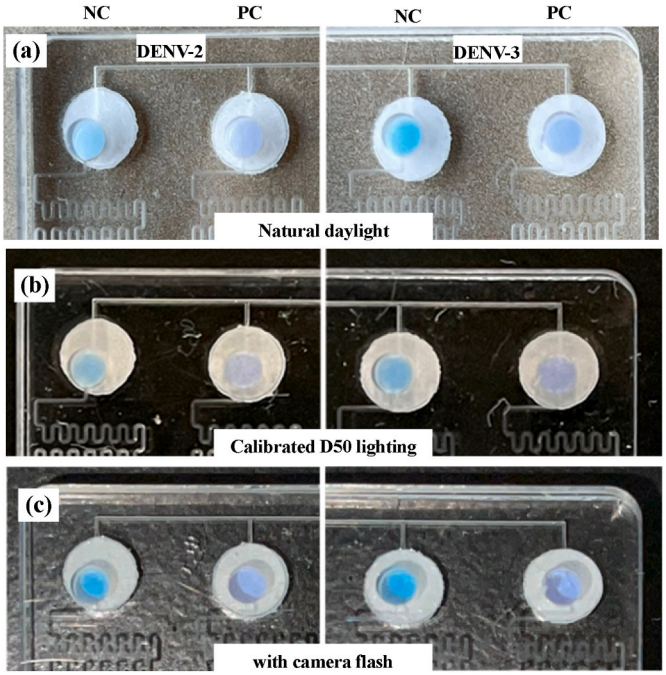

Fig. 9. Comparison of color contrast for NC and PC samples of two separate RT-PCR products performed on-chip. The photo was taken with (a) natural daylight illumination, (b) calibrated D50 lighting and (c) with smartphone camera flash.

DENV-3 respectively, due to binding of the DNA from the RT-PCR products with the cyanine dye. As expected, only the expected bands showed up during gel electrophoresis, exhibiting absence of non-specific bands and indicating high specificity.

The sensitivity test in Fig. 8 showed that the limit of the detection of the DENV-2 and DENV-3 was 2000 ng/μL. The color change observed corroborates with the intensity of the band of the gel electrophoresis.

3.2. Results for on-chip experiments

The color contrast between the negative control (NC) samples and positive control (PC) samples was noticeable with the naked eye when observed under 3 different lighting conditions – natural daylight (Fig. 9a), calibrated D50 lighting (Fig. 9b), and camera flash (Fig. 9c).

The negative control (NC) samples for both DENV-2 and DENV-3 samples yielded an azure blue coloration while positive samples for both DENV-2 and DENV-3 showed blue turned to violet coloration. It was observed that the on-chip color contrast was well-perceived with both the naked eye and the camera.

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) (Fig. 3) 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.

4. Conclusions

This study reports a microfluidic chip designed with pre-loaded diluent 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. The RT-PCR product of DENV-2 and DENV-3 used in the experiment has been optimized and tested in the microfluidic chip to determine the color change from blue to violet for positive control (PC) samples and no change in blue color for negative control (NC) samples.

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 for facile DNA detection has the potential to be applied to other applications requiring efficient mixing and prolonged incubation such as PCR mix (product) preparation and detection of nucleic acids with other colorimetric assays requiring multi-step fluidic manipulation such as on-chip storage, reactions, and incubation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Leon Chan Cong Zhi reports financial support was provided by APP Systems Services Pte Ltd, industry partner of Singapore Institute of Manufacturing Technology.

Acknowledgement

The authors would like to acknowledge and thank our industry partner, APP Systems Services Pte Ltd, for providing the funding to complete this study.

References

1. Bhatt S, Gething PW, Brady OJ, et al. The global distribution and burden of dengue. *Nature*. 2013;496:504–507. https://doi.org/10.1038/nature12060.
2. Brady OJ, Gething PW, Bhatt S, et al. Refining the global spatial limits of dengue virus transmission by evidence-based consensus. *PLoS Neglected Trop Dis*. 2012;6. https://doi.org/10.1371/journal.pntd.0001760.
3. Yacoub S, Farrar J. Dengue. In: Farrar J, Hotez PJ, Junghanss T, Kang G, Lalloo D, White NJ, eds. *Manson's Tropical Infectious Diseases*. W.B. Saunders; 2014:162–170.
4. Ng CFS, Lum LCS, Ismail NA, Tan LH, Tan CPL. Clinicians' diagnostic practice of dengue infections. *J Clin Virol*. 2007;40:202–206. https://doi.org/10.1016/j.jcv.2007.08.017.
5. Chen RF, Yang KD, Wang L, Liu JW, Chiu CC, Cheng JT. Different clinical and laboratory manifestations between dengue haemorrhagic fever and dengue fever with bleeding tendency. *Trans R Soc Trop Med Hyg*. 2007;101:1106–1113. https://doi.org/10.1016/j.trstmh.2007.06.019.
6. National Environment Agency Quarterly Dengue Surveillance Data 2018-2022 Available online: https://www.nea.gov.sg/dengue-zika/dengue/quarterly-dengue-surveillance-data.
7. Yung CF, Lee KS, Thein TL, et al. Dengue serotype-specific differences in clinical manifestation, laboratory parameters and risk of severe disease in adults, Singapore. *Am J Trop Med Hyg*. 2015;92:999–1005. https://doi.org/10.4269/ajtmh.14-0628.
8. Wong ML, Medrano JF. Real-time PCR for MRNA quantitation REVIEW. *Biotechniques*. 2005;39:75–85. https://doi.org/10.2144/05391RV01.
9. Kim SK, Cho H, Jeong J, Kwon JN, Jung Y, Chung BH. Label-free and naked eye detection of PNA/DNA hybridization using enhancement of gold nanoparticles. *Chem Commun*. 2010;46:3315–3317. https://doi.org/10.1039/b926940a.
10. Abdul Rahman S, Saadun R, Azmi NE, et al. Label-free dengue detection utilizing PNA/DNA hybridization based on the aggregation process of unmodified gold nanoparticles. *J Nanomater*. 2014;2014. https://doi.org/10.1155/2014/839286.
11. Tseng D, Mudanyali O, Oztoprak C, et al. Lensfree microsopy on a cellphone. *Lab Chip*. 2010;10:1787–1792. https://doi.org/10.1039/c003477k.Lensfree.
12. Chen Y, Zhang L, Chen G. Fabrication, modification, and application of poly(methyl methacrylate) microfluidic chips. *Electrophoresis*. 2008;29:1801–1814. https://doi.org/10.1002/elps.200700552.
13. Wilhelmsson LM, Nordén B, Mukherjee K, Dulay MT, Zare RN. Genetic screening using the colour change of a PNA-DNA hybrid-binding cyanine dye. *Nucleic Acids Res*. 2002;30:3–6. https://doi.org/10.1093/nar/30.2.e3.
14. Smith JO, Olson DA, Armitage BA. Molecular recognition of PNA-containing hybrids: spontaneous assembly of helical cyanine dye aggregates on PNA templates. *J Am Chem Soc*. 1999;121:2686–2695. https://doi.org/10.1021/ja9837553.
15. Xu M, Xing S, Xu X, Fu P, Xu W, Zhao C. Label-free colorimetric aptasensor for highly sensitive and selective detection of proteins by using PNA/DNA hybrids and a cyanine dye. *Anal Methods*. 2018;10:3824–3829. https://doi.org/10.1039/c8ay01071d.
16. Zhytniakivska O, Girych M, Trusova V, et al. Spectroscopic and molecular docking studies of the interactions of monomeric unsymmetrical polycationic fluorochromes with DNA and RNA. *Dyes Pigments*. 2020;180, 108446. https://doi.org/10.1016/j.dyepig.2020.108446.