Simultaneous detection of multiple HPV DNA via bottom-well microfluidic chip within an infra-red PCR platform

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Portable Polymerase Chain Reaction (PCR) devices combined with microfluidic chips or lateral flow stripes have shown great potential in the field of point-of-need testing (PoNT) as they only require a small volume of patient sample and are capable of presenting results in a short time. However, the detection for multiple targets in this field leaves much to be desired. Herein, we introduce a novel PCR platform by integrating a bottom-well microfluidic chip with an infra-red (IR) excited temperature control method and fluorescence co-detection of three PCR products. Microfluidic chips are utilized to partition different samples into individual bottom-wells. The oil phase in the main channel contains multi-walled carbon nanotubes which were used as a heat transfer medium that absorbs energy from the IR-light-emitting diode (LED) and transfers heat to the water phase below. Cyclical rapid heating and cooling necessary for PCR are achieved by alternative power switching of the IR-LED and Universal Serial Bus (USB) mini-fan with a pulse width modulation scheme. This design of the IR-LED PCR platform is economic, compact, and fully portable, making it a promising application in the field of PoNT. The bottom-well microfluidic chip and IR-LED PCR platform were combined to fulfill a three-stage thermal cycling PCR for 40 cycles within 90 min for Human Papilloma Virus (HPV) detection. The PCR fluorescent signal was successfully captured at the end of each cycle. The technique introduced here has broad applications in nucleic acid amplification and PoNT devices. *Published by AIP Publishing.* <https://doi.org/10.1063/1.5023652>

I. INTRODUCTION

The ability to provide on-site analysis results rapidly is invaluable for monitoring the epidemiology of serious infectious diseases, food hygiene, food additives, and pesticide residues. This is also especially important for developing countries with poor infrastructure as they have a higher need to easily identify harmful elements. Miniaturized PCR instruments^{1–5} have shown great potential in this ability, and they provide the conventional PCR functions on a portable device that can be used in any condition and are capable of rapid identification of target genes. Compared with bench-top PCR thermocyclers, many miniaturized PCR devices have exhibited superior performance in aspects such as cost, ramping speed, portability, integration level, and efficiency.

Sample partitioning is a critical step in many biological and chemical experiments, especially in digital PCR,^{6–8} drug screening analysis,^{9–11} and single cell analysis.^{12–16} Several methods exist to achieve this goal, such as the creation of hydrophilic/hydrophobic regions on the same surface,^{17–19} utilization of the physical property of microfluidic channels and wells,²⁰ and utilization of the shear force between two immiscible phases.^{21–23}

Flow focusing, which uses two immiscible phases to form a large quantity of emulsions, is one of the most popular methods in the field for the generation of small individual volumes of samples.^{24–28} This method has shown great results in the mixing study,²⁹ multi-emulsion

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droplets generation,³⁰ cell encapsulation,³¹ gene analysis,³² and enzyme or protein expression.³³ However, this method also has limitations, namely, the high dependency on the precise control of pump injection speed and need for more than two pumps, which hampers its applications in point-of-need testing (PoNT) devices.⁶

The self-digitization side-chamber assay^{8,34} and high-density microfluidic bottom-well assay,³⁵ introduced by Daniel T. Chiu *et al.*, have shown great potential in digital PCR and single cell studies. These assays utilized nanoliter side chambers or bottom-wells to contain the samples for subsequent fluorescent detection. However, in the above studies, the fluorescent signal in each chamber is detected and quantified using the TyphoonTM imager which is delicate and non-portable. This is necessary because each chamber only contains 2 nl of sample, which would require a high-resolution camera and optic amplification technology to capture. While in the field of PoNT, qualitative or semi-quantitative results can fulfill the majority of its applications. Thus, by enlarging the well volume from nanoliter to microliter, it not only reduces the cost of the optic detection module but also renders the entire system to be more compact and portable.

In this study, we introduce a bottom-well microfluidic chip upon the infrared mediated PCR platform to fulfill the testing of three different types of HPV nucleic acid amplification for 40 cycles within 90 min. Infrared (IR) heating in PCR systems has been reported by other researchers for more than a decade, such as the Landers group at the University of Virginia.^{36–38} Real-time detection of IR mediated PCR in single and multi-color detection formats was demonstrated by the Roper group.^{39,40} These method usually utilize a tungsten lamp or an IR lamp combined with a long-pass filter and convex lens to heat up the water phase directly. The novelty of this work originates in its use of an infrared (IR) light that directly and rapidly heats the oil contained in the carbon nanotubes. Compared to other direct heating light sources, light-emitting diode (LED) arrays are cheap, compact, and programmable with sufficient power. Additionally, the design does not require the use of any optical components since the LED array is placed right upon the microfluidic device to cover the entire fluidic surface. Cyclical rapid heating and cooling necessary for PCR are achieved by the alternative switching of power states for the IR-LED and USB mini-fan via a pulse width modulation (PWM) scheme. In this platform, we chose a blue-light LED and a camera to detect the fluorescent signals of PCR products. In addition, the bottom-well microfluidic chip utilizes the advantages of the digital PCR chip in sample partitioning that avoids cross-contamination. By increasing the droplet volume from nanoliter to microliter, it is also easier for the camera to detect the fluorescent signals.

II. MATERIALS AND METHODS

A. Design and preparation of the microfluidic chip

The microfluidic chip is consisted of a cover layer and a bottom layer (Fig. 1). The cover layer contains an inlet and an outlet, while the bottom layer contains the main channel and bottom wells. The bottom layer is produced from a mold (Fig. S1 in the [supplementary material](#)) which was 3D printed with the Form 1+ printer (Formlabs, USA). The inverse structures of the 3D printed templates were formed by pouring elastic polymer PDMS [poly (dimethylsiloxane), Sylgard 184 silicone elastomer kit, Dow Corning Corp., Midland, MI] onto the mold and peeling it off when it is cured. The main channel width and height are 2 mm (Wm) and 0.5 mm (Hm), and the bottom-well dimensions are all 1 mm × 2 mm × 1 mm (w × l × d). Three reverse primers, for the detection of HPV 16, 18, and 33, are added to the designated wells by pipetting. Then, the primers are sealed with a paraffin film after it dries. The paraffin film is to seal the primers from degradation before detection. In addition, the paraffin film can also prevent cross-contamination because the sample will go into each well when it is loaded. With paraffin sealing the primers, samples will not dissolve the primers or bring it to other wells. When the samples are heated, the paraffin films will melt, and only then, the samples can dissolve and react with the primers. The cover layer of the microfluidic chip was pierced twice with a flat head needle at the proper positions to form the inlet/outlet ports. These two PDMS pieces were bonded together after plasma treatment (plasma cleaner, Harrick Plasma Inc., Ithaca, NY, USA) for 50 s.

FIG. 1. The scheme of the bottom-well PCR microfluidic chip. (a) An overview of the bottom-well PCR microfluidic chip design. (b) An illustration of the reagent storage in the bottom-wells of the PCR microfluidic chip. (c) The final product image of the bottom-well PCR microfluidic chip.

The PDMS microfluidic chip was first soaked in mineral oil (30.0 cSt; light, embryo tested, Sigma Aldrich, St. Louis, MO) and then placed in a vacuum chamber to expel any excess air in the channel, wells, and PDMS material. Before sample digitization, the polytetrafluoroethylene (PTFE) tubes were filled with an oil phase [mineral oil, 0.02% Span 80 and 4 mg/ml multi-walled carbon nanotube (MWNT)]. Then, a 40 μ l PCR sample was sucked into the tube by a syringe pump. Next, the PCR sample and oil phase were injected into the PDMS chip to form sample digitization (Fig. S2 in the [supplementary material](#)). The PTFE tubes were unplugged from the chip, and a thermocouple was inserted into the chip. Finally, the inlet and outlet of the chip were sealed with ARsealTM adhesive tape (Adhesives Research, Inc., Glen Rock, PA).

B. The design of the PCR platform

The IR PCR platform consisted of an infrared LED (20 W, 840 nm), a blue-light LED (10 W, 460 nm), a USB fan (MIUI, China), a camera (HuaXinWei, China), two band-pass filters (460 nm and 530 nm), a holder, a thermocouple (TT-K-36-SLE, ETA), a data acquisition card (USB-DAQ-XF103, Antech Enterprise Limited), a thermocouple cold junction compensation circuit, a LED circuit, and a fan circuit [Fig. 2(a)]. The holder was 3D printed as the fixture for the IR-LED, blue-light LED, filters, and camera (Fig. S3 in the [supplementary material](#)). It was also sprayed with black paint to reduce ambient light.

The principle of the PCR platform is illustrated in the three-dimension graphic in Figs. 2(b) and 2(c) (rendered with Autodesk Maya). The voltage signal for the infrared LED was controlled with Proportional-Integral-Differential (PID) algorithms, and a pulse-width modulation (PWM) control was established to vary the voltage signal duty cycles. With a higher LED voltage signal duty ratio, more power would be generated to heat up the multi-walled carbon nanotube (MWNT). The USB mini-fan was used to cool the microfluidic chip between the denaturation and annealing processes. At the end of each extension process, the blue-light LED was triggered to excite the SYBR green in the PCR samples, and images were captured with the camera. Two band-pass filters were used to reduce signal noise.

C. Sample preparation

Patient cervical swab specimens were collected from volunteers at the International Peace Maternity & Child Health Hospital (Shanghai, China) and were de-identified. The pretreatment

FIG. 2. The scheme of the IR PCR platform. (a) The physical illustration of the IR PCR platform. (b) The working principle of the IR PCR platform. (c) The demonstration of temperature control for on-chip heating, cooling, and fluorescence detection.

method for the samples was in accordance with previous publications.⁴¹ The samples collected tested positive for either one or a combination of HPV 16, 18, or 33. Since the detection of HPV 16, 18, and 33 shared the same forward primer, the only difference is reverse primers. Thus, we used dry reagent storage to pre-add the reverse primers in different wells as demonstrated in Fig. 1(b). The patient's sample and the rest of the PCR reagents were mixed before loading.

Common forward primer: 5'-AAGGGCGTAACCGAAATCGGT-3'

HPV16 reverse primer: 5'-GTTTGCAGCTCTGTGCATA-3'

HPV18 reverse primer: 5'-GTGTTCAAGTCCGTGCACA-3'

HPV33 reverse primer: 5'-GTCTCCAATGCTTGGCACA-3'

The primer sequences amplified a 140 bp fragment. The 40 μ l PCR sample mixture was composed of 20 μ l of 2 $\times$ Fast SYBR[®] Green Master Mix (Lot No: 1212079, Applied Biosystems, USA), 1.0 μ l of forward primer (10 μ M), 2 μ l of pretreated patient sample, and 17.0 μ l of deionised (DI) water. The thermal cycling protocol was set to preheat at 94 $^{\circ}$ C for 5 min, followed by 40 cycles of 94, 59, and 72 $^{\circ}$ C for 30, 30, and 15 s, respectively, with a final extension step at 72 $^{\circ}$ C for 2 min.

D. Fluent simulation of sample digitization and heat transfer

Three-dimensional simulation of the sample digitization and a heat transfer process in multiple wells were conducted with the Fluent software (Workbench 17.0, ANSYS Inc., Pennsylvania, United States). The influence of viscous/interfacial properties (Ca), varying dimensions (Wm, Hm), and the overhang of the main channel (W) were studied by Thomas Schneider and his coworkers.³⁵ However, the influences of the well length/depth (l, w) and the velocity of the oil phase on sample digitization were lacking. The setup in the Fluent simulation included the mesh and volume of fluid (VOF) solver with similar boundary conditions to those of Thomas' paper, except for the interfacial tension value of water-oil which was set to 0.018 N/m, the contact angle of the main channel and well was set to 150 $^{\circ}$, and the volume of water plug was equal to the well volume.

In heat transfer simulation, PDMS (density $\rho = 1030$ kg/m³, specific heat $C_p = 1500$ kJ/kg K, and thermal conductivity $\lambda = 0.2$ W/m K) was used as a microfluidic chip material, water ($\rho = 998.2$ kg/m³, $C_p = 4182$ kJ/kg K, $\lambda = 0.6$ W/m K, and $\mu = 1.003 \times 10^{-3}$ kg/m s) and silicone

oil (50 cSt; $\rho = 840 \text{ kg/m}^3$, $C_p = 1942 \text{ kJ/kg K}$, $\lambda = 0.145 \text{ W/m K}$, $\mu = 0.049 \text{ kg/m s}$) were used as the aqueous solution/oil phase, and air ($\rho = 1.225 \text{ kg/m}^3$, $C_p = 1006.43 \text{ kJ/kg K}$, $\lambda = 0.0242 \text{ W/m K}$, and $\mu = 1.7894 \times 10^{-5} \text{ kg/m s}$) was set as the ambient phase. The solution method was implemented with a coupled scheme (pressure-velocity coupling). The spatial discretization was implemented by a first order unwind scheme for the turbulent kinetic energy/turbulent dissipation rate and second order upwind set for energy.

III. RESULTS AND DISCUSSION

A. Sample retention analysis

The sample digitization is based on the interplay between channel/well geometries and viscoelastic forces. Since the volume of wells in this study is 3-fold larger than the previous study,³⁵ we expect profound variance of liquid behavior between these studies. Thus, it is essential to perform a spate simulation on the liquid behavior with the geometries in this study.

The volume of the water phase, which was plugged in the main channel, equals 10 times of a single well volume. Since the geometry of wells and the velocity of injection speed varied among different conditions, it is necessary to define a normalized time. We define that the moment when the water phase reaches the first well as t_1 , when the water phase reaches the second well as t_2 , etc. [Fig. 3(a)]. The retention ratio is defined as the volume of the water phase in the well divided by the total volume of the well.

Figure 3(b) shows the retention ratio as a function of time and is plotted for different wells. Thus, the first well was chosen to demonstrate its retention ratio in different conditions. The maximum retention ratio decreases with the increase in the l/w ratio [Fig. 3(c)]. It seems that a smaller l/w ratio makes it harder for the water phase to enter the bottom wells and harder to be expelled by the following oil phase. On the other hand, the retention effect which traps the water phase inside the well seems to improve significantly with the decrease in the l/w ratio. However, if the total volume of the water phase and the main channel length were taken into consideration, it would conclude that the larger l/w ratio is preferred to improve the retention effect.

The increase in the d/w ratio has no observable effect on the maximum retention ratio [Fig. 3(d)], and larger d/w ratios are easier to trap the water phase in the bottom wells. Figure 3(e) shows that the injection speed has little influence on the retention effect but no obvious influence on the retention ratio. This indicates that the need of a pump for sample injection could be eliminated, and this prompts the application of current microfluidic chips in point-of-care testing (POCT) devices.

B. Heat transfer analysis

Figure 4(a) illustrates the heat transfer setup. The main channel was filled with oil, while the wells were filled with water. The well geometry in the heat transfer simulation was $1 \text{ mm} \times 2 \text{ mm} \times 3 \text{ mm}$.

In the heat-up simulation, the oil temperature was set to be constant at 95°C . The temperature of the PDMS and water was set to room temperature, which was 25°C . The whole system was set to be in an ambient condition of stagnant air. Based on the results from the heat-up study [Fig. 4(c)], it infers that the heat transfer between oil and water phases is rapid which can occur in less than 0.5 s. However, the heat convection that occurred at the well/PDMS surface or the PDMS/air surface decreases the temperature of the bottom of the well prominently.

The well volume was divided into three sections. The first, second, and third region represents the top 1/3, top 2/3, and the volume of the entire well, respectively. The average temperature of the three sections is shown in Fig. 4(b). Our simulation results indicated that the average temperature of the first region can be up to 10°C higher than the average temperature of the whole well. Thus, a smaller d/w ratio can speed up the heating rate and reduce the total heat capacity of the whole microfluidic chip. Combining the simulation of the d/w ratio on sample retention performance, it would be preferred to set the height of bottom wells to 1 mm. Moreover, the PCR chip only needs to be heated up from room temperature to 95°C in the

FIG. 3. Flow analysis of the three dimensional water/oil phase in the microfluidic chips. (a) Scheme of sample digitization in the bottom-well microfluidic chips. The contour represents the volume of the water phase. 1 represents pure water, while 0 represents pure oil. (b) The sample retention ratio difference between the first three wells in correlation with elapsed time. (c) The influence of the well length/width on the sample retention ratio. (d) The influence of the well depth/width on the sample retention ratio. (e) The influence of inlet velocity on the sample retention ratio.

preheat process, which lasts for 300 s in order to decrease the temperature difference within the wells. In addition, the wells in the real chip would contain a mixture of water and oil, which could also decrease the temperature difference within the wells.

In the cool down simulation (Fig. S5 in the [supplementary material](#)), the PDMS temperature was set to 65 °C and the oil/water temperature was set to 90 °C (without fans) and 95 °C (with fans). The wind speed was set to 0.5 m/s. As indicated in Figs. 4(d) and 4(e), the temperature difference in oil and different sections of the well volume can be neglected. The temperature decreased faster with operating fans compared to no fans operating due to enhanced heat convection effects [Fig. 4(f); Figs. S6 and S7 in the [supplementary material](#)].

C. Fluorescence detection of HPV

Based on the results of flow simulation and heat transfer simulation, the geometry of the bottom well was set to be 1 mm × 2 mm × 1 mm. The temperature ramping speed of this platform

FIG. 4. Heat transfer analysis of the three dimensional water/oil phase in microfluidic chips. (a) Scheme of the model setup in ANSYS. (b) The temperature difference of the three sections in the same well during the heat-up process. (c) The temperature distribution of the simulation diagram in the heat-up process. (d) and (e) The temperature difference of the three sections in the same well in the cool-down process without a fan (d) and the cool-down process with a fan (e). (f) The temperature distribution simulation diagram of the cool-down process.

is illustrated in Fig. 5(d). Compared with the previous design,⁴¹ the bottom well PCR microfluidic chip exhibited a rapid heat-up speed of 2.5 °C/s. This is due to the design where the oil phase is directly in contact with the water phase. Thus, the heat that the MWNT absorbed from the IR-LED can transfer to the water phase without a medium. However, the ramping speed of the cool-down process is slower than the previous design⁴¹ because it is only affected by the heat convection between air and PDMS. As the circulating oil phase reduces the PCR sample retention ratio to zero, the peristaltic pump is not suitable for this type of bottom well chip.

A representation of the visual and quantitative fluorescence level through the 40 PCR cycles is shown in Figs. 5(a)–5(c). Figure 5(a) shows a sample with a positive result for HPV16 and

FIG. 5. Fluorescence detection of HPV upon the infra-red PCR platform. (a) The fluorescence detection of HPV 16 exhibits positive results. (b) The fluorescence detection of HPV 16 and 18 exhibits positive results. (c) The fluorescence curve of HPV 16 and 18 exhibits positive results. (d) The temperature ramping speed of the PCR platform. The control wells work as the negative control for PCR detection (no reverse primers were added into the control wells). Data plots in Fig. 5(c) represent $\text{Mean} \pm \text{S.D.}$ from 4 detections.

Fig. 5(b) a sample with positive results for HPV 16 and 18, all of which matched the results provided by the hospital. The fluorescence curve of the sample from Fig. 5(b) is shown in Fig. 5(c), and the fluorescence of different wells within the same sections was consistent, proving the ability of this platform to accurately detect target DNA.

IV. CONCLUSIONS

A bottom-well microfluidic chip was utilized to partition PCR samples and fulfilled 40 cycles of three-stage PCR within 90 min. The microfluidic chip was fabricated with a 3D printed mold rather than multi-layer soft photolithography. By using LEDs and a USB mini-fan, the design of the IR-LED PCR platform is effectively compact and portable. Cyclical rapid heating and cooling necessary for PCR are achieved by alternative power switching of the IR-LED and USB mini-fan with a pulse width modulation scheme. The ramping rate of the introduced platform in the current study is 2.5°C/s for heating and -0.9°C/s for cooling. A PCR fluorescent signal was captured at the end of each cycle. This platform can successfully detect three types of HPV DNA, and the results are in correspondence with the hospital tests. This PCR platform shows great potential for in-field and PoNT of genetic or contagious diseases. Compared to conventional lab equipment, the size of the current device is only $13\text{ cm} \times 15\text{ cm} \times 12\text{ cm}$ and only weighing at 520 g. Therefore, the present setup offers a good solution for accurate and portable point-of-need PCR devices.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for the fabrication process of the holder, the sample digitization condition, and the temperature distribution simulation. A movie which simulates the sample digitization is also available online.

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