ISOTHERMAL REAL-TIME POLYMERASE CHAIN REACTION DETECTION OF HERPES SIMPLEX VIRUS TYPE 1 ON A LIGHT-ACTUATED DIGITAL MICROFLUDICS PLATFORM

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ABSTRACT

We report on a light-actuated digital microfluidics platform for real-time, isothermal helicase-dependent polymerase chain reaction (PCR). This platform is used to detect Herpes Simplex Virus Type 1 (HSV-1). Reagents in droplets are individually addressed, mixed, transported and arrayed on-chip before the PCR process. 16 droplets were formed from mixing 400 nl PCR master mix droplets with 35 nl HSV-1 viral lysate droplets, arrayed into a 4x4 matrix, and amplified simultaneously, all on chip. Amplification and detection of HSV-1 can be achieved within 45 minutes. No cross contamination was observed.

KEYWORDS

Digital Microfluidics, Electrowetting-on-Dielectric, EWOD, Light-Actuated Digital Microfluidics, Polymerase Chain Reaction, Isothermal Polymerase Chain Reaction, Herpes Simplex Virus

INTRODUCTION

Microfluidics-based polymerase chain reaction (PCR) systems have gained much interest in recent years because of its potential to miniaturize and automate the multi-step PCR processes in molecular biology. It has been demonstrated in EWOD (electrowetting on dielectric)-based digital microfluidics [1]. EWOD was used to shuttle droplets back and forth between a hot and warm zones on chip. However, the different temperature zones limit the PCR droplets to a one-dimensional array, and to date, only 4 simultaneous PCR droplets have been demonstrated [1]. Microdroplet-PCR can pack a large number of surfactant-stabilized, oil-emulsified droplets for digital PCR and large-scale enrichment for targeted sequencing. However, it does not have the flexibility to manipulate individual droplets [2]. Isothermal PCR, such as helicase-dependent nucleic acid amplification (HDA) [3], does not require separate temperature zones and is attractive for microfluidics.

Herpes simplex, caused by the Herpes Simplex Virus (HSV), is a commonly found sexually transmitted disease; symptoms may include meningitis, sores, and skin infections. Herpes Simplex Virus Type 1 (HSV-1) is typically linked to oropharynx infections whilst HSV-2 is typically linked to neonatal or genital infections [4]. Cell culturing of lesion swab content, such as the Enzyme-Linked Virus Inducible System (ELVIS®, Diagnostics Hybrids Inc., Athens, OH) [5], are the most common way of testing HSV currently. However, cell culture-based diagnostics takes a long time (16 hours or more) for results turn-around. Furthermore, cell culturing laboratories are required for diagnosis. Recently, HDA dependent detection of Herpes Simplex Virus has been demonstrated by BioHelix Corporation (Beverly, MA) [6, 7], where individual patient sample can be rapidly tested using portable devices. However, in these devices, reagent volume used is large and each device is only capable of testing one sample.

In this paper, we report on a Light-Actuated Digital Microfluidics (LADM) platform for real-time, helicase-dependent PCR amplification and detection of HSV-1. Similar to EWOD-based digital microfluidics, LADM offers reduced sample/reagent volume, faster reaction time, and higher automation. In addition, LADM can be easily scaled up to larger array format and a wider range of droplet volume. HSV-1 viral lysate is shown to be detected via HDA amplification on-chip within 45 minutes.

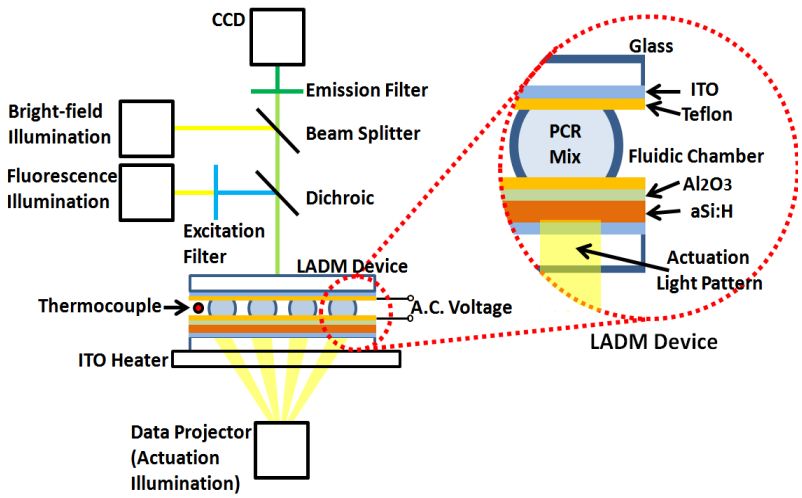

Fig. 1: The experimental setup for real-time isothermal PCR on LADM. Optical patterns from the projector control the droplet motion on LADM. An ITO heater is positioned below the device, and a thermocouple is inserted into the fluidic chamber to provide uniform heating and temperature feedback control. The fluorescence imaging system allows monitoring of the PCR process in real time.

(Inset) LADM Device schematic. Incident light from projector creates localized area of high conductivity in the aSi:H photoconductive film. This switches the voltage drop from the aSi:H layer to the oxide layer, and creates localized electric field concentration in the illuminated region. A net force then acts on the PCR mix droplet, translating it towards the light pattern.

DEVICE FABRICATION & OPERATION

The schematic of the LADM device is shown in the inset of Fig. 1, it consists of an indium-tin-oxide (ITO)-coated glass substrate, a 1 μm thick photoconductive a-Si:H layer deposited via plasma-enhanced chemical vapor deposition (PECVD) (Oxford Plasmalab 80plus), a 150-nm layer of dielectric Al_2O_3 deposited by atomic layer deposition (Picosun Sunale R150), and a 25 nm film of hydrophobic layer formed by spin coating 0.2%-Teflon (3000rpm, 30s). The top electrode consists of a Teflon-coated ITO glass wafer. Details of the LADM device has been reported previously [8].

The microfluidic chamber is formed between the top and bottom plates separated by a 500-μm-thick spacer. AC bias (40 Vppk, 10 kHz) is applied to the top and bottom ITO substrates. A commercially available projector (Dell 4210X DLP) is focused onto the device substrate (Fig. 1). Optical patterns are generated on a computer and sent to the projector. In the absence of light, the externally applied voltage exists primarily across the highly resistive a-Si:H layer. Upon illumination, the conductivity of the a-Si:H increases by more than 100x. This causes the externally applied voltage to drop across the electrically insulating layers (oxide). This imparts an opto-electrowetting force which causes the droplet to move toward the illuminated region. As a result, the illuminated area acts as a virtual electrode. The total manipulation area on chip is 1.5 cm x 1.1 cm. Bright-field illumination and a CCD camera (Sony, XCD-X710) are used for visualization and recording. Fluorescence excitation and read-out are incorporated into the set-up.

METHODS

The fluidic chamber is first flooded with silicone oil (1.0 cSt DMS Trimethylsiloxy terminated Polydimethylsiloxane). Droplets are introduced into the fluidic chamber via a syringe pump (KD Scientific, 780210) and Teflon tube. Temperature in the fluidic chamber is monitored by a thermocouple (Omega Engineering, 5SC-GG-K-30-36) and set by a close-loop controller (Omega Engineering, CN7533) regulating the electrical power supplied to a planar ITO-heater attached to the bottomed of the device.

Helicase-dependent PCR kit (IsoAmp®III tHDA) and blank viral transport medium were acquired from Biohelix Corporation (Beverly, MA), HSV-1 (MacIntyre Strain) Purified Viral Lysate was acquired from Advanced Biotechnologies (Columbia, MD), all viral lysate were centrifuged at 3100 rpm for 10 minutes and supernatant extracted for use. Forward and reverse primers were designed to target and amplify a 100 bp region of the HSV glycoprotein B gene [6], and were acquired from Integrated DNA Technologies (Coralville, IA). The primer sequences are shown in Table 1. EvaGreen Fluorescence dye was acquired from Biotium Corp. (Hayward, CA) and ROX reference dye was acquired from Life Technologies (Carlsbad, CA). Isothermal PCR master mix is prepared with components according to One-Step qHDA (Step B.1) of the IsoAmp®III tHDA kit manual [9], except without the component of DNA Template. 0.2% Pluronic F-68 (Sigma Aldrich, St. Louis, MO) surfactant is added to all reagents to prevent non-specific protein adsorption to the device surface [10].

Table 1: Sequence of Primers

Forward Primer	5'-TTCAAGGAGAACATCGCCCCGTACAA-3'
Reverse Primer	5'-TAAACTGGGAGTAGCGGTGGCCGAAC-3'

RESULTS

Array Formation and Amplification

A 4x4 array was formed by opto-electrowetting, as shown in Fig. 2. 400 nl PCR master mix droplets were dispensed from a tube and transported at 2 mm/s to merge with HSV-1 droplets of 35 nl containing 1.69 x 10^4 viral-particles/nl (prior to lysis), the final HSV-1 concentration was hence 1.36 x 10^3 viral-particles/nl. The merged droplet was then mixed by rolling back and forth [11] and the mixed droplet was transported to a spot in the 4x4 array.

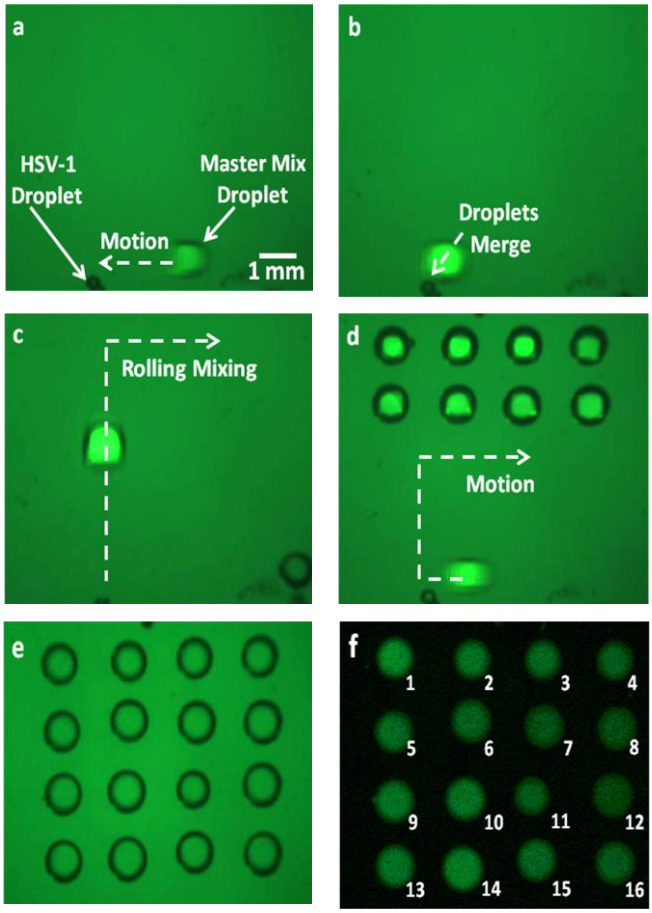

Fig. 2: Array formation. (a)-(c) A droplet of 400 nl isothermal PCR master mix is dispensed from a tube and subsequently transported by a light pattern at 2mm/s to merged with another 35 nl droplet containing HSV-1 viral lysate, the merged droplet is then mixed by rolling on-chip and positioned into array. (d)-(e) Dispensing, merging, mixing and position is repeated to form a 4x4 array. (f) Fluorescence signal of droplets after amplification. Labeled droplet number corresponds to droplet # of amplification curve in Fig. 3.

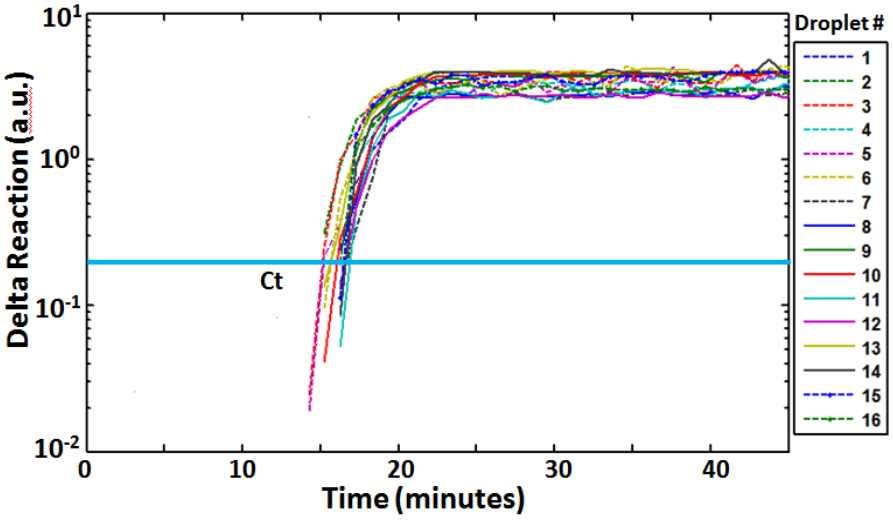

Fig. 3: Real-time isothermal PCR amplification curves of the 4x4 droplet array with PCR mix containing 1.36 x 10^3 viral-particles/nl (droplet # as shown in Fig 2(f). At threshold C_t, the mean amplification time is 16.0 minutes with standard deviation of 0.74 minutes.

The array then underwent isothermal PCR amplification at 64°C for 45 minutes. The real-time amplification curves and final fluorescence signal of the 16 droplets array is shown in Fig. 3. At threshold Ct, the mean amplification time is 16.0 minutes with standard deviation of 0.74 minutes. The array size is 4x larger than that achieved with EWOD shuttling between temperature zones [1]. The 16 on-chip droplets were extracted after the reaction and gel electrophoresis confirmed that the amplified product was 100 bp (data not shown).

Different Concentration Amplification

A 2x2 array with two different viral concentrations, differing by 10x, was formed by opto-electrowetting. Two 800 nl PCR master mix droplets were dispensed from a tube and transported at 2 mm/s to each merge with a HSV-1 droplet of 80 nl containing 1.69 x 10^4 viral-particles/nl (prior to lysis), resulting in a HSV-1 concentration of 1.45 x 10^3 viral-particles/nl. The merged droplets were then mixed and transported to the spots in the array. Two other 800 nl PCR master mix droplets were mixed with 80 nl droplets with 1.69 x10^3 viral-particles/nl, resulting in a HSV-1 concentration of 1.45 x 10^2 viral-particles/nl. The final array positioning is shown in Fig. 4 insert.

The array then underwent isothermal PCR amplification at 64°C for 45 minutes and the real-time amplification curves is shown in Fig. 4. At threshold Ct, the mean amplification times are 16.75 and 23.55 minutes respectively for 1.45 x 10^3 and 1.45 x 10^2 viral-particles/nl droplets.

Cross Contamination Study

A 4x4 array was formed using pre-mixed droplets of 400 nl each. 8 of the droplets formed were positive controls which contained PCR master mix and 1.45 x 10^3 viral-particles/nl HSV-1 viral lysate. The other 8 droplets were negative control containing PCR master mix and blank viral transport medium.

Positive and negative control droplets were dispensed from tubes sequentially and transported into array position. Fig. 5 (a)-(c) shows how positive and negative control droplets cross path on-chip. After arraying, the droplets then went through isothermal PCR amplification at 64°C for 45 minutes. The final fluorescence signal on-chip is shown in Fig. 5 (d).

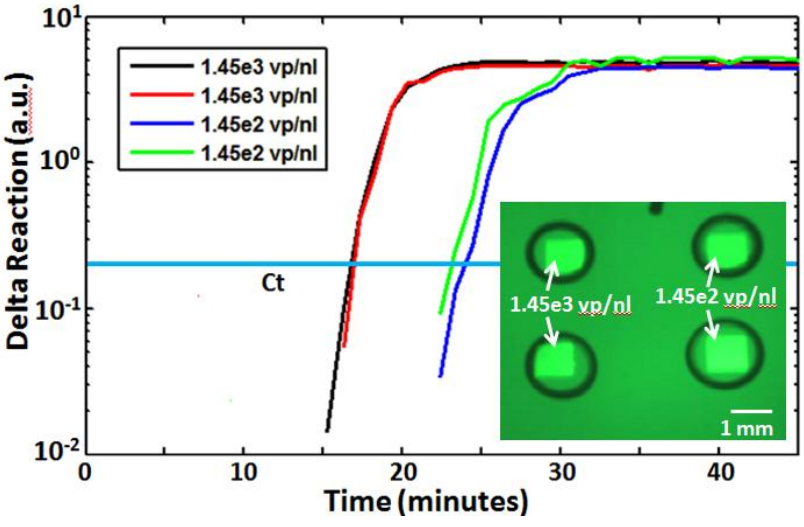

Fig.4: Real-time isothermal PCR amplification curves of 4 droplets of 880 nl each, with two different viral concentrations. (insert) Droplets on the left contain 1.45 x 10^3 viral-particles/nl, while those on the right contain 1.45 x 10^2 viral-particles/nl. At threshold Ct, the amplification time for 1.45 x 10^3 and 1.45 x 10^2 viral-particles/nl are 16.75 and 23.55 minutes, respectively.

Fig. 5(d) shows that no negative droplets (columns 2 and 4, indicated as "N") displayed amplification despite crossing paths with positive control droplets (columns 1 and 3, indicated as "P"). In Fig. 6, the average fluorescence reading for the 8 positive and 8 negative control droplet, normalized over the baseline fluorescence reading is plotted over time, showing amplification only for the positive control droplets.

To ensure that the negative control reagent was not inhibiting the amplification process, viral lysate was added to negative control reagent such that the viral content within reagent became 1.45 x 10^3 viral-particles/nl. This reagent displayed amplification thus confirming the negative control reagent was not inhibiting the reactions. Consequently, we confirm that there are no cross contamination on chip.

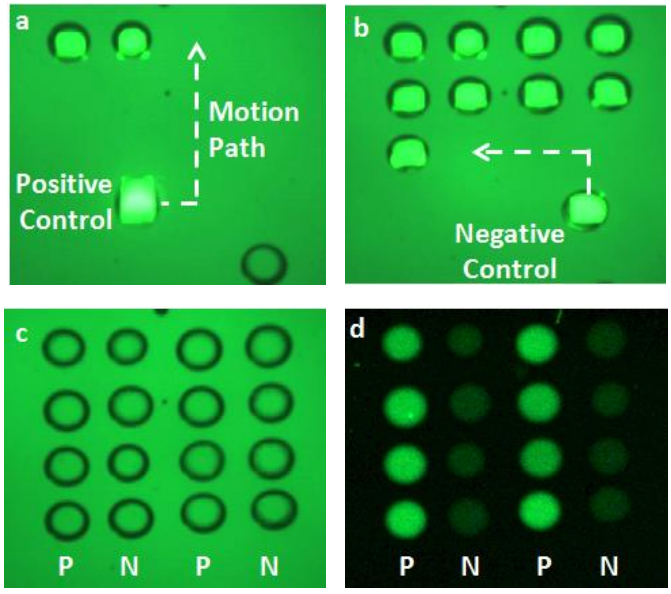

Fig.5:Cross contamination study. (a)-(c) Positive and negative control droplets were dispensed from tubes and transported into array position, A negative control droplet moves over the path previously used by a positive control droplet (with 1.45 x 10^3 viral-particles/nl). First and third columns are positive control droplets while second and fourth columns are negative control droplets. (d) Final amplified product, showing no cross contamination.

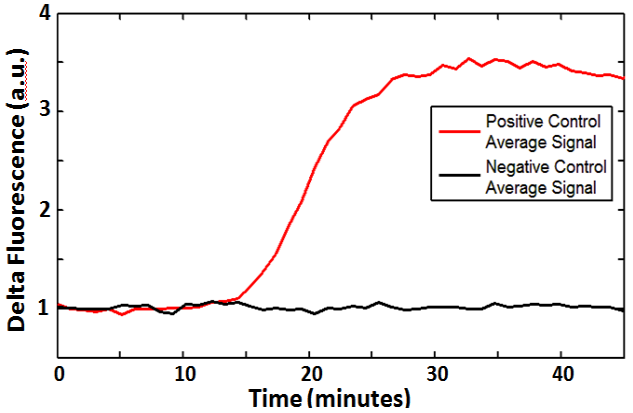

Fig.6: Average fluorescence reading for the 8 positive and 8 negative control droplets, normalized over baseline fluorescence reading.

CONCLUSION

In conclusion, we have used a Light-Actuated Digital Microfluidics device to perform real-time helicase-dependent isothermal polymerase chain reaction for the detection of Herpes Simplex Virus Type 1. A 16-droplet array was formed to demonstrate on-chip dispensing, mixing, transporting and HSV-1 nucleic acid amplification. HSV-1 detection can be performed in 45 minutes without cross contamination on chip.

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