GENERATION AND SELECTIVE RETRIEVAL OF MICRO DROPLETS IN AN ARRAY FOR MICRO-PCR USING A GLASS-SILICON-GLASS CHIP

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ABSTRACT
In this study, we fabricate a glass-silicon-glass chip for the generation of a water-droplet array. Using this array, we succeeded in performing polymerase chain reaction (PCR) within the hundreds of trapped water droplets. Then, we selectively retrieve a specific PCR droplet with a bubble formed by heating an aluminum micro pad with an infrared laser.

INTRODUCTION
The most popular nucleic acid amplification method is polymerase chain reaction (PCR), and this simple method is in advance adapted to a microfluidic field. Developments of on-chip PCR devices are focused in several important factors such as substrate materials and fluidic designs. Biocompatibility and thermal conductivity of the substrates are crucial in the efficiency of PCR. Designs of the PCR chip is also very important factors for the reaction speed and cross-contamination.

To fabricate PCR devices, many kinds of materials have been used as the substrates such as polymer and silicon. Due to the biocompatibility, excellent optical transparency and flexibility, polydimethylsiloxane (PDMS) have increasingly been used in a biochip field. Despite these advantages, PDMS has low chemical resistance to organic solvents and sometimes shows undesired absorption of biomolecules and fluorescent dyes. Also, the permeability of PDMS is not attractive when performing PCR; evaporation of the solution through the PDMS causes the change of the concentration. On the other hand, glass and silicon show high thermal conductivity, good chemical resistance and a non-absorption property of non-specific materials. Although the fabrication process needs expensive equipment compared to that with PDMS, the fabricated devices of silicon and glass can be easily recycled with a simple washing step [1,2].

Designs of PCR chips can be divided in two types: stationary chamber-based chips and continuous flow chips. Compared to stationary PCR chips, continuous flow chips are useful for fast PCR [3]. However, they usually have problems on (i) a cross-contamination, (ii) the poor controllability of the flow and temperature gradient, and (iii) the difficulty in the continuous observation of a specific target [4].

On the other hand, in the stationary chips, simultaneous observation of all the targets is possible. Also, separated chambers of a stationary chip are free from cross-contamination. However, most of recent stationary chips have been fabricated with open-chip architectures composed of etched chambers that needs large volume of PCR solution and covers for the protection of solution evaporation. Therefore, various type of research has been performed to construct stationary chips having a small volume microfluidic architecture without any covering process [5].

Here, we propose a glass-silicon-glass microfluidic chip for PCR. The 'Fishbone-like' etched silicon slide is designed to construct separated stationary chambers of PCR solution. Small microchambers are used as small batch reactors for PCR. Closed microchip and oil can protect evaporation of PCR mixture. Oil types, injection speed are optimized and selective retrieval of a specific product is successfully performed.

EXPERIMENTAL METHODS
Chip fabrication
We prepare three slides for the fabrication of the chip. The upper glass slide contains inlet and outlet holes. The middle silicon wafer is etched to make the channel and chamber walls. The bottom glass is prepared having an aluminum spots for droplet retrieval. To fabricate aluminum patterns, firstly, the S1818 photoresist is spread on the bottom glass and arrays of aluminum patterns are manufactured using a photolithography. After the lithography, extra aluminum is deposited onto the glass and etched. Then, three substrates are bonded using the anodic bonding process. Figure 1 shows scheme images of all components, a real image of the completed chip and a scanning electron micrograph (SEM) of the architecture inside of the device. After bonding of slides, PDMS molds for easy insertion and removal of solutions using silicon tubes are bonded by oxygen plasma treatment at the inlet and outlet regions of the upper glass.

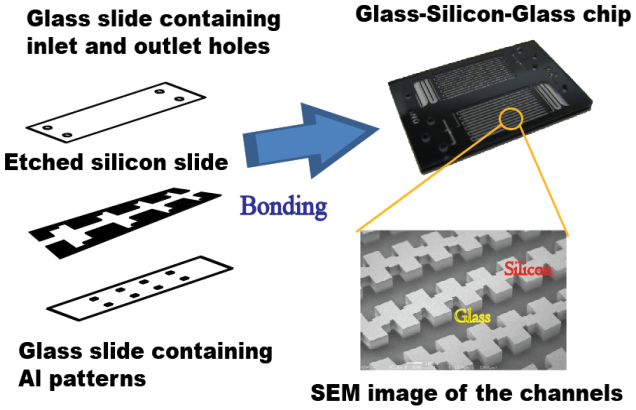

Figure 1. Fabrication of a glass-silicon-glass chip for PCR and selective retrieval. A SEM image shows the shapes of the silicon structure and glass surface. The size of each chamber is 100 x 100 x 115μm.

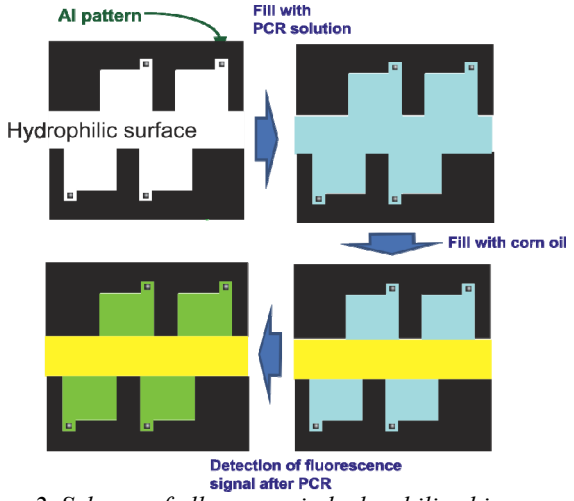

Figure 2. Scheme of all process in hydrophilic chip.

PCR amplification

The pUC19 plasmid DNA having 1.2bp is used as a template. The solution is composed of 50000 unit of template, 0.2 mM of dNTPs, 2.5 µM of each primer (forward: 5`-GTTTTCCCAGTCACGACGTTG-3` and reverse: 5`-ATGACCATGATTACGCCAAGC-3`), 2 mM $MgSO_4$, 2 unit/µL of Taq DNA polymerase (Invitrogen) in a volume of 50 µL. 1 µL SybrGreen I (Invitrogen) which intercalates into the double strand DNA and shows green fluorescence for the detection of PCR products is added to the mixture. After denaturing at 94°C for 7 min, the PCR is carried out for 30 cycles of 94 °C for 15 s, 55°C for 30 s, and 70°C for 1 min followed by final extension for 5 min at 70°C. PCR amplification is carried out on the 'Peltier-device' composed of Pt-100 heater, cooler, control panel and DC adapter purchased from 'VICS Japan'. Temperature is controlled using 'Tera-term' computer program.

Hydrophilic chip

Figure 2 shows the whole process in hydrophilic chip. First, prepared PCR mixture is injected in the channels and inside of the chip is fully filled. Then, oil is pumped into the channels for fabrication of a PCR array. We use two kinds of oil; corn oil, and mineral oil. Also we diversify of oil condition using 0.1% a surfactant, Span-80. And then the microfluidic chip is set on the 'Peltier-device' and PCR is carried out. During the PCR, we observe the midterm results of fluorescence intensity.

Hydrophobic chip

Figure 3 shows the whole process in hydrophobic chip. Before introducing of PCR solution, inside channels and chambers are rendered hydrophobic with octadecytimethoxysilane treatment. Modification solution is composed of 2% octadecytrimethoxy silane, and 0.5% butylamine and toluene. The modifier is injected into the chip and incubated for 20 min at room temperature. Modification solution is blown out following washing step with pure toluene. After the modification, PCR mixture solution is injected into the chip. Because of hydrophobic property of the chip, air in the chambers is maintained without flushing situation by PCR solution. Remained air in the chambers should be excluded for the formation of PCR mixture array.

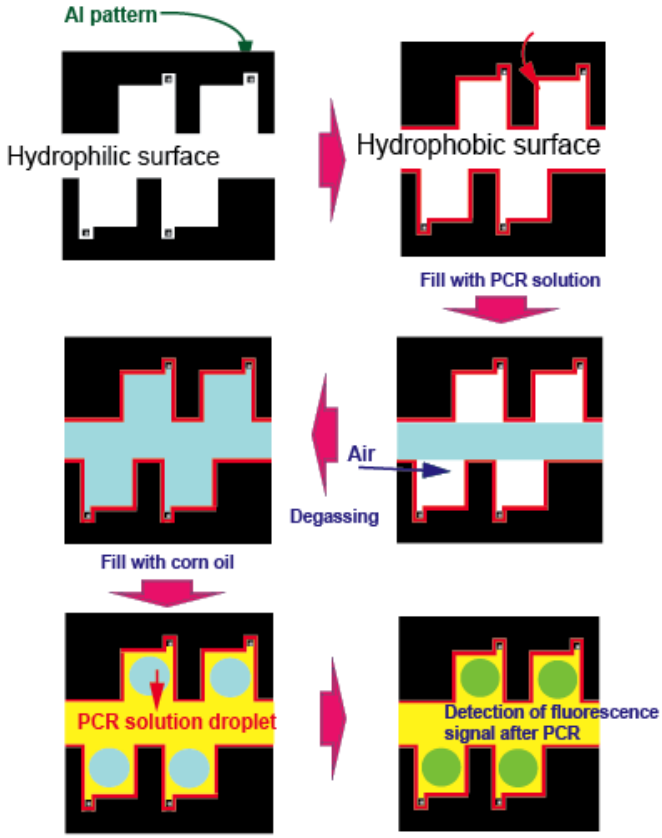

Figure 3. Scheme of all process in hydrophobic chip.

Figure 4 shows the degassing step in this chip. PCR mixture is injected into the channels and extra solution is located at the output. Inlet tube is plugged using a clip and aspiration is performed using a vacuum chamber. During the reducing the air pressure, air bubbles and PCR solution are removed from the chip.

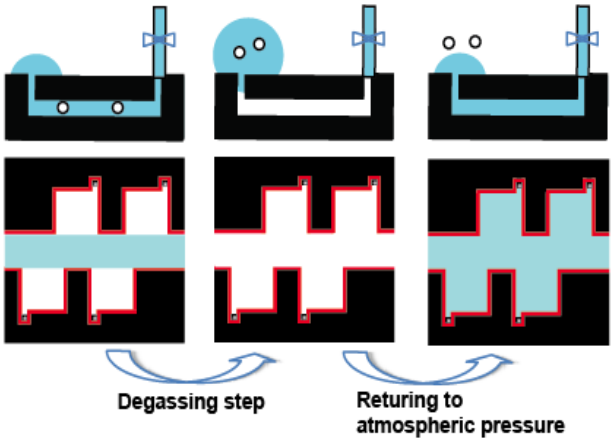

Figure 4. Degassing step. Sufficient water is injected into the channels and extra solution is located at the output. After blocking of inlet, the chip is degassed. Air and solution inside of the channels are removed during the decreasing of pressure. During the returning of pressure, water solution is inserted into the whole volume of the chip.

After empting the inside of the chip, pressure is recovered to atmospheric pressure. During the pressure recovering, only the PCR solution at the outlet region is flowed into the chambers. Therefore, all the channels and the chambers are filled with PCR solution. Following this step, oil is injected into the chip, and whole the walls of the channels and chambers are soaked with oil inducing fabrication of water droplets at the chambers. PCR is performed with the same method of hydrophilic chip.

Retrieval of water droplets

After PCR, we selectively retrieve a specific water droplet. The chip is located onto a microscope and laser controlled using a manipulator is focused on a specific aluminum pattern[6]. After focusing, we irradiate an infrared laser (near 1064 nm, 2.2 W) to the pattern and heating result generates an air bubble that pushes water-bubble containing PCR products. The specific bubble is then carried to the end of channel with the main stream and collected.

RESULTS

Fabrication of PCR array, PCR results and the retrieval process in hydrophilic chip

Because of hydrophilic property of the channels, the water solution is confused inside of the chamber not forming water droplets. Figure 5 (a) shows the optical image of water-oil phase. As shown in a fluorescent image of Figure 5 (b), there is little fluorescence at all area before PCR. After more than 30 thermal cycles of temperature change, observed fluorescence intensity at only water-phase regions is outstandingly increased as shown in Figure 5 (c).

Fluorescence intensity is increased after 25 cycles at this experiment. Increase of fluorescence intensity is affected with the quantitative amount and length of double helix DNA, irradiation time of exciting light, and product size. In this experiment, we obtained a shot product (111bp); therefore it takes a large number of cycles to observe

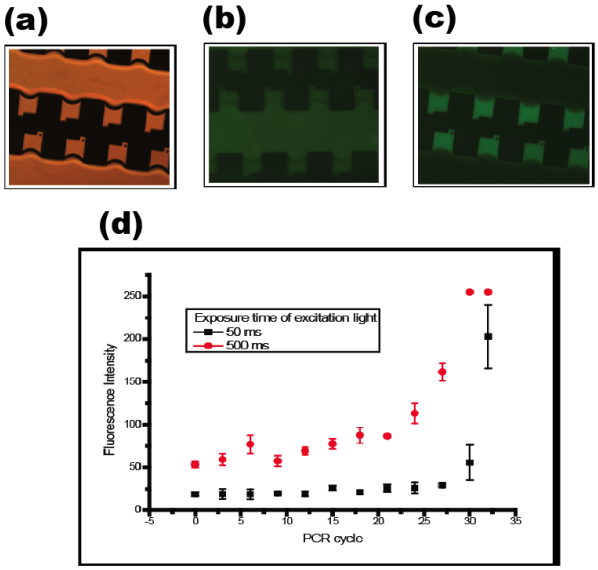

Figure 5. Results in hydrophilic channels: (a) An Optical image of water-oil phase. (b) A fluorescence image before PCR. (c) A fluorescence image after PCR. (d) A graph of the intensity vs. PCR cycle.

increased signal compared to that before reaction. By controlling of various factors such as product size and observing conditions, the time to decide the accomplishment of the PCR can be optimized

After confirmation of PCR results, we carry out a retrieval process of one specific product in a specific chamber using laser heating method. However, during the air bubble formation inside of the chamber, the PCR solution is not escaped from the chamber. Because the silicon and glass substrate is hydrophilic, water runs up the hydrophilic walls. This phenomenon brings out the cross-contamination of PCR droplets. In conclusion, in hydrophilic chip, it is impossible to archive a specific product.

Fabrication of water droplets, PCR results and the retrieval process in hydrophobic chip

Figure 6 shows the results containing from preparation to observation steps. In Figure 6(b), air bubbles which remained after PCR mixture insertion of Figure 6(a) are removed after degassing step. Figure (c) and (d) show the fabricated water droplets after corn-oil injection. Compared to water shape of hydrophilic chip, oil is flowed through the whole walls and glass substrates. Therefore, oil wrap up the PCR solution in each chamber, and water droplets are fabricated.

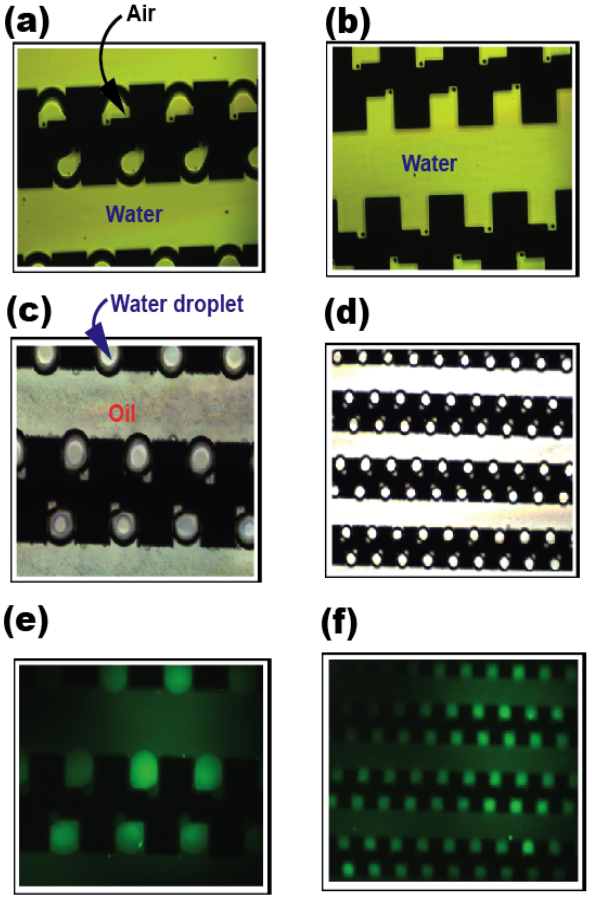

Figure 6. Optical/fluorescence images in hydrophobic channels: (a) An optical image after injection of water. (b) An optical image after degassing. (c) and (d) Optical images after oil injection. (e) and (f) Fluorescence images after PCR.

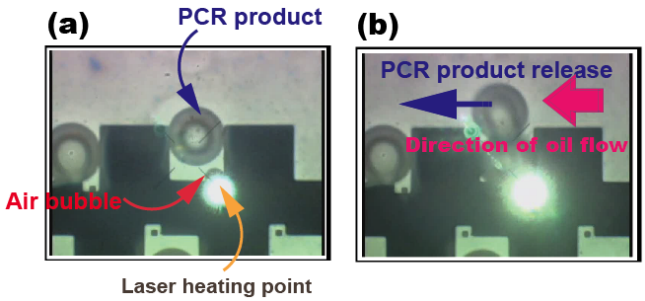

Figure 7. Selective retrieval of a specific PCR product from a microchamber: (a) Fabrication of air bubble by heating an aluminum spot using laser. (b) Retrieval of PCR product by air bubble.

Fabricating condition and size of water droplets are involved precisely with the pumping speed of oil. Water in all chambers is pushed away from the chambers below than 7 μm/min. More than above speed, mono-disperse water droplets are formed, and the size of water droplet is decreased as the speed of oil injection is increased.

PCR is performed with same method with hydrophilic chip after water droplet fabrication. Figure 6 (e) and (f) show the PCR result of water droplets. Detected fluorescent intensities of water droplets are not much in it of the hydrophilic chip.

After observation of PCR results, we try to retrieve one specific water droplet using a laser. In mineral oil, air bubble is not generated because of its high boiling point. On the other hand, in corn oil, air bubble is generated well as shown in Figure 7. The air bubble is fabricated on the Al pattern and it takes about 30 sec for fully removal of a water droplet from the chamber. Removed selected PCR product in the corn-oil containing 0.1% surfactant is acquired at the output, while water droplet is fused with a droplet of the next chamber in the corn oil without surfactant. Generated air bubble was remained in the chamber replaced of water bubble after stopping of laser irradiation.

After finishing the selection step, the chip is washed using 90% chloroform and 10% methanol solution. By simple heating for complete removal of remained washing solution, we reuse the chip to perform same process, and all the steps are successfully achieved again.

CONCLUSION

Our work shows various advantages over current micro-PCR stationary systems; (i) The microfluidic chip needs small amount of mixture, (ii) selective retrieval of a specific product is possible, and (iii) there is no evaporation without additional covering step since the PCR solutions are contained within the device and surrounded by oil. More efficient real-time, rapid and simultaneous detection is possible by controlling the experimental conditions such as chamber size and fluorescence related factors. Based on this result, we can analyze arbitrary biomolecules using our retrieval method combined with additional analytical tools such as primer-labels or aptamer- conjugated particles.

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