Application of Magnetic Microspheres for Pyrosequencing on a Digital Microfluidic Platform

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Abstract

One of the key advantages to a lab-on-a-chip system based on digital microfluidics is the reconfigurability and reusability of the same chip for different tasks. The ability to manipulate magnetic microspheres in solution on a lab-on-a-chip will expand the variety of tasks that can be performed if the microspheres are used as reagent immobilization surfaces. Because the microspheres can be transported in solution and immobilized by a magnetic field, the use of microspheres on-chip will not alter the reconfigurability or reusability of the chip. The retention of 8 μm diameter magnetic microspheres during droplet splitting has been demonstrated on a digital microfluidic platform. A droplet of deionized water was merged with a droplet containing magnetic microspheres, and Neodymium-Iron-Boron magnets were placed on the cover plate of the electrowetting chip. When the combined droplet was split, the magnetic microspheres were isolated in the daughter droplet in closest proximity to the magnets. Retention rates of microspheres during droplet splitting were observed at and above 88% retention. This result has important implications for the eventual goal of performing a substrate-based process such as pyrosequencing. The ability to retain magnetic microspheres during droplet splitting will allow the microspheres to combine with and split from various reagents as well as to be washed of reaction by-products or excess reagent without removing the microspheres from the chip.

Introduction

One of the key advantages to a lab-on-a-chip based on digital microfluidics is the reconfigurability of the same chip for different tasks. Unlike continuous-flow microfluidic systems which require permanently etched channels, digital microfluidic operations are based on the movement of discrete droplets.[1] The use of magnetic microspheres in lab-on-a-chip systems will greatly expand the variety of tasks that can be performed on-chip by creating a removable substrate to which components of an assay may be attached. This would allow a procedure such as solid-phase pyrosequencing to be performed by a lab-on-a-chip.

Pyrosequencing is real-time DNA sequencing-by-synthesis and is described by Ronaghi et al.[3-4] Pyrosequencing holds several advantages over the more traditional Sanger sequencing, or dideoxy chain termination method. The Sanger method uses fluorescently labeled analogs of dideoxynucleoside triphosphate to interrupt DNA synthesis followed by gel electrophoresis to separate the resulting DNA fragments by size.[2] Pyrosequencing allows real-time sequencing using natural nucleotides, which have a lower rate of misincorporation than labeled nucleotides, and does not require gel electrophoresis, making it less labor-intensive than the Sanger method. In solid-phase pyrosequencing, the DNA that is to be sequenced is amplified via PCR, and biotinylated PCR products are captured on streptavidin coated super paramagnetic beads. The immobilized DNA is then denatured so that a single DNA strand remains attached to the bead, and a sequencing primer is hybridized to this strand. Each of the four DNA nucleotides is added sequentially to the template DNA in the presence of DNA polymerase, ATP sulfurylase, and luciferase.[3] If a nucleotide is incorporated into the template DNA strand, a pyrophosphate molecule is released and detected via the light producing enzymatic luminometric inorganic pyrophosphate detection assay (ELIDA), shown in Figure 1. The pyrophosphate that is released is equal in molarity to the number of nucleotides incorporated into the template, and so analysis of the pyrogram produced allows determination of the number of nucleotides which were incorporated during any of the nucleotide additions.

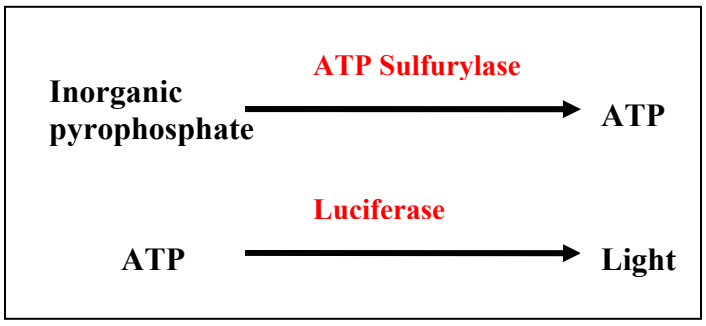

Figure 1: ELIDA reactions

Pyrosequencing currently has many applications, including determination of single-nucleotide polymorphisms, resequencing of PCR products, microbial typing, and analysis of secondary DNA structures such as hairpins.[4] There are many advantages to performing pyrosequencing on a digital microfluidic platform. Reduced reagent volume will result in decreased cost. Unlike continuous-flow microfluidic devices, digital microfluidic devices do not require large amounts of liquid to prime channels prior to use.[7] Since the DNA templates will be washed between each nucleotide addition, there will be no build-up of reaction products, and longer DNA read lengths will be possible. The overall time required for pyrosequencing will be reduced because of reduced incubation time for each nucleotide with the DNA template.[4] As well, the nucleotide incorporation and light detection steps can be decoupled, allowing for individual optimization of each step.

Several key steps must be accomplished before pyrosequencing can be conducted on a lab-on-a-chip. Current pyrosequencing technique utilizes DNA templates attached to magnetic microspheres, which can easily be put on an electrowetting chip in solution. On a digital microfluidic platform, pyrosequencing could be accomplished by merging reagent or wash droplets with the droplet containing the magnetic microspheres and then resolving the double-volumed droplet through droplet splitting. All of the solution removed from the beads after each nucleotide addition would be collected and analyzed with a CMOS photo-detector to determine how many (if any) nucleotides were incorporated during that particular addition. It is essential that none of the magnetic beads are displaced into the departing daughter droplet during droplet splitting because this would cause a decrease in the light signal produced by the ELIDA reaction.[3] Since the molarity of the inorganic pyrophosphate that is released upon successful incorporation of a nucleotide is equal to the molarity of the incorporated nucleotide, the loss of beads to which template DNA is attached could result in incorrect analysis of the pyrogram.[3]

Experimental Methods

Digital microfluidics is based on the concept of electrowetting, which is the decrease in solid-electrolyte interfacial tension that occurs when a potential difference is applied between a solid and an electrolyte, as shown in Figure 2.[5] By placing an aqueous droplet between a continuously grounded top plate and an electrowetting chip with addressable electrodes, the droplet can be transported due to the imbalance of interfacial tension that accompanies the application of an electric field on one side of the droplet.[6] An approximate volume of the droplet can be calculated by squaring the size of the electrodes and multiplying this result by the gap height between the electrode chip and cover plate. An ideally sized droplet will slightly overlap the edges of the electrodes on either side of the electrode it sits on.

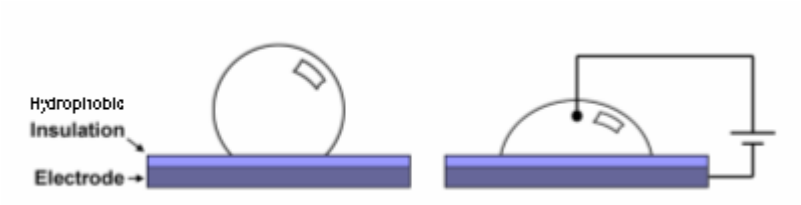

Figure 2: Electrowetting. The application of a voltage to the droplet creates a charge at the liquid/insulator interface, resulting in a wetting force at the contact line that changes the contact angle of the droplet.

The space between the top plate and the electrowetting chip is filled with 1 cSt silicone oil, which helps to contain evaporation of the droplet and also facilitates droplet transport.[7] The droplet is insulated from the electrodes by a ~800 nm layer of Parylene C. The cover plate and the chip with electrodes are both coated with a ~50 nm layer of Teflon AF 1600, which makes their surfaces hydrophobic and enables droplet transport. A side view of the electrowetting chip setup is shown in Figure 3.

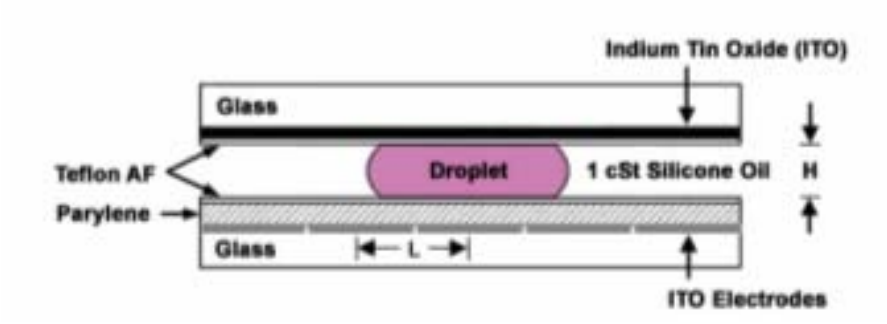

Figure 3: Side view of electrowetting chip

An electrowetting chip with 1.5 mm pitch electrodes (L) and a 175 μm gap height (H) was used to demonstrate magnetic bead retention during droplet splitting. These chips had linear arrays of twenty-two electrodes. Droplets were placed on the chip manually with a 0.1-2.5 μL micropipette. The electrodes were activated using a customized test-clip with spring-loaded pins and a computer-controlled custom-built electronic interface.[6] Fluorescent magnetic microspheres (Bangs Laboratories COMPEL Magnetic Microspheres, 8.46 μm mean diameter) were diluted and screened with a Neodymium-Iron-Boron magnet prior to placement on the chip. The screening consisted of applying the magnet to the outside of a plastic tube containing a 1:1 dilution of magnetic microspheres to deionized water. The solution and any microspheres which did not respond to the magnet were removed and the remaining microspheres were redispersed in deionized water. A significantly higher rate of microsphere retention was observed when pre-screened microspheres were used. A Zeiss Axiovert 100A fluorescence microscope was used to view the experiments. Because this microscope is inverted, chips used for experiments were limited to those which were patterned with (transparent) ITO electrodes.

Droplet splitting was accomplished by arranging a large droplet (approximately twice the volume of one droplet) across three electrodes and deactivating the center electrode, as shown in Figure 4. The volume of the combined droplet that worked best for splitting when magnetic microspheres were present was found to be 788 μL. Because the gap height between the top and bottom glass plates was created using a hand-made spacing device, the gap height was not constant across the entire chip. As a result, it was difficult to produce a consistent automated splitting routine. Pressure differences resulting from the gap height inconsistency caused the droplets to favor some electrodes over others, which made splitting a droplet using the same sequence but on different groups of electrodes nearly impossible.

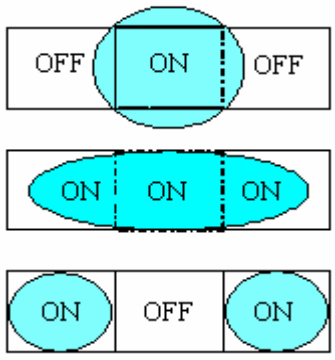

Figure 4: Ideal Droplet Splitting Protocol

Neodymium-Iron-Boron magnets (l=w=h=3mm) were used to immobilize the magnetic microspheres during droplet splitting. The magnet(s) were placed directly on top of the glass cover plate. Different magnet configurations were experimented with in order to obtain high rates of microsphere retention during splitting. For each experiment, a droplet of microsphere solution was placed on the chip and a magnet arrangement was placed on the top plate. The magnets were adjusted until all of the microspheres appeared to be corralled in an area within the droplet. Placing three magnet cubes adjacent to one another to create a magnetic-field well proved effective for microsphere retention (Figure 5). .

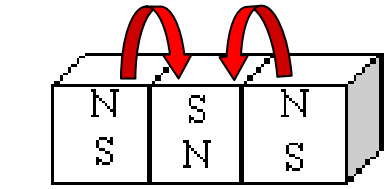

Figure 5: Magnetic-field well created by alternating North and South poles of cube magnet

In order to investigate droplet washing, experiments were transferred to an electrowetting chip with 500 μm pitch electrodes and a built-on spacer which created a consistent gap height of 90 μm. A top view of this chip is shown in Figure 6. These chips contained eight reservoirs which could be filled with reagents through a hole in the top plate. Droplets were formed on the chip from the solution in the reservoirs. The size of the droplets actuated depended on the timing of the actuation sequence and on the electrodes used in the sequence. For ideal splitting, the individual droplets should be small enough that when they are combined, they can be split by using only three electrodes as shown in Figure 4.

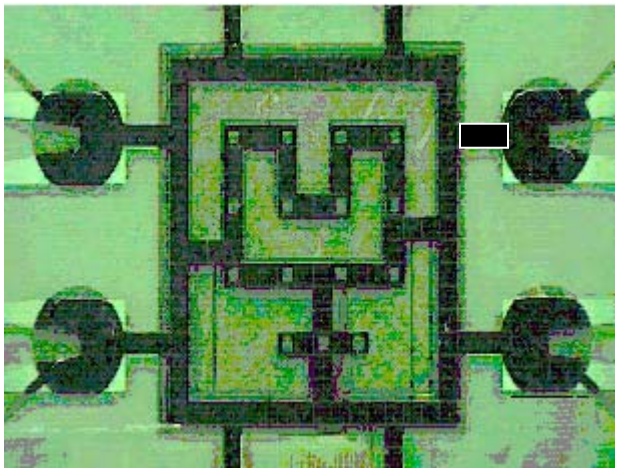

Figure 6: Top view of chip with gasket

Because the electrodes adjacent to the reservoirs are longer than the electrodes in the center of the chip, it was difficult to create droplets that were small enough to be split apart when combined. This is because during droplet splitting, the volume of the "neck" that forms between the emerging droplets is split equally between the droplets. Splitting a droplet off from the reservoir involved formation of the neck on the larger electrode resulting in a larger volumed droplet. In order to form small enough droplets, experiments were transferred to a second-generation chip identical in its properties to the chip shown in Figure 6 but without the elongated electrodes adjacent to the reservoirs.

Washing experiments were performed by actuating a droplet of 2 mM fluorescein in phosphate buffered saline (PBS, .1 M, pH 7.4), actuating a droplet of .001% Triton X in PBS, and transporting the pure PBS droplet through the fluorescein droplet, as shown in Figure 7.

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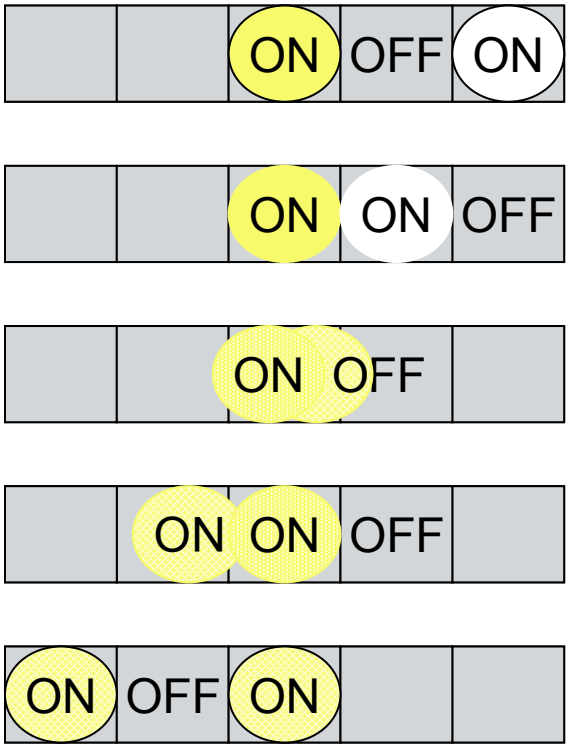

Figure 7: Droplet washing experiment protocol

Results

MANUAL SPLITTING AND BEAD RETENTION

The image sequence shown in Figure 8 is a non-automated merge-and-split of a clean droplet with a droplet containing magnetic microspheres. The wash solution was combined with the microsphere droplet, the solution was transferred to the opposite side of the microspheres, and clear solution was split off from the other side of the microsphere mass. The reflection seen behind the droplet containing microspheres is the Neodymium-Iron-Boron magnets that are on the top plate. The magnet configuration which was used for this experiment is shown in Figure 9.

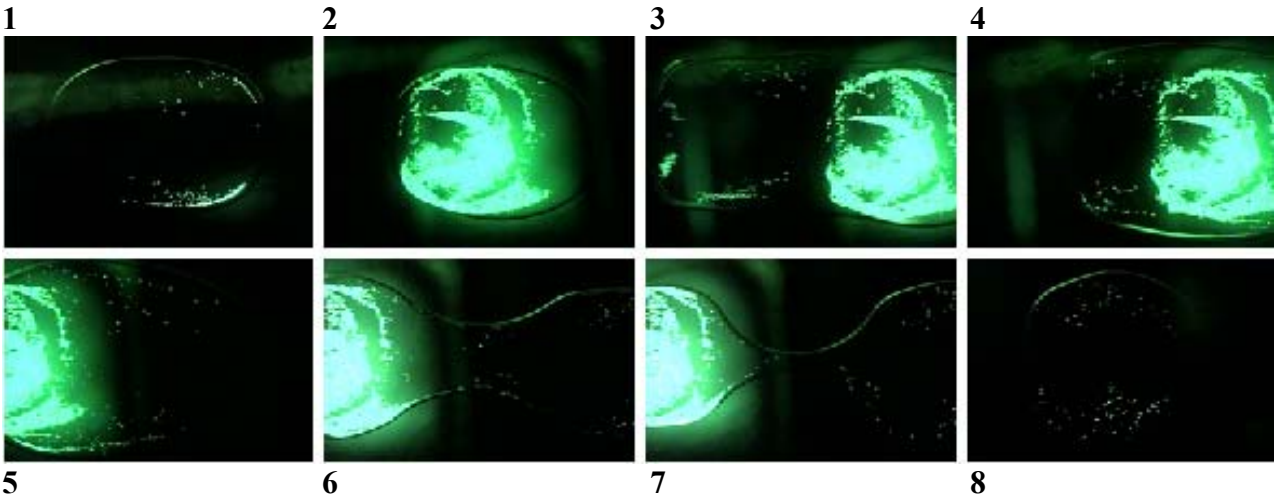

Figure 8: Non-automated merge-split sequence: 1. Wash droplet 2. Microsphere droplet 3-5. Droplet Merge 6-7. Droplet split 8. Clear droplet after split

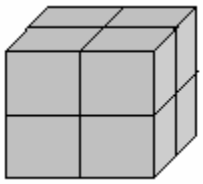

Figure 9: Magnet configuration

In order to keep the microspheres from being displaced during the formation of the neck between the emerging daughter droplets, the permanent magnets must be positioned such that the magnetic field is far enough away from where the neck forms. When the center electrode is deactivated and the neck begins to form, microspheres around the periphery of the magnetic field circulate around the edge of the forming daughter droplet and move toward the neck, as indicated in Figure 10). If the bulk of the microspheres are

immobilized too close to the neck, these displaced microspheres may be transferred to the other daughter droplet.

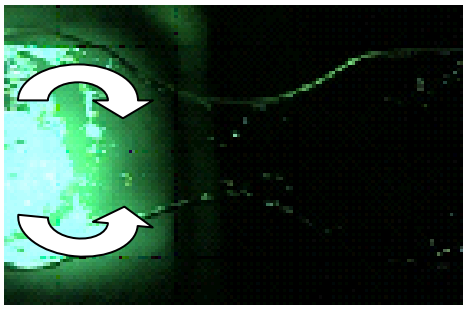

Figure 10: Direction of microsphere movement during droplet split

DROPLET WASHING

The droplet washing routine shown in Figure 7 was performed twice. The droplet which was left on the electrode that was always on was combined with another clear PBS droplet. During both washing simulations, almost immediate mixing of the two droplets was observed. The two daughter droplets resulting from the combine-and-split were observed to be identical in color, indicating that 2:1 dilution occurred.

Discussion

One of the more difficult aspects of the magnetic microsphere retention experiments was placement of the permanent magnets so that all of the microspheres were immobilized. Preliminary experiments with non-screened magnetic microspheres revealed some microspheres that seemed unaffected by the presence of the magnets. In order to determine if this was due to lack of magnetic field strength, a droplets of diluted microsphere solution were observed. Even with fewer microspheres present, there were still microspheres that were not attracted to the permanent magnets, which indicated that the problem was not lack of magnetic field strength. After screening the microsphere solution with a large permanent magnet, there were no observable microspheres that were unresponsive to the magnet. The best rates of magnetic microsphere retention were observed when the magnetic field was spread over an area rather than pinpointed. This appeared to allow more microspheres access to the same level of magnetic field as opposed to the pinpointed field, which immobilized only a portion of the microspheres. Immobilizing the microspheres too close to the perimeter of the droplet resulted in the microspheres being displaced when the droplet was moved

The presence of the magnetic microspheres coupled with the permanent magnet changed the behavior of droplet transport and splitting. When a magnet was immobilizing the microspheres, the droplet containing them could not be transported away from the magnet—the force of the magnet on the microspheres would cause a portion of the microspheres in solution to split away from the main droplet in an effort to move closer

to the magnet. Therefore, in order to transport a droplet of microspheres, the magnet must be removed.

The presence of a mass of immobilized microspheres affected the way droplets were split. Normally, droplets are split as shown in Figure 4. When the magnetic microspheres were present, the protocol shown in Figure 11 could be used. If the portion of the merged droplet touching the electrode farthest right was not large enough to allow a split (fourth image in sequence), the electrode the microspheres were on could be briefly turned off and back on. This allowed enough overlap on the rightmost electrode to accomplish splitting without disturbing the microspheres. The inability to automate this splitting process was most likely a result of the top plate being uneven rather than the presence of the beads.

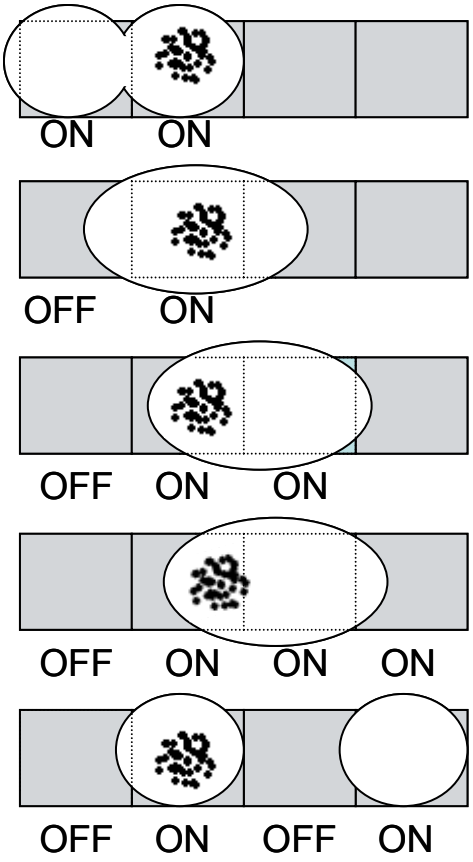

Figure 11: Splitting protocol with magnets present

The washing experiments indicate that a serial dilution would be required to wash magnetic microspheres. Combining two droplets, even for a brief amount of time, results in solution mixing rather than solution exchange. Therefore, in order to completely refresh the solution surrounding the microspheres between nucleotide additions, multiple wash droplets would have to be used to dilute the solution. Increasing the number of

wash droplets combined with and split from the microspheres will increase the chance that microspheres could get carried away in waste droplets, and so serial dilution would require a near perfect rate of microsphere retention for each splitting step. This will likely be difficult to guarantee, so a different method for washing that will decrease the chance of losing microspheres will need to be developed. One option may be to immobilize the magnetic microspheres securely enough to pull them out of solution, and then run wash droplets over the microspheres.

Conclusions

The successful demonstration of magnetic microsphere retention during droplet splitting on a digital microfluidic platform is an important first step towards performing solid-phase pyrosequencing on a lab-on-a-chip. The next logical step to accomplishing this goal is the automation of the merge-split sequence shown in Figure 7. Automation of a splitting routine that incorporates 100% magnetic microsphere retention must be accomplished before pyrosequencing can be tested on the digital microfluidic platform because droplet combination and subsequent splitting will be the method used to allow nucleotides and enzymes access to the DNA on the microspheres. As well, a washing protocol that allows a smaller chance of microsphere loss than serial dilution must be developed. The results presented in this paper indicate that pyrosequencing on a digital microfluidic platform will be accomplished and that the goal of a 500 base pair read will become a reality within the next two years.

[1] Paik, P., Pamula, V.K., Fair, R.B. 2003. Rapid droplet mixers for digital microfluidic systems. *Lab on a Chip*. **3,** 253-259.
[2] Nelson, David L., Cox, Michael M. 2005. Lehninger Principles of Biochemistry. 296-297
[3] Ronaghi, M. , Karamohamed, S., Pettersson, B., Uhlen, M., and Nyren, P. 1996. Real-time DNA sequencing using detection of pyrophosphate release. *Analytical Biochemistry*. **242**, 84-89.
[4] Ronaghi,M. 2001. Pyrosequencing sheds light on DNA sequencing. *Genome Research*. **11**. 3-11.
[5] Beni, G., and Hackwood, S. 1980. Electro-wetting displays. *Applied Physics Letters*. **38,** 207-209.
[6] Pollack, M.G., Shenderov, A. D., Fair, R. B. 2002. Electrowetting-based actuation of droplets for integrated microfluidics. *Lab on a Chip*. **2**, 96-101.
[7] Srinivasan, V., Pamula, V. K., Fair, R. B. 2004. An integrated digital microfluidic lab-on-a-chip for clinical diagnostics on human physiological fluids. *Lab on a Chip*. **4**