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An integrated digital microfluidic lab-on-a-chip for clinical diagnostics on human physiological fluids†‡

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Clinical diagnostics is one of the most promising applications for microfluidic lab-on-a-chip systems, especially in a point-of-care setting. Conventional microfluidic devices are usually based on continuous-flow in microchannels, and offer little flexibility in terms of reconfigurability and scalability. Handling of real physiological samples has also been a major challenge in these devices. We present an alternative paradigm—a fully integrated and reconfigurable droplet-based "digital" microfluidic lab-on-a-chip for clinical diagnostics on human physiological fluids. The microdroplets, which act as solution-phase reaction chambers, are manipulated using the electrowetting effect. Reliable and repeatable high-speed transport of microdroplets of human whole blood, serum, plasma, urine, saliva, sweat and tear, is demonstrated to establish the basic compatibility of these physiological fluids with the electrowetting platform. We further performed a colorimetric enzymatic glucose assay on serum, plasma, urine, and saliva, to show the feasibility of performing bioassays on real samples in our system. The concentrations obtained compare well with those obtained using a reference method, except for urine, where there is a significant difference due to interference by uric acid. A lab-on-a-chip architecture, integrating previously developed digital microfluidic components, is proposed for integrated and automated analysis of multiple analytes on a monolithic device. The lab-on-a-chip integrates sample injection, on-chip reservoirs, droplet formation structures, fluidic pathways, mixing areas and optical detection sites, on the same substrate. The pipelined operation of two glucose assays is shown on a prototype digital microfluidic lab-on-chip, as a proof-of-concept.

Introduction

Microfluidics is the enabling technology behind a whole new class of miniaturized analysis systems for chemical and biological applications.[1] Clinical diagnostics is one of the most promising applications for such microfluidic lab-on-a-chip systems, especially in a point-of-care setting. Clinical diagnostics refers to the measurement of clinically significant analytes in physiological fluids for either the prevention or treatment of disease. All the benefits of miniaturization such as smaller sample requirement, reduced reagent consumption, decreased analysis time and higher levels of throughput and automation are realized in this application. Smaller patient sample volume minimizes the invasiveness of sample drawing procedures and reduces iatrogenic blood losses particularly in geriatric and pediatric patients (especially neonatal babies), and in certain intensive care situations. Reduced reagent consumption significantly lowers costs, which is an important concern in clinical laboratories today. Analysis times are also effectively shortened due to the high levels of parallelism possible in microfluidic systems. All these benefits make the lab-on-a-chip technology ideal for near-patient and point-of-care testing.

The use of microfluidic lab-on-chip technology for clinical applications has been reviewed extensively by Tüdõs *et al.*[2] and Verpoorte.[3] Currently almost all microfluidic devices are based on continuous fluid flow in permanent microchannels in glass, plastic or other polymers. Though pumps based on electrokinetic phenomena (electrophoretic separation and electroosmotic pumping) dominate academic research,[3,4] there is a trend towards the use of alternative fluid actuation mechanisms, since many common samples are not directly compatible with electrokinetic phenomena. For instance, physiological fluids with high ionic strength, such as blood and urine, cannot be pumped using electroosmosis due to excessive Joule heating.[5] Among alternative active pumping mechanisms, centrifuge-based devices, which are independent of physicochemical properties of fluids, have attracted a lot of attention in the past few years.[5,6] Researchers at the University of Cincinnati have also developed a disposable plastic biochip incorporating passive microfluidics with embedded on-chip power sources and an integrated biosensor array to detect glucose, lactate, and O_2 in whole blood.[7]

In contrast to academic research, most commercial microfluidic devices for clinical diagnostics rely on external pressure sources (syringe pumps) or passive mechanisms (capillary action/gravity) for actuation. Micronics' ORCA Microfluidics platform[8] combines various pressure driven microfluidic elements, such as the diffusion based H-Filter and T-Sensor platforms, in a disposable microchip for point-of-care immunoassays on whole blood. The Biosite Triage Cardiac system[9] measures cardiac markers in whole blood in a microcapillary-based device for point-of-care testing. One of the most popular microfluidic instruments for point-of-care testing is the handheld I-Stat analyser,[10] which measures blood chemistry (glucose, blood gases, electrolytes, urea and more) in whole blood. Almost all the devices mentioned above have their microfluidic module as single-use, disposable, and based on continuous-flow.

Continuous-flow based microfluidic devices offer very little flexibility in terms of scalability and reconfigurability, and are usually application specific. They also require relatively large volumes of liquid for priming the channels prior to use. An alternative approach towards microfluidics is to manipulate the liquid as unit-sized discrete microdroplets. Due to the architectural similarities with digital microelectronic systems, we have often referred to this approach as "digital" microfluidics. Digital microfluidic systems have several advantages over continuous-flow systems, the most important being reconfigurability and scalability of architecture.[11]

† *The Science and Application of Droplets in Microfluidic Devices*.
‡ Electronic supplementary information (ESI) available: five video clips showing: high-speed transport of a droplet of blood across 4 electrodes; sample injection into an on-chip reservoir using an external pipette; droplet formation from an on-chip reservoir using only electrowetting forces; droplets moving in-phase on a 3-phase transport bus; and a pipelined glucose assay, showing sample and reagent droplet formation, mixing, splitting and colorimetric reaction. See http://www.rsc.org/suppdata/lc/b4/b403341h/

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Electrowetting[11] and dielectrophoresis[12] are the two most commonly used techniques for microdroplet actuation. Electrowetting is primarily a contact line phenomenon, and refers to electric field-induced interfacial tension changes between a liquid and a solid conductor. On the other hand dielectrophoresis is a bulk phenomena caused as a result of polarization induced in a dielectric liquid by a non-uniform electric field. Dielectrophoresis typically uses high frequency AC voltages (> 50 KHz), which can cause significant Joule heating in aqueous samples, even at moderate ionic strengths. In contrast, there is negligible Joule heating in electrowetting, since it can use DC or low frequency AC (< 100 Hz) and aqueous droplets of potassium chloride (KCl) with ionic strengths as high as 1 M have been transported without any problems.[11] Electrowetting therefore appears to be applicable for a wider matrix of samples, as compared to dielectrophoresis.

The use of electrowetting for dispensing, transport, splitting, merging and mixing of aqueous droplets, has been shown previously.[11,13–15] We have also previously demonstrated the transport of enzyme laden droplets without loss in activity, and a complete colorimetric enzyme-kinetic glucose assay (using standard solutions) on an electrowetting chip.[16] In this paper we extend the use of electrowetting device to actuate and analyze human physiological samples.[17] The basic compatibility of the human physiological fluids, such as whole blood, serum, plasma, urine, saliva, sweat and tear, with the electrowetting system is first evaluated using reliable and repeatable transportability as the criteria. A glucose assay is then performed on samples of serum, plasma, urine and saliva, and the values are compared with those obtained using a reference method. Finally, a lab-on-a-chip architecture for assaying multiple analytes on a monolithic device is presented, along with preliminary results and future directions for realizing a fully automated lab-on-a-chip platform for clinical diagnostics.[18]

Materials and methods

Electrowetting set-up

The electrowetting system comprises of a photolithographically patterned metal electrode array (chrome or indium tin oxide) on a glass substrate and a continuous ground plane (indium tin oxide on glass) parallel to it. A spacer of known thickness (H) separates the electrode array and the ground plane and the droplets are sandwiched between the two. 1cSt silicone oil (DMS-T01, Gelest, Morrisville, Pennsylvania, USA) is used as the filler medium surrounding the droplets to prevent evaporation and facilitate transport. The electrode array is insulated from the droplet by layer of Parylene C (~ 800 nm) and both the surfaces are hydrophobized by a thin layer of Teflon AF 1600 (~ 50 nm). Fig. 1 shows the vertical cross-section of a typical electrowetting setup. The fabrication and operation of the electrowetting system are described in.[11]

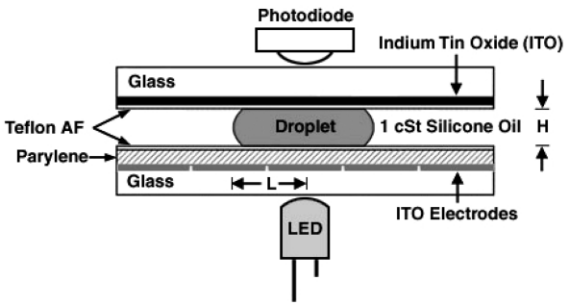

Fig. 1 Vertical cross-section of the electrowetting setup along with the optical absorbance measurement instrumentation.

Optical absorbance measurement instrumentation

Optical absorbance measurements were done in a plane perpendicular to that of the electrowetting chip. The set-up consisted of a green LED (545 nm, RadioShack) and a photodiode, which is a light-to-voltage converter (TSL257, Texas Advanced Optoelectronic Solutions), as shown in the schematic in Fig. 1. The voltage output of the photodiode $V(t)$ is directly proportional to the light intensity incident on it. The absorbance $A(t)$ is calculated from this measured intensity using the formula $A(t) = \ln(V_0/V(t))$, where V_0 is the blank reading corresponding to zero absorbance.

Chemicals

Glucose oxidase (G-6125), peroxidase (P-8125), 4-aminoantipyrine (4-AAP, A-4382), *N*-ethyl-*N*-sulfopropyl-*m*-toluidine (TOPS, E-8506), and 100 mg dL^{-1} glucose standard (Sigma 16–11) were purchased from Sigma Chemicals (St Louis, Missouri, USA). The glucose reagent was constituted with glucose oxidase (6 U mL^{-1}), peroxidase (6 U mL^{-1}), 4-aminoantipyrine (6 mM) and TOPS (6 mM), in 0.1 M phosphate buffered saline (Sigma, pH 7.0). The constituted reagent was stable without any visible coloration for 2 days when stored at 4 °C. Blood samples were obtained from the Duke University Medical Center.§All experiments reported in this paper were performed at room temperature.

Human physiological fluid transport

Clinical diagnostics in humans is commonly performed on physiological body fluids such as whole blood, serum, plasma and urine. Other fluids such as saliva, sweat, and tears have also been used in specific contexts. Real physiological samples have always been a major challenge in conventional microfluidic devices,[3] and it is therefore important to establish the compatibility of these fluids with the electrowetting system.

Biocompatibility of the electrowetting system

The transport of non-biological electrolytes using electrowetting has been demonstrated both in air[19] and in other immiscible media such as silicone oil.[11] On the contrary the transport of fluids containing proteins, such as enzyme-laden reagents and human physiological fluids is not as straightforward. This is because most proteins tend to adsorb irreversibly to hydrophobic surfaces, and contaminate them. In the electrowetting system, the liquid droplet is sandwiched between two hydrophobic (Teflon AF-coated) plates. Any contact between the liquid droplet and this Teflon AF surface, will therefore contaminate the surface. In addition to contamination, protein adsorption can also render the surface permanently hydrophilic.[20] This is detrimental to transport, since electrowetting works on the principle of modifying the wettability of a hydrophobic surface. Therefore any contact between a liquid droplet containing proteins and the Teflon surface should be avoided to prevent contamination and enable transport. As a consequence, air is not a suitable filler medium for assays involving proteins, since the droplet will always be in contact with the Teflon surface.[19] Silicone oil with its low surface tension and spreading property is an ideal alternative. From visual observations and electrical capacitance measurements, during the transport of droplets in silicone oil, we have inferred the presence of a thin film of oil, encapsulating the droplet. This oil film isolates the droplet from the Teflon surfaces, minimizing adsorption and facilitating transport.

Though the oil film is yet to be extensively characterized, its stability appears to decrease with lowering of the interfacial tension between the liquid (droplet) and oil. Since the liquid–oil interfacial tension typically decreases with increasing protein content, the

§ Blood samples were obtained with IRB clearance and the research is not subject to the Common Rule (45CFR46.102(f)) and HIPAA (45CFR164.500(a)).

stability of the oil film is also lower in such cases. A less stable oil film implies more adsorption of proteins on to the Teflon surface and consequently droplets having high protein concentrations are expected to be more difficult to transport and may require higher actuation voltages. The oil film may also be squeezed out by the droplet at high voltages due to electrostatic pressures. Therefore, the actuation voltage has to be carefully chosen to facilitate transport of the droplets while maintaining the oil film.

Measure of droplet transportability

Due to the discrete nature of operation of a digital microfluidic system, the maximum switching frequency, which we define as the highest rate at which a droplet can be moved across two adjacent electrodes, is the measure of the transport performance of the system. Higher the switching frequency, more the number of discrete fluidic operations that can be performed per second and higher the throughput of the system. It has been shown previously that the average speed of a droplet at a particular voltage remains constant with physical scaling.[11] The average speed is defined as the product of switching frequency and electrode pitch. Therefore the switching frequency is inversely proportional to the electrode pitch and can be increased by physically scaling the system down.

On-chip biological assay

The glucose assay, which is one of the most common and frequently performed assays in clinical diagnostics, was chosen to demonstrate the feasibility of performing biological assays on real samples. A colorimetric enzyme-kinetic method based on Trinder's reaction (eqn. (1)) was used to measure the concentration of glucose.

$$\begin{aligned} &\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2 \\ &2H_2O_2 + \text{4-AAP} + \text{TOPS} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O \end{aligned} \quad (1)$$

Conventional colorimetric glucose assay methods use sample dilution factors (sample volume divided by total volume of assay mixture) that are typically greater than 100. However such large dilution factors are not easily realizable on a droplet-based system due to chip real-estate concerns and reduced mixing efficiency. A dilution factor of 2 (1 sample droplet and 1 reagent droplet) is most easily implemented on an electrowetting system and was used in the glucose assay experiments reported in this paper. The method is linear up to a concentration of 100 mg dL^{-1},[16] which was sufficient for the experiments reported in this paper. The values obtained on the electrowetting system were compared to those obtained using a reference method on the same sample. The reference method uses the same assay reaction, but was performed on a bench-top spectrophotometer (Gensys 20) using a more conventional dilution factor of 100 as compared to the dilution factor of 2 on the electrowetting chip.

Results and discussion

Physiological fluid transport

The maximum switching frequency of a droplet of whole blood, serum, plasma, urine, saliva, sweat, tear and buffer (0.1 M PBS, pH = 7) was evaluated as a function of the applied voltage. The electrode pitch is L = 1.5 mm and the spacer height is H = 500 μm with 1cSt silicone oil as the filler fluid. The volumes of the droplets were between 1.3 μL and 1.5 μL. Fig. 2 plots the maximum switching frequency of droplets of the various fluids as a function of the actuating voltage. All the fluids could be actuated at frequencies of 20 Hz using less than 65 V. The general trend that can be observed from the graph is that the fluids with less or no protein such as buffer and saliva transport more easily than the ones with higher protein content such as whole blood or serum, which is consistent with what we expected. The transport of a droplet of whole blood was sustainable for ~25,000 continuous droplet transfers at 10 Hz (~40 min) using an actuating voltage of 52 V. After 25,000 cycles the droplet was slower to respond and required a higher voltage to maintain the same switching frequency. This performance degradation could either be due to insulator degradation or gradual adsorption of proteins on to the Teflon surface. The transport of other physiological fluids was sustainable for as much or in some cases even more cycles than whole blood, depending on the protein content. An important issue that has not been addressed is the viability of blood cells under electrowetting conditions, and further experiments are required to evaluate this.

Glucose assay

Serum, plasma, urine and saliva were assayed for glucose on the electrowetting device and compared with values obtained using a reference methodology. Saliva and urine samples were spiked with glucose since the original concentrations were too low to be measured in our system. On both the systems 100 mg dL^{-1} glucose was used as the calibrating concentration. Droplets of sample/calibrant (1.5 μL) and reagent (1.5 μL) were pipetted manually on to the electrowetting chip (L = 1.5 mm, H = 500 μm), and merged and physically mixed by shuttling the coalesced droplet across multiple electrodes for 15 s. The time for mixing protocol is higher than what is required and can be reduced to less than 5 s.[14,15] At the end of the mixing phase, the absorbance is measured for at least 30 s, using the LED-photodiode set up described earlier. The rate of change of absorbance (slope of absorbance *versus* time graph) is proportional to the glucose concentration in the physiological sample C_{SAMPLE} which is calculated as

$$C_{SAMPLE} = (\text{Rate}_{SAMPLE}/\ \text{Rate}_{CALIB}) \times 100 \text{ mg dL}^{-1}$$

where Rate_{SAMPLE}, and Rate_{CALIB} are the absorbance rates measured for the sample and the calibrant (100 mg dL^{-1}) respectively.

Table 1 compares the results from the on-chip assay with the reference values. The total error in the glucose concentration is 3 mg dL^{-1} for plasma, 8 mg dL^{-1} for serum, 3 mg dL^{-1} for saliva and 16 mg dL^{-1} for urine. The concentrations measured agree well with the reference values, except for urine where there is a

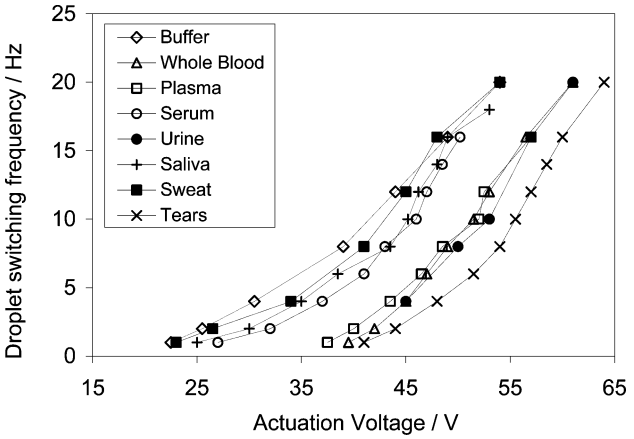

Fig. 2 Switching frequency of droplets of various physiological fluids as a function of applied voltage.

Table 1 Comparison of glucose concentrations in serum, plasma, saliva and urine, obtained on the electrowetting system with a reference method.

	Measured glucose concentration/mg dL^{-1}	
Sample type	Reference method	Electrowetting method
Plasma	83	80
Serum	108	100
Saliva	27	24
Urine	25	9

significant deviation. This is likely due to interference by uric acid, which reacts with hydrogen peroxide in the Trinder's reaction.[21] The interference may not be significant in the reference method because of the 100 fold dilution, which reduces the concentration of uric acid below interfering levels.

Integrated lab-on-a-chip architecture

The different components of a digital microfluidic lab-on-a-chip, such as droplet dispensing, droplet manipulation, physical mixers, biochemical reactors, and detection have already been demonstrated individually or in a partially integrated fashion. In this section we propose a novel lab-on-a-chip architecture which integrates all these components, to demonstrate for the first time a fully integrated and automated electrowetting-based digital microfluidic device.

Fig. 3 shows the high-level schematic of the lab-on-a-chip for analysis of multiple analytes on a digital microfluidic platform. The fluidic components of the lab-on-a-chip include reservoirs, droplet transport pathways or buses, and a waste area. The function of the reservoir is to store a large volume of liquid (samples, reagents, controls and calibrants), from which unit-sized droplets can be generated. A reservoir can also be designated as a waste area, to discard the droplets after use. The droplet pathways consist of contiguous electrodes, which connect different areas of the chip. These electrodes can be used either simply for transport or for other more complex operations such as mixing and splitting. By using a transparent conductor such as indium tin oxide, these electrodes can also be used as optical detection sites.

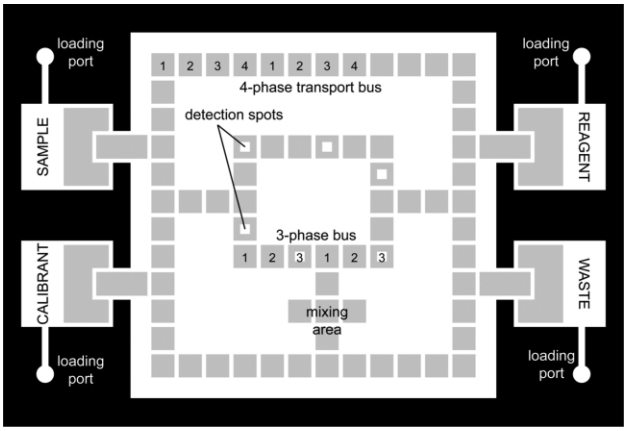

Fig. 3 High-level architecture of a fully integrated digital microfluidic lab-on-chip for clinical diagnostics.

A prototype lab-on-a-chip with sample injection elements, reservoirs (and waste), droplet formation structures, fluidic pathways, mixing areas and optical detection sites, was fabricated to test the various components of the architecture both individually and in an integrated fashion. The lab-on-a-chip consisted of 7 reservoirs, a waste area, an outer transport bus, an inner storage bus with optical detection sites, and a mixing area, as shown in Fig. 3. The reservoirs were physically defined by patterning the spacer material and are 2.67 mm in diameter. The electrode pitch is $L = 500$ μm and the spacer thickness is $H = 90$ μm.

Sample injection

Sample injection is the interface between the microfluidic device and the macroscopic world. The sample (or reagent) is injected into the reservoir through a loading hole in the top plate, designed to fit a small volume (< 2 μL) pipette tip. The loading hole is connected to the reservoir by a narrow channel (shown in Fig. 3) which offers a large resistance to prevent the liquid from spontaneously flowing back into the hole. This resistance is initially overcome by the pipetting action to fill the reservoir with the liquid. We have successfully used this technique to dispense liquids into the reservoirs, though care needs to be taken to avoid the introduction of air bubbles while pipetting.

Droplet dispensing

Droplet generation is the most critical component of an electrowetting-based lab-on-a-chip. In the proposed architecture, the droplets are formed from an on-chip reservoir in three steps—1. A liquid column is extruded from the reservoir by activating a series of electrodes adjacent to it. 2. Once the column overlaps the electrode on which the droplet is to be formed, all the remaining electrodes are deactivated to form a neck in the column. 3. The electrode in the reservoir is then activated to pull the liquid back and break to neck completely to form a droplet.

Fig. 4 shows these steps in the formation of KCl droplets (~ 20 nL each) from an on-chip reservoir at 50 V. The droplet volume variability was calculated to be less than 2% (standard deviation) from similar dispensing experiments.[13]

Fluidic droplet pathways

The fluidic droplet pathways or buses connect various areas of the chip. In order to minimize the number of electrical contacts, the electrowetting chip uses a multiphase bus for the fluidic pathways. In an n-phase bus every nth electrode is electrically connected, and droplets are always spaced apart by $(k \times n - 1)$ electrodes, where k is any integer. The mixing region still requires independently controllable electrodes. The prototype lab-on-a-chip uses a 4-phase

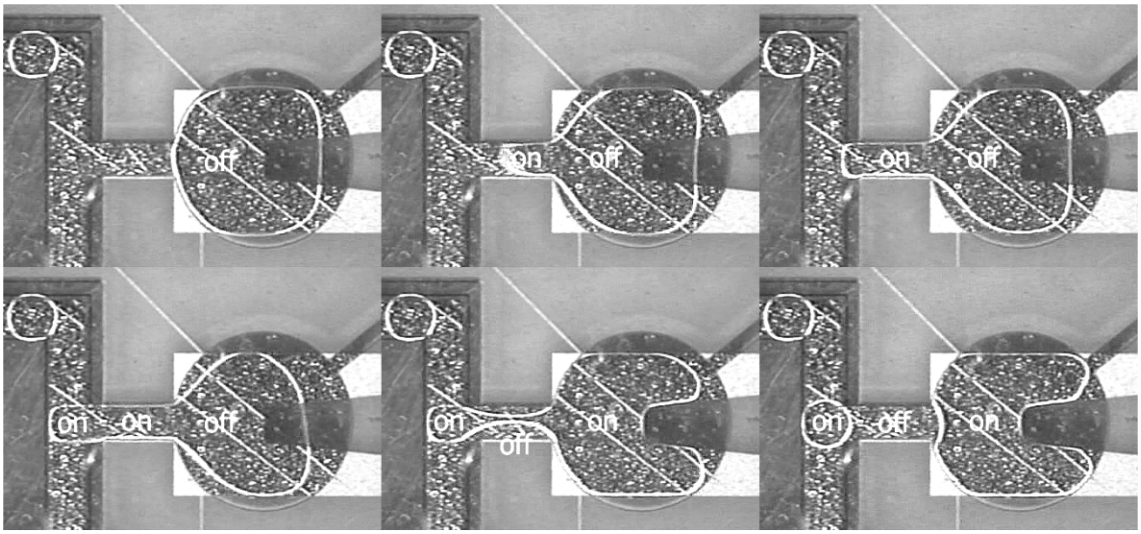

Fig. 4 Droplet formation from an on-chip reservoir (0.1 M KCl) using only electrowetting forces.

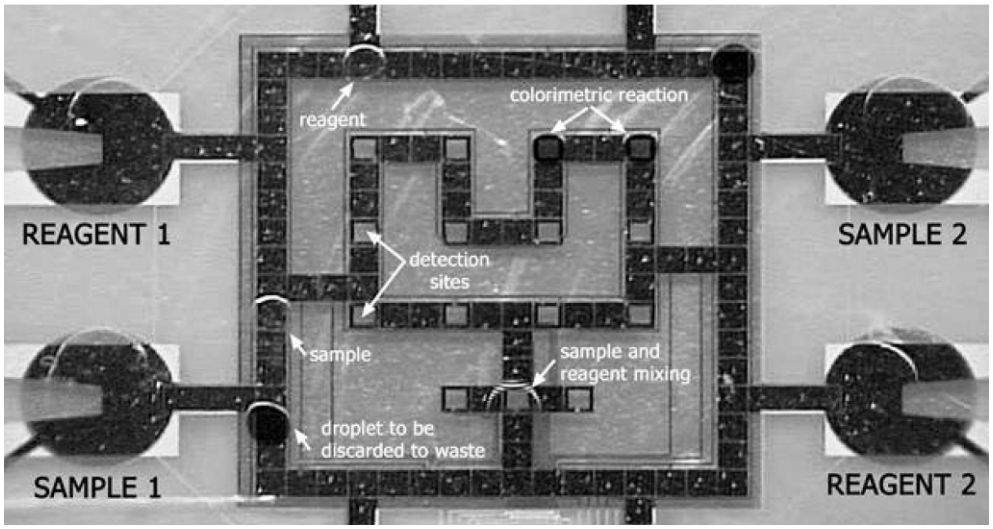

Fig. 5 A snapshot during the course of a pipelined glucose assay on a digital microfluidic lab-on-a-chip.

outer transport bus and a 3-phase inner storage bus, as shown in Fig. 3. The use of a multiphase bus (requiring operations to be synchronized) and a single dedicated mixing area imposes constraints on the extent of parallelism achievable using this architecture. An optimized pipelining strategy is therefore needed to achieve maximum throughput.

Pipelined glucose assays

Two pipelined glucose assays are performed on the prototype digital microfluidic lab-on-a-chip. Two droplets each of glucose sample (standard solution) and reagent are first formed from 4 different reservoirs. While the first sample and reagent are being mixed the second set of droplets are transported towards the mixing area. The mixed droplet is split into two and one droplet is moved to a storage area for detection, while the other droplet is sent to waste. The second sample and reagent droplets are mixed while detection in the first assay happens.

Fig. 5 shows a snapshot of the chip during the operation of a real pipelined assay. The figure shows colorimetric reactions on two droplets in the 3-phase inner storage bus, sample and reagent mixing, and another sample and reagent droplet being transported on the 4-phase outer bus towards the mixing area, and a droplet being moved to the waste area—all occurring simultaneously. This experiment demonstrates the full capability of the lab-on-a-chip, and this is the first time such high-levels of integration and automation have been realized on any droplet-based system.

Conclusions

In this paper we demonstrate for the first time the use of electrowetting to actuate human physiological fluids. Micro-droplets of human whole blood, serum, plasma, urine, saliva, sweat and tears, were transported at frequencies of 20 Hz using less than 65 V. The transport is also sustainable for at least 25,000 continuous cycles. The use of silicone oil as the filler fluid prevents biofouling, and enables the transport of physiological fluids by forming a thin film between the droplet and the hydrophobic Teflon AF surfaces. A glucose assay was also performed on serum, plasma, urine, and saliva and the values agree well with reference measurements, expect for urine where there is a significant difference due to interference by uric acid. A lab-on-a-chip architecture for pipelined analysis of multiple analytes on a monolithic device is presented, along with preliminary results on sample injection, droplet formation, and multiphase droplet transport. A fully automated and integrated glucose assay is also shown to demonstrate the pipelined operation of the lab-on-chip, as a proof-of-concept. This is the first time such high-levels of integration and automation have been realized on any droplet-based system. Future directions include extending the prototype lab-on-a-chip for real samples. Analytical performance parameters such as specificity, sensitivity, precision, recovery and interferences, also need to be measured to evaluate clinical applicability. Sample preparation needs to be addressed especially if the lab-on-a-chip is to be used in a point-of-care setting.

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