Chemical and Biological Applications of Digital-Microfluidic Devices

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Editor's note:
Digital-microfluidic technology offers a revolutionary platform for many chemical and biological applications. Learn how to manipulate droplets and process chemical and biological samples on chip for clinical diagnostics, gene sequencing, airborne chemical detection, and tissue engineering.
—Krishnendu Chakrabarty, Duke University

DIGITAL-MICROFLUIDIC LAB-ON-A-CHIP (LoC) technology offers a platform for developing diagnostic applications with the advantages of portability, sample and reagent volume reduction, faster analysis, increased automation, low power consumption, compatibility with mass manufacturing, and high throughput. In addition to diagnostics, digital microfluidics is finding use in airborne chemical detection, DNA sequencing by synthesis, and tissue engineering.

In this article, we review efforts to develop various LoC applications using electrowetting-based digital microfluidics. We describe these applications, their implementation, and associated design issues. The ''Related work'' sidebar gives a brief overview of microfluidics technology.

Electrowetting technology

Electrowetting is the phenomenon whereby an electric field modifies the wetting behavior of a polarizable or conductive liquid droplet in contact with a hydrophobic, insulated electrode.[1] Figure 1 illustrates this effect. Applying a voltage to a series of adjacent electrodes that can be turned on or off creates an interfacial tension gradient that can manipulate droplets. Droplets are usually sandwiched between two parallel plates; the bottom plate is the chip surface, which houses the addressable electrode array, and the top is either a continuous ground plate or a passive top plate (the chip's characteristics determine the top plate's nature).

In coplanar designs, both the buried activation electrode and the exposed electrodes that ground the droplet are located on the bottom surface.[2,3] As Figure 2 shows, the top plate is not required for coplanar devices, but we advise using it to contain the oil and the droplets. Also, manufacturers or users can customize the passive top plate with specific chemistry or structures appropriate for each application. Thus, the top plate can be a disposable component.

We have demonstrated electrowetting-based systems for manipulating microliter- and nanoliter-size droplets in LoC protocols.[4–6] Using the electrowetting principle, we can transport discrete droplets in a highly controlled way over an array of electrodes. We can reconfigure the array to transport the droplets or hold the droplets as virtual reaction chambers where mixing takes place. To view videos of electrowetting-induced droplet motion, visit http://www.ee.duke.edu/research/microfluidics and http://cjmems.seas.ucla.edu/index.html.

The LoC chip surface is coated with an insulating layer of parylene C (about 800 nm), and both the top and bottom surfaces are covered with a Teflon-AF thin

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Related work

Much of the reported work on lab-on-a-chip (LoC) microfluidic devices has focused on miniaturization of analytical methods and protocols to improve performance and throughput. Researchers have demonstrated the benefits of miniaturization, such as smaller sample requirements, reduced reagent consumption, decreased analysis time, and higher levels of throughput and automation. Most LoC devices have aimed at performing chemical or biological protocols on chip, with pre-prepared samples processed off chip. Thus, few researchers have reported work on integrating front-end functions such as sample collection, analyte extraction, preconcentration, and filtration, with the required analytical operations then performed on the chip.[1]

Currently, almost all microfluidic devices are based on continuous fluid flow in permanent microchannels in glass, plastic, or other polymers. De Mello and Beard present a review of sample pretreatment with continuous-flow microfluidic systems.[1] However, continuous-flow-based microfluidic devices offer very little flexibility in scalability and reconfigurability, and they are usually application specific.

An alternative approach to microfluidics is to manipulate the liquid as unit-sized discrete microdroplets. Because of its architectural similarities to digital microelectronic systems, we often refer to this approach as ''digital'' microfluidics. Digital-microfluidic systems have several advantages over continuous-flow systems; the most important are reconfigurability and architecture scalability.[2] Electrowetting[2] and dielectrophoresis[3] are the two most commonly used microdroplet actuation techniques, although other methods have been demonstrated, such as thermocapillary actuation[4] and surface acoustic wave actuation.[5] Electrowetting is primarily a contact line phenomenon consisting of electric-field-induced interfacial tension changes between a liquid and a solid conductor.

The use of electrowetting for dispensing, transporting, splitting, merging, and mixing aqueous droplets has appeared in the literature previously.[2,6–10] Researchers have addressed the problem of chip surface contamination through biomolecular adsorption by using a silicone oil medium[9] or by controlling the application of actuation voltages.[11] Fair et al. demonstrated sample collection and preconcentration on the same chip.[12] Chatterjee et al. demonstrated that a variety of organic and inorganic substances can be actuated in droplet form.[13] Their results suggest that actuation of non-conducting liquids might not occur through electrowetting but rather through some other mechanism such as dielectrophoresis, as others have suggested.[14,15]

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film (about 50 nm) to ensure a continuous hydrophobic platform, which is necessary for smooth droplet actuation. A spacer separates the top and bottom plates, resulting in a fixed gap height. The gap is usually flooded with silicone oil that acts as a filler fluid, preventing droplet evaporation and reducing surface contamination.[1] Figure 3 shows a typical LoC platform with multiple electrodes, reservoirs, and detection sites. The photo is a snapshot of two pipelined glucose assays in process.[5]

Unlike a continuous-flow microfluidic platform, a digital-microfluidic platform operates under software-driven electronic control, eliminating the need for mechanical tubes, pumps, and valves. Digital platform protocols work similarly to traditional bench-top methods, except that they use more automation and significantly smaller sample sizes. Users can merge, split, transport, mix, and incubate droplets by programming electrodes to carry out specific tasks. A digital-microfluidic platform offers many advantages for real applications. It

- *has no moving parts.* All operations take place between the two plates under direct electrical control without use of pumps or valves.
- *requires no channels.* The gap is simply filled with liquid; channels exist only in a virtual sense and can be instantly reconfigured through software.
- *controls many droplets independently* because the electrowetting force is localized at the surface.
- *controls or prevents evaporation* with the oil surrounding the droplets.
- *uses no ohmic current.* Although capacitive currents exist, the device blocks direct current, minimizing sample heating and electrochemical reactions.
- *works with a wide variety of liquids*—that is, most electrolyte solutions.
- *makes close to 100% utilization of the sample or reagent possible* by wasting no fluid for priming channels or filling reservoirs.
- *is compatible with microscopy.* Glass substrates and transparent indium-tin-oxide (ITO) electrodes make the chip compatible with observation from a microscope.
- *is extremely energy efficient*—using nanowatts to microwatts of power per transfer.
- *achieves high droplet speeds*—up to about 25 cm/s.

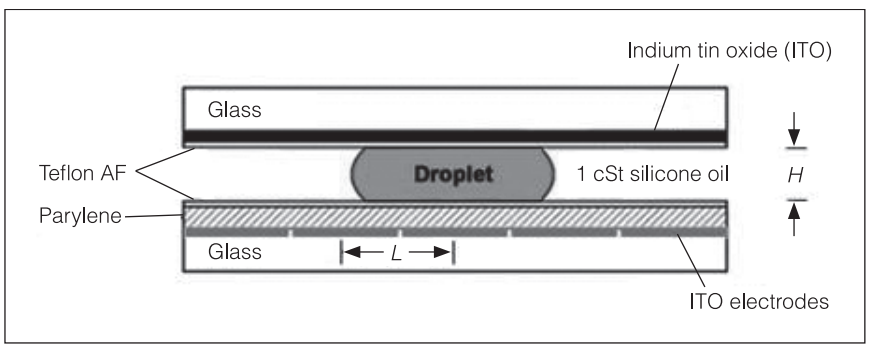

Figure 1. Electrowetting effect on a digital-microfluidic platform. Because of the conductive top plate and the individually addressed buried electrodes in the bottom plate, applying a voltage can actuate the droplet from one electrode position to the next.

- *uses droplet-based protocols functionally equivalent to bench-scale wet chemistry.* Thus, users can simply scale down, automate, and integrate established assays and protocols.
- *permits maximum operational flexibility.* Direct computer control of each step allows conditional execution steps.

Applications and design issues

In most diagnostic and chemical detection applications, a key challenge is the preparation of the analyte for presentation to the on-chip detection system. In diagnostics, raw physiological samples must be introduced onto the chip and then further processed by lysing blood cells and extracting DNA. For massively parallel DNA sequencing, scientists can prepare samples off chip, but they must perform synthesis steps in a sequential on-chip format through automated control of buffers and nucleotides to extend the read lengths of DNA fragments. In real-time airborne-particulate-sampling applications, the process of sample collection from an air stream must be integrated into the LoC analytical component. One way to accomplish this is with a collection droplet that scans an exposed impacted surface and then is introduced into a closed analytical section. In tissue-engineering applications, the challenge is to build high-resolution (less than 10 microns) 3D tissue constructs with embedded cells and growth factors by manipulating and maintaining live cells on the chip platform.

Here, we highlight these new applications, including detection of airborne sulfates obtained by air sampling, DNA pyrosequencing, and a biomimetic manufacturing process for soft-tissue engineering.

On-chip assays

On-chip assays for determining the concentrations of target analytes is a natural application for digital microfluidics. Work in this area has focused on multiplexed assays, which measure multiple analytes

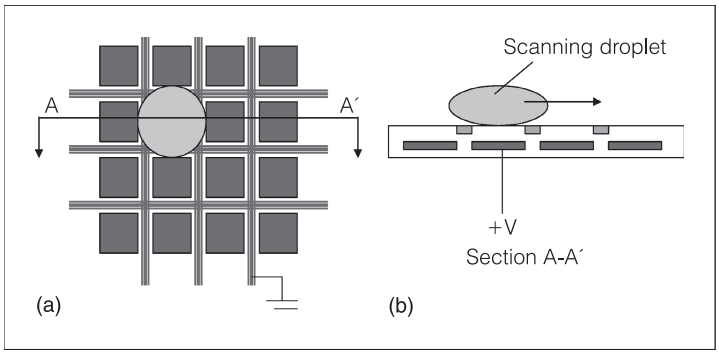

Figure 2. Coplanar actuation array for droplet scanning: top view (a) and side view of section A-A (b). Surface electrodes provide electrical contact to the droplet, making a top contact plate unnecessary. (This figure is reproduced, with permission, from R.B. Fair et al., "Integrated Chemical/Biochemical Sample Collection, Pre-concentration, and Analysis on a Digital Microfluidic Lab-on-a-Chip Platform," *Lab-on-a-Chip: Platforms, Devices, and Applications*, L.A. Smith and D. Sobek, eds., *Proc. SPIE*, vol. 5591, 8 Dec. 2004, pp. 113–124.)

in a single sample, as Figure 3 shows. Initially, scientists must determine the compatibility of each chemical substance with the electrowetting platform. Compatibility issues include the following:

- Do the liquid's viscosity and surface tension allow droplet dispensing and transport by electrowetting?

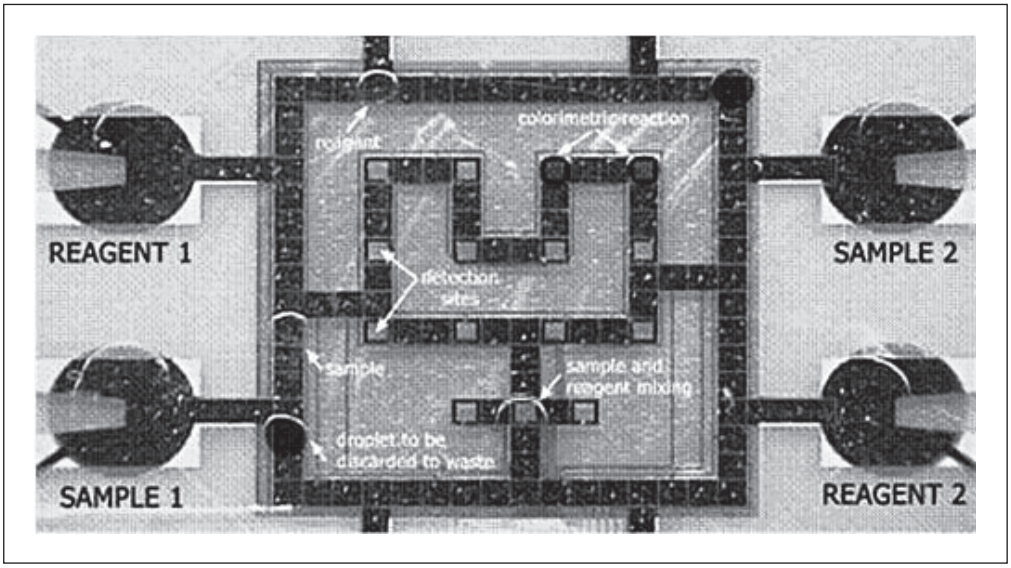

Figure 3. Fully automated and integrated operation of a multiplexed assay lab on a chip (LoC), with two droplets (samples 1 and 2) undergoing pipelined glucose assays.

- Will the droplet's contents foul the chip's hydrophobic surfaces?
- In systems with a silicone oil medium, will the chemicals in the droplet cross the droplet-oil interface, thus reducing the droplet's content?

Three examples of successful on-chip assay are glucose, TNT, and sulfate assays. These three examples use some or all of the following on-chip process steps:

- Load prediluted sample and reagent into on-chip reservoirs.
- Dispense droplets of analyte solutions and reagents.
- Transport droplets.
- Mix analyte solution and reagent droplets.
- Detect reaction products.

Glucose assay

The in vitro measurement of glucose in human physiological fluids is of great importance in clinical diagnosis of metabolic disorders. Srinivasan, Pamula, and Fair have demonstrated an LoC for glucose assay using a colorimetric enzyme-kinetic method based on Trinder's reaction,[7] which determines glucose concentration.[5]

Figure 4 shows a schematic drawing of the assay detection system. We perform on-chip glucose assay in three steps: dispensing, mixing, and detection. First, we pipette droplets of the glucose sample and the reagent onto the electrowetting chip. The LoC then merges and physically mixes the sample and the reagent by shuttling the coalesced droplet across three electrodes for 15 seconds, at a switching rate of 8 Hz and an actuation voltage of 50 V. The time for the mixing protocol is more than is required and can be reduced to less than five seconds.[4] At the end of the mixing phase, the 545-nm light-emitting-diode (LED) and photodiode setup measures the absorbance for at least 30 seconds. Electrowetting forces hold the mixed droplet stationary during absorbance measurement. Because absorbance measurement begins 15 seconds after the droplets merge, the measured reaction rate might not exactly equal the initial reaction rate.

This device makes detection of glucose concentrations possible in the range of 25 mg/dl to 300 mg/dl, using dilution factors as low as 2 and 3, in less than 60 seconds. The results compare favorably with conventional measurements on a spectrophotometer, implying no significant change in enzyme activity under electrowetting. Reproducibility of the measurement for the same sample concentration over multiple measurements on the same chip was less than 2%, indicating excellent control of droplet volumes and no cross-contamination.[8]

Glucose assay design issues

Any efficient or moderate-throughput microfluidic architecture inevitably requires droplets to share microfluidic resources on the chip (transport lanes, mixers, and incubators). Cross-contamination potentially can occur whenever droplets containing different samples are manipulated in the same chip area. Researchers have demonstrated the transport of nonbiological electrolytes using electrowetting both in air[9] and in other immiscible media such as silicone oil.[5,10] In contrast, the transport of fluids containing proteins, such as enzyme-laden reagents and human physiological fluids, is not as straightforward, because most proteins 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 the Teflon AF surface will therefore contaminate the surface.

In addition to contaminating the surface, protein adsorption can also render it permanently hydrophilic.[11] This effect is detrimental to transport because electrowetting works on the principle of modifying a hydrophobic surface's wettability. Therefore, to prevent contamination and enable transport, we must avoid contact between a liquid droplet containing proteins and the Teflon surface. Thus, air is a less desirable filler medium for assays involving proteins because the droplet will always be in contact with the Teflon surface.[9]

Researchers have reported exceptions for the use of air medium systems in applications in which it is desirable to deposit proteins on surfaces, such as matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).[12] However, for glucose assays, 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 film isolates the droplet from the Teflon surfaces, minimizing adsorption and facilitating transport.

Perhaps the most critical step in performing an assay is dispensing analytes and reagents from the on-chip reservoirs. The solution content can significantly affect the process of forming a droplet, particularly if it's a protein solution. The problem appears to be protein absorption in the liquid reservoirs, which have a far larger surface area than the electrodes.[5]

Dilution of sample volumes is an important step in almost all bioanalytical systems. We dilute the sample primarily for two reasons: to reduce the effect of interfering substances and to increase the linear range of device operation. Research has investigated the problem of obtaining on-chip dilution in a digital-microfluidic system.[13] Dilution requires mixing and splitting two unit droplets of different concentrations to produce an intermediate concentration. Unlike a continuous-flow system, this approach obtains a range of dilution factors through multiple passes of the two-droplet mixing and splitting, in various combinations. The accuracy and repeatability of the dilution process depends on volume variations in droplet splitting and concentration variations due to incomplete mixing. The errors in the dilution factor are about 15% for dilution factors up to 4, and about 25% for dilution factors between 4 and 8. Droplet volume variations in dispensing and splitting contribute to about 80% of this error.

Measurement repeatability is one of the most important performance metrics of an analytical method. The two aspects of precision are within-run precision and day-to-day precision.[8] For the electrowetting system, within-run precision is mainly affected by droplet volume errors, cross-contamination between experiments, and measurement errors. Srinivasan performed glucose assays at three different glucose concentrations.[8] He formed sample and reagent droplets in succession, merged and mixed them, and then monitored absorbance of the reaction products for 60 seconds, using the LED-photodiode setup (Figure 4). Figure 5 shows absorbance as a function of time for nine assays. The percentage coefficient of within-run variation for the three concentrations was within 2%.

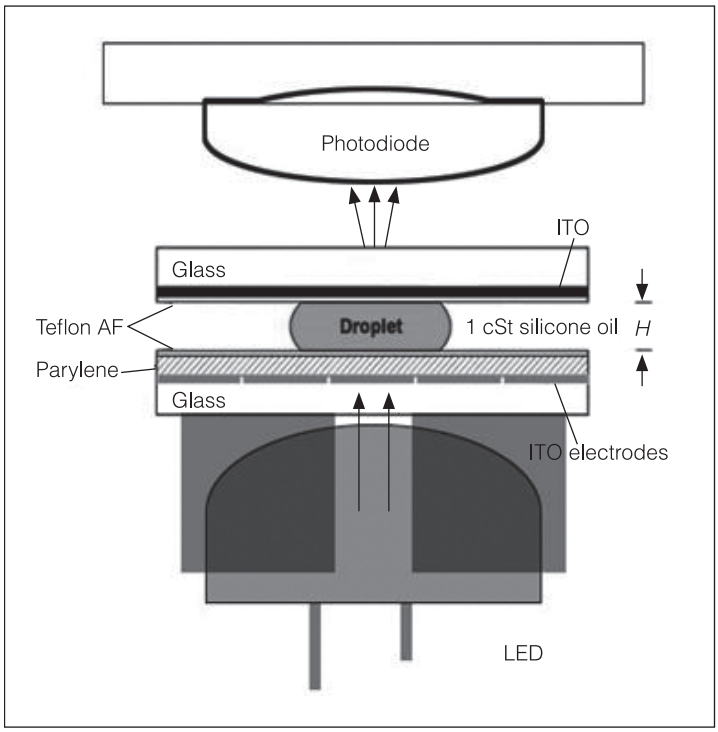

Figure 4. Optical absorbance measurement instrumentation monitors color change due to on-chip colorimetric reactions. (cSt is centiStokes.)

TNT assay

An estimated 100 million land mines buried in 65 countries throughout the world pose an enormous humanitarian problem, killing and mutilating thousands of civilians. The focus of mine detection is to find and destroy these mines. In addition to land mine detection, many countries also need chemical detection of explosives to assess soil and water contamination. TNT poses a human health risk even at very low parts-per-billion concentrations in ground water. Experts suspect it to be a carcinogen as well as being highly toxic to humans, other animals, and plants. Thus, the world has a significant unmet need for rapid, portable, on-site detection of land mines, unexploded ordnance, and soil contaminants.

Researchers obtained preliminary results using an LoC to detect commercial-grade 2,4,6-trinitrotoluene (TNT) and pure 2,4-dinitrotoluene (DNT).[14] They performed assays in three steps: dispensing, electrowetting-enabled mixing, and colorimetric detection. They dispensed droplets of TNT and potassium hydroxide (KOH) by pipette onto the electrowetting chip. Applying voltages to the appropriate electrodes merged the two droplets, and transporting the merged droplet mixed it still further by enhancing the mixing flow. At the completion of mixing, the LoC measured absorbance with an LED. All reactions occurred at room temperature. Figure 6 shows the measurement results.

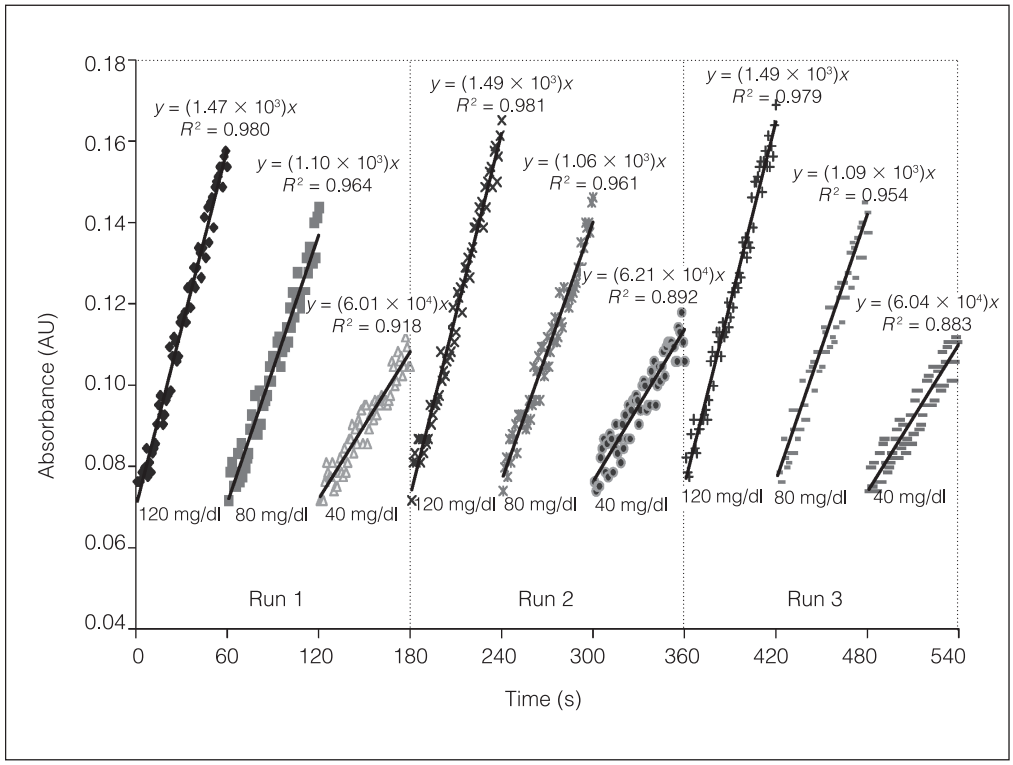

Figure 5. Absorbance measured as a function of time for nine serial glucose assays—three different glucose concentrations (40 mg/dl, 50 mg/dl, and 120 mg/dl) assayed three times each.[8] Here, AU is absorbance units, *y* is the slope of the linear curve fit, *R* is a measure of the deviation of the data from a linear fit, the abscissa *x* here represents time, and the equations represent a linear curve fit to the data.

The researchers estimated the on-chip method's detection limit from the results shown in Figure 6, assuming that the chip performs the calibration or the reference reading using only a single concentration. The lowest detectable concentration corresponds to about 2.6 μg/ml. If a cubic meter of sampled air contains 1 μg of TNT (1 femtogram/ml of air), and all of this particulate is collected on the chip's sampling surface and concentrated in a 1-μl droplet, the droplet will contain a TNT density of 1 μg/μl, or 1,000 μg/ml, well above the assay's detection limit. This means that detection sensitivities in the low attograms (10^{-18} g) of TNT per ml of air are possible. This level is competitive with results obtained by Nomadics Inc. with amplifying fluorescent polymers.

Figure 7 shows the absorbance spectra of the color products resulting from the TNT and DNT assays. The absorbance peaks of the colored complexes formed by the reaction of TNT and DNT with KOH are mutually independent. This means that we can quantitatively estimate TNT even in the presence of DNT, which is common when TNT is dissolved in soil.

TNT assay design issues

Nitroaromatic compounds such as TNT and DNT react with nucleophiles (bases) such as hydroxides and alkoxides, to form colored Jackson-Meisenheimer complexes. Acetone, acetonitrile, and methanol have been the most popular solvent choices for TNT analysis, even though researchers have demonstrated this reaction in various organic solvents. All three solvents are currently incompatible with oil-medium electrowetting systems because they are miscible with silicone oil. Dimethyl sulfoxide is another versatile solvent that dissolves most aromatic hydrocarbons, including nitroaromatics such as TNT, and yet is immiscible with silicone oil. Thus, it is compatible with the electrowetting platform. DMSO is also completely miscible in all proportions with water and has a low order of toxicity. For these reasons, DMSO was the solvent used to develop the TNT reactions just described. DMSO is also known to enhance the stability of the Jackson-Meisenheimer complex.[15]

Although alkoxides such as sodium methoxide or potassium methoxide are usually used as the base to react with nitroaromatics in DMSO, they are not suitable for a field method because of their high reactivity with water and their toxicity. That is why the researchers used KOH as the base in the TNT assay.

Sulfate assay

Another assay developed on a digital-microfluidic platform is atmospheric sampling of sulfate particles. Atmospheric particulate matter contributes to adverse health effects, visibility reduction, and global climate change, all with significant socioeconomic implications.[16–19] Knowledge of aerosol composition as

a function of size is critical to understanding the origin, properties, and health effects of particulate matter. Scientists usually measure size-segregated chemical aerosol composition with cascade impactors. Cascade impactors collect aerosol on several stages with progressively smaller cutoff diameters. The aerosol is impacted on aluminum or Teflon filter substrates, which are washed in ultrapure water, and the extract is analyzed. Because the preparation, sampling, and analysis steps are manual, impactors require considerable labor to obtain one sample, making their use extremely expensive.

Researchers have proposed a scanning droplet method for automated on-chip measurement of airborne particulate matter.[2,19] The scanning droplet method is used with a standard cascade impactor. Technicians perform sample collection by impacting airborne analyte particles directly on the electrowetting chip's surface. The surface is then washed with a microdroplet of solution. The droplet is digitally directed across the impaction surface, dissolving sample constituents. Because of the very small droplet volume used to extract the sample from a wide collection area, the resulting solution is relatively concentrated, and thus the analyte can be detected after a short sampling time (1 minute). After the washing phase, the droplet is mixed with reagents that produce colored reaction products. The LoC quantitatively determines the analyte's concentration by measuring absorption at target wavelengths using a simple LED and photodiode setup. Target applications include quasi-real-time sampling of airborne contaminants and bioagents, and detection and quantification of nitroaromatic explosive particles.

Our recent work has aimed at implementing the methyl thymol blue (MTB) method of sulfate determination.[20] Sulfate is the major aerosol component in the atmosphere, comprising 30% to 80% of fine-aerosol mass.[21] We based the colorimetric detection of sulfate on Madsen and Murphy's traditional MTB method,[20] with some modifications to the reagent composition. Ethanol is the traditional reagent solvent, but it proved incompatible with the digital-microfluidic platform because of its solubility in silicone oil.[22] After testing several alternative solvents, we obtained the best results with 40% methanol. We measured absorbance at 608 nm for sulfate detection. The measurements show a linear relationship for sulfate concentration ranging from the detection limit of 0.5 mg/l to 150 mg/l.

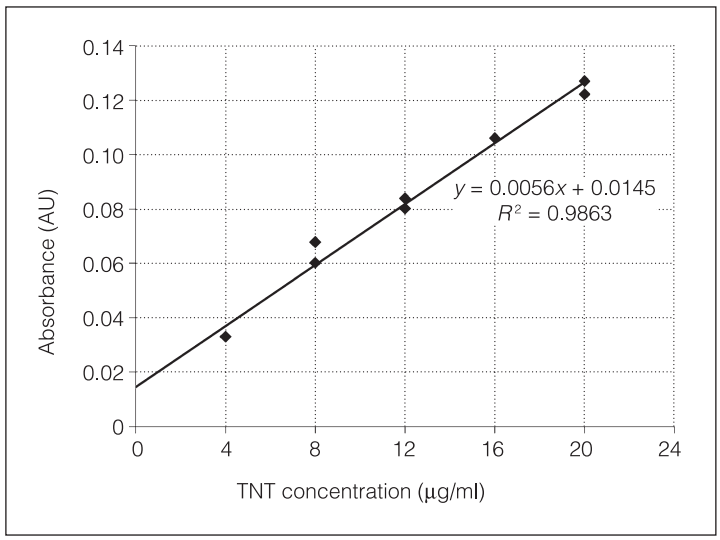

Figure 6. Calibration curve of absorbance versus TNT concentration, demonstrating a linear relation on the electrowetting chip.[14]

Sulfate assay design issues

For sulfate assay, we must perform droplet scanning and sample collection in air without a top plate, so as not to perturb the impactor air flow. To prevent evaporation, the scanning droplet can be clad in an oil encasement that travels through air with the actuated droplet. We have demonstrated oil cladding by transporting a droplet through the interface between the oil medium and the air.[2]

Figure 8 shows a demonstration of the scanning-droplet particle collection method.[2] We diluted

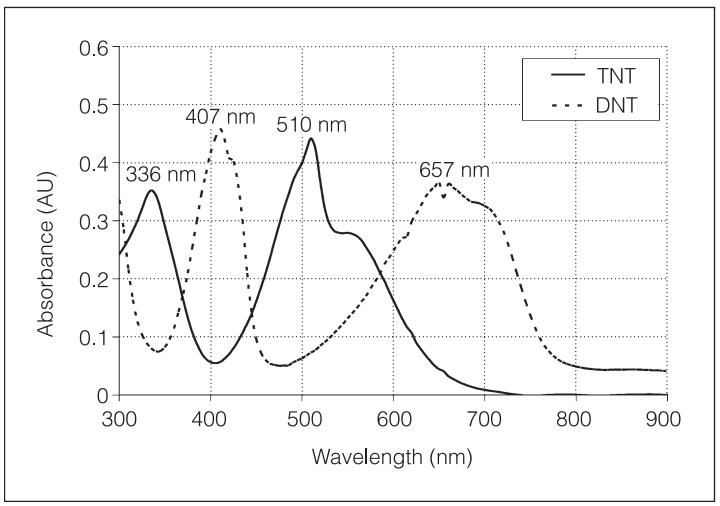

Figure 7. Absorbance spectrum of colored complexes formed in the reaction of TNT and DNT with KOH, using dimethyl sulfoxide (DMSO) as the solvent.[14]

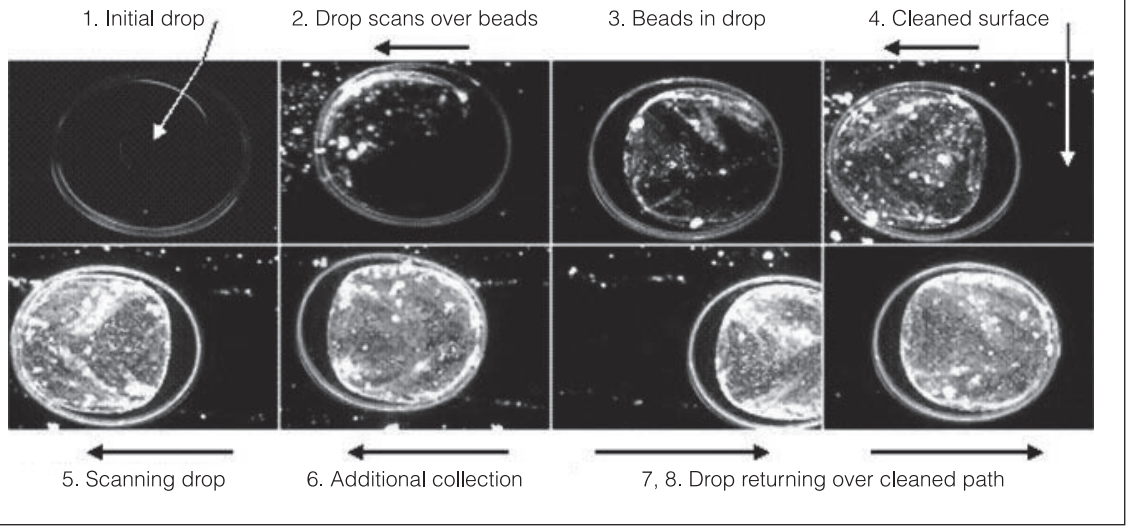

Figure 8. Scanning-droplet particle collection process. Arrows indicate direction of droplet scan, and frame numbers show the time sequence. The droplet picks up the deposited 1-micron beads on the surface, leaving behind a cleaned swath. The droplet does not redeposit beads once it reverses its scan direction. (This figure is reproduced, with permission, from R.B. Fair et al., "Integrated Chemical/Biochemical Sample Collection, Pre-concentration, and Analysis on a Digital Microfluidic Lab-on-a-Chip Platform," *Lab-on-a-Chip: Platforms, Devices, and Applications*, L.A. Smith and D. Sobek, eds., *Proc. SPIE*, vol. 5591, 8 Dec. 2004, pp. 113–124.)

a solution containing 1-micron-diameter polystyrene beads and deposited it on the hydrophobic Teflon surface of an electrowetting chip. After the solvent dried, a 1-µl water droplet clad with a thin oil film scanned the bead-coated surface through air. The time sequence in Figure 8 starts with the upper left frame, which shows the virgin droplet. The droplet scan direction in frames 1 through 6 is right to left, and in the last two frames (7 and 8), the droplet scans left to right back over the cleaned surface. The droplet picks up beads efficiently, and does not redeposit them on the hydrophobic surface after collecting them. When sample collection is complete, the collection droplet must move through an air-oil interface into the LoC's analytical section. We have not yet demonstrated the design and reliability of an appropriate on-chip oil-air interface system.

Integrating optical detectors on a digital-microfluidic platform is relatively easy, especially because the platform contains plates and see-through, indium-tin-oxide electrodes. However, optical absorption detection scales poorly with miniaturization because Beer's law incorporates a path length dependence.[23] In the detector shown in Figure 4, the optical path length is 100 to 300 microns, which is 30 to 100 times smaller than in conventional systems (10 mm). This small path length poses serious sensitivity issues.

DNA pyrosequencing

The number of bases in the GenBank genetic sequence database has increased exponentially, with a doubling period of approximately 18 months, and the database currently contains about 3×10^{10} bases, equivalent to the content of 10 human genomes (a base is a nucleotide on a DNA strand). In 10 years, the database will contain the equivalent of approximately 1,000 human genomes, and in 20 years, the equivalent of 100,000 human genomes. Achieving the productivity necessary for continued exponential growth of sequence information will require new, intrinsically scalable sequencing methods with no inherent operational limits.

Researchers have proposed using digital-microfluidic devices in several competing technologies to reduce reagent costs, which, along with instrument cost, are the primary cost of Sanger-based sequencing (the most common sequencing method, developed by Fred Sanger). These technologies include mass spectrometry,[24] nanopore sequencing,[25–27] sequenc-

ing by hybridization,[28–32] sequencing by synthesis,[33] single-molecule sequencing,[34,35] miniaturized electrophoresis,[36–38] and miniaturized pyrosequencing (see http://www.454.com).

On-chip sequencing by synthesis

We are currently evaluating the digital-microfluidic platform for performing miniaturized sequencing by synthesis. Sequencing-by-synthesis methods involve enzymatic extension by polymerase through iterative addition of labeled nucleotides, often in an array format. The process begins with the addition of a known nucleotide to the DNA (or RNA) strand of interest. The reaction to incorporate a nucleotide is carried out by DNA polymerase. Upon nucleotide incorporation, inorganic pyrophosphate (PPi) is released. The enzyme ATP sulfurylase converts the PPi to adenosine triphosphate (ATP), which then provides energy for the enzyme lucerifase to oxidize luciferin. One of the byproducts of this final oxidation reaction is light generated by the reaction at approximately 560 nm. Figure 9 shows this sequence. A photodiode, a photomultiplier tube, or a charge-coupled device (CCD) camera can easily detect the light.[39] Because we know the order in which the nucleotide addition occurs, we can determine the unknown strand's sequence by formation of its complementary strand. The entire pyrosequencing process takes 3 to 4 seconds per nucleotide added.[39]

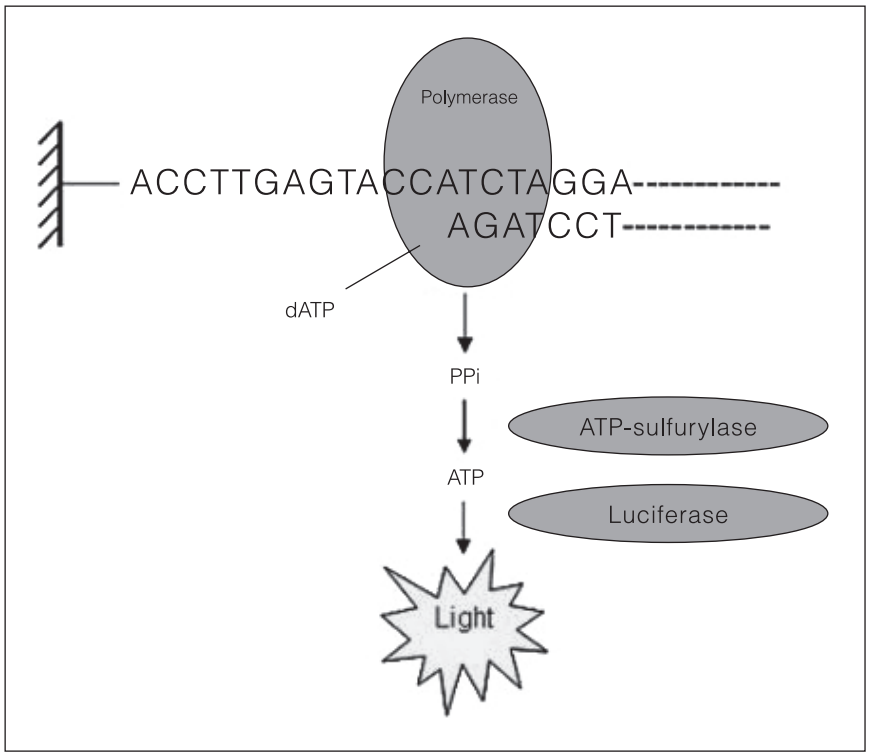

Figure 9. Solid-phase pyrosequencing (sequencing done on a solid platform, as opposed to a liquid phase). After incorporation of a nucleotide—in this case, deoxyadenosine triphosphate (dATP)—a washing step removes excess substrate. For the two strings shown, one is arbitrary DNA, and the other is complementary to the first string. (ATP is adenosine triphosphate; PPi is inorganic pyrophosphate.)

Figure 10 shows a preliminary digital-microfluidic pyrosequencing platform. The chip has reservoirs to house nucleotides and enzymes. The pyrosequencing protocol is easy to integrate in such a system. The on-chip chain of events is as follows:

- The reservoir dispenses a droplet containing nucleotides of a single type and polymerase, and the droplet goes to site S, which contains immobilized single-stranded DNA.
- The droplet incubates, allowing time for polymerization.
- The droplet leaves site S and reacts with luciferase.
- The lucerifase-containing droplet goes to a detector array (not shown in Figure 10).

While the detector array works to detect a light signal from the droplet, the next nucleotide-containing droplet enters site S, so that DNA synthesis occurs simultaneously with detection. This protocol allows optimized synthesis reactions, and because synthesis is physically separated from detection, the system has potentially no inherent limitation on its read length.

Unlike traditional pyrosequencing approaches, this approach does not limit the system to detecting light before the next nucleotide is added. The rate-limiting step in the scheme is the conversion of PP_i to ATP-by-ATP sufurylase. This reaction takes approximately 1.5 seconds, and the incorporation of a base takes less than 0.5 seconds. The luciferase completes the light generation in less than 0.2 seconds.[39,40] In the existing solid-phase pyrosequencing protocol, all these reactions must take place before the next nucleotide can be introduced.

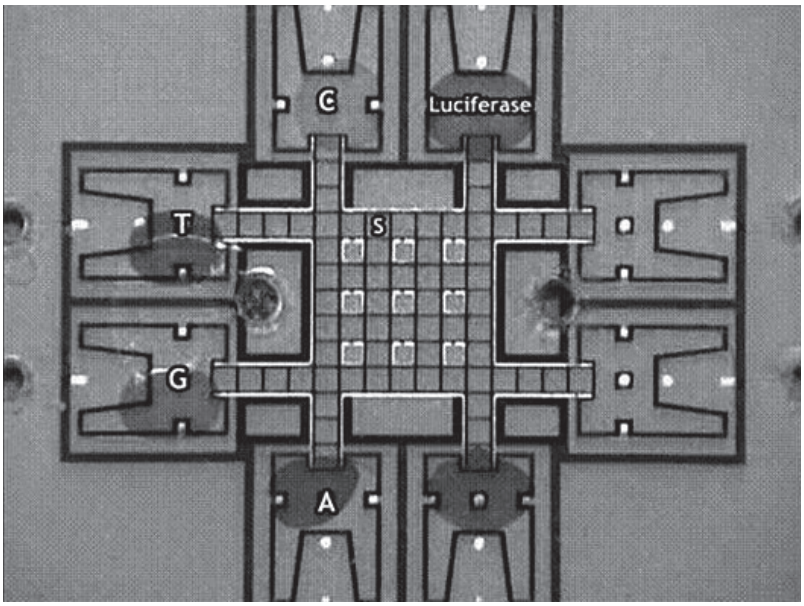

Figure 10. Advanced Liquid Logic's prototype pyrosequencing chip, with on-chip reservoirs for dispensing reagents and nucleotides A, T, C, and G. The DNA is attached to sample location S.

Pyrosequencing design issues

One of the chief design issues for performing pyrosequencing on an electrowetting chip platform is DNA immobilization through surface attachment. Because the platform's top plate is glass, it opens new possibilities for surface chemistry and enables convenient chip reuse. This feature makes attachment to the top plate appealing and preferable. However, the top plate must be hydrophobic for droplet transport, and we have typically used Teflon coatings. Thus, the challenge is to attach DNA to Teflon.

On-chip DNA immobilization is feasible with gold particles, although droplet transport over gold particles dislodges them.[41] A better method is to use streptavidin, which binds well to aminosilane and glutaraldehyde-treated glass top plates. This method requires patterning the Teflon film to create small access windows to the treated glass surface. Unlike gold, which is noncovalent, streptavidin binds covalently to the Teflon surface, ensuring that the protein remains attached after droplet transport. Additionally, biotinylated DNA is guaranteed to bind well to streptavidin because streptavidin has an extremely high affinity for biotin. Furthermore, commercial pyrosequencing relies on a streptavidin substrate to bind biotinylated DNA.[39] Thus, we can be sure that the reagents used for pyrosequencing won't hinder the streptavidin-biotin interaction.

To scale up pyrosequencing so that it handles multiple DNA fragments in parallel, we must address basic scientific and engineering issues involved in the scaling of an electrowetting-based microfluidic platform to handle picoliter droplets. Scaling droplet volumes by a factor of 1,000 to 1 million from the current scale translates to linear-dimension scaling of electrode pitches from hundreds of microns to 10 microns and switching times of electrode potentials from 50 Hz to 10 kHz. Such scaling is critical to massively parallel on-chip DNA sequencing at high clock rates. Thus, sequencing a 1-million-base-pair-long genome would require 1,000 parallel sequencing channels at 1,000 base pair reads per channel.

On-chip tissue engineering

Tissue engineering (TE) is evolving as a potential method for the repair and reconstruction of diseased or damaged tissues.[41,42] Doctors have used TE successfully to replace a blood vessel in pediatric surgery.[43] Nevertheless, the technology faces many challenges. The primary unmet needs are the following:

- manufacturing techniques that can mimic tissue and extracellular matrix architecture with high resolution (less than 10 microns) for tissues such as myocardium (heart muscle), blood vessels, bone, and nerves;
- digital automation methods for delivery of cells and growth factors into tissue scaffolds (artificial structures that support the tissue);[44] and
- manufacturing techniques to place vascular structures in engineered tissue, in which a lack of nutrient transport currently limits the size and cellular content of implants.[41,42]

TE's major goal is to synthesize or regenerate functional tissue and organs. Currently, scientists and doctors attempt to do this by combining cells with an existing tissue scaffold, culturing them in a controlled mechanical-biochemical environment, and implanting them in the body. The scaffold should not only mimic

the tissue's biological functions but also provide mechanical support of the tissue during the reconstruction process, maintain the tissue's initially fabricated 3D shape, and protect the tissue from handling during implantation and in vivo loading. A promising method of generating a tissue scaffold is electrowetting printing—embedding cells and growth factors in hydrogels and printing the hydrogel-containing cells onto the hydrogel-containing growth factors.[45] Work is underway to develop a new manufacturing system for 3D tissue scaffold and tissue construct fabrication using biomimetic structure modeling, electrowetting, solid freeform fabrication, and biomaterials.

A KEY REQUIREMENT FOR fabricating the hydrogel scaffold is placing the materials at specific locations on the chip surface. The hydrogel solution is initially in solution form, and once it meets a cross-linker solution, it solidifies. Thus, the hydrogel's constituents must be transported or printed on the surface at the desired location and then reacted. The first experiments performed by us addressed whether a 2% sodium alginate solution (with a viscosity of 250 centipoise) and a calcium chloride cross-linker solution could be actuated, dispensed, and reacted on an electrowetting chip with a silicone oil medium. On-chip reservoirs dispensed each solution, and the chip then combined the two droplets by actuating them into each other. When the droplets moved together, they rapidly became difficult to actuate, indicating they had changed to a gel. This is the first known demonstration of the formation of a solid phase in an on-chip chemical reaction. At present, more than 25 groups worldwide are working on droplet-based electrowetting science and technology, and the number of papers published each year in this specialty is growing exponentially. While researchers are making progress in furthering the understanding of electrowetting phenomena from a fundamental perspective, other efforts are exploring the applications, novel device structures, and CAD methods possible in digital microfluidics.[46] Efforts in all these areas are necessary to put the field on a solid footing and to find the unique niche that the technology can fill.

Investigators have conducted extensive research on the basic principles and operations underlying the implementation of electrowetting-based digital-microfluidic systems. The result is a substantial microfluidic toolkit of automated droplet operations, a sizable catalog of compatible reagents, and demonstrations of a few important biological assays. However, a lack of good on-chip sample preparation methods currently is the greatest impediment to commercial acceptance of microfluidic technologies, including digital microfluidics. Other issues include system integration and interfacing to other laboratory formats and devices; packaging; reagent storage; a way to maintain temperature control of the LoC during field operation; and a scalable, compatible detector technology.

Finally, there has been no attempt to implement molecular separation on a digital-microfluidic platform. The number and variety of analyses being performed on chip have increased, along with the need to perform multiple-sample manipulations. It is often desirable to isolate components that produce a signal of interest, so that they can be detected. Currently, mass separation methods, such as capillary electrophoresis, are not part of the digital-microfluidic toolkit, and integration of separation methods presents a significant challenge. ■

Acknowledgments

Funding from the following sources has enabled technology development and collaborative efforts across our separate disciplines: the Defense Advanced Research Projects Agency, the National Science Foundation, the National Institutes of Health, the Lord Foundation, GlaxoSmithKline, and Duke University Medical Center.

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