RAPID PATHOGEN DETECTION USING HANDHELD OPTICAL IMMUNOASSAY AND WIRE-GUIDED DROPLET PCR SYSTEMS

by

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ABSTRACT

This work introduces technology for rapid pathogen detection using handheld optical immunoassay and wire-guided droplet PCR systems. There have been a number of cases of foodborne or waterborne illness among humans that are caused by pathogens such as *Escherichia coli* O157:H7, *Salmonella typhimurium*, Influenza A H1N1, and the norovirus. The current practices to detect such pathogenic agents are: cell/viral culturing, immunoassays, or polymerase chain reactions (PCRs). These methods are essentially laboratory-based methods that are not at all real-time and thus unavailable for early-monitoring of such pathogens. They are also very difficult to be implemented in field, preventing early detection opportunities.

This dissertation is divided into three papers that present methodologies towards the expeditious detections of infectious pathogens and the miniaturization of these detection systems towards field-deployable and point-of-care applications. Specifically, the work presented focuses on two methodologies: (1) light scatter detection using immunoagglutination assays with optimized Mie light scatter parameters in a real biological matrix consisting of plant tissue, and (2) wire-guided droplet manipulations for rapid and improved sample analysis, preparation, and PCR thermocycling. Both of these methods carry a collective objective towards providing high impact technologies for addressing the issues of food-related outbreaks and overall public safety.

In the first paper, the direct and sensitive detection of foodborne pathogens from
fresh produce samples was accomplished using a handheld lab-on-a-chip device, requiring little to no sample processing and enrichment steps for a near-real-time detection and truly field-deployable device. The detection of *Escherichia coli* K12 and O157:H7 in iceberg lettuce was achieved utilizing optimized Mie light scatter parameters with a latex particle immunoagglutination assay. The system exhibited good sensitivity, with a limit of detection of 10 CFU mL$^{-1}$ and an assay time of <6 min. Minimal pretreatment with no detrimental effects on assay sensitivity and reproducibility was accomplished with a simple and cost-effective KimWipes filter and disposable syringe. Mie simulations were used to determine the optimal parameters (particle size $d$, wavelength $\lambda$, and scatter angle $\theta$) for the assay that maximize light scatter intensity of agglutinated latex microparticles and minimize light scatter intensity of the tissue fragments of iceberg lettuce, which were experimentally validated. This introduces a powerful method for detecting foodborne pathogens in fresh produce and other potential sample matrices. The integration of a multi-channel microfluidic chip allowed for differential detection of the agglutinated particles in the presence of the antigen, revealing a true field-deployable detection system with decreased assay time and improved robustness over comparable benchtop systems.

In the second paper, we demonstrate a novel method of wire-guided droplet manipulations towards very quick RT-PCR. Because typical RT-PCR assays take about 1–2 h for thermocycling, there is a growing need to further speed up the thermocycling to less than 30 min. Additionally, the PCR assay system should be made portable as a point-
of-care detection tool. Rapid movements of droplets (immersed in oil) over three different temperature zones make very quick PCR possible, as heating/cooling will be made by convective heat transfer, whose heat transfer coefficients are much higher than that of conduction, the latter of which is used in most conventional PCR systems. A 30-cycle PCR of a 160 bp gene sequence amplified from 2009 H1N1 influenza A (human origin) was successfully demonstrates in 6 min and 50 sec for a very large 10 μL droplet (with additional 4 min for reverse transcription). The proposed system has a potential to become a rapid, portable, point-of-care tool for detecting influenza A.

In the third paper, a wire-guided CNC apparatus was used to perform droplet centrifugation, DNA extraction, and VQ-PCR thermocycling on a single superhydrophobic surface measuring 25 mm by 55 mm and a multi-chambered PCB heater. This methodology exhibited no limitations on the complexity and configuration of procedures that it can perform, making it versatile and far-reaching in its applications. The only modification required for adding or implementing changes for a new protocol is through simple pre-defined programming. The highly adaptive and flexible system was used to execute easily pre-programmed droplet movements and manipulations for the rapid detection of *Escherichia coli* from PCR detection. Serial dilutions were performed to simulate a diluted field sample with a high level of accuracy. Centrifugation of the diluted sample containing *E. coli* demonstrated a novel approach to sample pre-treatment. Furthermore, the extraction of DNA from the sample droplet containing *E. coli* was also performed on the same superhydrophobic surface as the previous 2 steps, requiring less
than 10 min. Following extraction, the genetic material was amplified using wire-guided droplet PCR thermocycling, successfully completing 30 cycles of Peptidase D (a long 1500 bp sequence) in 10 min. The droplet centrifugation process was determined to greatly improve the positive band intensity over the non-centrifuged sample. Thus, this work demonstrates the adaptability of the system to replace many common laboratory tasks on a single platform (through re-programmability), in rapid succession (using droplets), and with a high level of accuracy and automation.
INTRODUCTION

*Escherichia coli* O157:H7, *Salmonella* spp., Influenza A H1N1 2009, and the norovirus have been found in many different food and water samples in the past couple of decades. In the U.S., for example, it has been estimated that foodborne illness contributes to between 63 and 88 million annual cases of acute gastroenteritis [1], resulting in 325,000 hospitalizations and 5,000 deaths annually [2]. Influenza A H1N1 2009 is a highly infectious disease that initiated a global pandemic in April 2009 [3]. As of 25th of April 2010, the World Health Organization (WHO) has reported at least 17,919 deaths of H1N1 2009 worldwide and reported confirmed laboratory cases in more than 214 countries and overseas territories and communities [4]. H1N1 2009 is defined as an acute febrile respiratory illness, in which the most common symptoms were fever, coughing, and sore throats [5]. In June 2009, the WHO raised the level of influenza alert from phase 5 to phase 6, indicating that a global pandemic was underway [6]. Currently, polymerase chain reaction (PCR) technology and viral cultures are the only CDC (US Centers for Disease Control and Prevention) recognized and FDA (US Food and Drug Administration) approved methods for the detection of influenza A subtype (H1N1) virus [7], requiring methods to significantly speed up these protocols rather than introducing new techniques. However, while these CDC requirements pertain to detecting at a post-consumer level, pre-consumer level detection for the prevention of outbreaks has not been widely established, opening the doors for novel approaches for early pathogen detection in fresh produce and related agriculture.
Fresh produce (including fruits and vegetables) has received more attention in food safety area in 1990s and 2000s. In 2002, for example, the number of cases of produce-associated illness was almost equal to all of those reported for beef, poultry, and seafood combined [8]. Expanding global distribution and consumption patterns, in combination with pathogen-contaminated irrigation water, will likely lead to a continued increase in produce-associated outbreaks [2]. This has been demonstrated by recent outbreaks of Escherichia coli O157:H7, Salmonella spp. and the norovirus that were linked to contaminated water used to irrigate fresh produce before harvest [9].

The lack of portable, real-time biosensors for these pathogens resulted in a significant time lag (a couple of days to a week) between the first outbreak and its identification. This time lag is due to the fact that the specimens need to be delivered to a remote laboratory (this delivery itself may take a day or two) and that the subsequent analyses take an additional few hours up to a couple of days. Furthermore, the time and labor associated with these laboratory-based analyses have limited the number of sampling/analyses before outbreaks hit. In many cases, the remedy for pathogen contamination is not implemented until there is an outbreak detected post-consumer level. If there is a means to speed up the testing by eliminating the need for an off-site laboratory, regular and routine testing would eliminate any problems for pathogens to reach the consumer level. The work presented in this manuscript addresses the need for a point-of-care and rapid detection of pathogens in two ways: (1) handheld E. coli detection system, and (2) wire-guided droplet manipulations.
1. **Handheld E. coli detection system**

Recently, there have been numerous novel detection methods introduced that are capable of detecting pathogens in near-real-time, exhibiting excellent sensitivity and reproducibility, and have been made portable for true point-of-care diagnostics. However, the criteria of what defines ‘portable’ can vary considerably. A portable system may describe an apparatus that can be transported by one person, but is still rather bulky, weighing more than a few kilograms, and generally cumbersome to transport, especially in the field. Also, the system may still require an AC outlet and/or laptop computer for signal processing. Whereas, a handheld device should be defined as a single unit that is small enough to be held and operated by one hand, weighing as little as possible, preferably less than 1 kg. It should be also battery-operated and have its own microprocessor, display, and integrated controls.

Although these characteristics are essential for a handheld apparatus, the major hurdle for these systems is the ability to detect the antigen in a real biological matrix with little to no preparation, which can be accomplished in the field. Thus, there is a reoccurring concern regarding whether novel, portable, and near-real-time pathogen detection systems can detect the target within a sample matrix. Consequently, there exists a great demand for a handheld pathogen detection system that not only exhibit all the requirements of conventional laboratory testing, but is also field deployable in the sense that the steps required to go from picking the crop in the field to displaying a result on an LCD screen is minimal and expeditious.
Particle immunoagglutination assays offer a rapid and sensitive platform for pathogen detection down to the single cell level [27,28,29]. Highly carboxylated microspheres enhance diffusional mixing in microfluidic devices and provide excellent reproducibility [30], and allow for long-term stability of reagents through lyophilization, which is essential for field-deployable applications [31]. Furthermore, this methodology is also effective for detecting antigens in a real biological matrix, utilizing light scattering measurements at a fixed angle from incident light delivery based on Mie scattering regime [32].

Because the refractive index $n$ of typical plant cell walls is reported as 1.425 [34], closer to that of water ($n = 1.33$) compared to immunoagglutinated particles ($n = 1.59$), the plant cell induced light scattering should be less significant in comparison to the scatter from the agglutinated particles. Additionally, the refractive index of $E. coli$ is very similar to that of water, with $n = 1.397$, and thus do not directly contribute to light scattering in latex immunoagglutination assays. However, the sizes of plant cells, which can be as large as 100 µm, are significant compared to $E. coli$ (ca. 1 µm) [35,36], and therefore may scatter some light due to their big size, despite their low $n$, under Mie regime.

The reproducibility and sensitivity of particle immunoagglutination assays can be improved through the use of lab-on-a-chip (LOC) microfluidic devices. A lab-on-a-chip is a device that integrates several laboratory functions onto one small platform, typically only millimeters or centimeters in size. LOCs normally involve the handling of very small fluid volumes; this introduces the area of "microfluidics" that deals with the
behavior, precise control, and manipulation of fluids that are constrained to a small, sub-millimeter scale.

LOC technology utilizes a network of channels and wells that are etched onto glass or polymer chips to build mini-laboratories. Pressure or electrokinetic forces move small volumes in a finely controlled manner through the channels. The LOC enables sample handling, mixing, dilution, electrophoresis, staining, and detection on a single integrated system. The main advantages of the LOC are: ease-of-use, speed of analysis, low sample and reagent consumption, and high reproducibility due to standardization and automation. LOC-based biosensors are a perfect medium to make portable and real-time biosensing of foodborne and waterborne pathogens possible.

![Fig 1. An LOC contains a network of channels and wells. Image source: Wikimedia Commons.](image-url)
The LOC has primarily been investigated to replace the need for routine and high-throughput medical diagnostics. To use the LOC for field applications, the device should be made fully portable and capable of real-time detection. To this end, the following must be demonstrated:

(1) Automated liquid handling (mixing, transport, and separation if necessary). Y- or T-junction channels have been used to accomplish liquid mixing, coupled with several different designs of passive/pulse/serpentine mixer designs. Liquid transport is made by applying either voltage (electroosmotic flow), external pressure (syringe pumping), or capillary flow.

![Diagram of sample/reagent injection and suction in immunoassay LOC devices.](image)

**Fig 2.** Sample/reagent injection and suction in immunoassay LOC devices.
(2) **Minimal sample pre-treatment.** Food samples (i.e. spinach or lettuce) need to be ground and filtered/centrifuged to remove large particles (plant cells and tissue fragments). Water samples (i.e. recycled water) need to be concentrated since the probability/concentration of waterborne pathogens in a certain sample is typically very low. A typical procedure involves: centrifuging multiple times, resuspension of pellets with a vortex mixer and/or a sonicator, liquid column chromatography, cell lysis, and nucleic acid extraction. These complicated procedures should be minimized into one or two simple steps using small and simple equipment such as a mini-centrifuge (battery-powered) or a syringe with a filter, so that the sensor can be used by non-experts with minimal processing time.

![Image 1](image1.png)  ![Image 2](image2.png)

**Fig 3.** Left: iceberg lettuce is ground in a bowl. Right: simple filtration of ground lettuce suspension in phosphate buffered saline (PBS) with KimWipes (laboratory paper wiper). Images from the author's laboratory, not yet published.
(3) *Fast.* The usual ELISA takes about a few hours in a laboratory. A typical PCR (including cell lysis and RNA extraction) also takes about a few hours in a laboratory. A normal cell culture test typically takes more than 24 hours. These times do not take the sample delivery time into consideration. "Real-time" detection, strictly speaking, indicates that the detection should be made simultaneously with sampling, but it would be safe to define less than five minute detection as real-time sensing.

**Fig 4.** Desktop testing unit (DTU) from Pathogen Detection Systems (PDS), now part of Veolia Water Solutions & Technologies. Although the device can be placed on a desktop, it is still sizeable and not truly portable (9 kg / 20 lb) and requires AC power and an external computer. Overnight cell culture is still necessary (> 24 hr). Image from www.pathogendetect.com.
(4) **Fully integrated system.** The entire system should be incorporated into a single package, for the ease of use and equipment delivery. Many other biosensor systems (including LOCs) require separate equipment for pre-treatment and/or detection. Almost all commercial biosensor systems (including LOCs) require an external computer. A true fully-integrated system should not require any extra equipment. At minimum, it should have its own user interface (just a few buttons with no keyboard) and an integrated LCD display for system operation and test results.

(5) **Battery-powered.** AC outlet is not always available in field situations. Therefore, the system should be operated fully via battery power.

(6) **No refrigerator required.** The reagents needed to complete the assays, such as the antibodies, nucleic acids, or enzymes, typically require refrigeration. In field situations, however, these reagents need to be packed in an ice box or lyophilized (freeze-dried) as powder for storage in room temperature.

(7) **Very low limit of detection (i.e. high sensitivity).** The limits of detection (LODs) for common ELISA tests can be as low as 10's pg proteins per mL of sample. LODs for common PCR can theoretically be at the level of single cell per 10-100 µL of sample, equivalent to 10-100 cells (normally represented by colony forming units; CFU) per mL of sample. About the same levels of LODs are expected for LOCs, but the actual limits have been a few or a few tens of nanogram proteins per mL of sample or a few hundred or million cells per mL of sample ($10^2$-10$^6$ CFU mL$^{-1}$) [6-10].
2. *Wire-guided droplet manipulations*

Manipulating small droplets has been the focus of much attention in recent years. The use of small droplets allows for significantly lower reaction volumes and decreased assay times for many common laboratory procedures. Furthermore, it has been established that complex and reconfigurable bioanalysis and biorecognition is only possible with droplets [10]. The two primary modes of droplet manipulations are: (1) to use discrete liquid plugs in pre-defined microchannels (Figure 1[11,12], or (2) to use droplets sitting on an open, flat surface [13,14]. Although the former (liquid-plug type) has been popular in digital microfluidics, the latter (open-surface type) has more potential as its reaction protocol can be reprogrammed to whatever combination one can conceive. Furthermore, while complex and reconfigurable algorithms can be implemented into pre-defined microchannel systems through the use of elastomeric microvalves [15,16], these microfluidic large-scale integrations (mLSI) systems typically require an array of external pneumatic solenoid valves, access to compressed air or a portable air compressor, and a control system with computer in order to choreograph precise, controlled droplet manipulations, requiring the addition of more components based on the complexity of the pre-defined microchannel layout.

The manipulation of droplets on an open, flat surface has been demonstrated most notably with magnetofluidics [17], but also includes electrowetting-based microfluidics (Figure 1) [18] and, more recently, wire-guided microfluidics [19]. In magnetofluidics, droplets containing paramagnetic particles move over a superhydrophobic surface under the influence of an external magnetic field. However, paramagnetic particles need to be
designed as not to interfere with biological reactions, a capability that has not yet been confirmed. Another common technique is electrowetting-on-dielectrics (EWOD), which allows for precise droplet movement, splitting, and merging. However, this method is comparatively more difficult to fabricate and operate, and has limitations with diffusional mixing and contamination from increased wetting on the surface.

![Image of PCR demonstrations in digital microfluidics: EWOD (top) and magnetofluidics (bottom). Reprinted from [17] and [18].]
Wire-guided droplet manipulations offer a simpler method for controlling droplets on an open surface. Whether we are able to manipulate a droplet or not depends primarily on the surface tension of the droplet, which is closely associated with its contact angle. Figure 2 in the bottom right graphically defines the liquid contact angle $\theta$. In most cases, the liquid is water or another aqueous solution. A liquid drop sitting on a certain surface maintains its shape due to the equilibrium in its surface tensions, specifically at its three-phase borderline. At this line, three different surface tensions are in exact balance, $\gamma_{SL}$ (solid-liquid), $\gamma_{SV}$ (solid-vapor) and $\gamma_{LV}$ (liquid-vapor).

![Diagram](image.png)

**Fig 6.** Magnetofluidics vs. wire-guide droplet manipulation. Reprint from [19].
Although a clean, metal wire was initially used to guide a droplet on a superhydrophobic surface (deriving the term wire-guided), the wire can be replaced with a variety of materials and sizes to modulate the force of the droplet to the wire (Wa), making the system highly adaptable to a wide range of droplet volumes and properties. Furthermore, the use of a syringe needle or disposable pipette tips can be used to perform precise droplet splitting and mixing with an attached vibration motor to the linear actuated syringe plunger. The syringe needle can also be used to form pendant droplets on the ends of syringe needles to perform rapid PCR thermocycling.

PCR is a well-established method for the detection and amplification of DNA and RNA with an unlimited sensitivity and unparalleled specificity. It is an essential tool in medical research and clinical medicine, used extensively for the detection of infectious disease organisms as well as detecting mutations in genes. Reverse transcription PCR (RT-PCR) is a variant of PCR in which an RNA strand is first transcribed to complementary DNA (cDNA) using the enzyme reverse transcriptase, which is then amplified using traditional PCR.

PCR works by copying a specific DNA sequence of interest. The process of PCR amplification requires three important steps: denaturing, annealing, and extension. Each of these steps is performed at three specific temperatures. Commercial PCR systems generally incorporate a high powered Peltier module in order to heat and cool the chamber in which the samples reside. This is the major disadvantage of current systems: conduction is the least efficient method for heating and cooling when comparing the three modes of heat transfer (conduction, convection and radiation). Additionally, most
commercial systems utilize a single heating block. The time required to reach the desired temperatures usually comprises a significant portion of the total PCR cycling time.

There has been significant interest towards the reduction of PCR cycling times, primarily through the implementation of microfluidic devices, in which small volumes of fluid travel through microchannels. Conventional methods of conductive heat transfer for heat cycling mean that it often takes a couple of minutes to complete a single cycle (and thus over an hour to finish typical 25–30 cycles of PCR). However, if this liquid is made to move over three different temperature areas within the microfluidic channel, the time required for heating and cooling may be significantly reduced, thus leading to a faster PCR assay. Additionally, the liquid volume that is needed to be heated and cooled is so small that the required heat transfer can be completed in a much shorter amount of time. The first such demonstration was made by Kopp et al. [20], where a single serpentine microfluidic channel travels through three different temperature zones to achieve 20-cycle PCR. In this manner, less than 20 min or even less than 10 min 20-to-30-cycle PCR has become a possibility. This concept has been combined with microfluidic cell lysis [21], capillary electrophoresis to confirm PCR products [22,23], or fluorescence microscopy for real-time monitoring of PCR products [24]. Liquid flows through a microchannel continuously, or as discrete liquid plugs within a microchannel. Although this microchannel PCR is faster than conventional PCR assays, it has several limitations in terms of reconfigurability or adaptability; for example, the number of cycles is set by the microchannel layout (if microvalves are not employed); sample-dependent pre-processing steps such as cell lysis, DNA/RNA extraction and/or reverse transcription
(RT) are rather difficult to be implemented into microchannel layout, as it is not reconfigurable. Additionally, these are closed systems, which mean that once liquid is inserted into the microchannel inlets, the process cannot be interrupted or augmented until it has completed its course or has been expelled from the system.

**Fig 7.** Continuous-flow PCR on a chip. Reprinted from [20].
The reconfigurability and reprogrammability feature of droplet microfluidics allows for the strong adaptability and expandability over the microfluidic microchannels. Specifically, these systems can be easily modified to be combined with cell lysis, DNA/RNA extraction and subsequent detection and sequencing of PCR products. Therefore, droplet microfluidic platforms are very adaptable to the variety of available and newly emerging PCR protocols. Unfortunately, attempts to perform PCR in droplet microfluidics format have been relatively rare, and focused primarily on electrowetting-on-dielectrics. Magnetofluidics does not involve complicated fabrication, as all you need is a superhydrophobic surface. However, proper heat transfer via a superhydrophobic surface can be inefficient, and the effect of magnetic fields and the presence of magnetic particles on PCR thermocycling and subsequent identification of its products remain to be investigated and resolved [25]. Wire-guided microfluidics is the simplest method for droplet manipulation and does not require the addition of particles that may interfere with the PCR thermocycling. Wire-guided microfluidics can be implemented on a variety of droplet environments, including superhydrophobic surfaces [19] and oil submersion, of which the latter is presented in this dissertation.

It is possible to significantly reduce the time for PCR thermocycling by employing wire-guide droplet microfluidics. A syringe needle tip is used to control the movement of a droplet submerged in silicone oil over three temperature regions. The 3-axis computer-numerically-controlled (CNC) system offers the benefit of reconfigurability and reprogrammability of droplet (digital) microfluidics. The submersed droplet continuously moves in silicone oil, which allows for forced convective heat
transfer from the surrounding oil to the droplet. This method of very quick PCR will allow for the rapid analysis of viral RNA for the purpose of detecting and preventing the spread of infectious diseases before they become pandemic.

This method should not be confused with droplet or nanodrop PCR [26], where the PCR assay is performed in a very small droplet to speed up the conduction-based heat transfer, which is not moving. This “stationary” droplet PCR has succeeded in speeding up the thermocycling significantly faster than other conventional methods. However, with this tiny volume (typically in nanolitre range), it becomes very difficult to confirm its products by traditional means such as gel electrophoresis, subsequent imaging and/or gene sequencing. Additionally, there is a concern in assay reproducibility and reliability, as aliquoting the low-concentration sample into nanolitre volume may occasionally result in no target molecule in the droplet. In wire-guide microfluidics, however, the volume of the droplet can be made large enough (in this case, 10 μL) to enable confirmation of its products (gel imaging and gene sequencing) as well as ensuring the assay reliability.

Fig 8. Nanodrop PCR. Reprinted from [26].
Wire-guided droplet manipulations address the problem of significantly decreasing assay time and introducing a system towards point-of-care diagnostics, while simultaneously falling within the criteria set forth by the CDC as a currently approved method. However, the detection of pathogens from fresh produce has not been fully established, allowing for the opportunity to introduce new methods of detection that incorporate a variety of existing and new technologies. There is a general consensus that when dealing with consumable agricultural commodities, such as, but not limited to, meat, poultry, and fresh produce, that early detection is an essential requirement towards the prevention of outbreaks for curtailing widespread risk to public safety and huge economic losses.
PRESENT STUDY

Appendix A summarizes the development and optimization of a miniaturized, handheld, lab-on-a-chip device for the direct detection of foodborne pathogens from fresh produce, requiring little to no sample processing/enrichment steps. *Escherichia coli* O157:H7 EDL933 and K12 were detected in near-real-time from a plant tissue sample utilizing optimized Mie light scatter parameters with a latex particle immunoagglutination assay. The device exhibited good sensitivity and reproducibility with a detection limit of 10 CFU mL\(^{-1}\) and an assay time of <6 min, which includes sample preparation of raw lettuce. Microscope images of the solution samples containing prepared plant tissue show sufficient filtration for this type of assay with a simple and cost-effective KimWipes filter. Mie simulations were used to maximize light scatter intensity of agglutinated latex microparticles and to minimize light scatter intensity of the sample matrices. The optimal parameters (particle size *d*, wavelength *λ*, and scatter angle *θ*) for the assay, involving *Escherichia coli*-contaminated iceberg lettuce, were determined using this approach and experimentally validated, introducing a powerful method for detecting foodborne pathogens in fresh produce and other potential sample matrices. The integration of a multi-channel microfluidic chip allowed for differential detection of the agglutinated particles in the presence of the antigen, while simultaneously excluding the light scatter from the plant cellular debris, decreasing assay time and improving robustness. Additionally, the incorporation of a microfluidic device improved sensitivity and linearity of the calibration curve.
Two sample preparation methods were studied and evaluated in a simulated field study based on the maximum percent intensity change over blank, as well as protocol complexity and assay time. Preparation of the plant tissue sample by grinding had a two-fold improvement in scatter intensity over washing. However, washing yielded a significant reduction in assay time, from ~5 min (grinding) versus ~1 min (washing). Specificity studies also demonstrated high specificity of *E. coli* O157:H7 EDL933 to O157:H7 antibody conjugated particles, with no cross-reactivity to K12. This suggests the adaptability of the system for use with a wide variety of pathogens, so long as the antibodies are properly designed and evaluated for antigen cross reactivity.

This device provides an easy-to-use, ultra-portable platform for use in the field, while maintaining the same sensitivity as its laboratory analog. The device and methodology can be adapted easily to any other food samples, including spinach, tomato, ground beef and eggs. The device will bring a huge impact on the safety of consumers and the agricultural industry by providing a cost-effective and rapid analysis tool on the front lines of preventing food-borne outbreaks.

Appendix B successfully demonstrates wire-guided droplet RT-PCR cycling of 2009 H1N1 influenza A in 10 min 50 s. Specifically, 30 cycles of heat cycling were performed in 6 min 50 s, demonstrating the effectiveness of utilizing wire-guide droplet manipulation in conjunction with convective heat transfer. With further optimization of the system, this heat cycling time is expected to be reduced to less than 3–4 min. Simulation studies were employed to develop a multi-chambered heat cycler and to establish the effectiveness of wire-guide droplet PCR with standard PCR assay solution
volumes. DNA sequencing confirmed the product created in the wire-guide droplet PCR system. The computer controlled wire-guided manipulator was constructed for general use in a variety of droplet microfluidics applications. However, when considering commercialization potential for the methods introduced in this paper, the system can be easily miniaturized and simplified. For example, the entire 3-chamber design with embedded temperature sensors can be constructed on a single circuit board incorporating a simple 2-dimensional table for moving the syringe. The entire system can be made relatively small and has the potential for portability due to the low power consumption of the PCB heaters. Alternatively, for high-throughput applications, an array of syringe needles can be used to run multiple PCR assays. Implementing a rotating circular chamber design with multiple heating chambers would allow for the continuous and uninterrupted heat cycling of many solution samples with different start and end times simultaneously. With further optimization, the wire-guide droplet PCR will prove to be an effective method for significantly accelerating PCR assays with the advantages of reconfigurability and reprogrammability necessary for the variety of infectious disease protocols, while still allowing for post analysis procedures to be performed.

Appendix C summarizes the work using a wire-guided CNC apparatus to execute pre-programmed droplet movements and manipulations on a superhydrophobic surface measuring 25 mm by 55 mm and a multi-chambered PCB heater for the rapid detection of *Escherichia coli* from PCR detection. This methodology exhibited no limitations on the complexity and configuration of procedures that it can perform, making it versatile and far-reaching in its applications. The only modification required for adding or
implementing changes for a new protocol is through simple pre-defined programming, which can be easily accomplished by any user with minimal programming knowledge. Because of this characteristic, the user has the ability to start and stop the assay/s at any time, manually control the droplet system with a computer, make adjustments to the protocol on-the-fly, and resume where it left off. To demonstrate the capabilities of this methodology and current system, serial dilutions were performed to simulate a diluted field sample with a high level of accuracy. Centrifugation of the diluted sample containing *E. coli* with a 10 μL droplet spinning around a 22-gauge blunt ended stainless steel syringe needle at 2300 rotations per minute demonstrated a novel approach to sample pre-treatment. This technique resulted in a mean increase in the concentration of *E. coli* contained within the sample droplet by more than 3 fold. Additionally, Fluent simulations were used to validate the centrifugation of *E. coli* particles and to track their migration over time, showing that effective centrifugation of particles occurred within 3 minutes. Furthermore, the extraction of DNA from the sample droplet containing *E. coli* was also performed on the same superhydrophobic surface as the previous 2 steps, requiring less than 10 minutes. This approach utilized the disposable pipette tip to cleverly extract the precipitated DNA from the sample droplet residing on the superhydrophobic surface. Following extraction, the genetic material was amplified using wire-guided droplet PCR thermocycling, which incorporates pendant droplets hanging from a syringe needle and moving through separately heated silicon oil chambers, utilizing forced convective heat transfer over a large surface area, to successfully complete 30 cycles of a long ~1500 bp sequence in 10 minutes. The results
of these sequentially executed processes were analyzed using gel electrophoresis, in which the droplet centrifugation was determined to greatly improve the positive band intensity over the non-centrifuged sample. And, although the system used in this work represents a benchtop apparatus, the method employed is not highly-complex, and thus alludes to the ability for further miniaturization, making a potentially portable device for performing a nearly limitless number of protocols, including all of their variations. Thus, this work demonstrates the reconfigurability of the system to replace many common laboratory tasks on a single platform (through re-programmability), in rapid succession (using droplets), and with a high level of accuracy, leading towards an all-in-one, portable lab on a chip system.
FUTURE WORK

The method of wire-guided droplet manipulations presented in this dissertation utilizes a general benchtop system using Cartesian coordinates. However, the methodology of guiding a small droplet with a wire is not limited to this system. Future work includes the use of a much smaller and simpler device that is battery operated and portable or even hand-held. Using a polar coordinate system, as opposed to Cartesian coordinate, will simplify the system and reduce the amount of motors/electronics required. This will facilitate the further miniaturization of the system into something handheld or possibly the size of a large watch or PDA. Furthermore, by using immunoglobulin M (IgM), centrifugation of the antigen can be significantly improved through immunoprecipitation, the results of which were verified through simulation.

Gel electrophoresis was used primarily to verify experimental results, and thus real-time detection through the implementation of an LED and photodiode will be incorporated. In the interest of portability, the reduction of PCB trace width is needed to improve the heat to battery consumption ratio. Furthermore, CFD simulations indicate that a droplet size of 1 μL, which is still capable of real-time detection, requires less than 3 min for 30 cycles. Additionally, throughput will be increased through implementation of multiple syringe needles, which is possible by rotating about a central axis and using a circular PCB heater.
In regards to the handheld *E. coli* detection system: future work includes expanding Mie scatter optimization to a variety of complex sample matrices, including meat and poultry. Improved LOC designs and particle lyophilization studies will allow for multiplex pathogen analysis from a single sample. Additional research also includes the use of paper microfluidics as a potential replacement for the LOC devices for latex particle immunoagglutination assays, which would significantly reduce cost and assay complexity.
REFERENCES


APPENDIX A:

DIRECT AND SENSITIVE DETECTION OF FOODBORNE PATHOGENS WITHIN FRESH PRODUCE SAMPLES USING A FIELD-DEPLOYABLE HANDHELD DEVICE

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Abstract

Direct and sensitive detection of foodborne pathogens from fresh produce samples was accomplished using a handheld lab-on-a-chip device, requiring little to no sample processing and enrichment steps for a near-real-time detection and truly field-deployable device. The detection of *Escherichia coli* K12 and O157:H7 in iceberg lettuce was achieved utilizing optimized Mie light scatter parameters with a latex particle immunoagglutination assay. The system exhibited good sensitivity, with a limit of detection of 10 CFU mL$^{-1}$ and an assay time of <6 min. Minimal pretreatment with no detrimental effects on assay sensitivity and reproducibility was accomplished with a simple and cost-effective KimWipes filter and disposable syringe. Mie simulations were used to determine the optimal parameters (particle size $d$, wavelength $\lambda$, and scatter angle $\theta$) for the assay that maximize light scatter intensity of agglutinated latex microparticles and minimize light scatter intensity of the tissue fragments of iceberg lettuce, which were experimentally validated. This introduces a powerful method for detecting foodborne pathogens in fresh produce and other potential sample matrices. The integration of a multi-channel microfluidic chip allowed for differential detection of the agglutinated particles in the presence of the antigen, revealing a true field-deployable detection system with decreased assay time and improved robustness over comparable benchtop systems. Additionally, two sample preparation methods were evaluated through simulated field studies based on overall sensitivity, protocol complexity, and assay time. Preparation of the plant tissue sample by grinding resulted in a two-fold improvement in scatter intensity over washing, accompanied with a significant increase in assay time: ~5 min (grinding)
versus ~1 min (washing). Specificity studies demonstrated binding of *E. coli* O157:H7 EDL933 to only O157:H7 antibody conjugated particles, with no cross-reactivity to K12. This suggests the adaptability of the system for use with a wide variety of pathogens, and the potential to detect in a variety of biological matrices with little to no sample pretreatment.

*Keywords: Escherichia coli* K12; *Escherichia coli* O157:H7; latex immunoagglutination assay; Mie light scattering; Microfluidic device; Lab on a chip; Iceberg lettuce

1. Introduction

The unavailability of a portable real-time biosensor for food-borne pathogens results in a significant time lag (a couple of days to a week) between the first outbreak and its identification. This time lag is due to the delivery of the specimens to a remote laboratory (typically takes a day or two) and the subsequent analyses (may take an additional few hours up to a couple of days). Additionally, the time and labor associated with these laboratory-based analyses have limited the number of sampling and analysis before the outbreaks hit. In many cases, the remedy for pathogen contamination is not acted until there is an outbreak detected post-consumer level. If there is a means to speed up the testing by eliminating the need for an off-site laboratory, regular and routine testing would eliminate any problems for pathogens to reach the consumer level.

The most commonly used detection methods of food-borne and waterborne pathogens include: (1) conventional culturing and colony counting, (2) enzyme-linked
immunosorbent assay (ELISA) and (3) polymerase chain reaction (PCR) (Munster et al., 2005). In addition, there are numerous novel detection methods capable of detecting pathogens in near-real-time. These methods exhibit excellent sensitivity and reproducibility, and have been made portable for true point-of-care diagnostics. However, the criteria of what defines ‘portable’ vary considerably. A portable system may describe an apparatus that can be transported by one person, but is still rather bulky, weighing more than a few kilograms, and generally cumbersome to transport, especially in the field. Also, the system may still require an AC outlet and/or laptop computer for signal processing. Whereas, a handheld device should be defined as a single unit that is small enough to be held and operated by one hand, weighing as little as possible, preferably less than 1 kg. It should be also battery-operated and have its own microprocessor, display, and integrated controls.

However, the major hurdle in developing a field-ready detection system is not the portability, nor the ease of operation (although both of these are quite essential), but rather the ability to detect the antigen in a real biological matrix with little to no preparation, which can be accomplished in the field. Thus, there is a reoccurring concern regarding whether novel, portable, and near-real-time pathogen detection systems can detect the target within a sample matrix. Consequently, there exists a great demand for a handheld pathogen detection system that not only exhibit all the requirements of conventional laboratory testing, but is also field deployable in the sense that the steps required to go from picking the crop in the field to displaying a result on an LCD screen is minimal and expeditious.
Particle immunoagglutination assays offer a rapid and sensitive platform for pathogen detection down to the single cell level (Lucas et al., 2007, Han et al., 2008, Heinze et al., 2009). Highly carboxylated microspheres enhance diffusional mixing in microfluidic devices and provide excellent reproducibility (Lucas et al, 2006), and allow for long-term stability of reagents through lyophilization, which is essential for field-deployable applications (Kwon et al., 2010). Furthermore, this methodology is also effective for detecting antigens in a real biological matrix, utilizing light scattering measurements at a fixed angle from incident light delivery based on Mie scattering regime (Heinze et al., 2010). Mie light scatter intensities are described as:

\[
i_1 = \left| \sum_{n=1}^{\infty} \frac{2n+1}{n(n+1)} (a_n \pi_n \cos \theta + b_n \tau_n \cos \theta) \right|^2
\]

\[
i_2 = \left| \sum_{n=1}^{\infty} \frac{2n+1}{n(n+1)} (a_n \pi_n \cos \theta + b_n \tau_n \cos \theta) \right|^2
\]

(1)

where \( i_1 \) and \( i_2 \) = complex scattering amplitudes for two orthogonal directions of incident polarization, \( n \) = refractive index, and \( \pi_n \) and \( \tau_n \) = angular dependent functions expressed in terms of Legendre polynomials. The scattering coefficients \( a_n \) and \( b_n \) are given by:

\[
a_n = \frac{\psi_n(z)}{\zeta_n(z)} A_n \quad \text{and} \quad b_n = \frac{\psi_n(z)}{\zeta_n(z)} B_n
\]

(2)

where \( A_n \) and \( B_n \) = quantities that depend on the ratios and logarithmic derivatives of the Riccati-Bessel functions, \( \zeta_n \) and \( \psi_n \). Riccati-Bessel functions are given by:
where \( J_{n+1/2}(z) \) = the half-integer-order Bessel function of the first kind, \( Y_{n+1/2}(z) \) = the half-integer-order Bessel function of the second kind, and \( z = \pi d/\lambda \), the size parameter of the scatterer (Cai et al., 2008). Hence, for Rayleigh scatter, when the particle diameter \( d \) is much smaller than the wavelength \( \lambda \), the wavelength becomes the dominant parameter. For Mie scatter, when the particle diameter is equal to or greater than the wavelength, the particle diameter is instead the dominant parameter. Therefore, equations (2) and (3) indicate that Mie scatter intensity is highly dependent upon the particle size \( d \) and less dependent upon wavelength \( \lambda \) compared to the Rayleigh scatter regime. Equation (1) also shows high dependency upon the scattering angle \( \theta \).

Because the refractive index \( n \) of typical plant cell walls is reported as 1.425 (Gausman et al., 1974), closer to that of water \( (n = 1.33) \) compared to immunoagglutinated particles \( (n = 1.59) \), the plant cell induced light scattering should be less significant in comparison. Additionally, the refractive index of \( E. coli \) is very similar to that of water, with \( n = 1.397 \), and thus do not directly contribute to light scattering in latex immunoagglutination assays. However, the sizes of plant cells, which can be as large as 100 \( \mu \)m, are significant compared to \( E. coli \) (ca. 1 \( \mu \)m; Institute for Biomolecular Design 2006, CDC 2009), and therefore may scatter some light due to their big size, despite their low \( n \), under Mie regime.
In this work, a particle immunoagglutination assay utilizing a microfluidic device is incorporated into a handheld, all-in-one device. The microfluidic chip adopts a multi-channel design to simultaneously detect the extent of light scatter from immunoagglutinated particles over the plant cells through a differential operational amplifier circuit with parallel incident light sources and avalanche photodiodes. Furthermore, this study aims to isolate the light scattering of immunoagglutinated particles from that of plant cells by using Mie scatter simulations to quickly determine the optimal parameters (particle size, wavelength of light source, angle of detection) that will maximize the assay sensitivity and robustness. These simulations are then experimentally verified by measuring the normalized changes in light scattering at different wavelengths (375 nm, 470 nm, and 650 nm) and microparticle diameters (510 nm and 920 nm) and evaluated as an approach for the system to adapt to different foodborne pathogens and sample matrices. The device is evaluated for sensitivity and specificity to pathogenic versus non-pathogenic strains of *E. coli* as well as exposed to simulated field studies to compare the sensitivity and labor requirements of two field-ready pre-treatment steps, grinding and washing, of iceberg lettuce.

2. Materials and methods

2.1. Handheld detection system

The completed device (Figure 1) includes a rigid plastic enclosure containing a battery compartment, avalanche photodiodes (APDs) and 650 nm light sources with collimating lens, positioning stages for photodiodes and light sources, circuit board for
signal conditioning, amplification, and analog to digital signal conversion for data processing, microcontroller, LCD display, and external controls and power switch. The enclosure was designed using Solidworks 2010 (Solidworks Corp., Concord, MA, USA) and then stereolithographically printed using a Dimension 1200ES 3D printer (Stratasys, Inc., Eden Prairie, MN, USA) in an acrylonitrile butadiene styrene (ABS) polymer. The system is powered by a 9 V disposable, or alternatively, a 6 A·h rechargeable lithium ion battery obtained from SparkFun Electronics (Boulder, CO, USA). The power source is conditioned with a NCP1400-5V DC-DC converter, outputting a low ripple 5 V source into an Arduino Duemilanove microcontroller (SparkFun Electronics). The microcontroller is also connected to a serial-enabled liquid crystal display (LCD) and momentary mini button pad set for operator controls and data recording (SparkFun Electronics).
**Fig A-1.** A) Battery-operated handheld detection system with integrated microprocessor, display, and controls. B) Internal compartment showing differential op-amp circuit, microcontroller, battery compartment, and sampling region. C) Positioning stages for APD’s and laser diodes with collimating lenses. D) Multi-channel microfluidic device with negative and positive detection regions for differential measurement in biological matrix.
A two-well slide or microfluidic device is inserted or removed from the device via a sliding tray mechanism on the side of the device, also printed from ABS. The sliding tray locks into place once fully inserted, maintaining consistent positioning of the sample space between tests. The internal testing region of the device, which includes the sample tray, laser diodes, and APDs, is thermally and optically isolated from the device circuitry and power source by multiple walls to reduce signal drift and error.

Light irradiation to the agglutinated latex microparticles was accomplished using two 3.3 mm laser diode modules with plastic lens housings and 7 mm acrylic aspheric collimating lenses obtained from Lasers4U, Co. (Walnut Creek, CA, USA). This configuration resulted in a front focal point of approximately 5 mm. The laser diode modules are held in a friction fitting positioning stage, also printed in ABS, affixed to the bottom of the enclosure. This configuration allows for z-axis adjustment of the laser diode modules and collimating lenses for an adjustable front focal point. Thus, the diameter of the spot size on the two-well slide and microfluidic device can be adjusted. Power to the laser diode modules are simultaneously moderated by a LTC1477 digital switch (SparkFun Electronics) controlled by the microcontroller. The intensity of each laser module is independently adjusted by a 10 kΩ potentiometer. Calibration of the laser diode intensities were performed using an Ocean Optics USB4000 UV-Vis miniature fiber optic spectrometer (Ocean Optics, Inc., Dunedin, FL, USA).

The scattered irradiant light source was detected using two UV-Vis silicon avalanche photodiodes (APD) with 1.0 mm active areas obtained from Edmund Optics (Barrington, NJ, USA). The APDs were mounted at a fixed 45° angle from the sample
plane, with 3-axis orthogonal adjustment. The distance between the centers of the photodiode active areas is congruent with the distance between the centers of the laser diode modules, and similarly with the detection regions of the microfluidic device.

2.2. Circuits for APDs and light sources

The differential voltage signal from the APDs are amplified and conditioned through multiple operational amplifier stages (op-amp), processed with a 24-bit analog to digital converter (ADC), and statistically analyzed by the microcontroller software. The entire amplifier circuit is powered through an ICL 7660 CMOS switched-capacitor voltage converter obtained from Digi-Key Corporation (Thief River Falls, MN, USA), for powering the op-amps. The positive and negative voltage sources feed the five TL082P operational amplifiers, each with a gain of 10, and a zero adjust stage with a 20 kΩ potentiometer. The op-amp circuit outputs the amplified differential voltage between the two APDs into an LTC2400 24-bit ADC, which utilizes an LT1021-5 5 V reference voltage IC (Digi-Key Corporation). The digital signal then feeds into the microcontroller for data processing and output.

The entire APD and light source circuit was constructed on one double-sided photoresist coated copper clad circuit board. Circuit boards, resist etching solution, and ferric chloride solution were obtained from Marlin P. Jones & Assoc. (MPJA; Lake Park, FL, USA). Circuit design, optimization, and trace routing were performed in FreePCB (Free Software Foundation, Inc., Boston, MA, USA) and printed onto clear transparencies using a 2400 dpi laser printer. The coated copper-clad board was then
exposed to an incandescent 100 W bulb and dissolved in photoresist developer (MPJA) at 46°C for 6 min. The board was then transferred to 0.240 g mL\(^{-1}\) ferric chloride bath (MPJA) at 55°C for 10 min. Remaining photoresist was removed using pure anhydrous acetone and drilled with a 0.025 inch (0.635 mm) bit.

2.3. Microfluidic device

The master mold for the microfluidic device was constructed using standard photolithography methods. Each channel is 1 mm wide and 40 μm deep and contains an inlet and outlet (Figure 2). SU-8 resist (MicroChem Corp., Newton, MA, USA) was spin-coated onto glass slides, exposed by UV using an ABM Mask Alignment System (ABM, Inc., Scotts Valley, CA, USA) over a chrome mask created with a Heidelberg Micro PG 101 Direct Write System (Heidelberg Instruments Mikrotechnik GmbH, Tullastrasse, Heidelberg, Germany), and developed. Polydimethylsiloxane (PDMS) was poured onto the positive master mold and cured in a convection oven for 1 h. The PDMS was then peeled off of the master mold, revealing two channels, and bonded to a glass slide via oxygen plasma treatment for 2 min (Plasma Preen Cleaner/Etcher; Terra Universal, Fullerton, CA, USA). Teflon tubing and a syringe attached to the channel inlets were used to fill and evacuate the chamber.
Fig A-2. Differential detection of *E. coli* in iceberg lettuce using a multi-channel microfluidic device with antibody-conjugated and non-antibody-conjugated microparticles in a latex particle immunoagglutination assay.
2.4. Microparticle preparation

920 nm nominal mean diameter, high-acid content, poly(styrene/15% acrylic acid) latex microparticles (Bangs Laboratories Inc., Fishers, IN, USA) were used in these assays. Rabbit antibodies to *Escherichia coli* K12 (Sigma-Aldrich, St. Louis, MO, USA) and O157:H7 (Meridian Life Sciences, Inc., Saco, ME, USA) were covalently attached to the latex microparticles according to the protocol by Bangs Laboratories (Bangs Laboratories, 2008). The covalent coupling process began by suspending microparticles in activation buffer, MES (C$_6$H$_{13}$NO$_4$S, 50 mM, pH 6.0, Sigma-Aldrich). The microparticle suspension was washed twice in activation buffer (MES) by centrifuge at 14,000 rpm for 15 min, and suspended in MES. Carbodiimide (Sigma-Aldrich) was added and mixed at room temperature for 15 min. It was washed twice and suspended in coupling buffer, PBS (50 mM, pH 7.4, Sigma-Aldrich), and then introduced to the antibody solution, also suspended in PBS. These were allowed to mix overnight on a rocker at 4°C. Following overnight mixing the solution was washed 3 times with PBS and re-suspended in the quenching solution, hyrdoxylamine (40 mM with 1% w/v BSA, Sigma-Aldrich) and mixed for 30 min. This was washed and suspended in the storage buffer, PBS-BN (PBS with 1% w/v BSA and 0.05% w/v sodium azide) and stored at 4°C until used. The antibody concentration to yield 100% coverage was calculated using the equation $S = (6/\rho D)(C)$, where $S$ is the concentration required for complete saturation, $6/\rho D$ is the surface area to mass ratio based on microparticle diameter ($\text{m}^2 \text{g}^{-1}$), and $D$ is the diameter (µm) of the microparticle.
2.5. Lettuce solution preparation

The lettuce solution was prepared from fresh iceberg lettuce acquired from local markets. These lettuce samples were *E. coli*-negative as confirmed by culturing samples in brain heart infusion broth and performing standard plate counting methods. The plant tissue samples were ground and mixed with 50 mM PBS (pH 7.4; Sigma-Aldrich) in a ratio of 1 g (wet weight) to 2 mL of PBS. 5 g of iceberg lettuce were hand-shredded and added to a mortar and pestle and ground. The ground lettuce was then added to a disposable 50 mL tube and 10 mL of PBS (10 mM and pH 7.4) was added and the entire solution was vortexed at high speed for 30 s. This mixture was filtered using a KimWipes delicate task wiper (Kimberly-Clark, Neenah, WI, USA), with a measured average pore size of 13 μm, and 10 mL disposable syringe; the mixture was pulled through the KimWipes and into the syringe, filtering out large lettuce particles. This solution was deposited into centrifuge tubes and stored at 4°C until used.


The *Escherichia coli* K12 was cultured in 37 mg mL\(^{-1}\) brain heart infusion broth (6 mg brain heart infusion, 7 mg peptic digest of animal tissue, 14.5 mg pancreatic digest of casein, 5 mg NaCl, 2 mg dextrose and 2.5 mg Na\(_2\)HPO\(_4\); Remel, Lenexa, KS, USA), with lyophilized powders of *Escherichia coli* K12 (Sigma-Aldrich) at 37°C for 6 h. A combination of 25 mg *E. coli* flakes with 10 mL of brain heart infusion broth was used. For the specificity study, *E. coli* O157:H7 EDL933 was cultured in 25 mg mL\(^{-1}\) LB broth (5 mg yeast extract, 10 mg tryptone and 10 mg NaCl; growcells.com, Irvine, CA, USA),
for the same duration and temperature. The antigen solutions were serially diluted with 10 mM PBS (pH 7.4). The colony-forming units (CFU) of each serial dilution were evaluated by standard plate counting methods.

2.7. *E. coli* on lettuce for simulated field study: grinding vs. buffer wash preparations

A simulated field study comparing the effectiveness of two different methods of preparing the lettuce solution for testing required the culturing of *E. coli* directly on fresh lettuce instead of the previous preparation method. The lyophilized *E. coli* powders were added to brain heart infusion broth (25 mg to 10 mL) and incubated at 37°C for 1 h. This culture solution was then added to a 50 mL tube containing 5 g of fresh iceberg lettuce and left at room temperature overnight (approximately 24 h). Multiple containers were prepared simultaneously in identical fashion. One lettuce-*E. coli* sample was then ground, combined with PBS, and filtered in the same manner as before. The other sample was not ground, but the same 10 mL PBS was added and mixed thoroughly (shaken) with the lettuce-*E. coli* sample and then a disposable syringe was used to withdraw a sample for use in testing. No KimWipes was necessary for buffer wash preparation because there would not be large lettuce pieces.

2.8. Microfluidic immunosensing on benchtop detection system

For the benchtop set-up, a microfluidic chip was positioned using 3-axis translation stages (Edmund Optics) and secured on a platform with an ultraviolet light
emitting diode (375 nm UV LED) light source (Ocean Optics) and optical fiber connected to a miniature spectrometer (Ocean Optics) and analyzed with SpectraSuite (Ocean Optics) to generate a photon intensity value. The details about this benchtop detection system can be found in our previous works (Heinze et al., 2009). The test samples were prepared with the antibody conjugated microparticles, lettuce sample and *E. coli* dilutions (or lettuce-*E. coli* solution for the preparation comparison). 20 µL of antibody conjugated microparticles and 60 µL of *E. coli*-spiked or -contaminated lettuce solution was injected into the channel of the microfluidic device. A measurement was recorded and a standard curve was generated for the normalized photon intensity value at each serial dilution of *E. coli*. Additionally, negative controls were conducted with microparticles not conjugated with antibodies.

### 2.9. Microfluidic immunosensing on handheld detection system

The mixing of antibody conjugated microparticles and *E. coli*-spiked or -contaminated lettuce solutions and subsequent insertion into the microfluidic device through a syringe were conducted in a similar method to the benchtop protocol, with the addition of non-antibody conjugated microparticles for use in the blank microchannel. To ensure measurements were not affected by APD heating, the system was turned on and programmed to collect data during a certain time interval (less than 30 sec) and then automatically shut off and allowed to cool for 1-2 min. Any potential heat generated by the laser diodes, LCD backlighting, or circuitry is minimal and reproducible for this time period. This reproducibility was confirmed by using a blank tray to measure a baseline
signal before each concentration was tested: if the baseline signal was greater than 0, the system would be allowed to cool further. The algorithm was programmed so that once the baseline signal returned to 0, it would automatically initiate the test sequence so that all data was recorded on an exact time frame throughout the experiment to reduce inaccuracies due to temporal variability. In real world, the additional steps in the protocol would not be ideal, however, this could be easily resolved by either using anti-drift APD’s or incorporating a temperature sensor and adjust the output through the microcontroller algorithm.

The LTC2400 used in the handheld detection system was able to convert approximately 2 readings at 24-bit resolution every second. The algorithm averaged a total of 50 measurements over 25 sec and warned if any extreme outliers were detected that would indicate a potential bubble or problem at the test region of the microfluidic chip. This averaged signal reading was then either displayed directly in raw intensity value, or calculated against a standard curve to display a concentration in CFU mL\(^{-1}\), as used for the simulated field study.

### 2.10. Mie scattering simulation method

Light scattering simulations were performed using online software (Prahl, 2007) with parameters of particle diameter, refractive index of medium (phosphate buffered saline; PBS), real and imaginary refractive indices of microparticles and plant cellular
debris, wavelength of incident light, and concentration of the suspension. Scatter intensity simulations were conducted for a range of scatter angles from incident light source.

Average particle size of plant cells was determined by analyzing a compilation of 9 sequential microscope images using ImageJ (National Institute of Health, Bethesda, MD, USA) to determine the mean particle size and concentration. The cells were stained using toluidine blue (1% w/v aqueous solution, RICCA Chemical Company, Arlington, TX, USA) with standard staining procedures. The particle diameter was assumed to be nearly spherical for simulation studies.

3. Results and discussion

3.1. Mie scatter simulation with plant tissue sample

To determine the effect of plant tissue on light scattering, a series of microscope images were taken of the sample in various stages of preparation and subsequent scatter simulations were performed. Figure 3-A shows the plant tissue sample before grinding of intact cells with a mean diameter of 80.6 μm. Figure 3-B shows the plant tissue sample just after grinding and before filtering. The average particle size was 13.5 μm, and it was evident that a majority of the large particles were composed of cellular debris. It is important to note that the average particle size for the ground and unfiltered sample solution does not include the lettuce “pulp.” Figure 3-C shows the plant tissue sample after filtering with a KimWipes general task wiper, in which all of the “pulp” and other remaining large plant particles were removed from the sample solution. The microscope image suggests that the majority of the plant debris and cell wall was removed during the
filtration step. The average particle size was 0.77 μm for the filtered lettuce sample. As shown in Figure 5-D, the colorations of the samples before and after KimWipes filtering are about the same, indicating the overall amount of lettuce fragments did not change significantly. It eliminated only the large “pulps.”
Fig A-3. A) Raw iceberg lettuce. B) Ground iceberg lettuce before KimWipes filtering. C) Ground iceberg lettuce after KimWipes filtering. D) Mie simulation results of lettuce particles (0.1433 spheres µm$^{-3}$) and carboxylated 920 nm microparticles (0.01% w/v; triplet: 0.04766 spheres µm$^{-3}$, singlet: 0.1433 spheres µm$^{-3}$) using a 650 nm light source.
Figure 3-D exhibits the Mie scatter properties of the filtered lettuce particles \((n = 1.425)\) and the 920 nm microparticles \((n = 1.59)\) as singlets and triplets using a 650 nm light source. The simulations take into account the decrease in particle concentration from agglutination. Thus, the triplet model results in \(1/3\) the particle concentration as the singlet model. At a forward scattering angle of 45°, the 920 nm singlet shows a minimal increase in light scatter intensity over the lettuce particles (~0.01 AU), and the 920 nm triplet shows a significant increase over the lettuce particles (~0.05 AU) as well as the 920 nm singlet (~0.04 AU). These simulations demonstrate that the detection of \(E. coli\) in a real biological matrix under these minimal pretreatment steps for ground iceberg lettuce is possible by choosing an appropriate scattering angle. They also reveal that the forward light scattering angle is an essential optimization parameter, since a scattering angle of 5° in either direction from 45° would have no detectable change in light scatter intensity and substantial scattering from lettuce particles. Furthermore, while backscatter (180°) is commonly used, simulations results show a much weaker signal compared to forward light scatter (Heinze et al., 2010).

3.2. Particle size and wavelength optimization with Mie scatter

In the interest of optimizing the light scatter intensity of the assay, an analysis of the microparticle size and light source wavelength towards light intensity was conducted using simulation software, and the results were verified with experimental data. Figure 4 shows both Mie scatter simulations (top) and experimental results (bottom) obtained with the benchtop detection system using both 510 nm microparticles with 375 nm UV and
470 nm blue light sources, and 920 nm microparticles with 375 nm UV, 470 nm blue, and 650 nm red light sources. In Mie scatter regimes, the scatter intensity is greatly compromised when the wavelength is equal to or greater than the particle size. Therefore, the 650 nm red light source was not used with 510 nm microparticles based on simulations and experimental data from the 470 nm blue light source.
Fig A-4. Mie simulation for 510 nm microparticles with 375 nm and 470 nm light sources and 920 nm microparticles with 375 nm, 470 nm, and 650 nm light sources and experimental results confirming 920 nm microparticles and 650 nm light source as optimal parameters for detecting *E. coli* in iceberg lettuce.
The experimental results for the 510 nm microparticles show a moderate difference in percent change in intensity with the 375 nm (~8% change) over the 470 nm (<1% change) light source. This is supported by simulations for 375 nm showing a moderate increase in scatter intensity at 45° between the singlet and triplet models (where the triplet identifies agglutination from the presence of the antigen), and no increase in scatter intensity for the 470 nm. Although the simulations indicate that a forward scattering angle of 46-47° would be better suited, it is important to note that these simulations only demonstrate triplet models. From previous work, a 45° forward scattering angle was determined to be optimal, which take into account all the various models exhibited by the assay. The experimental results for the 920 nm microparticles show that the 375 nm, 470 nm, and 650 nm wavelengths produce large intensity changes of ~14-16%. However, while all 3 wavelengths showed high sensitivity, the 470 nm wavelength exhibited the greatest variation, followed by 375 nm, and then by 650 nm. This can be explained through the slopes of the singlet models for each wavelength. For 470 nm, 45° falls on an anti-node, making it the most sensitive to changes in particle size (and angle). At 375 nm, the slope decreases significantly, and at 650 nm, the slope broadens out even further. Thus, while 375 nm had the largest increase in scatter intensity, 650 nm exhibited the least signal variation. Therefore, a microparticle size of 920 nm and a light source of 650 nm wavelength were chosen as the optimal parameters for maximum assay sensitivity with minimal signal variation. Component cost, heat generation, and power consumption were also taken into consideration, as the red light
source is the cheaper and generates less heat \(E = h c / \lambda\) where \(E = \) energy, \(h = \) Planck’s constant and \(c = \) speed of light) than blue or UV.

### 3.3. Handheld detection system with microfluidic device

The handheld detection system merges all of the individual and bulky components of the benchtop system into a handheld, battery-operated device, for point-of-care analysis. The parameters of the system to maximize the Mie light scatter intensity have been incorporated to optimize the latex particle immunoagglutination assay. Figure 1-B shows the apparatus with the top cover removed. The entire device measures 12.8 cm x 20.8 cm x 5.2 cm (W x L x H) and weighs 543.90 g including the battery pack and microfluidic device. However, there is an abundant amount of space inside of the case to accommodate future plans of a miniature pumping apparatus and waste disposal for the microfluidic device. It is very feasible that such a device could be further miniaturized into something the size of a cell phone or equivalent, or be designed as an attachment for a cellular device.

An important component of the handheld detection system is the simultaneous differential detection of positive and negative samples in a single assay. This was accomplished by implementing a multi-channel design with parallel light sources and photodiodes, coupled with a differential op-amp circuit, to detect a signal change based predominantly on antibody-antigen bonding, and not residual plant cellular debris and other particulates present in the sample. Figure 1-C shows the central compartment of the device which houses the photodiodes and light sources. This portion was designed to
be thermally isolated from the battery and circuitry, as well as being a light-tight box. For certain incidences, signal drift was observed between measurements due to APD heating. When this occurred, the system was either allowed to further cool (preferred method), or the system simply re-zeroed the signal through the ADC algorithm if ultimately it was not able to achieve zero (only occurred during high environmental temperature). For the sake of accuracy, the system was always allowed to cool; however, the second approach would be suitable for a real-world scenario in the field where adequate cooling may be absent.

Figure 5-A shows the positive standard curve and negative control using the handheld detection system with the multi-channel microfluidic device. The results show good linearity with a limit of detection of 10 CFU mL⁻¹ (E. coli log CFU mL⁻¹ = 1) with good statistical significance (p<0.05) over blank. The inherent “dip” associated with particle immunoagglutination assays is still evident, but has been repeatedly demonstrated to become less pronounced through the use of microchannels instead of microscope two-well slides or multi-well slides (Heinze et al., 2011).
Fig A-5. A) Positive standard curve with antibody conjugated particles and negative control with non-antibody conjugated particles for *E. coli* in iceberg lettuce using handheld detection system with multi-channel microfluidic device. B) Simulated field study comparing wash and grind preparation procedures with *E. coli* cultured directly onto iceberg lettuce (*E. coli* concentrations were calculated using standard curve from A). C) Diagram representing axial diffusion and perpendicular diffusion of microparticles in a microchannel. D) Images of ground iceberg lettuce before and after filtering, and buffer washed.
Linearization of the calibration curve for the handheld device is an essential requirement for field applications. A common approach for benchtop devices is to systematically dilute the sample to determine which side of the curve the target concentration resides, and then to back-calculate the concentration based on the amount of diluent added. While effective, this approach is not ideal for real-time detection, and may induce some error from its inherent methodology for indirectly calculating the original concentration. A more optimal approach for a handheld system is to use a microfluidic device. Because of the size of the microfluidic channels, the samples exhibit strictly laminar flow, in which diffusion is the predominant mode of particle mixing (Figure 5-C). Specifically, axial diffusion is the mass transfer of interest (Han et al., 2007). During injection of the sample into the microfluidic channel, the velocity profile of the cross-sectional area induces agglutinated particles of smaller mean diameter to reside towards the center, where the velocity is greater, whereas the larger agglutinated particles will reside primarily along the walls, where the velocity is lower. Thus, the agglutinated particles adopt a form of temporal history in which the smaller particles will reach the detection region at a different time than the larger particles. This was observed as a slight decrease in signal intensity, evident in the concentrations exhibiting a greater difference in particle size variation (E. coli log CFU mL\(^{-1}\) = 3, 4). Due to the time sensitive nature of particle immunoagglutination assays, the control of time-wise measurements by the system was an essential design criterion. Therefore, the averaged intensity measurement over a consistent time scale improved assay reproducibility/linearity and increased sensitivity by observing a broader temporal field
of agglutinated particles. This methodology, performed through a finely tuned algorithm, proved superior to the benchtop detection system, which was not possible due to software limitations.

3.4. Simulated field study

Sample preparation is an important area of investigation to optimize sensitivity, reproducibility, and assay time for the handheld detection system to be used in the field. Two preparation methods were investigated, grinding and washing, of iceberg lettuce with *E. coli* cultured directly onto the surface to simulate a contaminated water source. Grinding the lettuce in a mortar and pestle may promote better separation of the *E. coli* from the lettuce surface, but may also destroy the *E. coli* in the process or increase the concentration of plant debris in the biological matrix, sacrificing sensitivity. Alternatively, washing the lettuce in PBS may prevent unwanted damage to the pathogen and also improve the signal to noise ratio by eliminating the presence of plant cellular debris from the sample, but may decrease the overall sensitivity due to poor separation of the *E. coli* from the lettuce surface.

Figure 5-B is the result of a simulated field study in which 3 independent trials comparing wash and grind preparation procedures. The concentration values were calculated based on the standard curve generated in Figure 5-A. The variation in intensity change from trial to trial was due to different lettuce samples and slightly different incubation times. However, it is apparent that the grinding method had a
consistent improvement in percent change of signal intensity over blank, with an average improvement of two-fold over washing.

Although grinding the lettuce samples yielded higher sensitivity, it came at the cost of longer assay time with additional equipment required (mortar and pestle/KimWipes filter). The use of the grinding protocol may be less than ideal in real-world situations out in the field, where the buffer wash would decrease assay time and reduce cost by eliminating additional equipment. The typical preparation time for the grinding protocol was approximately ~5 min, whereas washing was ~1 min, a reduction of 80%. A more practical approach may be to include a combination of both methods: shredding of the lettuce into smaller pieces and then washing them. Such a protocol could potentially eliminate the need for additional equipment and assay time, while maintaining excellent sensitivity.

3.5. Specificity results

For a handheld detection system to be used in field for rapidly identifying the presence of harmful pathogens, a false positive reading will have detrimental economic effects. To ascertain the ability of the system to identify pathogenic from non-pathogenic strains in a plant tissue sample, a study was conducted based on antibody specificity towards different strains of *E. coli*. Figure 6 shows results with *E. coli* O157:H7 EDL933 and K12 with particles conjugated with polyclonal antibodies specific to only *E. coli* O157:H7. The results show a relatively weak change in signal intensity for *E. coli* O157:H7 EDL933 (~1.5%), however, they reveal that there is no non-specific binding to
K12 within the biological matrix. The low change in signal intensity could be attributed to inferior antibodies with weak binding affinities. A similar study was also conducted with K12 polyclonal antibody conjugated particles showing minimal non-specific binding of O157:H7 EDL933 to the particles. However, the K12 polyclonal antibodies were not identified as being tested for cross-specificity. With the use of a properly designed antibody, the assay shows excellent specificity towards pathogenic strains of *E. coli* in the presence of plant cellular debris.
Fig A-6. Specificity results with *E. coli* O157:H7 EDL933 and K12 with O157:H7 antibody-conjugated microparticles showing no cross-reactivity of O157:H7 antibodies to K12 antigens.
4. Conclusions

This work demonstrates the development and optimization of a miniaturized, handheld, lab-on-a-chip device for the direct detection of foodborne pathogens from fresh produce, requiring little to no sample processing/enrichment steps. *Escherichia coli* O157:H7 EDL933 and K12 were detected in near-real-time from a plant tissue sample utilizing optimized Mie light scatter parameters with a latex particle immunoagglutination assay. The device exhibited good sensitivity and reproducibility with a detection limit of 10 CFU mL\(^{-1}\) and an assay time of <6 min, which includes sample preparation of raw lettuce. Microscope images of the solution samples containing prepared plant tissue show sufficient filtration for this type of assay with a simple and cost-effective KimWipes filter. Mie simulations were used to maximize light scatter intensity of agglutinated latex microparticles and to minimize light scatter intensity of the sample matrices. The optimal parameters (particle size \(d\), wavelength \(\lambda\), and scatter angle \(\theta\)) for the assay, involving *Escherichia coli*-contaminated iceberg lettuce, were determined using this approach and experimentally validated, introducing a powerful method for detecting foodborne pathogens in fresh produce and other potential sample matrices. The integration of a multi-channel microfluidic chip allowed for differential detection of the agglutinated particles in the presence of the antigen, while simultaneously excluding the light scatter from the plant cellular debris, decreasing assay time and improving robustness. Additionally, the incorporation of a microfluidic device improved sensitivity and linearity of the calibration curve.
Two sample preparation methods were studied and evaluated in a simulated field study based on the maximum percent intensity change over blank, as well as protocol complexity and assay time. Preparation of the plant tissue sample by grinding had a two-fold improvement in scatter intensity over washing. However, washing yielded a significant reduction in assay time, from ~5 min (grinding) versus ~1 min (washing). Specificity studies also demonstrated high specificity of *E. coli* O157:H7 EDL933 to O157:H7 antibody conjugated particles, with no cross-reactivity to K12. This suggests the adaptability of the system for use with a wide variety of pathogens, so long as the antibodies are properly designed and evaluated for antigen cross reactivity.

This device provides an easy-to-use, ultra-portable platform for use in the field, while maintaining the same sensitivity as its laboratory analog. The device and methodology can be adapted easily to any other food samples, including spinach, tomato, ground beef and eggs. The device will bring a huge impact on the safety of consumers and the agricultural industry by providing a cost-effective and rapid analysis tool on the front lines of preventing food-borne outbreaks.

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APPENDIX B:

VERY QUICK REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION FOR DETECTING 2009 H1N1 INFLUENZA A USING WIRE-GUIDE DROPLET MANIPULATIONS

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Abstract

Reverse transcription polymerase chain reaction (RT-PCR) is currently a gold standard in identifying influenza A virus, especially H1N1 flu. Typical RT-PCR assays take about 1–2 h for thermocycling, and there is a growing need to further speed up the thermocycling to less than 30 min. Additionally, the PCR assay system should be made portable as a point-of-care detection tool. There have been attempts to further speed up the PCR assays by reducing its volume. There have also been attempts to use droplet microfluidics technology to PCR, primarily to automate the PCR enrichment processes and take advantage of its small volume. In all these attempts, heating and cooling is made by conduction heat transfer. Rapid movements of droplets (immersed in oil) over three different temperature zones make very quick PCR possible, as heating/cooling will be made by convection heat transfer, whose heat transfer coefficients are much higher than that of conduction. We used our newly-invented method of wire-guide droplet manipulations towards very quick RT-PCR. Computational fluid dynamics (CFD) simulation of our system revealed that heating/cooling for each temperature change takes 1–4 s for a 10 μL droplet, as compared to >30 s in the other quick PCRs. Theoretically a 30-cycle process can take as short as 13 s × 30 cycles = 6 min 30 s. The entire system was made as a single instrument, with the components made by a milling machine and a rapid prototyping device. No additional equipment and external computers are required. With this newly developed system, 160 bp gene sequence was amplified from 2009 H1N1 influenza A (human origin). The 30-cycle process took as short as 6 min 50 s for a 10 μL droplet (with additional 4 min for reverse transcription). Its product was confirmed
by traditional gel electrophoresis, subsequent imaging as well as gene sequencing, which has been very difficult with the other stationary droplet/nanodrop approaches. The proposed system has a potential to become an extremely rapid, portable, point-of-care tool for detecting influenza A.

**Introduction**

Influenza A H1N1 2009 is a highly infectious disease that initiated a global pandemic in April 2009 [1]. As of 25th of April 2010, the WHO has reported at least 17,919 deaths of H1N1 2009 worldwide and reported confirmed laboratory cases in more than 214 countries and overseas territories and communities [2]. H1N1 2009 is defined as an acute febrile respiratory illness, in which the most common symptoms were fever, coughing, and sore throats [3]. In June 2009, the WHO raised the level of influenza alert from phase 5 to phase 6, indicating that a global pandemic was underway [4]. Currently, polymerase chain reaction (PCR) technology and viral cultures are the only CDC (US Centers for Disease Control and Prevention) recognized and FDA (US Food and Drug Administration) approved methods for the detection of influenza A subtype (H1N1) virus [5].

PCR is a well-established method for the detection and amplification of DNA and RNA with an unlimited sensitivity and unparalleled specificity. It is an essential tool in medical research and clinical medicine, used extensively for the detection of infectious disease organisms as well as detecting mutations in genes. Reverse transcription PCR
(RT-PCR) is a variant of PCR in which an RNA strand is first transcribed to complementary DNA (cDNA) using the enzyme reverse transcriptase, which is then amplified using traditional PCR.

PCR works by copying a specific DNA sequence of interest. The process of PCR amplification requires three important steps: denaturing, annealing, and extension. Each of these steps is performed at three specific temperatures. Commercial PCR systems generally incorporate a high powered Peltier module in order to heat and cool the chamber in which the samples reside. This is the major disadvantage of current systems: conduction is the least efficient method for heating and cooling when comparing the three modes of heat transfer (conduction, convection and radiation). Additionally, most commercial systems utilize a single heating block. The time required to reach the desired temperatures usually comprises a significant portion of the total PCR cycling time.

There has been significant interest towards the reduction of PCR cycling times, primarily through the implementation of microfluidic devices, in which small volumes of fluid travel through microchannels. Conventional methods of conductive heat transfer for heat cycling mean that it often takes a couple of minutes to complete a single cycle (and thus over an hour to finish typical 25–30 cycles of PCR). However, if this liquid is made to move over three different temperature areas within the microfluidic channel, the time required for heating and cooling may be significantly reduced, thus leading to a faster PCR assay. Additionally, the liquid volume that is needed to be heated and cooled is so small that the required heat transfer can be completed in a much shorter amount of time. The first such demonstration was made by Kopp et al. [6], where a single serpentine
microfluidic channel travels through three different temperature zones to achieve 20-cycle PCR. In this manner, less than 20 min or even less than 10 min 20-to-30-cycle PCR has become a possibility. This concept has been combined with microfluidic cell lysis [7], capillary electrophoresis to confirm PCR products [8,9], or fluorescence microscopy for real-time monitoring of PCR products [10]. Liquid flows through a microchannel continuously, or as discrete liquid plugs within a microchannel. Although this microchannel PCR is faster than conventional PCR assays, it has several limitations in terms of reconfigurability or adaptability; for example, the number of cycles is set by the microchannel layout (if microvalves are not employed); sample-dependent pre-processing steps such as cell lysis, DNA/RNA extraction and/or reverse transcription (RT) are rather difficult to be implemented into microchannel layout, as it is not reconfigurable. Additionally, these are closed systems, which mean that once liquid is inserted into the microchannel inlets, the process cannot be interrupted or augmented until it has completed its course or has been expelled from the system.

Droplet microfluidics (sometimes referred as “digital” microfluidics) are another area of significant interest for speeding up the PCR cycling process. These include electrowetting-based microfluidics [11], magnetofluidics [12], and wire-guide microfluidics [13]. The reconfigurability and reprogrammability feature of droplet microfluidics allows for the strong adaptability and expandability over the microfluidic microchannels. Specifically, these systems can be easily modified to be combined with cell lysis, DNA/RNA extraction and subsequent detection and sequencing of PCR products. Therefore, droplet microfluidic platforms are extremely adaptable to the variety
of available and newly emerging PCR protocols. Unfortunately, attempts to perform PCR in droplet microfluidics format have been relatively rare, and focused primarily on electrowetting-on-dielectrics (EWOD), which is comparatively more difficult to fabricate and operate [14]. Magnetofluidics does not involve complicated fabrication, as all you need is a superhydrophobic surface. However, proper heat transfer via a superhydrophobic surface can be inefficient, and the effect of magnetic fields and the presence of magnetic particles on PCR thermocycling and subsequent identification of its products remain to be investigated and resolved [15]. Wire-guide microfluidics is the simplest method for droplet manipulation and does not require the addition of particles that may interfere with the PCR thermocycling. Wire-guide microfluidics can be implemented on a variety of droplet environments, including superhydrophobic surfaces [13] and oil submersion, of which the latter is introduced in this paper.

This paper presents a novel method to significantly reduce the time for PCR thermocycling by employing wire-guide droplet microfluidics. A syringe needle tip is used to control the movement of a droplet submerged in silicone oil over three temperature regions. The 3-axis computer-numerically-controlled (CNC) system offers the benefit of reconfigurability and reprogrammability of droplet (digital) microfluidics. The submersed droplet continuously moves in silicone oil, which allows for convective heat transfer from the surrounding oil to the droplet. This method of very quick PCR will allow for the rapid analysis of viral RNA for the purpose of detecting and preventing the spread of infectious diseases before they become pandemic.

This work should not be confused with droplet or nanodrop PCR [16], where the
PCR assay is performed in a very small droplet to speed up the conduction-based heat transfer, which is not moving. This “stationary” droplet PCR has succeeded in speeding up the thermocycling significantly faster than other conventional methods. However, with this tiny volume (typically in nanolitre range), it becomes very difficult to confirm its products by traditional means such as gel electrophoresis, subsequent imaging and/or gene sequencing. Additionally, there is a concern in assay reproducibility and reliability, as aliquoting the low-concentration sample into nanolitre volume may occasionally result in no target molecule in the droplet. In wire-guide microfluidics, however, the volume of the droplet can be made large enough (in this case, 10 μL) to enable confirmation of its products (gel imaging and gene sequencing) as well as ensuring the assay reliability.

Materials and methods

Temperature controller and PCB heater

Temperature monitoring and heating of the silicone oil in the chambers were conducted on a single-sided printed circuit board (PCB) with exposed copper traces. Positive photoresist-coated copper-clad boards, resist etching solution, and ferric chloride solution were obtained from Marlin P. Jones & Assoc. (MPJA; Lake Park, FL, USA). Photoresist template was created using Adobe Photoshop CS4 (San Jose, CA, USA). Trace width (w, in mm) for the heating element was calculated using equations from IPC-2221, generic standard on printed circuit boards [17]:
\[ w = \frac{[I/k(\Delta T)^b]^{1/c}}{h \times 1.378} \]

where I is current in amperes (A), \( \Delta T \) is temperature rise in °C, h is thickness as represented by how many ounces (oz) of copper to cover one square foot of circuit board, the constant 1.378 has the unit of mils oz\(^{-1} \), where mils is 1/1000 of an inch and k, b and c are constants resulting from curve fitting to the IPC-2221 curves (for IPC-2221 external layers: k = 0.048, b = 0.44 and c = 0.725).

The photoresist template design was printed onto a clear transparency using a 2400 dpi laser printer. The coated copper-clad board was then exposed to an incandescent 100 W bulb and dissolved in photoresist developer (MPJA) at 46 °C for 6 min. The board was then transferred to ferric chloride (0.240 g mL\(^{-1} \) bath (MPJA) at 55 °C for 10 min. Remaining photoresist was removed using pure anhydrous acetone and drilled using a 0.025 inch (0.635 mm) drill bit.

Temperature monitoring for the PCR chambers was conducted using DS18B20 1-Wire temperature sensors, an Arduino Duemilanove microcontroller, and a serial-enabled liquid crystal display (LCD) obtained from Sparkfun Electronics (Boulder, CO, USA). The PCR chamber was created through rapid prototyping from a Dimension 1200ES (Stratasys; Eden Prairie, MN, USA) using a high temperature acrylonitrile butadiene styrene (ABS) polymer. Silicone oil (catalog number S159-500, Fisher Scientific; Pittsburgh, PA, USA) was used to fill the PCR chambers, which were heated with 2 A benchtop power supplies (MPJA). Solid-state relays, also acquired from MPJA, were used to switch power to the heating elements and were controlled by the Arduino through
a transistor circuit using common NPN bipolar junction transistors (BJT; 2N2222), diodes (1N4148), and 1 kΩ resistors. A proportional-integral-derivative (PID) algorithm was implemented to control the heating of the silicone oil. This setup allowed for precise temperature control to within ±0.25 °C of the target temperatures.

**Computer-controlled wire-guided manipulator**

The computer-controlled wire-guided apparatus was fabricated and assembled for manipulating the PCR solution droplets in the silicone oil (Fig. 1). The base was constructed of aluminum onto which Thomson 1/2 inch (12.7 mm) linear rails and twin ball bearing pillow blocks (Thomson; Radford, VA, USA) were mounted, allowing precise movement along the X, Y, and Z axes. Computer-controlled linear movement of each axis was performed with Nema 17 stepper motors obtained from Automation Direct (Cumming, GA, USA), connected to 1/2–10 inch (12.7–10 mm) Delrin® lead screw assemblies obtained from Precision-CNC-Router (Melbourne, FL, USA). Stepping of each motor was performed with an Arduino Mega microcontroller and Easydriver stepper motor controllers utilizing A3967 chips (Sparkfun Electronics). The stepper motors were powered by a 0–30 V, 3 A bench-top power supply (MPJA). All of the mechanical components of the system were connected by ABS structures formed from rapid prototyping (Dimension 1200ES).
Fig B-1. Droplet PCR Apparatus.
For droplet insertion and extraction, disposable syringes were used with Luer-Lok blunt-ended needles (OK International; Garden Grove, CA, USA). 1 mL disposable plastic syringes with Luer-Lok tips were obtained from Fischer Scientific, and fitted into a quick-release plastic holder attached to the Z-axis of the apparatus. Precise droplet insertion/extraction from a 14 gauge (1.628 mm inner diameter) blunt-ended needle was accomplished with a 5 V mini linear step motor (MPJA) attached to the syringe plunger.

**Primer design**

RNA polymerase subunit (PA gene) nucleotide sequences of H1N1 influenza A virus with human origin (GB CY061288.1 and CY061152.1), swine origin (GB AB514935.1), and avian origin (GB GU168243.1) were pooled from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). These RNA sequences were then subjected to multiple alignment analysis using ClustalX [18] to search for conserved regions among 3 different species such that consensus primers could be designed. Selected primers were then analyzed for appropriate melting temperature as well as any possible hairpin or self-dimerization by using OligoAnalyzer 3.1 (IDT Corporation, Coralville, IA, USA). These primers are PAh1n1_583F (5’-CAGTCCGAAAGAGGGAAG-3’) and PAh1n1_745R (5’-GAAAGCTTGCCCTCAATGCGAAG-3’); resulting in a product of about 160 base pairs (bp).
RNA extraction

NATtrol influenza A H1N1 virus external run control (catalog number NATFLUAH1N1-ERCM) from ZeptoMetrix Corporation (Buffalo, NY, USA) in medium was used as a target. This virus sample is 2009 H1N1 flu with human origin, purified/modified by the manufacturer. Its RNA was extracted using the E.Z.N.A. viral RNA kit (catalog number R6874-01, Promega Bio-tek; Madison, WI, USA) through the spin column protocol. Briefly, 500 μL of QVL lysis buffer was mixed with 150 μL cell media, then precipitated with absolute ethanol. The mixture was then passed through a spin column, and then cleaned with provided RWB buffer from the kit. Finally, RNA was eluted with elution buffer. RNA was then subjected to one-step RT-PCR.

RT-PCR

Reverse transcription PCR (RT-PCR) was first performed under a conventional PCR machine to ensure proper design of primers and proper extraction of RNA. RT-PCR was run according to what was recommended by the AccessQuick™ RT-PCR system (catalog number A1701, Promega Bio-Tek, Madison, WI, USA). Once positive results were verified, we employed our wire-guide droplet PCR system to detect the presence of influenza A H1N1 virus.

AccessQuick™ RT-PCR system kit was used for the wire-guide droplet PCR reaction. 10 μL droplets were used in 30 PCR cycles. After initial extraction of the PCR solution into the syringe, the system inserted the droplet into the 37 °C chamber for 4 min for reverse transcription. After formation of the cDNA template, the chamber would heat
to 54 °C degrees during an initial 30 s denaturing step in the 95 °C chamber. The droplet began the cycles in the 95 °C chamber for denaturing, 54 °C chamber for annealing of primers, and then 72 °C chamber for extension of the products. The final step consisted of final annealing for 20 s.

During each of these stages, the system would move the inserted droplet in a circular motion to aid in convective heat transfer. The chambers were connected with narrow bridges to allow the syringe needle to stay submerged in the silicone oil, preventing the possibility of evaporation or droplet loss due to the effects of surface tension from inserting and removing the needle from the oil.

**PCR analysis**

PCR product was determined through gel electrophoresis and fluorescent imaging of gel. PCR products were applied into 2% low melting agarose gel (catalog number E-3126-25, ISC BioExpress; Kaysville, UT, USA), using a Fisher Scientific (catalog number FB200) power supply at 80 V and 1 A for 60 min in a 1× tris-acetate-EDTA (TAE) buffer solution (catalog number BP13324, Fischer Scientific). The gel was soaked in about 1 μg mL−1 ethidium bromide for ca. 6 min and imaged using a Gel Doc 1000 imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

**DNA preparation and sequencing**

PCR products from conventional and wire-guide droplet system were cleaned with QIA quick PCR purification kit (catalog number 28106, Qiagen, Valencia, CA,
USA) for sequencing to ensure proper detection of influenza A H1N1 virus. Amplified PCR products were sequenced using the Applied Biosystems 3730 DNA analyzer (Carlsbad, CA, USA). Sequences are then viewed and corrected using Chromos Lite V 2.01 (Technelysium Pty Ltd, Southport, Queensland, Australia) and finally blasted against GenBank database.

**CFD modeling**

The commercial software (FLUENT®6.3 and GAMBIT®1.3; FLUENT Inc., Lebanon, NH, USA) was used to build mathematical grids for our PCR system using the finite volume method (FVM) [19]. A 3-D model was used in this study rather than a 2-D model as our system was not symmetric in shape.

The grid size used in the simulation consisted of 285,376 cells, 876,984 faces and 305,725 nodes. The shape of all meshes was hexagonal to decrease the number of meshes. First-order schemes were used as it is known to provide better convergence of calculations than the second-order, although it provides less accurate results due to the increased error in numerical discretization [20]. A steady-state solution was used because both the outdoor temperature and the amount of supplied heat to each chamber were fixed. To simulate heat transfer in the system, the energy equation option was chosen in FLUENT [19]. To consider the convective heat flow inside the chamber by heating, the viscous heating and buoyancy heating options were chosen. Since the Reynolds number of the flow around the μL-sized droplet was much smaller than 5000, the turbulence model was not used in this simulation. Since the droplet is relatively small and do not
mix with oil, and its movement is forced by external wire-guide manipulation not by surface tension, it can be treated as a solid sphere and thus the volume of the fluid model was not used. Outdoor temperature was set to the measured room temperature, 25 °C.

Results and discussion

Multi-chamber design

Several different designs of multi-chambers were considered, initially with three chambers connected in-line. This in-line chamber system worked fine for PCR thermocycling, and corresponding CFD simulation revealed that each chamber could maintain its designated temperature even though they are connected through “tunnels”. However, this design was inefficient because the droplet travelled through the middle chamber twice in one cycle, and was dismissed.

The next design was a rectangular/circular one as shown in Fig. 1. Corresponding CFD simulation (Fig. 2) shows that steady state for the three necessary PCR cycling temperatures, 95 °C, 54 °C and 72 °C are achievable in a connected design, when the actual temperature of the oil is set to 105 °C (10 °C higher), 55 °C (1 °C higher) and 80 °C (8 °C higher), respectively. Temperatures near the connecting tunnels are not very different from the other parts of the chamber, indicating the effect of heat transfer through the tunnels can be disregarded.
Fig B-2. CFD simulation of three-chambered PCR cycler. FLUENT simulation showing steady state temperatures of silicone oil heated by PCB modules in ABS polymer chamber.
As the temperature sensors were placed near the outer wall (as shown in Fig. 1 and 2), significant heat loss to the ambient air (25 °C) is expected through the walls of the chambers, especially at high temperatures (105 °C and 80 °C), which led to a lower temperature reading by about 10 °C. Therefore, the set temperatures of a PID controller were set to 95 °C, 54 °C and 72 °C (the actual temperatures within droplets), which would heat up the nearby oil to 105 °C, 55 °C and 80 °C, respectively.

While the temperature variation parallel to the heating element did not vary more than ±1.0 °C, except near the walls, the depth of the thermocouple resulted in a more significant temperature variation of ±2.0 °C. Thus, the depth of the thermocouple placement for the temperature controller in relation to the depth of the droplet was considered to ensure proper heating of the droplet PCR solution. It is important to note that the mixing motion during the heat cycling process was not included in the CFD simulation of the silicone oil. Most likely this mixing assisted in the temperature distribution.

Convective heat transfer to a droplet

In addition to the chamber design, CFD modeling was also performed on the droplet that travels through three chambers (Fig. 3). The parameters of the simulation consisted of a 10 μL droplet traveling at a rate of 2 cm s⁻¹ through uniformly heated silicone oil. The simulation reveals the effect of convective heat transfer on the PCR droplet, at the surface and throughout its volume.
**Fig B-3.** CFD simulation of a 10 μL droplet in silicone oil. Snapshot of FLUENT simulation showing the temperature gradient of droplet traveling through silicone oil. Left top: 72 °C droplet entered into 95 °C chamber (actual \( T = 105 \) °C). Right top: The fully heated droplet at 92 °C is now entering into 54 °C chamber (actual \( T = 55 \) °C). Bottom: The droplet is fully cooled down at 62 °C.
The simulation from the convective heat transfer to a droplet were datalogged and used to construct a temperature versus time graph (Fig. 4). This graph shows the temperature of the droplet at the center, surface, and overall volume. Heating of the droplet from 72 °C to 95 °C takes approximately 2 s (in the 95 °C chamber; actual temperature is 105 °C), cooling from 95 °C to 62 °C takes approximately 4 s (in the 54 °C chamber; actual temperature is 55 °C), and heating from 62 °C to 72 °C takes approximately 1 s (in the 74 °C chamber; actual temperature is 80 °C). Therefore, the overall time spent heating and cooling the droplet for one PCR cycle is theoretically 7 s. However, because particles will primarily accumulate at the oil to water interface, these times may be significantly reduced. If the droplet is additionally maintained at the correct temperatures for 2 s per chamber, then the overall PCR cycle takes only 13 s. Thus, to perform 30 cycles would only require 6 min 30 s with a 10 μL droplet. The droplet size may be decreased to further speed up the heat cycling process. There has been much focus to reduce the size of the droplet, even to the nanoliter scale [16]. However, 10 μL droplets were chosen for these experiments to closely match the volumes used in typical PCR assays, as well as to allow further analysis to be conducted, such as gel electrophoresis, and DNA sequencing. Without these post-analysis procedures, the product cannot be definitively determined, possibly resulting in misdiagnosis. The CDC requires that positive results for H1N1 2009 using their real-time RT-PCR protocol must be sent to them for further analysis [5].
Fig B-4. CFD simulation results for a 10 μL droplet traveling through silicone oil. Each plot represents the surface-averaged (“surface”) and volume-average (“volume”) temperature s as well as at the droplet center. Graph indicates theoretical PCR cycle can be conducted in 13 s, and 30 cycles in 6 min 30 s.
Droplet insertion and retraction

With this new design, the droplet stayed “hanging” (i.e. pendant drop) throughout the entire thermocycling. This configuration did not create any problems within each chamber, as the droplet only needed to stay in the chamber for 4–5 s, thus did not require fast transportation. Within each chamber, the speed of the wire-guide movements were set to 2 cm s\(^{-1}\), the same as the CFD modeling. When the droplet moves from one chamber to another, however, the transport should be made as quick as possible to speed up the entire process. (This transportation time was not taken into consideration in the above CFD simulation.) This high speed transition from chamber to chamber, however, resulted in the droplet falling off the needle.

To prevent the droplet from falling off the needle during this transport, the system would retract the droplet from the oil inside the needle prior to movement, and then re-insert after the chamber-to-chamber transition completed. This protocol significantly reduced the time needed for thermocycling, which will be reported later in this section. Extracting additional oil after the droplet to provide a barrier from the air was considered and tested during initial chamber designs, however this also created intermittent droplet loss and was dismissed.

The linear step motor for the syringe plunger was calibrated to insert and extract precisely 10 μL of PCR solution into each chamber. The droplet was completely evacuated from the syringe needle while thermocycling in each chamber. Conductive heat loss or gain from the stainless steel syringe needle towards the droplet was considered, but because the droplet was a complete pendant drop, the contact area of the
droplet with the syringe needle in relation to the contact area of the droplet to the
surrounding silicone oil was significantly smaller. Additionally, because the droplet and
syringe needle moved together and were in direct contact, the method of heat transfer
between the two was conduction, whereas the heat transfer between the droplet and the
silicone oil was primarily convection, which provides a much higher rate of heat transfer.
Thus the effects of the syringe needle on the thermocycling temperatures were dismissed
in the CFD analysis and experimental protocol.

Gel electrophoresis and DNA sequencing

Primers were specifically designed to work across human, swine, and avian
strains of influenza A H1N1. Fig. 5 shows a gel image of about 160 bp products of
influenza A H1N1 (human origin), in which 30 cycles were successfully performed in 6
min 50 s (Fig. 5, lane 3), with an additional 4 min reverse transcription step. This
minimal reverse transcription time was determined through series of PCR experiments.
Additionally, 30 cycles in 8 min 10 s (Fig. 5, lane 2) and in 10 min (Fig. 5, lane 1) are
also shown as well as a negative control experiment (Fig. 5, lane 4) in which the PCR
cycling was performed under the same conditions. Results show a decrease in PCR
amplification from the 8 min 10 s heat cycling to 6 min 50 s. However, the signal is still
relatively strong and can be considered a positive result from the negative control.
Obviously the longer thermocycling correlates to the brighter band suggesting that time is
essential for Taq polymerase to amplify DNA. If there were more DNA in the target
sample or simply prolonging the reverse transcription time, 20 PCR cycles would be
sufficient, which would require only $13 \text{ s} \times 20 \text{ cycles} = 4 \text{ min 20 s}$. In addition, if a smaller droplet is used, e.g. 1 μL, the time for each cycle can be further reduced down a few second scale, possibly leading to <3 min 20 PCR cycles.
Fig B-5. Wire-guide droplet RT-PCR results. The shortest 30 cycles were completed in 6 min 50 s with 4 min of reverse transcription. L is the 1-kb plus ladder, lanes 1–3 are the 2009 influenza A H1N1 viruses, and lane 4 is the negative control.
Although the PCR products are at the right band size (Fig. 5), further verification is required to validate the content of the band. PCR products were cleaned and submitted for sequencing. The genomic content of the band is then blasted against the nucleotide collection database on The National Center for Biotechnology Information. The query sequence is 99% to 100% homologous to various samples of 2009 H1N1 influenza A virus with an E-value of $3 \times 10^{-50}$ (Table 1) suggesting that our wire-guided droplet manipulation PCR system is working and able to detect virus in 10 min and maybe less.
Table 1. DNA sequencing results. DNA sequencing confirms that the product from wire-guide droplet RT-PCR is 99% to 100% homologous to influenza A H1N1 virus.

<table>
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<tr>
<th>Accession</th>
<th>Influenza A virus description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query coverage</th>
<th>E-value</th>
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<td>219</td>
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<td>100%</td>
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<tr>
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<td>219</td>
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<td>$3.00 \times 10^{-54}$</td>
<td>100%</td>
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<tr>
<td>CY044906.1</td>
<td>A/New York/3611/2009(H1N1)</td>
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<td>219</td>
<td>95%</td>
<td>$3.00 \times 10^{-54}$</td>
<td>100%</td>
</tr>
<tr>
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<td>215</td>
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<td>$2.00 \times 10^{-52}$</td>
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Conclusion

This paper has successfully demonstrated the wire-guide droplet RT-PCR cycling of 2009 H1N1 influenza A in 10 min 50 s. Specifically, 30 cycles of heat cycling were performed in 6 min 50 s, demonstrating the effectiveness of utilizing wire-guide droplet manipulation in conjunction with convective heat transfer. With further optimization of the system, this heat cycling time is expected to be reduced to less than 3–4 min. Simulation studies were employed to develop a multi-chambered heat cycler and to establish the effectiveness of wire-guide droplet PCR with standard PCR assay solution volumes. DNA sequencing confirmed the product created in the wire-guide droplet PCR system. The computer controlled wire-guided manipulator was constructed for general use in a variety of droplet microfluidics applications. However, when considering commercialization potential for the methods introduced in this paper, the system can be easily miniaturized and simplified. For example, the entire 3-chamber design with embedded temperature sensors can be constructed on a single circuit board incorporating a simple 2-dimensional table for moving the syringe. The entire system can be made relatively small and has the potential for portability due to the low power consumption of the PCB heaters. Alternatively, for high-throughput applications, an array of syringe needles can be used to run multiple PCR assays. Implementing a rotating circular chamber design with multiple heating chambers would allow for the continuous and uninterrupted heat cycling of many solution samples with different start and end times simultaneously. With further optimization, the wire-guide droplet PCR will prove to be an effective method for significantly accelerating PCR assays with the advantages of
reconfigurability and reprogrammability necessary for the variety of infectious disease protocols, while still allowing for post analysis procedures to be performed.

References


wineH1Assay-2009_20090430.pdf.


APPENDIX C:

DROPLET CENTRIFUGATION, DNA EXTRACTION, AND VQ-PCR

THERMOCYCLING USING REPROGRAMMABLE WIRE-GUIDED

MANIPULATIONS ON A SINGLE SURFACE CHIP

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Abstract

A wire-guided CNC apparatus was used to perform droplet centrifugation, DNA extraction, and VQ-PCR thermocycling on a single superhydrophobic surface measuring 25 mm by 55 mm and a multi-chambered PCB heater. This methodology exhibited no limitations on the complexity and configuration of procedures that it can perform, making it extremely versatile and far-reaching in its applications. The only modification required for adding or implementing changes for a new protocol is through simple pre-defined programming, which can be easily accomplished by the operator. Because of this characteristic, the user has the ability to start and stop the assay/s at any time, manually control the droplet system with a computer, make adjustments to the protocol on-the-fly, and resume where it left off. The highly adaptive and flexible system was used to execute easily pre-programmed droplet movements and manipulations for the rapid detection of Escherichia coli from PCR detection. Serial dilutions were performed to simulate a diluted field sample with a high level of accuracy. Centrifugation of the diluted sample containing E. coli with a 10 μL droplet spinning around a 22-gauge blunt ended stainless steel syringe needle at 2300 rotations per minute demonstrated a novel approach to sample pre-treatment. This technique resulted in a mean increase in the concentration of E. coli contained within the sample droplet by more than 3 fold. Additionally, computational fluid dynamics (CFD) simulations were used to validate the centrifugation of E. coli as bacterial particles and to track their migration over time, showing that effective centrifugation of particles occurred within 3 min. Furthermore, the extraction of DNA from the sample droplet containing E. coli was also performed on the
same superhydrophobic surface as the previous 2 steps, requiring less than 10 min. This approach utilized the disposable pipette tip to cleverly extract the precipitated DNA from the sample droplet residing on the superhydrophobic surface. Following extraction, the genetic material was amplified using wire-guided droplet PCR thermocycling, which incorporates pendant droplets hanging from a syringe needle and moving through separately heated silicon oil chambers, utilizing forced convective heat transfer over a large surface area, to successfully complete 30 cycles of Peptidase D (a long 1500 bp sequence) in 10 min. The results of these sequentially executed processes were analyzed using gel electrophoresis, in which the droplet centrifugation process was determined to greatly improve the positive band intensity over the non-centrifuged sample. Thus, this work demonstrates the adaptability of the system to replace many common laboratory tasks on a single platform (through re-programmability), in rapid succession (using droplets), and with a high level of accuracy and automation.

**Introduction**

Manipulating small droplets has been the focus of much attention in recent years. The use of small droplets allows for significantly lower reaction volumes and decreased assay times for many common laboratory procedures. Furthermore, it has been established that complex and reconfigurable bioanalysis and biorecognition is only possible with droplets [1]. The two primary modes of droplet manipulations are: (1) to use discrete liquid plugs in pre-defined microchannels [2,3], or (2) to use droplets sitting on an open, flat surface [4,5]. Although the former (liquid-plug type) has been popular in
digital microfluidics, the latter (open-surface type) has more potential as its reaction protocol can be reprogrammed to whatever combination one can conceive. Furthermore, while complex and reconfigurable algorithms can be implemented into pre-defined microchannel systems through the use of elastomeric microvalves [6,7], these microfluidic large-scale integrations (mLSI) systems typically require an array of external pneumatic solenoid valves, access to compressed air or a portable air compressor, and a control system with computer in order to choreograph precise, controlled droplet manipulations, requiring the addition of more components based on the complexity of the pre-defined microchannel layout.

The manipulation of droplets of an open, flat surface has been demonstrated most notably with magnetofluidics. In magnetofluidics, droplets containing paramagnetic particles move over a superhydrophobic surface under the influence of an external magnetic field [8]. However, paramagnetic particles need to be designed as not to interfere with biological reactions, a capability that has not yet been confirmed. Another common technique is electrowetting-on-dielectrics (EWOD), which allows for precise droplet movement, splitting, and merging [9]. However, this method is comparatively more difficult to fabricate and operate, and has limitations with diffusional mixing and contamination from increased wetting on the surface.

Wire-guided droplet manipulations offer a simpler method for manipulating droplets on an open surface. Although a clean, metal wire was initially used to guide a droplet on a superhydrophobic surface (deriving the term *wire-guided*) [10], the wire can be replaced with a variety of materials and sizes to modulate the force of the droplet to
the wire \( W_o \), making the system highly adaptable to a wide range of droplet volumes and properties. Furthermore, the use of a syringe needle or disposable pipette tips can be used to perform precise droplet splitting and mixing with an attached vibration motor to the linear actuated syringe plunger. Using the wire-guided system to form pendant droplets on the ends of syringe needles has also been demonstrated for rapid PCR thermocycling, by using forced convective heat transfer through movement of the droplet in submerged silicon oil (VQ-PCR) [11].

This work demonstrates the use of wire-guided droplet manipulations to perform a series of laboratory tasks on either a single superhydrophobic surface measuring 25 mm x 55 mm or a multi-chambered PCB heater. The first step includes the execution of a series of 10-fold serial dilutions of a cultured *Escherichia coli* sample. Following dilution, the sample is then concentrated through a novel method of centrifugation by spinning the droplet around a metal syringe needle at a high rate by using a vibration motor under pulse width modulated (PWM) control. During centrifugation, the diluted sample at the center of the axis of rotation is drawn into the syringe, leaving a concentrated sample for further analysis. The sample then undergoes rapid DNA extraction, in which wire-guiding offers a clever approach to extracting and separating the precipitated genetic material. And afterwards, a genetic sequence is amplified through VQ-PCR thermocycling, and the results confirmed by gel electrophoresis. In summary, we are presenting a reprogrammable, reusable, cheap, and reliable wire-guided droplet manipulation system that can concentrate field samples, extract DNA, and
perform VQ-PCR thermocycling in microliter scale capable for subsequent DNA analysis.

Materials and Methods

Computer-controlled wire-guided manipulator

The computer-controlled wire-guided apparatus was fabricated and assembled for manipulating droplets on both the superhydrophobic surface (for serial dilution and DNA extraction) and in the silicon oil-filled PCR chambers for thermocycling. The base was constructed of aluminum onto which Thomson 1/2 inch (12.7 mm) linear rails and twin ball bearing pillow blocks (Thomson Industries, Radford, VA, USA) were mounted, allowing precise movement along the X, Y, and Z axis. Computer-controlled linear movement of each axis was performed with Nema 17 stepper motors obtained from Automation Direct (Cumming, GA, USA), connected to 1/2-10 inch (12.7-10 mm) Delrin® lead screw assemblies obtained from Precision-CNC-Router (Melbourne, FL, USA). Stepping of each motor was performed with an Arduino Mega microcontroller and Easydriver stepper motor controllers utilizing A3967 chips (Sparkfun Electronics, Boulder, CO, USA). The stepper motors were powered by a 0-30 V, 3 A bench-top power supply (MPJA). All of the mechanical components of the system were designed using Solidworks 2010 (Solidworks Corp., Concord, MA, USA) and then stereolithographically printed using a Dimension 1200ES 3D printer (Stratasys, Inc., Eden Prairie, MN, USA) in an acrylonitrile butadiene styrene (ABS) polymer.
For droplet insertion and extraction, disposable syringes were used with disposable pipet tips or modified Luer-Lok blunt-ended needles. 1 mL disposable plastic syringes with Luer-Lok tips were obtained from Fischer Scientific, and fitted into a quick-release plastic holder attached to the Z-axis of the apparatus. Precise droplet insertion, extraction, and manipulation was performed with either Beckman Span-8 black pipet tips (Beckman Coulter, Brea, CA, USA) or 14 gauge (1.628 mm inner diameter) blunt-ended needle (OK International; Garden Grove, CA, USA) and automated with a 5 V mini linear step motor (Marlin P Jones & Assoc., Inc., Lake Park, FL, USA) attached to the syringe plunger.

Periodic calibration of the system was performed using a series of infrared photo interrupters (Sparkfun Electronics) integrated into the X and Y planes. Black pipet tips were used as an opaque medium to trigger the photo interrupters. Calibration of the Z plane occurred during removal of the pipet tip.

Wire-guided droplet serial dilution

Serial dilution of a concentrated sample of *E. coli* culture was performed to demonstrate the application of the wire-guided droplet manipulation apparatus towards rapid and automated droplet splitting, merging, and mixing on a superhydrophobic surface. 20 μL droplets of PBS were deposited onto the surface. Following diluent deposition, 2 μL of *E. coli* culture were added to the first droplet and mixed using the attached vibration motor. The pipet was then discarded, during which the system simultaneously recalibrated itself to ensure device precision, and a new one obtained
automatically from the replaceable pipet box. 2 μL of the first dilution was then extracted and deposited into the second droplet and mixed. This process was continued 4 times during the demonstration, creating a $10^{-4}$ dilution from the original *E. coli* culture. The precision of the system to perform serial dilutions in droplet platform were evaluated using standard plate counting methods for *E. coli* and forward light scattering assay for microparticles (Bangs Laboratories Inc., Fishers, IN, USA).

**Wire-guided droplet centrifugation**

Concentration of the diluted sample was accomplished through the use of droplet centrifugation. The apparatus consisted of a 22-gauge blunt-ended syringe needle (OK International) attached to a vibration motor (Sparkfun Electronics) and controlled using pulse-width-modulation via the Arduino Mega microcontroller. The droplet was centrifuged for 5 min at which point the solution sample from the center of the droplet was extracted, leaving a concentrated sample. Standard colony plate counting methods were used to determine the concentration of *E. coli* in the sample before and after centrifugation. Furthermore, to determine the effect of evaporation towards sample concentration (not centrifugation), an extracted DNA sample solution was used and 3 case studies were compared by measuring the DNA concentration with a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA): 1) stock concentration, 2) concentration
after leaving droplet stationary on superhydrophobic surface for 5 min, 3) concentration after spinning droplet for 5 min using wire-guided droplet centrifugation.

A Casio Exilim EX-FH100 (Casio America, Inc., Dover, NJ, USA) was used to acquire high speed video capture of the droplet rotation about the syringe needle at 480 frames per second.

*Wire-guided droplet DNA extraction*

Following concentration of the *E. coli* sample, rapid DNA extraction was performed in droplet format. Nuclei lysis solution was added, mixed, and heated in the 80°C PCR thermocycler chamber for 5 min. The lysed sample solution was redeposited onto the superhydrophobic surface and allowed to cool. 70% isopropanol solution was added to precipitate the DNA. After an initial mixing, the pipet tip rotated slowly to allow precipitated DNA to adhere to the tip. The tip was then removed and air dried for 1 min, washed in 70% ethanol, and air dried again for 1 min. Finally, the tip was mixed in DNA rehydration solution for 3 mins.

*Wire-guided droplet VQ-PCR thermocycling*

PCR was first performed under a conventional PCR machine to ensure proper design of primers and to serve as positive controls. PCR was run according to what was recommended by The GoTaq Green Master Mix (catalog number M7122, Promega Bio-Tek, Madison, WI, USA). Once positive results were verified, we employed our wire-guided droplet PCR system to detect the presence of *E. coli*. 
AccessQuick® PCR system kit was used for the wire-guided droplet PCR reaction. A cocktail mixture which includes 4 μL of GoTaq Green, 1 μL of each 10 μM forward and reverse primers, 1 μL of DNA sample, and 3 μL of autoclaved water, for a total of 10 μL droplets, were used to run 30 PCR cycles. Following DNA extraction, the system automatically secured a 14-gauge blunt-ended syringe needle (modified for friction fit) and extracted the PCR-ready solution (DNA solution + TAQ solution + Primers). The droplet began the cycles in the 105°C chamber for denaturing, 55°C chamber for annealing of primers, and then 80°C chamber for extension of the products. The final step consisted of final annealing for 20 s. During each of these stages, the system would move the inserted droplet in a circular motion to aid in forced convective heat transfer. The chambers were connected with narrow bridges to allow the syringe needle to stay submerged in the silicone oil, preventing the possibility of evaporation or droplet loss due to the effects of surface tension from inserting and removing the needle from the oil.

Primer design

Nucleotide sequences of aminoacyl-histidine dipeptidase (pepD) and 16S ribosomal RNA from *Escherichia coli K12* were pooled from GenBank [12]. These sequences were then subjected to multiple alignment analysis using ClustalX [13] to search for a conserved region so that consensus primers could be designed. Selected primers were then analyzed for appropriate melting temperature as well as any possible hairpin or self-dimerization by using OligoAnalyzer 3.1 (IDT Corporation, Coralville, IA,
USA). Primers for pepD are 4F (5’- GGG AAT TCG TCG ACG TGT CTG AAC TGT CTC AAT T-3’) and 4R (5’- GAG CCG AAG CTT TTA CTT CGC CGG AAT TTC TT-3’) which results in about 1500 base pairs.

Conventional DNA extraction of Escherichia coli

DNA was extracted from E. coli that was cultured in Luria-Bertani (LB) media (catalog number MBPE-1050, Growcells; Irvine, CA, USA) overnight at room temperature to prevent cells from reaching death phase using Wizard Genomic DNA purification kit (catalog number A1120, Promega Bio-tek; Madison, WI, USA). Basically, cells were collected either by conventional centrifugation or centrifugation from the droplet manipulation system; and then subjected to the cell lysis solution to lyse cells and RNase solution to deactivate nucleases from breaking DNA and RNA. After an incubation period, DNA was precipitated with isopropanol and collected using centrifugation or the droplet manipulator. Samples were then washed with 70% ethanol and allowed to dry at room temperature for 15 minutes before rehydrate with rehydration solution. DNA was then subjected to conventional PCR for positive controls and VQ-PCR using droplet manipulations.

PCR analysis

PCR product was determined through gel electrophoresis and fluorescent imaging of gel. PCR products were applied into 2% low melting agarose gel (catalog number E-3126-25, ISC BioExpress; Kaysville, UT, USA), using a Fisher Scientific (catalog
number FB200) power supply at 80 V and 1 A for 60 min in a 1x tris-acetate-EDTA (TAE) buffer solution (catalog number BP13324, Fischer Scientific). The gel was soaked in about 1 μg mL⁻¹ ethidium bromide for ca. 6 min and imaged using a Gel Doc 1000 imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

**CFD Modeling**

In order to track *E. coli* particles inside the droplet and to estimate the appropriate time for droplet centrifugation prior to DNA extraction, the commercial software (FLUENT 6.3 and GAMBIT 1.3; FLUENT Inc., Lebanon, NH, USA) was used to build mathematical grids for our droplet PCR system using the finite volume method (FVM) [14]. A 2-D model was used in this study rather than a 3-D model as our system was symmetric in shape and it was assumed that the *E. coli* particles were generated and migratory at the face parallel to the direction of gravity due to centrifugation and perpendicular to the axis of rotation.

The grid size used in the simulation consisted of 70642 cells, 106277 faces and 35636 nodes. The shape of all meshes was triangular. First-order schemes were used as it is known to provide better convergence of calculations than the second-order, although it provides less accurate results due to the increased error in numerical discretization [15]. An unsteady-state solution was used in order to track the movement of the *E. coli* particles by time. Since the force of gravity from centrifugation was the dominant force of interest inside the droplet, neither the energy equation nor turbulent model options were chosen in FLUENT. Instead, a discrete phase model was used to track the *E. coli*
particles. It was assumed that size of the droplet was 4 mm in diameter. The boundary was divided evenly into 4 zones by distance from the bottom of the droplet (zone 1; 2-3mm from the bottom, zone 2; 1-2mm from the bottom, zone 3; 0-1mm from the bottom). Different gravitational forces were applied to each zone (zone 1; 58.86m/s², zone 2; 107.91m/s², zone 3; 215.82m/s²) based on the distance of that zone from the axis of rotation. For the migratory simulation, it was assumed that the *E. coli* particles started from the top of zone 1 and migrated towards the bottom. Five cases of simulations were conducted by changing the size of *E. coli* particle (diameter of 1, 2, 5, 7, 10μm) to simulate colony formation. The *E. coli* particles were assumed to be a solid sphere with density of 1.2 g cm⁻³. The time required for each *E. coli* colony to migrate to the bottom of the droplet was estimated for each case.

**Results and Discussion**

*Wire-guided serial dilution and centrifugation*

To demonstrate precise sample handling in a reconfigurable manner, serial dilutions were performed to simulate a diluted field sample. This diluted sample was then re-concentrated using wire-guided droplet centrifugation and the genetic material extracted using the same droplet manipulation assay surface as the serial dilutions with only a different pre-programmed algorithm, demonstrating the ease of reconfigurability of the system. Afterwards, the DNA was amplified using VQ-PCR. Figure 1 shows the entire apparatus to perform all the aforementioned protocols. The modular base allows for easy repositioning and addition of further components to the system. While only a
single confluent assay was demonstrated, the ability to run multiple assays simultaneously, utilizing the necessary delays between steps in the protocols, could easily be implemented.
Fig C-1. Wire-guided droplet manipulator apparatus. Modular design includes PCR chamber for VQ-PCR thermocycling and superhydrophobic surface for serial dilution, centrifugation, and DNA extraction.
Figure 2 illustrates a representative algorithm of the serial dilution protocol, in which an initial 20 μL droplet of PBS was mixed with 2 μL of *E. coli* cultured in LB broth, and serially diluted 4 times. Standard plate counting revealed low variance in final *E. coli* concentrations, which is attributed to the accuracy of the linear actuator in conjunction with the 1 mL disposable syringe needles. Clearing the surface for the next set of procedures was as simple as either extracting the droplets and then dumping the pipette tip, or simply guiding the droplet off of the surface into a KimWipe to be absorbed and later discarded.

To increase the concentration of the target antigen for improved PCR detection, wire-guided droplet centrifugation was performed through the use of a long, 22-gauge stainless steel blunt-ended needle upon which a vibration motor was securely attached (Figure 3). Careful consideration was used in the positioning of the vibration motor to generate the best circular motion path, as verified by high-speed imaging. To maximize the number of rotations per minute (RPM), a pulse-width-modulation (PWM) algorithm was used to isolate a resonant frequency that induced the most stable and rapid circular motion path. This frequency was found to change between samples due to variations in needle height from the surface, contact angle to the superhydrophobic surface due to variations in droplet content, and the connection between the needle and syringe. Therefore, small changes to the PWM frequency was made through manual adjustment via the user interface.
Fig C-2. A-H) Serial dilution protocol for E. coli sample. Wire-guided syringe needle manipulates, splits, and mixes sample on hydrophobic surface. Mixing performed with vibration motor.
After a specific period of centrifugation, the syringe automatically extracted the solution from the droplet. This extraction occurred at a much slower rate (~0.5 μL s⁻¹) to reduce potential turbulence induced by the extraction process and subsequent decrease in droplet diameter. The concentration of *E. coli* before centrifugation and in the final droplet was analyzed using standard plate counting methods. Figure 3-B reveals the concentration of *E. coli* in a 10 μL sample before and after droplet centrifugation revealing a 3.06 fold increase in the mean concentration after only 3 mins.

Computational fluid dynamic (CFD) simulations were employed to analyze the predictive movement of *E. coli* particles using varying single and multi-cell colony sizes (1, 2, 5, 7, 10 μm diameter colonies) and their location from the bottom versus time. When the diameter of the particle was 5, 7 and 10 μm, it took 120, 60 and 30 seconds respectively to settle down to the bottom of the droplet. For the cases of 1 and 2 μm, the particle never reached the bottom even after 300 seconds, however they did move by ~100 μm and ~300 μm, respectively, suggesting that this method is capable of centrifuging single *E. coli* colonies after only a short period of time. The simulation results indicate that longer periods of centrifugation for single colonies may be sufficient for complete centrifugation.
Fig C-3. A1-A4) Concentration of E. coli sample through droplet centrifugation. Pulse-width-modulated control of vibration motor induces circular motion path of droplet. B) Concentration of *E. coli* in 10 μL sample before and after droplet centrifugation revealing a 3.06 fold increase in mean concentration after 3 min.
The extraction and amplification of genetic material from *E. coli* was performed using the same apparatus configuration and on the same superhydrophobic surface. The only difference is the execution of a different preprogrammed algorithm. Figure 4 illustrates a representative algorithm of the DNA extraction protocol, in which the concentrated droplet of *E. coli* from the previous centrifugation step undergoes a series of lysing, precipitation, washing, and rehydration steps to prepare the genetic material for amplification and subsequent detection.
**Fig C.4.** A1-A8) Rapid DNA extraction of concentrated sample. B1) Concentrated sample of E. coli from droplet centrifugation step. B2) Nuclei lysis solution is added, mixed, and heated in lysis chamber at 80°C for 5 min. B3) IPA is added to lysed sample to precipitate DNA, which adheres to the pipette tip. B4) After drying from IPA, the DNA-saturated pipette tip is washed in EtOH and dried. B5) The DNA is resuspended in hydration solution. B6) The DNA solution is prepared for VQ-PCR thermocycling.
Figure 4-B presents a 6 step protocol for the extraction of DNA from *Escherichia coli*. An essential component of this protocol is step 3, in which the precipitated DNA/proteins following cell lysis is cleverly extracted from the droplet using the pipette tip as the substrate. Because the droplet is residing on the superhydrophobic surface, the predominant force is the electrostatic interaction of the genetic material towards the polymer pipette tip during removal, making the extraction process fast and easy, with minimal residual fluid needing to be evaporated. This same procedure was initially attempted in a 2 mL centrifuge tube, but when the precipitated material left the fluid and entered the air at the empty upper portion of the tube, the absence of a polar medium caused the material to immediately attach to the inside of the tube, making extraction difficult and inconsistent. Furthermore, rehydration of the extracted DNA in rehydration buffer from the pipette tip utilizing the vibration motor required only 3 mins. This process could be further sped up by using heat to reduce the rehydration time to less than a minute, making the entire DNA extraction process from start to finish in less than 10 mins.
**Fig C-5.** A1-A3) Droplet VQ-PCR thermocycler executes 30 cycles in 10 min with ~1500 bp sequence through forced convective heat transfer under silicon oil submersion. B) Gel electrophoresis results for sample undergoing droplet centrifugation, DNA extraction, amplification, and VQ-PCR. Lane 1 shows positive control of DNA extracted with culture media and lane 2 shows positive control of DNA extracted without culture media. Since culture media does not interfere with DNA extraction, lane 3 shows droplet with centrifugation and lane 4 shows droplet without centrifugation, demonstrating an increase in signal intensity of the PCR band through effective sample concentration by wire-guided droplet centrifugation.
Immediately following extraction, the droplet containing the genetic material underwent a 30-cycle thermocycling process using the VQ-PCR method that was previously established [11] (Figure 5-A). Due to the long genetic sequence being amplified (~1500 bp) the algorithm required a slightly longer extension time, requiring 10 mins overall to perform 30 cycles. Previous work demonstrated PCR amplification of a 160 bp sequence in 6 min 50 sec for 30 cycles. Figure 5-B is a gel image of the final results for the amplified ~1500 bp genetic sequence of *Escherichia coli*. The first lane is the ladder and confirms the ~1500 bp sequence being amplified. The second lane is the positive control with culture media, and lane three is the positive control without culture media. Pelletizing the *E. coli* with droplet centrifugation is not possible, and therefore the culture media could not be completely removed using this methodology; therefore the extent of inhibition of the DNA amplification was qualitatively determined by the positive controls in lane 2 and 3, showing that there is a minimal reduction in band intensity, resulting from a lower concentration of the amplified sequence, however, the strong positive band of lane 2 suggests that droplet PCR amplification in the presence of culture media remains a viable option.

Lane 4 is the result of the wire-guided droplet dilution, centrifugation, extraction, and amplification of *Escherichia coli* cultured in LB media. Lane 5 is the result using the identical processes of lane 4, minus the concentrating step from droplet centrifugation. The band intensity of lane 4 is much stronger than that of lane 5, further lending to the process of droplet centrifugation as a useful method for concentrating samples in the
interest of either increasing the positive signal of PCR amplification or to reduce the number of cycles required for a positive result, decreasing assay time.

Conclusions

This work demonstrates the use of a wire-guided CNC apparatus to execute pre-programmed droplet movements and manipulations on a superhydrophobic surface measuring 25 mm by 55 mm and a multi-chambered PCB heater for the rapid detection of *Escherichia coli* from PCR detection. This methodology exhibited no limitations on the complexity and configuration of procedures that it can perform, making it extremely versatile and far-reaching in its applications. The only modification required for adding or implementing changes for a new protocol is through simple pre-defined programming, which can be easily accomplished by any user with minimal programming knowledge. Because of this characteristic, the user has the ability to start and stop the assay/s at any time, manually control the droplet system with a computer, make adjustments to the protocol on-the-fly, and resume where it left off. To demonstrate the capabilities of this methodology and current system, serial dilutions were performed to simulate a diluted field sample with a high level of accuracy. Centrifugation of the diluted sample containing *E. coli* with a 10 μL droplet spinning around a 22-gauge blunt ended stainless steel syringe needle at 2300 rotations per minute demonstrated a novel approach to sample pre-treatment. This technique resulted in a mean increase in the concentration of *E. coli* contained within the sample droplet by more than 3 fold. Additionally, Fluent simulations were used to validate the centrifugation of *E. coli* particles and to track their
migration over time, showing that effective centrifugation of particles occurred within 3 mins. Furthermore, the extraction of DNA from the sample droplet containing *E. coli* was also performed on the same superhydrophobic surface as the previous 2 steps, requiring less than 10 mins. This approach utilized the disposable pipette tip to cleverly extract the precipitated DNA from the sample droplet residing on the superhydrophobic surface. Following extraction, the genetic material was amplified using wire-guided droplet PCR thermocycling, which incorporates pendant droplets hanging from a syringe needle and moving through separately heated silicon oil chambers, utilizing forced convective heat transfer over a large surface area, to successfully complete 30 cycles of a long ~1500 bp sequence in 10 mins. The results of these sequentially executed processes were analyzed using gel electrophoresis, in which the droplet centrifugation was determined to greatly improve the positive band intensity over the non-centrifuged sample. And, although the system used in this work represents a benchtop apparatus, the method employed is not highly-complex, and thus alludes to the ability for further miniaturization, making a potentially portable device for performing a nearly limitless number of protocols, including all of their variations. Thus, this work demonstrates the reconfigurability of the system to replace many common laboratory tasks on a single platform (through re-programmability), in rapid succession (using droplets), and with a high level of accuracy, leading towards an all-in-one, portable lab on a chip system.
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References


