



A smartphone-based particle diffusometry platform for sub-attomolar detection of *Vibrio cholerae* in environmental water

Taylor J. Moehling^{a,1}, Dong Hoon Lee^{b,1}, Meghan E. Henderson^a, Mariah K. McDonald^a, Preston H. Tsang^a, Seba Kaakeh^c, Eugene S. Kim^a, Steven T. Wereley^b, Tamara L. Kinzer-Ursem^a, Katherine N. Clayton^{b,d,**}, Jacqueline C. Linnes^{a,*}

^a Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN, 47907, USA

^b School of Mechanical Engineering, Purdue University, West Lafayette, IN, 47907, USA

^c Department of Biochemistry, Purdue University, West Lafayette, IN, 47907, USA

^d OmniVis LLC, Indianapolis, IN, 46201, USA

ARTICLE INFO

Keywords:

Particle diffusometry
Loop-mediated isothermal amplification
Smartphone
Biosensor
Vibrio cholerae
Point of use

ABSTRACT

Each year, 3.4 million people die from waterborne diseases worldwide. Development of a rapid and portable platform for detecting and monitoring waterborne pathogens would significantly aid in reducing the incidence and spread of infectious diseases. By combining optical methods and smartphone technology with molecular assays, the sensitivity required to detect exceedingly low concentrations of waterborne pathogens can readily be achieved. Here, we implement smartphone-based particle diffusometry (PD) detection of loop-mediated isothermal amplification (LAMP) targeting the waterborne pathogen *Vibrio cholerae* (*V. cholerae*). By measuring the diffusion of 400 nm streptavidin-coated fluorescent nanoparticles imaged at 68X magnification on a smartphone, we can detect as few as 6 *V. cholerae* cells per reaction (0.66 aM) in just 35 minutes. In a double-blinded study with 132 pond water samples, we establish a 91.8% sensitivity, 95.2% specificity, and 94.3% accuracy of the smartphone-based PD platform for detection of *V. cholerae*. Together, these results demonstrate the utility of this smartphone-based PD platform for rapid and sensitive detection of *V. cholerae* at the point of use.

1. Introduction

Approximately 1 in every 6 individuals worldwide do not have access to quality drinking water (Riley *et al.*, 2011). When individuals unknowingly consume water contaminated with pathogens, such as the cholera-causing bacteria *Vibrio cholerae* (*V. cholerae*), they can suffer from severe gastroenteritis resulting in dehydration and death within hours if left untreated. Annually, cholera leads to an estimated 3 million cases and thousands of deaths globally (Global Task Force on Cholera Control, 2017; World Health Organization, 2018). Early detection and monitoring of *V. cholerae* in the environment can prevent illness and death, particularly in low-resource settings (Ramírez-Castillo *et al.*, 2015). Even at concentrations of 1 cell/mL, *V. cholerae* is harmful for human health (Alam *et al.*, 2015). The gold standard technique, polymerase chain reaction (PCR), can only detect concentrations this low

after an 8-hour enrichment of the environmental sample (Alam *et al.*, 2015). Thus, there is a need for a sensitive biosensor that can rapidly detect waterborne pathogens, such as *V. cholerae*, directly from environmental water samples at exceedingly low concentrations.

In the past decade, many pathogen detection biosensors have emerged by translating standard optical methods onto portable platforms. Notably, smartphones possess both advanced computational power and quality image sensors that previously could only be found in expensive laboratory equipment (Hernandez-Neuta *et al.*, 2019; Steinhubl *et al.*, 2015). Along with these technical improvements, the usability and accessibility of smartphone technology are attractive features that promote translation of cumbersome optical techniques from the laboratory to the point of use (Zhang and Liu, 2016). For example, Zeng *et al.* combined smartphone technology with surface-enhanced Raman scattering (SERS) optical detection modality to

* Corresponding author. 206 S. Martin Jischke Drive, West Lafayette, IN, 47907, USA.

** Corresponding author. 1417 Commerce Ave Ste A, Indianapolis, IN, 46201, USA.

E-mail addresses: klclayton@omnivistech.com (K.N. Clayton), jlinnes@purdue.edu (J.C. Linnes).

¹ Indicates equal contribution.

enable detection of analytes down to 10^{-5} M at the point of use. This portable Raman spectrometer allows rapid, real-time analysis; however, its applications are limited due to poor sensitivity and specificity (Zeng et al., 2018). Wu et al. developed a dark-field smartphone microscope to be used in combination with an immunoassay for rapid and specific detection of *E. coli* in water samples down to 10 cells/10 mL after filtration (Wu et al., 2017). Although sensitive, immunoassays involve several time-sensitive user steps that limit the usability of such platforms in the field. Koydemir et al. utilized a smartphone-based fluorescence microscope for imaging and quantifying fluorescently labeled *G. lamblia* cysts in large volumes of water. The entire process, from sample preparation to image processing and quantification, takes only 1 h and has a limit of detection of 12 cysts in 10 mL of water (Koydemir et al., 2015). Unfortunately, this smartphone-based fluorescence microscopy technique is not suitable for detection of bacterial or viral pathogens because the system is optimized for larger microorganisms. Alternatively, integrating optical techniques with nucleic acid amplification assays allows for detection of a multitude of pathogens with excellent sensitivity and specificity (Hernandez-Neuta et al., 2019).

Nucleic acid amplification techniques, such as PCR, efficiently target and produce millions of copies of a nucleic acid sequence during a

thermal cycling process with high accuracy and sensitivity (Hernandez-Neuta et al., 2019). Several groups have combined the precise optics and computational power of smartphones with miniaturized thermal cyclers to conduct PCR assays that provide sensitive, specific, and accurate detection of pathogens (Jiang et al., 2014; Lee et al., 2011). However, PCR typically requires nucleic acid extraction and purification prior to initiation of the assay which increases the number of user steps and complicates microfluidic chip design.

Isothermal amplification methods can robustly amplify nucleic acids in complex sample matrices, thus simplifying and accelerating the sample-to-answer process (Phillips et al., 2019, 2018; Zanolli and Spoto, 2013). Researchers have demonstrated the utility of one such isothermal technique, loop-mediated isothermal amplification (LAMP), by combining the assay with smartphone technology to sensitively detect nucleic acid analytes in 1 h with minimal user intervention (Barnes et al., 2018; Damhorst et al., 2015; Kong et al., 2017). Even though LAMP is a selective and rapid amplification technique, current fluorescence detection methods depend on relative measurements to adjust for the variation between repeats (Duarte et al., 2013; Rolando et al., 2018).

By integrating LAMP with particle imaging, we can minimize the variability between repeats and take advantage of the high selectivity of

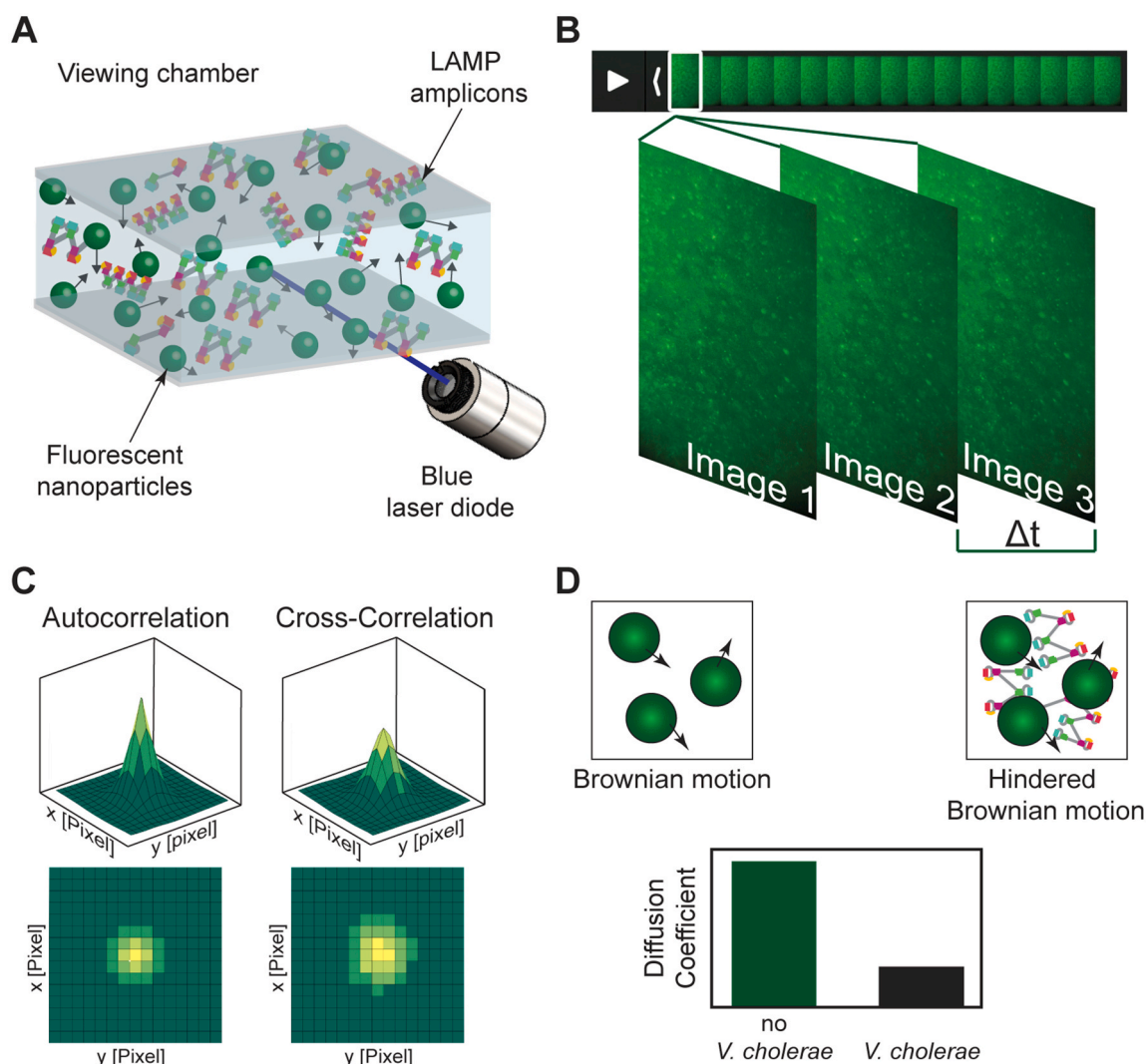


Fig. 1. Smartphone-based particle diffusometry. (A) 400 nm streptavidin-coated fluorescent nanoparticles are combined with LAMP amplicons and excited with a blue laser diode. (B) Brownian motion of the nanoparticles is recorded using sequential images from an iPhone 6 with a frame extraction rate of 15 frames per second. (C) 3D and 2D correlation peaks of sequential frames. (D) Relationship between nanoparticle motion and diffusion. In the presence of LAMP amplicons, nanoparticles experience hindered Brownian motion and decreased diffusivity, indicating the presence of *V. cholerae* in the sample. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

LAMP and the sensitivity of particle imaging. The polymerization that occurs during LAMP produces a change in viscosity that can be measured using an optical detection method called particle diffusometry (PD) (Clayton et al., 2016, 2017b, 2019, 2017a). PD can also measure changes in size; by labeling a LAMP primer with biotin and combining streptavidin-coated nanoparticles with the LAMP products, the inherent binding causes an increase in particle size and enhances PD measurements. After recording a video of the Brownian motion of nanoparticles that are added to the amplification products (Fig. 1A), the images can be extracted (Fig. 1B) and auto- and cross-correlated (Fig. 1C) to calculate a diffusion coefficient for the sample. The diffusion coefficient quantifies the Brownian motion of nanoparticles in solution and is affected by both sample viscosity and particle size (Fig. 1D). In our previous proof-of-concept work using a fluorescence laboratory microscope, we demonstrated that PD could be used to detect LAMP amplicons with a 10-fold improvement in sensitivity over end-point fluorescence detection (Clayton et al., 2019). We also established that PD is a statistically robust detection method; three diffusion coefficients are calculated for each sample.

Here, we demonstrate a smartphone-based PD platform for the rapid and specific detection of *V. cholerae* in environmental water samples using LAMP. We perform a 30-minute LAMP assay using a biotinylated primer and add 400 nm streptavidin-coated fluorescent nanoparticles to induce a change in particle size that alters PD measurements along with changes in solution viscosity. Using a 30-second video recording of the nanoparticles in solution, the smartphone algorithm computes the diffusion coefficient in less than 1 minute. The resulting diffusion coefficient suggests the presence of *V. cholerae* when diffusivity of the nanoparticles is low and absence of the pathogen when diffusivity is high. We establish the robustness of this smartphone-based PD platform, determine its selectivity and limit of detection (LOD) for *V. cholerae* in pond water, and compare its sensitivity and specificity to real-time fluorescence detection using 132 blinded samples.

2. Materials and methods

2.1. Bacteria culture

Both toxigenic *V. cholerae* strain N16961 (O1 serogroup) and non-toxigenic *V. cholerae* strain NRT-36 were provided by Dr. Afsar Ali at the University of Florida. *E. coli* strain DH5 α (NEB, Ipswich, MA) was also used for experimentation. All bacteria were stored at -80°C until ready to be cultured. All cultures were grown in Lysogeny Broth (LB) overnight at 37°C in a miniature incubating orbital shaker at 320 rpm (Thermo Fisher, Waltham, MA). An Ultrospec 10 (Biochrom, Cambridge, UK) cell density meter was used to measure the optical density (OD) of the cultures. Cultures were diluted to an OD₆₀₀ of 1, representing 10^9 cells/mL of *E. coli* and 5×10^8 cells/mL of both *V. cholerae* strains as determined by counting colony forming units of serially diluted cells.

2.2. Loop-mediated isothermal amplification (LAMP)

LAMP primers, provided in Table S1, were designed to target the cholera toxin A (*ctxA*) gene (sequence in Table S2) of toxigenic *V. cholerae* strains (Okada et al., 2010). There is one copy of the *ctxA* gene per *V. cholerae* genome. LAMP reactions were 15 μL total, consisting of the LAMP master mix and a biotinylated LF primer (concentrations shown in Table S3 and vendors shown in Table S4). All LAMP reactions contained 7.5 μL (50% v/v) of pond water. Pond water was collected from a local small (100 m wide and 200 m long) water source. 1.2 μL of template or water (molecular biology water (Invitrogen, Carlsbad, CA) for the negative control (NTC)) was spiked into the reactions prior to heating. *V. cholerae* cells lyse due to thermal effects alone at 65°C , so an additional cell lysis step was not necessary (Clayton et al., 2019). LAMP reactions were incubated at 65°C for 30 minutes using an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA).

Real-time fluorescence data was collected to track amplification. LAMP samples were stored at 4°C until analyzed with the smartphone-based PD platform. LAMP amplicons were also characterized via gel electrophoresis at 100 V for 60 min. The 2% agarose gels were stained with ethidium bromide and imaged using an ultraviolet light gel imaging system (c400, Azure Biosystems, Dublin, CA) with UV 302 settings and a 15-second exposure.

2.3. Particle preparation

Streptavidin-coated 400 nm Dragon green polystyrene fluorescent nanoparticles (Ex480/Em520 nm) were purchased from Bangs Laboratories (Fishers, IN). We used streptavidin-coated nanoparticles because they strongly bind to the biotin-labeled amplicons causing a change in both viscosity and size; therefore, improving sensitivity (Clayton et al., 2019). Nanoparticles were added to the 15 μL LAMP reaction at a final concentration of 1.84×10^9 particles/mL. The LAMP-nanoparticle solution was incubated at room temperature for 10 minutes to allow binding of the biotinylated amplicons to the streptavidin-coated nanoparticles.

2.4. Microfluidic chip assembly

The microfluidic chips were designed as a drawing exchange format (.dxf) in Autodesk Fusion 360 software and translated to the Silhouette Studio 2.0 Software (Silhouette America, Lindon, UT). Microfluidic chips were composed of four layers: 2 layers of 188 μm cyclic olefin polymer (COP) (Zeonor, Tokyo, Japan), 1 layer of 60 μm COP (Zeonor, Tokyo, Japan), and 1 layer of 120 μm double-sided tape. The 188 μm COP was cut with the Silhouette Cameo Craft Cutter (Silhouette America, Lindon, UT) with two passes using a 10-blade, force of 19, and a speed of ten (arbitrary units within the Silhouette software). The COP pieces were cleaned after cutting with 1) a MilliQ rinse and 2) a 2-minute sequential sonication in acetone, isopropanol, methanol, and MilliQ. After cleaning and drying with a nitrogen stream, the two layers of 188 μm COP were bonded together by thermal fusion using a Carver 4386 hydraulic press (Carver Inc., Wabash, IN) with 1.2 tons of pressure at 120°C . Double-sided tape with a 3.175 mm hole served as the sample well. The 60 μm COP was placed on top of the double-sided tape to enclose the sample and prevent evaporation. The assembled microfluidic chips were 25.4 mm by 22.10 mm and 0.556 mm thick.

2.5. Optics design

Three different elements were used to develop the optics within the smartphone-based PD platform: a spherical BK7 borosilicate lens with 500 μm diameter (Edmund Optics, Barrington, NJ), a blue laser diode (Laserland, Wuhan, China) powered by a lithium ion polymer battery (Adafruit, New York, NY), and #12 straw Cinegel film filter (Rosco, Stamford, CT).

The spherical 500 μm diameter BK7 lens has a 117 μm back focal length, 160 μm field of view, and produces a 68X magnification. All lens calculations were adapted from Cybulska et al. (2014). The iPhone 6 camera lens system used in this platform has a CMOS with one blue, one red, and two green pixel sensors (Bayer array). Because there are twice as many green pixel sensors than blue or red, the smartphone camera lens system can detect green light most effectively (Palum, 2001). Therefore, Dragon green (Ex480/Em520 nm) 400 nm streptavidin-coated nanoparticles (Bangs Laboratories, Fishers, IN) were selected for imaging. The size of the nanoparticles was selected based on the fixed optics of the iPhone 6 camera lens system and magnification provided by the spherical lens; 400 nm was the smallest particle size visible using the smartphone optics. These nanoparticles then dictated the laser and filter combination. To excite the nanoparticles, a 445 nm laser with an 80-mW power capacity (12 mm diameter and 35 mm length) was selected. Any light with a wavelength below 470 nm was

filtered out by the straw film filter. This straw film filter was selected after reviewing the color spectrums of all green and yellow Rosco film filters (Rosco, 2001). Rosco provides inexpensive cinematic lighting films that have been shown to be effective low-cost fluorescence filters for portable devices (Cybulski et al., 2014).

2.6. Particle diffusometry (PD) theory

PD involves recording a series of images of fluorescent particles undergoing Brownian motion in a quiescent volume and calculating the particle diffusion coefficient using correlation analysis (Clayton et al., 2016). Each individual image is partitioned into smaller interrogation areas (64×64 pixel²) containing, on average 8–10 particles (Lu and Sick, 2013). Within these interrogation areas, autocorrelation and cross-correlation of the images are computed for the entire series of images. Cross-correlation (s_c) involves correlating two temporally sequential images and autocorrelation (s_a) is performed by correlating a captured image with itself (Adrian and Yao, 1985). Using both autocorrelation and cross-correlation, the diffusion coefficient, D , can be calculated by the equation derived by Olsen and Adrian (2000)

$$D = \frac{s_c^2 - s_a^2}{16M^2\Delta t} \quad (1)$$

where M is the magnification and Δt is time step between images. We can compare the experimentally determined diffusion coefficient, D , from Eq. (1) to the theoretical value calculated from the Stokes-Einstein relationship in Eq. (2) (Chuang and Sie, 2014; Einstein, 1905).

$$D = \frac{kT}{6\pi\eta a} \quad (2)$$

Here, k is the Boltzmann constant, T is the absolute temperature, η is the viscosity, and a is the hydrodynamic radius of fluorescent particles that are imaged. Eq. (3) shows the relationship between the diffusion coefficient, viscosity, and hydrodynamic radius.

$$D \sim \frac{1}{\eta a} \quad (3)$$

LAMP produces polymerized DNA strands that cause a change in the viscosity of the surrounding solution. We use streptavidin-coated fluorescent nanoparticles that bind directly to the biotin-labeled LAMP amplicons to provide a change in size, in addition to viscosity. The change in both viscosity and size produces a more substantial difference in the diffusion coefficient measurements.

2.7. Smartphone-based platform development

The smartphone-based PD platform is composed of a 3D printed platform that houses the optics and a smartphone application built for the iPhone 6. The platform was designed to incorporate the optics and power source, secure the smartphone and microfluidic chip, and exclude external light during imaging. The smartphone application was created using Apple's development platform, XCode.

The platform, composed of six parts, was designed using SolidWorks (Fig. S1). Five parts were 3D printed with a Fortus 400mc 3D printer (Stratasys, Eden Prairie, MN) using acrylonitrile butadiene styrene (ABS). The aluminum plate that holds the external ball lens was machined. The platform was developed following Apple's accessory design guidelines to ensure the iPhone 6 fit securely into the platform. The external ball lens is situated directly below the camera lens system which aligns with the sample well of the microfluidic chip. The platform also contains a slot with a z-axis stage to insert the microfluidic chip; a bolt and nut (McMaster-Carr, Elmhurst, IL) with a 3D printed knob were used to adjust the z-axis stage to ensure the microfluidic chip was within the focal length of the external ball lens.

The smartphone application was developed following the principles

of PD introduced in Clayton et al. (2017b). Both Swift and Objective-C programming languages were used in the smartphone application development. The iOS application takes advantage of the iPhone's capability to record a 30 frames per second (FPS) video. By reducing the frame rate to 15 FPS, 300 frames on average are extracted from a 30-second video recording, leaving a 21-second video for analysis. The center 800×800 pixel² region is used for processing to minimize the distortion effects around the edges from the external ball lens. A 2×2 binning is then applied for signal improvement. This binning allowed for a high signal-to-noise ratio while maintaining a statistically relevant number of data points. Frames were processed, and auto- and cross-correlation analysis was performed using the in-house smartphone algorithm. Native software development kits (SDK) and application programming interfaces (API) were used to perform image processing and PD analysis. All steps were verified by comparing the code with the previously developed MATLAB code.

2.8. Smartphone-based particle diffusometry measurements

A 1.5 μ L aliquot of the LAMP-nanoparticle solution was added to the sample well within the microfluidic chip and a 1×1 cm² piece of 60 μ m COP was placed over the sample well to seal the sample, preventing evaporation. Three 1.5 μ L aliquots from each sample were loaded into three separate microfluidic chips for intra-sample measurement validation. Each microfluidic chip was inserted into the smartphone-based PD platform for imaging at room temperature on a stabilizing optical table to minimize external vibration. After turning the laser on, a 30-second video of nanoparticle Brownian motion was recorded. After the 60-second image analysis, three diffusion coefficients were displayed for each microfluidic chip. Three diffusion coefficient measurements from three different microfluidic chips were collected for each biological repeat (nine measurements), highlighting the statistical robustness of PD.

2.9. Experimental design

For all studies presented in this work, three individuals prepared biological samples and performed LAMP and three different individuals measured the PD of the samples on the smartphone-based platform. For the LOD experiments, 10-fold serial dilutions (6×10^0 - 6×10^4 cells/reaction) of *V. cholerae* N16961 whole cells were prepared and added to LAMP reactions. For the selectivity experiments, *V. cholerae* N16961, *V. cholerae* NRT-36, and *E. coli* DH5 α were all used as the template. *V. cholerae* NRT-36 was chosen to ensure that LAMP is only selective for toxigenic strains of *V. cholerae*. *E. coli* DH5 α was tested because it is also found in environmental water sources and we wanted to ensure no cross-reactivity of PD-LAMP (Ishii and Sadowsky, 2008). The bacteria were added to separate LAMP reactions at a concentration of 10^3 cells/reaction. The three individuals measuring PD on the smartphone-based platform were blinded to the sample contents for the selectivity experiments. All nine diffusion coefficients were plotted for each biological repeat for both experiments.

We wanted to compare smartphone-based PD with real-time fluorescence detection of LAMP to determine the sensitivity, specificity, and accuracy of the smartphone-based PD platform. The data were split into a training and test set. The 30 samples used in the training set were ordered by diffusion coefficient to determine the positive and negative diffusion coefficient thresholds. Real-time fluorescence results were recorded for each sample in the training set. For the test set, we used a double-blinded study design to approximate a field study. Neither the individual who performed LAMP nor the individual who performed PD on the smartphone-based platform knew the contents of the samples. A total of 132 samples, ranging from 0 cells/mL to 3.5×10^8 cells/mL, were used in the test set. If the relative fluorescence increased ten standard deviations above the baseline (the auto-threshold setting on the real-time PCR system) by the end of the 30-minute amplification

period, the individual that performed LAMP recorded that sample as positive by real-time fluorescence. If the relative fluorescence did not increase ten standard deviations above the baseline, the sample was considered negative by real-time fluorescence. We confirmed fluorescence results via gel electrophoresis. Each sample was then measured on the smartphone-based PD platform and the diffusion coefficients were recorded. The average of all nine diffusion coefficients was used to describe each sample for the double-blinded study.

2.10. Statistical analysis

For the LOD experiments, a one-way ANOVA post-hoc Dunnett's test was performed with multiple comparisons against a negative control with no template (NTC) using a 95% confidence interval. For the selectivity experiments, a one-way ANOVA post-hoc Dunnett's test was conducted with multiple comparisons against the positive control (toxigenic *V. cholerae*) with a 95% confidence interval. Quartile box-and-whisker plots were generated for the PD data for both experiments where the upper and lower bounds represent the 75th and 25th percentile about the median, respectively, and the minimum and maximum values are represented by the upper and lower whiskers. Individual data points for each sample were plotted on the quartile box-and-whisker plots.

A single, averaged diffusion coefficient (from 9 measurements) for each of the 132 test set samples from the double-blinded study was plotted and fitted with a bimodal Gaussian distribution curve.

A 2×2 contingency table was created to directly compare the smartphone-based PD platform and real-time fluorescence measurement of LAMP. From this contingency table, we calculated the sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of the smartphone-based PD platform (Baratloo et al., 2015). We created a receiver operating characteristic (ROC) curve to demonstrate the tradeoff between sensitivity and specificity and indicate the accuracy of the platform (Hajian-Tilaki, 2013). All figures were created using GraphPad Prism 7.

3. Results and discussion

3.1. Construction of optics

High resolution optics and robust construction are critical for PD measurements on a portable device. Therefore, we developed a 3D printed platform to house the optics for nanoparticle visualization that is perpendicular to the axis of gravity to improve platform stability. An external ball lens was incorporated to enhance the magnification of the iPhone 6 camera lens system to visualize the nanoparticles. The external

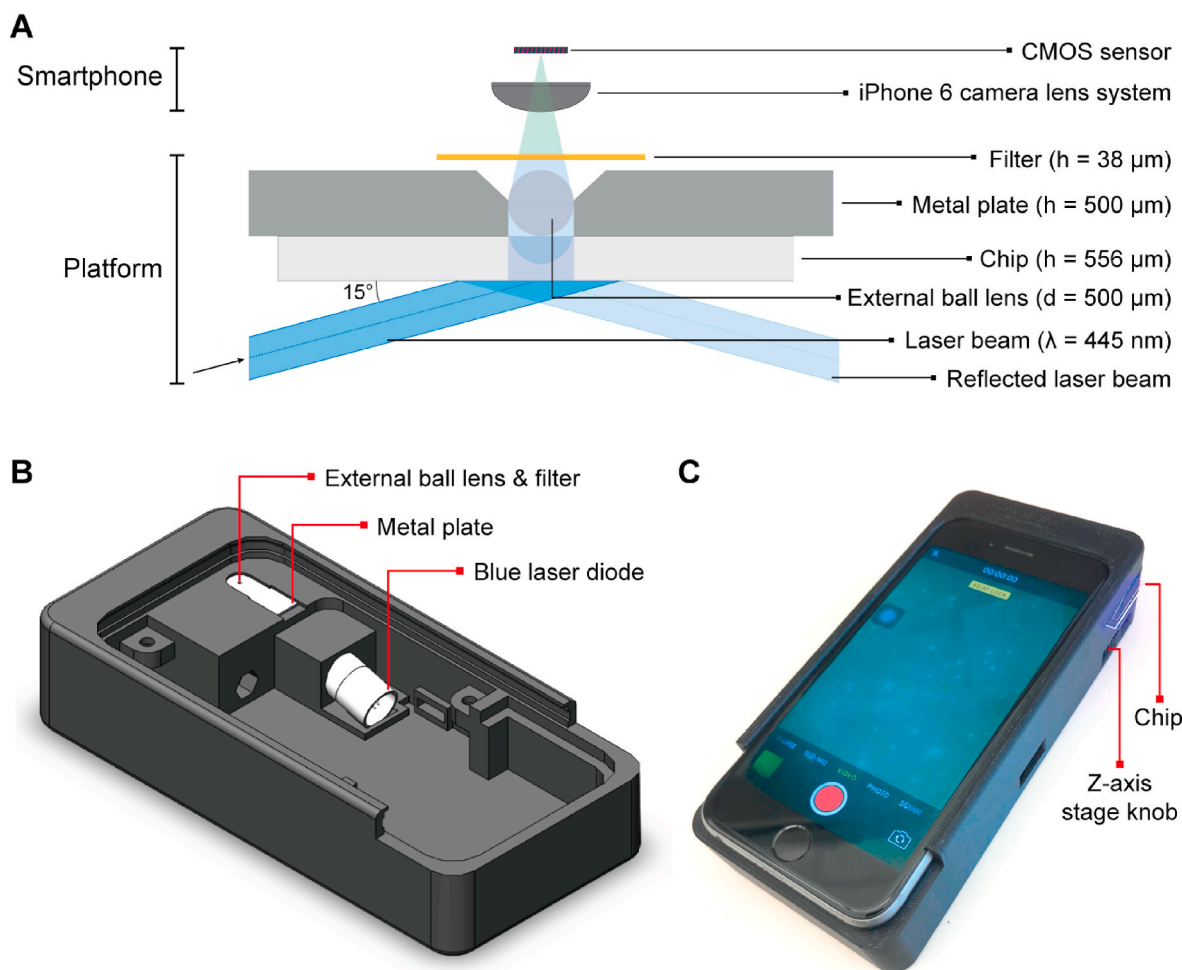


Fig. 2. Smartphone-based particle diffusometry platform. (A) Optics setup within the platform includes an external ball lens, filter, complementary metal-oxide-semiconductor (CMOS) sensor, iPhone 6 camera lens system, and laser at a 15° incident angle. (B) Schematic of the platform that incorporates the blue laser diode, metal plate, external ball lens, and filter. (C) Image of the integrated smartphone-based PD platform recording Brownian motion of 400 nm streptavidin-coated fluorescent nanoparticles in the microfluidic chip. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ball lens diameter is inversely related to magnification; thus, the 500 μm spherical glass ball lens provides a magnification of 68X which is sufficient for our application. The external ball lens back focal length (BFL) is 117 μm ; therefore, the topmost layer of the microfluidic chip must be less than the BFL (Cybulski et al., 2014). We selected optically transparent 60 μm cyclic olefin polymer (COP) as the top layer of the microfluidic chip. Lastly, a 445 nm blue laser diode was used to excite the fluorescent nanoparticles and a yellow filter was placed between the external ball lens and camera lens system to act as a high-pass filter, transmitting wavelengths above 470 nm to the iPhone 6 camera (Fig. 2A). Four hundred (400) nm fluorescent particles (Ex480/Em520 nm) are greater than four pixels in diameter under imaging conditions, which minimizes the diffractive effects and maximizes the signal-to-noise ratio (Aguirre-Pablo et al., 2017).

The external ball lens, iPhone 6 camera lens system, microfluidic chip, and blue laser diode were first aligned using the virtual construction tool in SolidWorks. The computer-aided design (CAD) model of the laser was placed at a 15° incident angle and fixed in position by designing the surrounding structure to support the laser (Fig. 2B). At the 15° incident angle, the fluoresced and scattered light is focused into the iPhone 6 camera lens system (Wei et al., 2013). The light passes through the optically transparent COP microfluidic chip containing the LAMP sample with nanoparticles and then through the external ball lens. We ensured the microfluidic chip fit snugly into the platform by designing a slot to match the width of the microfluidic chip and incorporating a z-axis stage. The z-axis stage also allows adjustment of the optical focus (Fig. 2C).

After confirming alignment in SolidWorks, the external ball lens was carefully placed in a metal plate and secured using epoxy. The collimator on the 445 nm blue laser diode was glued into the 3D printed

platform. The body of the laser was screwed into the stationary collimator. The combined effect of fluorescence and scattering of the nanoparticles makes this optical system ideal for imaging small fluorescent nanoparticles (Cybulski et al., 2014; Wei et al., 2013). Scattered light reduces the background noise by filtering out the oblique illumination on the sample plane (Wei et al., 2013). Further, the short working distance of the optical system keeps the platform compact. The cost of the consumables for the smartphone-based PD-LAMP system is \$0.78 (Table S4) and the reusable components of the platform cost less than \$265 (Table S5).

3.2. Image quality, recording, and processing

We designed a smartphone application to process the images captured with our optical system to perform PD analysis. Videos recorded on an iPhone 6, using high definition settings, have a resolution of 1920×1080 pixel² (Apple, 2019). Therefore, the resulting images depict the 400 nm streptavidin-coated fluorescent nanoparticles in water with high contrast and minimal background noise (Fig. 3A). The center 800×800 pixel² region is used for analysis to minimize distortion effects around the edges of the images from the external ball lens (Fig. 3B). This central region is processed to further differentiate nanoparticles from background noise (Fig. 3C). The images are then auto- and cross-correlated within the smartphone application to generate diffusion coefficients. All image processing was performed independently by the smartphone application without external processing support (network or hardwired). Next, we imaged the same nanoparticle solution using 40X magnification on an inverted epifluorescence microscope (Fig. 3D and E). We compared image quality and resulting diffusion coefficient measurements from the

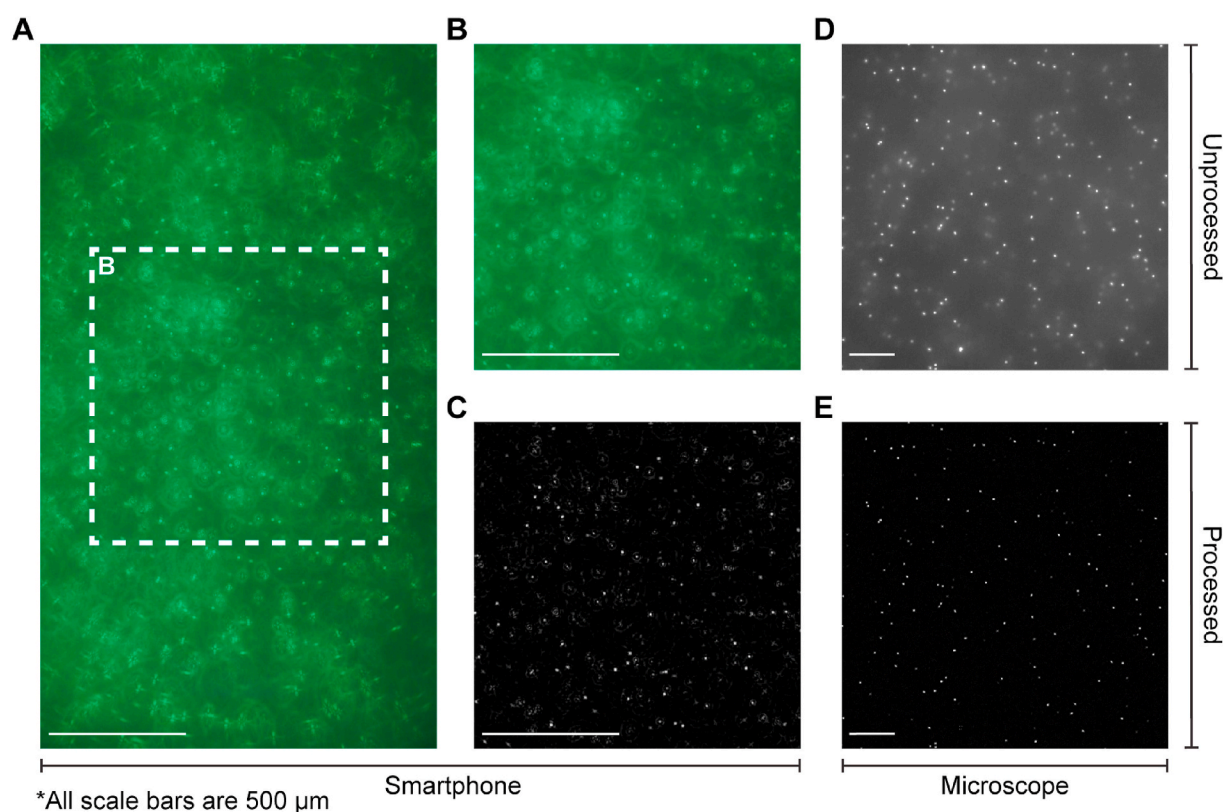


Fig. 3. Image quality comparison between smartphone and microscope. (A) Raw image of 400 nm streptavidin-coated fluorescent nanoparticles in water using smartphone-based optical system. (B) Central 800×800 pixel² used for analysis. (C) Final processed image taken with the smartphone. (D) Image of the same streptavidin-coated fluorescent nanoparticle solution using a 40X objective on an inverted epifluorescence microscope. (E) Resulting image from the microscope after processing. Note: all scale bars are 500 μm .

smartphone-based optical system ($9.83 \times 10^{-13} \text{ m}^2/\text{s} \pm 1.83 \times 10^{-14} \text{ m}^2/\text{s}$) with results obtained on the microscope ($1.02 \times 10^{-12} \text{ m}^2/\text{s} \pm 1.57 \times 10^{-14} \text{ m}^2/\text{s}$) and saw no significant difference ($p > 0.05$) (Figure S2).

3.3. Limit of detection in pond water

After measuring the diffusion coefficient of the nanoparticles in water, we performed PD on whole cells in environmental pond water. The pond water contains some sediment and other debris which add to sample matrix complexity and simulates the natural environment where *V. cholerae* is found (Alam et al., 2014; Cabral, 2010; Clayton et al., 2019; Reidl and Klose, 2002). If we use environmental samples with excessive amounts of sediment in the future, we may need to filter the water samples prior to performing PD measurements. We incorporated multiple users in the experimental design to demonstrate the repeatability of smartphone-based PD; three users performed LAMP and three different users executed measurements on the smartphone-based PD platform. LAMP assays were prepared with $6 \times 10^0 - 6 \times 10^4$ cells/reaction in pond water (where 50% of the total LAMP reaction volume was pond water). Fluorescence visualization of LAMP performed in a real-time PCR system demonstrated that samples with higher initial concentrations of *V. cholerae* amplify more rapidly than samples with lower initial concentrations (Fig. 4A) as indicated by the lower cycle threshold (C_T) values (Table S6). Gel electrophoresis was used to confirm amplification (Fig. 4B). There was a statistically significant difference in the diffusion coefficients from $6 \times 10^0 - 6 \times 10^4$ cells/reaction (0.66 aM – 6.6 fM) when compared to NTC (p-value < 0.01 for 6×10^0 and p-value < 0.0001 for 6×10^1 , 6×10^2 , 6×10^3 , and 6×10^4 cells/reaction) (Fig. 4C). This LOD of 6 *V. cholerae* cells/reaction, 0.66 aM or 400 cells/mL, falls within the concentration range that is commonly found in the environment (10 – 1000 cells/mL) (Alam et al., 2014). This environmentally relevant LOD suggests the applicability of this handheld smartphone-based PD platform for future point of use applications. The sub-attomolar concentration of *V. cholerae* measured on the smartphone-based PD platform is equivalent to the microscope results in our previous work (Clayton et al., 2019); however, unlike the microscope, the smartphone platform used here is both portable and low-cost.

3.4. Single-blinded selectivity in pond water

After establishing the LOD, we confirmed the selectivity of the PD technique by ensuring only toxigenic *V. cholerae* was detected and other environmental bacteria that may be present in water samples were not. We used *E. coli* and non-toxigenic *V. cholerae* to measure selectivity since both bacteria are commonly found in the same environment as toxigenic *V. cholerae* (Ishii and Sadowsky, 2008). It is important to note that non-toxigenic *V. cholerae* lacks the cholera toxin A (*ctxA*) gene targeted by the LAMP primers, and therefore, cannot produce the toxin responsible for severe symptoms and disease outbreaks in humans (Centers for Disease Control and Prevention, 2018).

We prepared LAMP assays with 10^3 cells/reaction of *E. coli*, non-toxigenic *V. cholerae*, and toxigenic *V. cholerae* in 50% pond water. To measure selectivity, we performed a single-blinded experiment with six different users to remove the potential for measurement bias. The three users performing LAMP knew the contents of each sample; however, the sample details were blinded to the three individuals taking measurements on the smartphone-based PD platform. There was no amplification of samples containing *E. coli* or the non-toxigenic strain of *V. cholerae* as indicated by gel electrophoresis (Fig. 5A) and fluorescence data (Table S7 and Fig. S3). These results demonstrate the selectivity of LAMP and coincide with previous literature regarding the target efficiency of LAMP primers (Besuschio et al., 2017; Nliwasa et al., 2016; Okada et al., 2010). There was a statistically significant difference in the diffusion coefficient of the NTC, *E. coli*, and non-toxigenic *V. cholerae* samples when compared to toxigenic *V. cholerae*, which resulted in a

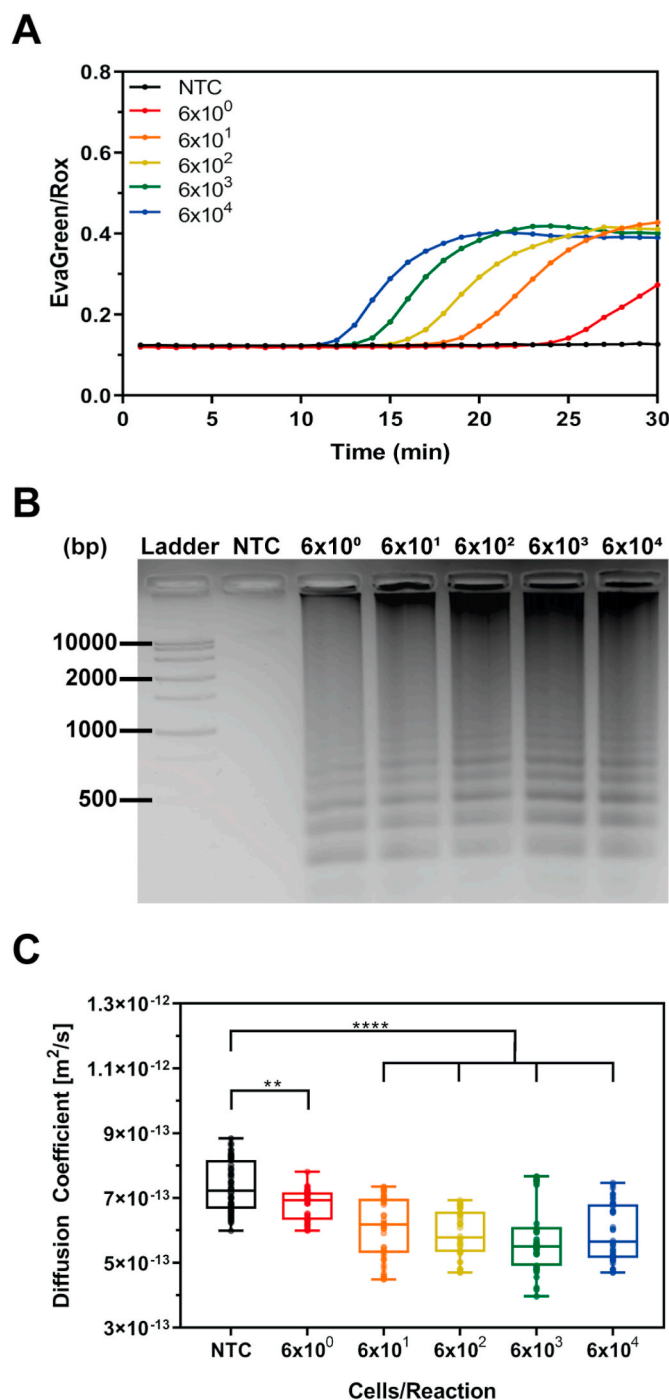


Fig. 4. LOD of *V. cholerae* in pond water. *V. cholerae* cells were spiked into pond water at concentrations ranging from $6 \times 10^0 - 6 \times 10^4$ cells/reaction. (A) Real-time fluorescence was monitored over a 30-minute LAMP reaction. (B) A 2% agarose gel confirms amplification and presents the banding pattern indicative of LAMP amplification for positive samples. (C) The diffusion coefficient measurements show a decreasing trend as a function of starting cell concentration with significance at 6×10^0 (** $p < 0.01$), 6×10^1 , 6×10^2 , 6×10^3 , and 6×10^4 (**** $p < 0.0001$) cells/reaction. Statistical analysis was a one-way ANOVA with Dunnett's post-hoc relative to NTC ($N = 5$, $n = 9$).

much lower diffusion coefficient than the off-target samples (Fig. 5B, p-value < 0.0001). This illustrates that off-target bacteria that may be present in a water sample will not interfere with diffusion coefficient measurements. Moreover, incorporating six users in the study design speaks to the repeatability of smartphone-based PD measurements.

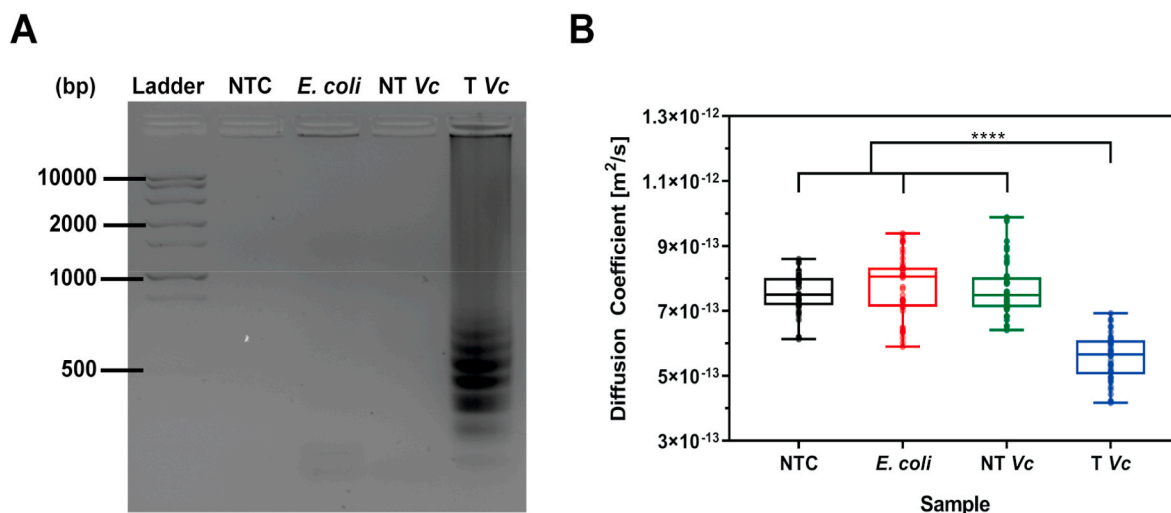


Fig. 5. Single-blinded selectivity in pond water. *E. coli*, non-toxicogenic (NT) *V. cholerae*, and toxicogenic (T) *V. cholerae* cells were spiked into pond water at 10^3 cells/reaction. (A) A 2% agarose gel confirms amplification and presents the banding pattern indicative of LAMP amplification only for toxicogenic *V. cholerae*. (B) The diffusion coefficient measurements show a statistically significant difference between NTC, *E. coli*, and non-toxicogenic *V. cholerae* (**** $p < 0.0001$) when compared to toxicogenic *V. cholerae*. Statistical analysis was a one-way ANOVA with Dunnett's post-hoc relative to toxicogenic *V. cholerae* ($N = 5$, $n = 9$).

3.5. Double-blinded study in pond water

To determine the sensitivity, specificity, and accuracy of the smartphone-based PD platform, we directly compared it to real-time fluorescence detection of LAMP amplicons. The data were split into a training and test set. The training set ($n = 30$) was used to determine the positive and negative diffusion coefficient thresholds. Using the PD (Fig. S4) and real-time fluorescence (Table S8) data collected for the training set, we determined that test set samples with an average diffusion coefficient less than $7.0 \times 10^{-13} \text{ m}^2/\text{s}$ would be considered positive for *V. cholerae* while samples with an average diffusion coefficient greater than $7.2 \times 10^{-13} \text{ m}^2/\text{s}$ would be considered negative for *V. cholerae*. From the training set and other preliminary testing on the smartphone-based PD platform, we realized that there was a small range of diffusion coefficients, from $7.0 \times 10^{-13} \text{ m}^2/\text{s}$ to $7.2 \times 10^{-13} \text{ m}^2/\text{s}$, for which results were inconclusive.

The test set was validated using a double-blinded study design in which all 132 samples (varying concentrations of *V. cholerae*) were unknown to all individuals performing LAMP and collecting smartphone-based PD data. We included multiple users in this study; three users performed LAMP and three different users conducted measurements on the smartphone-based PD platform. The frequency of test set samples plotted in a histogram over a range of averaged diffusion coefficients follows a bimodal Gaussian distribution (Fig. 6A). Using the thresholds identified from the training set, we determined that 59 samples were positive when measured by the smartphone-based PD platform while 65 were considered negative. PD measurements for 8 of the 132 samples (6.1%) fell into the inconclusive range (Fig. 6A). The percentage of samples determined to be inconclusive was similar to clinical trial results of several FDA-approved devices that also have a pre-determined inconclusive range (Alere, 2015). As we analyze more samples on the smartphone-based PD platform, we expect to narrow or even eliminate this inconclusive range.

To directly compare the smartphone-based PD platform and real-time fluorescence measurements, we constructed a 2×2 contingency table, seen in Fig. 6B. Overall, there were 56 true positives (TP), 3 false positives (FP), 5 false negatives (FN), and 60 true negatives (TN). The data indicates that false negatives are slightly more common than false positives using smartphone-based PD. Both false negatives and false positives can be mitigated in the future by altering the thresholds or

improving the optics within the smartphone-based PD platform. From the true and false negatives and positives, we calculated a sensitivity of 91.8% and a specificity of 95.2% for the smartphone-based PD platform (NCCLS, 1995). For comparison, the commercialized Crystal Vc Rapid Diagnostic Test for detection of *V. cholerae* in stool has a published sensitivity of 93.1% but a specificity of only 49.2% (Ley et al., 2012). The characterization of the smartphone-based PD platform demonstrates its ability to sensitively and specifically detect low concentrations of *V. cholerae* found in the environment.

Lastly, we created an ROC curve, as seen in Fig. 6C, to further evaluate the sensitivity and specificity of the smartphone-based PD platform when compared to real-time fluorescence data. ROC curves depict the tradeoff between sensitivity and specificity of a device. In the case of this smartphone-based PD platform, both sensitivity and specificity are high indicating that there is not a significant tradeoff. The area under the curve (AUC) is 0.943, or 94.3%, which represents the accuracy of the platform (Zhou et al., 2011). Others have demonstrated comparable ROC curve analyses for smartphone platforms (Lee et al., 2019; Yeo et al., 2016). Combined, these results highlight the excellent sensitivity, specificity, and accuracy of the smartphone-based PD platform for measuring LAMP amplicons.

4. Conclusions

This portable and compact optical system combines an external ball lens, iPhone 6 camera lens system, blue laser diode, and high-pass filter. The optical system produces a 68X magnification, sufficient for imaging the Brownian motion of 400 nm streptavidin-coated fluorescent nanoparticles bound to biotin-labeled amplification products. The smartphone application efficiently processes the recorded images and computes a diffusion coefficient for each sample that indicates the presence or absence of pathogens.

Smartphone-based PD is incredibly sensitive, detecting as few as 6 *V. cholerae* cells/reaction (0.66 aM) in pond water in just 35 minutes. The LOD of *V. cholerae* in pond water using smartphone-based PD is equivalent to the microscope-based PD measurements presented in our previous work. Smartphone-based PD selectively identified toxicogenic *V. cholerae* while off-target waterborne pathogens such as *E. coli* and non-toxicogenic *V. cholerae* did not interfere with measurements. When directly compared to real-time fluorescence detection of LAMP

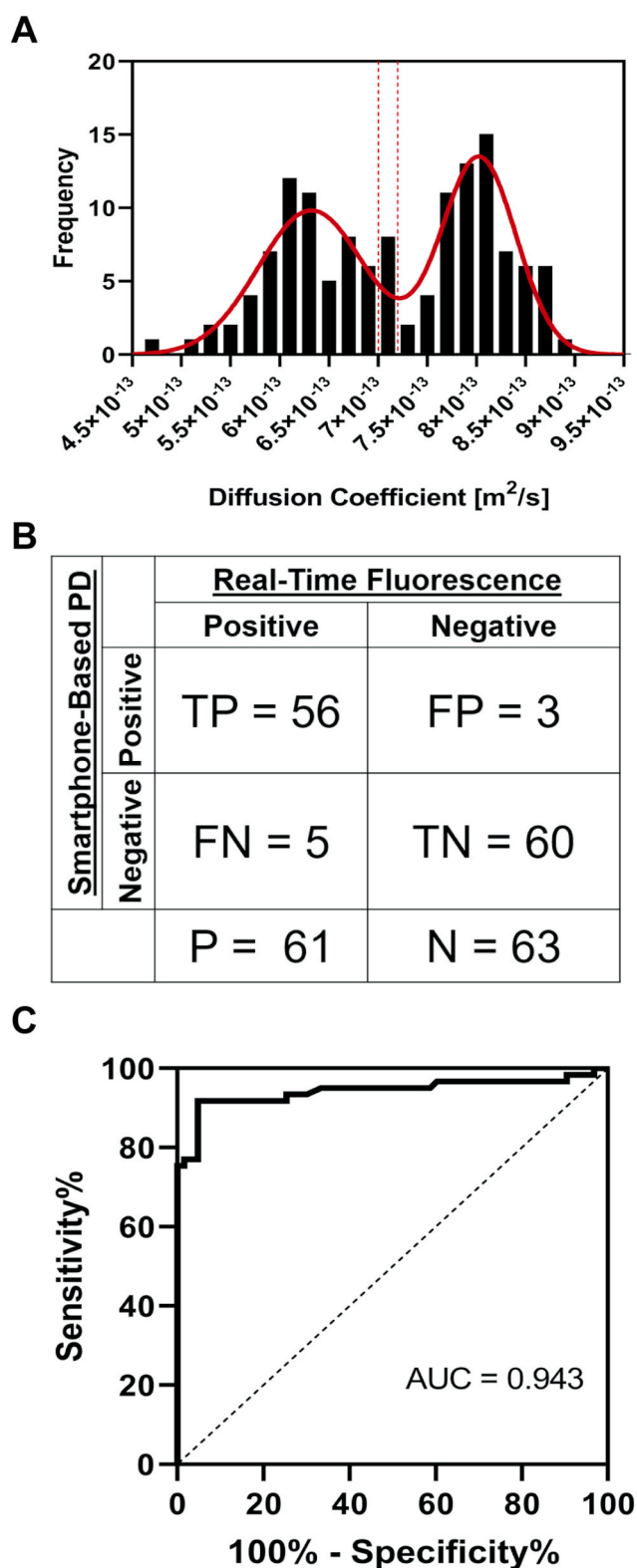


Fig. 6. Characterization of the smartphone-based PD platform. (A) Distribution of averaged diffusion coefficients for 132 samples in the double-blinded study. The red solid line demonstrates that this data follows a bimodal Gaussian distribution while the red dashed lines outline the inconclusive range. (B) 2×2 contingency table used to calculate sensitivity and specificity that is confirmed by (C) the ROC curve with an area under the curve (AUC) of 0.943. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

amplicons in a double-blinded study, smartphone-based PD had an overall sensitivity of 91.8%, 95.2% specificity, and accuracy of 94.3%. Furthermore, this portable platform requires only \$0.78 of consumables, making it affordable for low-resource areas. Altogether, these results demonstrate that smartphone-based PD is accurate, sensitive, and robust for detection of *V. cholerae* LAMP amplicons in pond water.

This handheld smartphone-based PD biosensor can serve as a platform for detection of other pathogens at the point of use. By simply modifying the LAMP primers to target a new gene and implementing sample preparation as needed, this platform could be used for detection of other bacteria, viruses, and even parasites from complex sample matrices. Future development includes incorporating a heating unit to perform the LAMP assay within the smartphone-based PD platform, programming the smartphone application to record geographical information for each test, and including sample preparation within the microfluidic chip for detection of bloodborne pathogens. Further, a sensor for monitoring the temperature variance in the surrounding environment would be beneficial. A fully integrated smartphone-based PD-LAMP platform will enable rapid sample-to-answer detection of pathogens at the point of use.

CRediT authorship contribution statement

Taylor J. Moehling: Methodology, Investigation, Validation, Formal analysis, Project administration, Visualization, Writing - original draft, Writing - review & editing. **Dong Hoon Lee:** Methodology, Investigation, Validation, Formal analysis, Software, Visualization, Writing - original draft, Writing - review & editing. **Meghan E. Henderson:** Investigation, Validation, Writing - review & editing. **Mariah K. McDonald:** Investigation, Validation, Writing - review & editing. **Preston H. Tsang:** Investigation, Validation, Writing - review & editing. **Seba Kaakeh:** Investigation, Validation, Writing - review & editing. **Eugene S. Kim:** Investigation, Writing - review & editing. **Steven T. Wereley:** Software, Supervision, Writing - review & editing. **Tamara L. Kinzer-Ursem:** Methodology, Supervision, Writing - review & editing. **Katherine N. Clayton:** Funding acquisition, Conceptualization, Methodology, Formal analysis, Software, Supervision, Writing - original draft, Writing - review & editing. **Jacqueline C. Linnes:** Conceptualization, Methodology, Formal analysis, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Steven T. Wereley, Tamara L. Kinzer-Ursem, Katherine N. Clayton, and Jacqueline C. Linnes are co-founders of OmniVis, LLC, a spinout company of Purdue University to translate the smartphone PD-LAMP technology. Dr. Clayton is presently the CEO of OmniVis, LLC.

Acknowledgements

We would like to thank Rivu Ghosh and Ethan Pollack for their help making microfluidic chips for these experiments and Elizabeth Phillips for her assistance in preparing samples for the double-blinded study. Further, we want to recognize Melinda Lake for providing feedback throughout the review process. We would like to acknowledge the NSF SBIR Phase I Award #1819970, Vodafone Americas Foundation Wireless Innovation Project Award, NIH NIBIB Award #R61AI140474, Purdue University Shah Family Global Innovation Lab, and the Purdue Research Foundation Trask Innovation Fund Award for their gracious funding to perform this work. Finally, the authors would like to thank Dr. Afsar Ali from the Department of Environmental and Global Health at the University of Florida for providing the *V. cholerae* bacterial strains.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bios.2020.112497>.

References

- Adrian, R.J., Yao, C.-S., 1985. *Appl. Optic.* 24, 44. <https://doi.org/10.1364/AO.24.000044>.
- Aguiñe-Pablo, A.A., Alarfaj, M.K., Li, E.Q., Hernández-Sánchez, J.F., Thoroddsen, S.T., 2017. *Nat. Sci. Reports* 7, 3714. <https://doi.org/10.1038/s41598-017-03722-9>.
- Alam, M.T., Weppelmann, T.A., Longini, I., De Rochars, V.M.B., Morris, J.G., Ali, A., Rashid, M., 2015. *PLoS One* 10, e0124098. <https://doi.org/10.1371/journal.pone.0124098>.
- Alam, M.T., Weppelmann, T.A., Weber, C.D., Johnson, J.A., Rashid, M.H., Birch, C.S., Brumback, B.A., Beau de Rochars, V.E.M., Morris, J.G., Ali, A., 2014. *Emerg. Infect. Dis.* 20, 356–363. <https://doi.org/10.3201/eid2003.131293>.
- Alere, 2015.
- Apple, 2019. https://support.apple.com/kb/SP705?viewlocale=en_US&locale=ko_KR (accessed 6.29.2019).
- Baratloo, A., Hosseini, M., Negida, A., El Ashal, G., 2015. *SBMU* 48–49.
- Barnes, L., Heithoff, D.M., Mahan, S.P., Fox, G.N., Zambrano, A., Choe, J., Fitzgibbons, L. N., Marth, J.D., Fried, J.C., Soh, H.T., Mahan, M.J., 2018. *EBioMedicine* 36, 73–82. <https://doi.org/10.1016/j.ebiom.2018.09.001>.
- Besuschio, S.A., nica Llano Murcia, M., Benatar, A.F., Monnerat, S., Cruz, I., Picado, A., de los, M.A., Curto, N., Kubota, Y., Wehrendt, D.P., Pavia, P., Mori, Y., Puerta, C., Ndung, J.M., Schijman, A.G., 2017. *PLoS Negl. Trop. Dis.* 11 <https://doi.org/10.1371/journal.pntd.0005779>.
- Cabral, J.P.S., 2010. *Int. J. Environ. Res. Publ. Health* 7, 3657–3703. <https://doi.org/10.3390/ijerph7103657>.
- Centers for Disease Control and Prevention, 2018. <https://www.cdc.gov/cholera/infection-sources.html> (accessed 10.18.2019).
- Chuang, H.-S., Sie, Y.-S., 2014. *Microfluid. Nanofluidics* 16, 65–72.
- Clayton, K.N., Berglund, G.D., Linnes, J.C., Kinzer-Ursem, T.L., Wereley, S.T., 2017a. *Anal. Chem.* 89, 13334–13341.
- Clayton, K.N., Lee, D., Wereley, S.T., Kinzer-Ursem, T.L., 2017b. *Lab Chip* 17, 4148–4159. <https://doi.org/10.1039/C7LC00507E>.
- Clayton, K.N., Moehling, T.J., Lee, D.H., Wereley, S.T., Linnes, J.C., Kinzer-Ursem, T.L., 2019. *Nat. Sci. Reports* 9, 1739. <https://doi.org/10.1038/s41598-018-38056-7>.
- Clayton, K.N., Salameh, J.W., Wereley, S.T., Kinzer-Ursem, T.L., 2016. *Biomicrofluidics* 10, 1–15. <https://doi.org/10.1063/1.4962992>.
- Cybulski, J.S., Clements, J., Prakash, M., 2014. *PLoS One* 9, e98781. <https://doi.org/10.1371/journal.pone.0098781>.
- Damhorst, G.L., Duarte-Guevara, C., Chen, W., Ghonge, T., Cunningham, B.T., Bashir, R., 2015. *Eng. (Beijing, China)* 1, 324–335. <https://doi.org/10.15302/J-ENG-2015072>.
- Duarte, C., Salm, E., Dorvel, B., Reddy, B., Bashir, R., 2013. *Biomed. Microdevices* 15, 821–830. <https://doi.org/10.1007/s10544-013-9769-5>.
- Einstein, A., 1905. *Ann. Phys.* 17, 549. <https://doi.org/10.1002/andp.19053220607>.
- Global Task Force on Cholera Control, 2017.
- Hajian-Tilaki, K., 2013. *Casp. J. Intern. Med.* 4, 627–635.
- Hernandez-Neuta, I., Neumann, F., Brightmeyer, J., Ba Tis, T., Madaboosi, N., Wei, Q., Ozcan, A., Nilsson, M., Tis, B.T., 2019. *J. Intern. Med.* 285, 19–39. <https://doi.org/10.1111/joim.12820>.
- Ishii, S., Sadowsky, M.J., 2008. *Microb. Environ.* 23, 101–108. <https://doi.org/10.1264/jsm.2.23.101>.
- Jiang, L., Mancuso, M., Lu, Z., Akar, G., Cesarman, E., Erickson, D., 2014. *Nat. Sci. Reports* 4. <https://doi.org/10.1038/srep04137>.
- Kong, J.E., Wei, Q., Tseng, D., Zhang, J., Pan, E., Lewinski, M., Garner, O.B., Ozcan, A., DiCarlo, D., 2017. *ACS Nano* 11, 2934–2943. <https://doi.org/10.1021/acsnano.6b08274>.
- Koydemir, H.C., Gorocs, Z., Tseng, D., Cortazar, B., Feng, S., Yan, R., Chan, L., Burbano, J., Mcleod, E., Ozcan, A., 2015. *Lab Chip* 15, 1284–1293. <https://doi.org/10.1039/c4lc01358a>.
- Lee, D., Chou, W.P., Yeh, S.H., Chen, P.J., Chen, P.H., 2011. *Biosens. Bioelectron.* 26, 4349–4354. <https://doi.org/10.1016/j.bios.2011.04.036>.
- Lee, W.-I., Park, Y., Park, J., Shrivastava, S., Son, Y.-M., Choi, H.-J., Lee, J., Jeon, B., Lee, H., Lee, N.-E., 2019. *Lab Chip* 19, 1502–1511. <https://doi.org/10.1039/C8LC01344F>.
- Ley, B., Khatib, A.M., Thiemer, K., von Seidlein, L., Deen, J., Mukhopadhyay, A., Chang, N.-Y., Hashim, R., Schmied, W., Busch, C.J.-L., Reyburn, R., Wierzb, T., Clemens, J.D., Wilfing, H., Enwere, G., Aguado, T., Jiddawi, M.S., Sack, D., Ali, S.M., 2012. *PLoS One* 7. <https://doi.org/10.1371/journal.pone.0036930>.
- Lu, L., Sick, V., 2013. *J. Vis. Exp.* <https://doi.org/10.3791/50559>.
- NCCLS, 1995.
- Nliwasa, M., Macpherson, P., Chisala, P., Kamdolozi, M., Khundi, M., Kaswaswa, K., Mwapasa, M., Msefula, C., Sohn, H., Flach, C., Corbett, E.L., 2016. *PLoS One* 11. <https://doi.org/10.1371/journal.pone.0155101>.
- Okada, K., Chantaroj, S., Taniguchi, T., Suzuki, Y., Roobthaisong, A., Puiprom, O., Honda, T., Sawanpanyalert, P., 2010. *Diagn. Microbiol. Infect. Dis.* 66, 135–139. <https://doi.org/10.1016/j.diagmicrobio.2009.09.004>.
- Olsen, M.G., Adrian, R.J., 2000. *Exp. Fluid* 29, S166–S174. <https://doi.org/10.1007/s003480070018>.
- Palum, R., 2001. *Society for Imaging Science and Technology*, pp. 239–245.
- Phillips, E.A., Moehling, T.J., Bhadra, S., Ellington, A.D., Linnes, J.C., 2018. *Anal. Chem.* 90, 6580–6586. <https://doi.org/10.1021/acs.analchem.8b00269>.
- Phillips, E.A., Moehling, T.J., Ejendal, K.F.K., Hoilett, O.S., Byers, K.M., Basing, L.A., Jankowski, L.A., Bennett, J.B., Lin, L.-K., Stanciu, L.A., Linnes, J.C., 2019. *Lab Chip* 19, 3375–3386. <https://doi.org/10.1039/c9lc00506d>.
- Ramírez-Castillo, F.Y., Loera-Muro, A., Jacques, M., Garneau, P., Avelar-González, F.J., Harel, J., Guerrero-Barrera, A.L., 2015. *Pathogens* 4, 307–334. <https://doi.org/10.3390/pathogens4020307>.
- Reid, J., Klose, K.E., 2002. *FEMS Microbiol. Rev.* 26, 125–139. <https://doi.org/10.1111/j.1574-6976.2002.tb00605.x>.
- Riley, M.R., Gerba, C.P., Elimelech, M., 2011. *J. Biol. Eng.* 5 <https://doi.org/10.1186/1754-1611-5-2>.
- Rolando, J.C., Jue, E., Schoepp, N.G., Ismagilov, R.F., 2018. *Anal. Chem.* 91, 1034–1042. <https://doi.org/10.1021/acs.analchem.8b04324>.
- Rosco, 2001. <https://us.rosco.com/en/mycolor> (accessed 7.27.2019).
- Steinhubl, S.R., Muse, E.D., Topol, E.J., 2015. *Sci. Transl. Med.* 7, 283–286. <https://doi.org/10.1126/scitranslmed.aaa3487>.
- Wei, Q., Qi, H., Luo, W., Tseng, D., Jung Ki, S., Wan, Z., Göröcs, Z., Bentolila, L.A., Wu, T.-T., Sun, R., Ozcan, A., 2013. *ACS Nano* 7, 9147–9155. <https://doi.org/10.1021/nn4037706>.
- World Health Organization, 2018.
- Wu, T.-F., Chen, Y.-C., Wang, W.-C., Kucknoor, A.S., Lin, C.-J., Lo, Y.-H., Yao, C.-W., Lian, I., 2017. *Sensors* 17. <https://doi.org/10.3390/s17061348>.
- Yeo, S.-J., Choi, K., Cuc, B.T., Hong, N.N., Bao, D.T., Ngoc, N.M., Le, M.Q., Hang, N.L.K., Thach, N.C., Mallik, S.K., Kim, H.S., Chong, C.-K., Choi, H.S., Sung, H.W., Yu, K., Park, H., 2016. *Theranostics* 6, 231–242. <https://doi.org/10.7150/thno.14023>.
- Zanolli, L.M., Spoto, G., 2013. *Biosensors* 3, 18–43. <https://doi.org/10.3390/bios3010018>.
- Zeng, F., Mou, T., Zhang, C., Huang, X., Wang, B., Ma, X., Guo, J., 2018. *Analyst* 144, 137–142. <https://doi.org/10.1039/c8an01901k>.
- Zhang, D., Liu, Q., 2016. *Biosens. Bioelectron.* 75, 273–284. <https://doi.org/10.1016/j.bios.2015.08.037>.
- Zhou, X.-H., McClish, D.K., Obuchowski, N.A., 2011. *Electronic Book Collection*. Wiley.