Microfluidic multi-angle laser scattering system for rapid and label-free detection of waterborne parasites

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Abstract: A microfluidic laser scattering system for rapid and label-free detection of single waterborne parasites in microfluidic flows was designed, fabricated and demonstrated. The key novelty of the system lies in the integration of functional modules involving pre-concentration, on-chip laser scattering detection, and pattern recognition. The silicon-based pre-concentration chip can concentrate 10 ml reagent water sample spiked with protozoa (oo)cysts into a volume of 200 μl in ~30 minutes. The concentrated sample is further channeled into the on-chip laser scattering detection module at a flow rate of 10 μl/min, which can collect the multi-angle scattering pattern of single flowing microparticles. The Zernike moment features of scattering patterns are extracted using principal component analysis, and classification of scattering patterns are performed using the linear discriminator analysis algorithm. As a result, Cryptosporidium parvum oocysts and Giardia lamblia cysts spiked in ~10 ml reagent water can be enumerated and identified within an hour without labeling, with a mean recovery efficiency of ~73% and average accuracies of 96%, 97%, 97% and 98% at concentrations of 10, 50, 100, 300 (oo)cysts per 10 ml water, respectively. We believe that this compact microfluidic laser scattering system has potential for rapid and label-free water quality monitoring in field and resource-limited environments.

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OCIS codes: (290.5850) Scattering, particles; (100.5010) Pattern recognition; (280.1415) Biological sensing and sensors
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1. Introduction

A safe supply of water, which is fundamental to public health, presents challenges for improving purification processes while accelerating water quality monitoring technologies. The challenges include proliferation of contamination events and increasing demands by consumers for high quality water.

Health-affected microbiological contaminants in drinking water may cause a fatal outbreak among people, especially in densely-populated cities. One main source of microbiological contaminants in water is protozoan parasite, among which Cryptosporidium parvum (C. parvum) oocysts and Giardia lamblia (G. lamblia) are two common waterborne parasites with complicated life cycles [1,2]. C. parvum oocysts (4-7 μm diameter) are smooth, thick-walled, spherical or slightly ovoid bodies containing four elongated sporozoites and a cytoplasmic residual body when fully developed. G. lamblia cysts have a characteristic oval shape and measure 11-14 μm in length and 7-10 μm in width, have four nuclei, a retracted cytoplasm, and clearly visible axostyles [3]. The C. parvum oocyst or G. lamblia cyst is protected by an outer shell that allows it to survive outside the body for long periods of time, making it very tolerant to chlorine disinfection.

Cryptosporidium and Giardia infection can occur through the ingestion of dormant microbial cysts in contaminated water and food, or by the fecal-oral route. The infection dose varies from isolate to isolate and from host species to host species. The symptoms of infections in humans or animals typically involve gastrointestinal disturbances, diarrhea, nausea, vomiting, low-grade fever, and even death for kids, elderly and immune-compromised individuals [4]. Waterborne protozoan outbreaks remain the major challenge in the worldwide drinking water supply. In May 1993, an epidemic of intestinal illnesses occurred in the Milwaukee, Wisconsin area resulting in 400,000 people getting sick and 104 deaths. It was caused by cryptosporidium and this unfortunate event brought nationwide attention to Cryptosporidium and Giardia in water supplies. Since then, many waterborne outbreaks of giardiasis and cryptosporidiosis have been reported (MacKenzie et al., 1994; Craun et al., 1998; Rebecca Sunenshine, 2016) [5–7].

The current standard method for protozoan parasite detection in drinking water consists of many tedious laborious steps. Specifically, sample collection, filtration, elution and concentration are performed sequentially, and the sample volume is reduced to ~10 ml. Subsequently, (oo)cysts are further separated from other particulates through immunomagnetic separation (IMS), which is an expensive procedure with considerable losses of (oo)cysts. Following these steps, many detection techniques are developed. Among them, immunofluorescence assay (IFA) is utilized in the most commonly used USEPA 1623 method [8]. Newly alternative detection methods, such as flow cytometry and polymerase chain reaction (PCR) are increasingly being used [9,10]. It is worth mentioning that all aforementioned methods are proved to be sensitive and specific. However, both USEPA 1623 and flow cytometry methods involve boring staining step, and they can be adversely influenced by the presence of autofluorescent algae and antibody cross-activity with other particulates; while PCR is susceptible to environmental inhibitors. Also, all the
aforementioned methods are high-cost and time-consuming, and can only process small sample volumes, making them not feasible for on-site and real-time monitoring and warning. There is a high demand for an alternative method following the concentration step, which provides label-free and rapid detection of waterborne protozoa, so remedial actions can be taken immediately to reduce risk of further infections.

To date, various optical approaches have been demonstrated for non-invasive and label-free detection of waterborne protozoan. C. H. Yang demonstrated a lensless sub-pixel sweeping microscopy (SPSM) technique for automatic and high-throughput imaging and identification of protozoan parasite (oo)cysts [11]. A. Ozcan developed a holographic microscope to image and detect pathogenic protozoan parasites [12]. H. Anis presented the application of coherent anti-Stokes Raman scattering (CARS) microscopy for label-free detection of waterborne pathogens at the single oocyst level [13]. A. K. Bhunia demonstrated a light scattering sensor capable of real-time detection and identification of multiple pathogen colonies without needing a labeling reagent or biochemical processing [14]. However, all the above methods rely on the detection and identification of quiescent species or single (oo)cysts in a small volume of water by acquiring either optical images or scattering signatures. These methods are not capable of providing real-time detection of protozoan parasites in large volumes of water sample.

To address these issues, in this paper, we demonstrate a label-free microfluidic laser scattering system for rapid detection of waterborne parasites. The system collects distinctive multi-angle laser scattering patterns of waterborne parasite spiked in 10 ml drinking water when the parasites pass through the interrogation region in the microfluidic chip, and they can subsequently be identified by classification of scattering patterns of single (oo)cysts using pattern recognition algorithm within an hour. The recovery efficiency and identification accuracy of the system was tested and found to be relatively high. We believe the method will be a significant milestone in water microbiology fields.

2. Materials and methods

2.1 Microfluidic laser scattering system

Figure 1 shows a configuration of the microfluidic multi-angle laser scattering system. The system consists of three key modules including water sample pre-concentration module, on-chip laser scattering detection module and pattern recognition module. In the pre-concentration module, 10 ml reagent water spiked with (oo)cysts is pumped into a pre-concentration chip using a syringe pump (LSP01-2A, LONGER Co., China), where (oo)cysts (P101 and P102C, Waterborne Inc., USA) can be concentrated resulting in a 200 μl water concentrate within tens of minutes. Next, 200 μl water concentrate is further injected into the sample inlet of the sheath-flow based microfluidic detection chip in the on-chip laser scattering detection module. Then, distinctive scattering patterns are collected when each
particle passes through the laser beam at the interrogation region individually. Finally, these patterns are sent into the pattern recognition module, where identification of (oo)cysts is performed by classification of scattering patterns using pattern recognition algorithms.

A schematic diagram of the on-chip laser scattering detection module is shown in Fig. 2. The module includes a delicately-designed laser scattering unit aligned with a sheath-flow based microfluidic chip, and the sample flow inlet of the detection chip connects to the backflow outlet of the pre-concentration chip. Laser light emitted from a fiber-pigtailed laser diode (LP488-SF20, Thorlabs Inc., USA) operating at a wavelength of 488 nm is focused into a tiny spot with diameter of approximately 200 µm at the interrogation region, where target particles are focused and pass sequentially through the laser beam. In the sheath-flow based microfluidic chip, a water sample spiked with (oo)cysts is focused by two flanking sheath flow streams from both sides of the sample flow. As a result, the sample water stream and particles can be confined to a narrow stream. In addition, a fiber is embedded in a groove perpendicular to the sample channel, and the groove and focused sample stream meets at the interrogation region. A photomultiplier tube (PMT) (H10722-20, Hamamatsu Photonics Co., Japan) located at the exit of the fiber is used to detect the side-scattered light. The chip is fixed on a 3D translation stages for accurate positioning of the target focused stream in the focal point of the incident beam.

As individual particles pass sequentially through the laser beam, the forward scattering light from the particle is collected by an aspherical lens L2 (NT67-245, EDMUND OPTICS, USA) and then mapped onto a complementary metal-oxide-semiconductor (CMOS) camera (MT9T031, Aptina, USA), which is triggered by the side scatter signal from the PMT. It’s noted that a home-made beamstop is glued to the center of a glass window (WL11050-C7, Thorlabs Inc., USA) placed in close proximity to the lens L2, and blocks the bright incident beam, which would otherwise saturate or even destroy the camera sensor.

2.2 Fabrication of pre-concentration chip and detection chip

The microfluidic pre-concentration chip was fabricated on a silicon substrate that is bonded with a flat glass, and the chip can sustain high pressure when working at a high flow rate [15]. Specifically, the silicon wafer was patterned by standard photolithography, and the structures were then transferred onto the silicon by deep reactive-ion etching (DRIE) process. Inlets and outlets were drilled on the glass with the help of an alignment equipment, and finally the flat glass was bonded with the structured silicon wafer using thermal fusion method. The PDMS-based microfluidic detection chip was typically fabricated using a soft lithography process. First, lithography was used to fabricate a microfluidic detection chip master composed of a
positive relief of SU-8 resin. Next, a fiber was aligned perpendicularly to the relief pattern, and Polydimethylsiloxane (PDMS) was poured onto the master and the aligned fiber, and the ensemble was cured. Once cured, the PDMS with embedded fiber was then peeled away and holes were pierced for tubing connection. The PDMS replica was permanently sealed with a PDMS-coated glass slide by oxidizing both the replica and the cover in a plasma charge.

2.3 Pattern recognition

The typical scattering patterns of *C. parvum* oocysts or *G. lamblia* cysts have elliptical or circular shapes, and simplistic features like area, perimeter and skewness are not sufficient enough for differentiating these patterns. The use of more complex Zernike moment features, which are efficient tools to quantify the content and boundary characteristics, is a good choice for feature extraction and differentiation of scattering patterns.

A Zernike moment of order *n* with repetition *m* for a digital image function of *f(x, y)* is defined by:

$$Z_{nm} = \frac{n+1}{\Pi} \sum_{x} \sum_{y} f(x,y) V_{nm}^*(r, \theta) dx dy, \quad x^2 + y^2 < 1$$  \hspace{1cm} (1)

where $V_{nm}$ is a polynomial in *r* and *θ* with powers *n* and *m*, *n* is a non-negative integer, [m] is less than or equal to *n* subject to the constraint that *n* - [m] be an even number. *r* is the length of a vector from the origin to an (x, y) pixel, *θ* is the angle between vector *r* and the x axis in the counterclockwise direction, and

$$V_{nm}(r, \theta) = R_{nm}(r)e^{i\theta}$$ \hspace{1cm} (2)

where

$$R_{nm}(r) = \frac{(-1)^m}{s!} \sum_{s=0}^{\lfloor\frac{n-m}{2}\rfloor} \frac{(-1)^s (n-s)!}{\left(\frac{n+m}{2}\right) - s} \left(\frac{n-m}{2}\right) - s!} r^{n-2s}$$ \hspace{1cm} (3)

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**Fig. 3.** Graphical representation of radial zernike polynomials: (a) real part $Z_{2,2}$; (b) magnitude $Z_{2,2}$; (c) real part $Z_{8,2}$; (d) magnitude $Z_{8,2}$. 
The high-resolution scattering patterns were centered in the middle by using zero- and first-order geometrical moments for finding the centroid. After performing adaptive histogram equalization, the magnitudes of Zernike moment features were calculated [16], which includes not only low frequency shape information, but also high frequency details of the patterns. Examples of 2-D radial Zernike polynomials are shown in Fig. 3. Low order zernike moment $Z_{2,2}$ represents low frequency statistical information while higher order zernike moment $Z_{10,2}$ encodes higher frequencies. The larger the n-m difference, the more oscillations are present in the shape.

At the training stage, batches of scattering patterns from each kind of particles were used as the training data set. The resultant 120 features characterizing each pattern were calculated, and feature reduction was performed to select the most distinctive features using principal component analysis (PCA) [17]. At the recognition stage, classification of unknown scattering patterns was performed using linear discriminant analysis (LDA) method [18].

3. Results and discussion

3.1 Performances of pre-concentration chip

Pre-concentration chip was successfully fabricated and tested. The pre-concentration chip (Fig. 4(a)) consists of a sample inlet, a waste outlet, a backflow inlet, a backflow outlet, a filtration chamber, two side channels and microfilters. Microfilters are arranged around the chamber except the inlet side, and the two side channels and chamber are connected through two arrays of filters respectively. Figure 4(b) shows a SEM image of a pillar array with a height of $\sim$80 $\mu$m, and the gap size between the adjacent pillars is $\sim$3 $\mu$m. A large volume of sample flow is continuously injected from the sample inlet into the chamber, where water and the particles smaller than the gap size pass through the microfilters and are collected from the filtration outlet. Meanwhile, particles larger than the gap size are trapped by the filter. Next, a low volume of backflow (reversed flow) is injected from the backflow inlet, and the trapped particles are released from the filters and flow into the detection chip.

Before injection of water samples for analysis, a 0.01% Tween 20 solution is injected into the microfluidic pre-concentration chip and microfluidic detection chip respectively, reducing the number of polystyrene(PS) spheres (Fluoro-Max, Thermo Scientific Inc., USA) and...
(oo)cysts adhering to the walls of the syringe barrel and microfluidic chip. 10 ml reagent water spiked with fluorescent PS spheres with different sizes was first introduced into the pre-concentration chip within ~30 minutes, where the PS spheres were trapped by the filters. Subsequently, 200 μl backflow (reagent water) was injected from the backflow inlet rapidly. Figure 4(c) shows a micrograph of pillars with trapped PS spheres. Figure 4(d) shows a fluorescent image at a moment when the PS spheres were removed from the filters by back-flushing method, and the motion blur in the image arises due to the streaking of the rapidly flowing PS spheres.

Similar tests were repeated 100 times for water sample spiked with *C. parvum* oocysts and *G. lamblia* cysts at concentrations of 10, 50, 100, 300 (oo)cysts per 10 ml water, respectively. The (oo)cysts in the water from the backflow outlet were counted using a microscope, and the recovery efficiencies were calculated (defined as the ratio of (oo)cysts in the backflow outlet to the initial spiked (oo)cysts). The dependency of recovery efficiency on (oo)cysts concentration is shown in Fig. 5. As expected, there is no much difference in the recovery efficiency (65% to 80%) at different concentrations due to the fact that the number of the trapped (oo)cysts is much less than the number of pillars. Furthermore, the recovery efficiency of *G. lamblia* cysts is slightly higher than that of *C. parvum* oocysts due to a much larger size of *G. lamblia* cyst.

3.2 Scattering patterns and performance of the laser scattering detection module and the pattern recognition module

As stated above, the concentrated sample would subsequently flow into the on-chip laser scattering detection module, where the sheath-flow based microfluidic detection chip has a depth of ~200 μm and a focused sample stream width of ~100 μm. The particles suspended in the concentrated sample flow through the interrogation point one at a time due to hydrodynamic focusing mechanism and a relatively small number of particles present in the sample concentrates. A sequence of scattering patterns could be collected when each particle passes through the focused laser beam. It’s noted that only the pattern with the highest average gray intensity of all pixels, in which the measured particle lasts in the center of the beam, is considered for further analysis. The background pattern, in which no particle passes through the beam, may significantly deteriorate the scattering patterns, and it may vary every once in a while. In light of this situation, the background pattern was collected and updated.
every one minute, and it was subtracted from the captured scattering pattern to obtain the effective scattering pattern with significantly improved signal to noise ratio. Without loss of generality, 200 μl backflow spiked with PS microspheres, *C. parvum* oocysts, *G. lamblia* cyst, and *E. coli* at different concentrations (i.e., 10, 50, 100, and 300 per 200μl), were injected into the microfluidic detection chip respectively at a flow rate of 10 μl/min, and on average ~96% of spiked micro-particles were detected (i.e., an scattering pattern sequence was recorded when a particle was detected), with a negligible loss primarily due to adhesion of the particles to the microfluidic detection chip and chip accessories.

The effective scattering patterns of individual PS microspheres with different sizes are shown in Fig. 6(a), Fig. 6(b), Fig. 6(c) and Fig. 6(d). The black disk in the center of the patterns represents the shadow of the beamstop, as stated above. It can be seen that the scattering patterns of PS spheres consists of a bright central disk accompanied with alternate bright and dark bands of decreasing intensity, and PS spheres of larger size produce a scattering pattern with many more orders of rings, and the experimental results matches well with the theoretical predictions based on Lorentz-Mie theory. Figure 6(e), Fig. 6(f), Fig. 6(g), Fig. 6(h) shows the effective scattering patterns of *C. parvum* oocyst, *G. lamblia* cyst, *E. coli*, and impurities (suspended, e.g., clay and sand), respectively. Different particulates generate unique scattering patterns. Pathogenic microbes present distinctive patterns that differ greatly from impurities, which generate highly irregular scattering patterns. Scattering patterns of waterborne parasites (e.g., *C. parvum* and *G. lamblia*) are composed of less regularly alternate dark and bright rings than those of PS microspheres, and the rings of the scattering pattern of *G. lamblia* appears to be elliptical due to the ellipsoidal shape of the cyst.
Approximately 200 scattering patterns from each of the four kinds of particulates (C. parvum oocyst, G. lamblia cyst, E.coli, and unknown impurity), were collected and used for analysis. For each of the four species, we use 80% of the data set (i.e., 160 patterns from each species) for training and validation, and the remaining 20% of the data set (i.e., 40 patterns) for test. In this work, we assessed the identification accuracy via 10-fold cross-validation. Specifically, we trained on all subsets (144 patterns per each species) except one to construct the classifier, and the excluded single subset (16 patterns per each species) was retained as the validation data. The cross-validation process was repeated 10 times, with each of the 10 subsets used exactly once as the validation data. We calculated the Zernike moment features for each pattern, and determined the principal components for these feature vectors. Distributions of the first and 60th order principal components of feature vectors are shown in Fig. 7(a) and Fig. 7(b), respectively. It can be seen that the first principal component is more informative for classification. The first three principal components of some patterns were plotted, and different species were marked by different colors (Fig. 8), which are well separated. The identification results of the optimized classifier are illustrated in Table 1, and the overall cross-validation accuracy is 97.5%. LDA algorithm gives an average classification accuracy of 100% for E.coli, and ~95% for C. parvum oocyst and G. lamblia cyst.
### Table 1. Confusion matrix of classification results

|       | Predicted | Actual | C. parvum | G. lamblia | E. coli | Impurity |
|-------|-----------|--------|-----------|------------|---------|----------|
| C. parvum | 152       | 8      | 0         | 0          |         |          |
| G. lamblia | 8        | 152    | 0         | 0          |         |          |
| E. coli  | 0         | 0      | 160       | 0          |         |          |
| Impurity | 0         | 0      | 0         | 160        |         |          |

3.3 System performance characterization

A graphic user interface with embedded pattern recognition algorithm was developed and integrated with the system, and it can show the number of *C. parvum* oocyst and *G. lamblia* cyst in real time. To explore the system’s performance, we tested flow-cytometer enumerated (oo)cysts spiked water samples, each 10 ml in volume. The tests were based on four different (oo)cyst concentrations (i.e., 10, 50, 100, and 300 per 10 ml) and three different ratios of *C. parvum* oocyst to *G. lamblia* cyst (i.e., 1:9, 1:1, 9:1), and each concentration with each ratio was measured 5 times, with each test verified by a bench-top microscope. Each spiked sample was introduced into the system and went through a sequence of steps in sequence, i.e., pre-concentration (~30 minutes), on-chip laser scattering pattern acquisition and pattern recognition (~20 minutes). As a result, on average a recovery efficiency of ~73% was achieved, which is primarily influenced by the significant loss of (oo)cyst in the pre-concentration chip. The average accuracies of the system, (i.e., accuracy $\bar{CR} = \frac{1}{C} \frac{1}{R} \sum_{r=1}^{C} \sum_{j=1}^{R} \left( TP_{rj} + TN_{rj} \right) / n$, where $C$ and $R$ are the number of concentrations and the number of ratios, respectively; $TP_{rj}$ and $TN_{rj}$ refer to True Positives and True Negatives at $i_{th}$ concentration and $j_{th}$ ratio), were measured as 96%, 97%, 97% and 98% at concentrations of 10, 50, 100, 300 (oo)cysts per 10 ml water, respectively. The performances are quite consistent with those of pre-concentration chip and laser scattering detection module described in Sections 3.1 and 3.2. The results illustrate the success of microfluidic multi-angle laser scattering system to effectively detect and quantify waterborne parasites.

4. Conclusions and discussions

Label-free detection and identification of waterborne parasites in water in a rapid and continuous way offers a valuable defense against widespread illness. In this paper, a microfluidic laser scattering system capable of concentrating large volumes of water sample and acquiring laser scattering pattern of single micrometric sized particles in microfluidic flow conditions is demonstrated. The system enables automated detection and identification of *C. parvum* oocysts and *G. lamblia* cysts in large volumes of water (~10 ml) by classification of scattering patterns using PCA and LDA algorithms. We demonstrate that *C. parvum* oocysts and *G. lamblia* cysts spiked in 10 ml reagent water can be effectively enumerated and identified within an hour without labeling, with a mean recovery efficiency of ~73% and average accuracies of 96%, 97%, 97% and 98% at concentrations of 10, 50, 100, 300 (oo)cysts per 10 ml water, respectively. The present approach can be readily extended to the detection and identification of various bacterium species by decreasing the gap size between the adjacent pillars of pre-concentration chip into ~1µm. Furthermore, we may filter
out uninterested particles with size bigger than that of the concerned one by passing water through commercial filter membranes before injecting it into the pre-concentration chip. We believe the presented method has great potential for label-free detection of bacteria and parasites pathogens in water and food industries.

**Funding**

National Natural Science Foundation of China (NSFC) (61505240); Jiangsu Natural Science Foundation (BK20140393); Jiangsu Industry-Academia-Research Joint Innovation Foundation (BY2014065); Applied Basic Research Programs of Suzhou City (SYG201414); Youth Innovation Promotion Association of CAS (2015258).

**Acknowledgements**

We thank Dr. Lei lei for his discussion in chip manufacturing.

**Disclosures**

The authors declare that there are no conflicts of interest related to this article.