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An optofluidic imaging to measure the biophysical signature of single waterborne bacterium

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Abstract

In this paper, for the first time, an on-chip optofluidic imaging system is innovated to measure the biophysical signature of single waterborne bacterium, including both the refractive index and morphology (size and shape), based on immersion refractometry. The key features of the proposed optofluidic imaging platform include (1) multiple sites for single bacterium trapping, which enable parallel measurements to achieve higher throughput, and (2) chaotic micromixer, which enable efficient refractive index variation of the surrounding medium. In the experiments, the distinctive refractive index of *Echerichia coli*, *Shigella flexneri* and *Vibrio cholera* are measured with a high precision of 5×10^{-3} RIU. The developed optofluidic imaging system has high potential not only for building up a database of waterborne bacterium biophysical signature, but also developing single bacterium detection in treated water that is real-time, label-free and low cost.

Keywords: Immersion refractometry, waterborne, bacteria, optofluidics

Introduction

Water safety is a major concern especially in densely populated cities because the presence of pathogenic microbial in drinking water may cause fatal outbreaks. One of the main sources of pathogenic contaminants in drinking water is bacterial contamination such as *Escherichia coli* (*E. coli*), *Shigella flexneri*, *Vibrio cholera and Salmonella enterica etc.***[1]** These pathogenic bacteria are commonly transmitted through the oral-fecal route and cause gastroenteritis, resulting in the combination of diarrhea, vomiting, abdominal pain and cramping.**[2]** Other diseases include typhoid caused by *Salmonella enterica* and also hepatitis.**[3-4]** The infection is severe and may be fatal for children, elderly and immune-compromised individuals such as HIV positive patients.

Up to now, waterborne bacteriological outbreak prevention remains the major challenge in drinking water supply worldwide. In 1998, municipal drinking water in Alpine, Wyoming, USA was contaminated with *E. coli* O157:H17 and the outbreak caused 157 people to fall sick.**[5]** In 2000, *E. coli* O157:H17 contaminated drinking water in Walkerton, Ontario, Canada which led to 2,300 illness and 7 deaths.**[6]** Another commonly seen pathogenic bacteria species is *Legionella pneumophila*. In 2008, an outbreak due to drinking water contamination occurred in New Jersey, USA. **[7]**. Based on World Health Organization (WHO) and United States Environmental Protection Agency (USEPA), *E. coli* is exploited as the biological indicator for water sample analysis because the presence of *E. coli* in water samples is an indication of fecal pollution and possible presence of enteric pathogens.**[8]** The current standard method used for bacteria detection in drinking water is the USEPA Method 1604.**[9]**

It consists of four major steps: sample collection, preconcentration, bacteria culture and detection. Several technological gaps exist in this method that hinders its effectiveness in preventing bacteria outbreak. For example, this method is not applicable for on-site monitoring because laboratory facilities are essential for bacteria culture and colony inspection. In addition, the processing time of the current method is at least 10 hours. Such prolonged processing time for pathogenic bacteria is ineffective to prevent and increase the threat of bacteriological contamination outbreak on the public. By the time the culture detection results are available, the public may already be exposed to a health threat. Such culture method also does not provide specific identification on the bacterial species unless specific enzyme fluorescence detection technique is used. In recent years, alternative methods have been developed to provide the need for a rapid and accurate waterborne bacteria detection system. For example, Polymerase chain reaction (PCR) is a three step in-vitro technique based on the ability of the DNA polymerase enzyme to copy a strand of the bacteria DNA.**[10]** PCR is specific and faster than Method 1604. However, PCR is subjected to signal interference which limits it from accurately detecting the presence of low concentration of bacteria in water sample. Another method is immunoassay (ELISA method), which uses antigen-antibody reaction detection such as enzyme-linked immunosorbent assay.**[11]** The targeted bacteria are detected when specific antigen proteins in the water sample bind with the corresponding antibodies. A secondary antibody is linked to an enzyme that forms a colored precipitate, and the enzyme-linked fluorescent assay gives off light. Such method is relatively fast; however, it is not quantitative and also with low sensitivity. Most of the molecular

detection methods require water sample collection and delivery to qualified laboratories, which are time-consuming that usually takes around half a day, depending on the reservoir and laboratory accessibilities. Thus, it is not feasible for real-time and on-site detection. To efficiently avoid bacteria outbreak, a highly sensitive, low-cost ad real-time biosensor is essential and necessary.

To realize a label-free and real-time detection technique, an optical method based on the biophysical properties of bacteria may be a potential approach, such as size, shape and refractive index. The morphology of single bacterium can be easily measured under phase contrast or dark field microscopes. On the other hand, refractive index of a biological sample is correlated with the mass density of its internal constituents such as protein concentration, nucleus contents and cellular sub-organelles, which might be a distinctive parameter for different bacterial species.**[12]** Several refractometric techniques have been demonstrated for biological sample measurements, such as digital holographic microscopy,**[13]** surface plasmon technique,**[14]** light scattering measurement**[15]** and optical resonators.**[16-20]** Digital holographic microscopy and light scattering suffer from low precision (10^{-2} RIU), while optical resonators and surface plasmon techniques with adequate precision (10^{-3} – 10^{-4} RIU) are limited by low throughput and long acquisition time.

In this paper, an on-chip optofluidic imaging system is innovated to measure the biophysical signature of single waterborne bacterium that include size, shape and refractive index. The microfluidic chip consists of an array of trapping sites to ensure 50 bacteria being trapped, and extendable to hundreds, simultaneously for measurement. The refractive indices of the bacteria are measured by the null-method

of phase contrast imaging via immersion refractometry.**[21-22]** In this work, three different species of bacteria are investigated, i.e. *E. coli, Shigella flexneri* and *Vibrio cholera*. The novelty of this work is focused on innovating an optofluidic imaging system to measure the biophysical signature of single waterborne bacterium, which is significantly useful for drinking water industry. With the on-chip optofluidic imaging system, the biophysical signature of different bacteria can be measured to build up a database, which has high potential to innovate a real-time and label-free detection technique for waterborne bacteria. The detection of single waterborne bacterium based on the biophysical signature will be a significant milestone in drinking water industry.

Materials and methods

On-chip optofluidic imaging system

The optofluidic imaging system consists of a microfluidic chip to manipulate bacterial samples and vary the liquid medium, combined with a phase-contrast microscope, to realize immersion refractometry. Figure 1(a) shows the schematic illustration of the microfluidic chip with a trapping microchamber and an integrated micromixer. Bacterial-containing water samples are loaded into the microchannel. Several bacteria are trapped in the sample trapping area with an array of trapping sites. Each trapping site has a U-groove structure with a small gap of 500 nm.**[23]** Fig. 1(b) shows the trapping of *E. coli*. With the trapped bacteria, optofluidic immersion refractometry is employed to measure their refractive index by immersing them into a liquid medium and observing the optical contrast between the bacteria and the liquid. When light

passes through a single bacterium in the microfluidic chip, being immersed in a medium with lower refractive index, the light rays slowdown in phase relative to background light passing through the liquid medium. The optical path difference experienced by the light can be expressed as $OPD = (n_{bac} - n_{med}) \cdot t_{bac}$ where t_{bac} is the thickness of the bacteria sample, n_{bac} and n_{med} are the refractive indices of the bacterium and medium, respectively. However, in bright-field microscopy, the brightness contrast associated with the phase delay experienced is limited. In order to increase sensitivity of system in measuring refractive index, the phase is either advanced or retarded by ¼ of a wavelength such that the intensity contrast between the bacterium and the medium will be maximized. Therefore, the phase-contrast microscopy is employed to enhance the brightness contrast caused by the phase delay experienced. When the external medium has a refractive index lower than that of the bacterium ($n_{bac} < n_{med}$), the bacterium appears to be darker as shown in Fig. 2a. Whereas when the external buffer medium has a higher refractive index ($n_{bac} > n_{med}$), the bacterium appears to be brighter as shown in Fig. 2c. Once the refractive index of the buffer medium is equal to that of the bacterium ($n_{bac} = n_{med}$), the bacterium appears to be invisible as shown in Fig. 2b. Hence, this null method can be employed to measure the refractive index of the bacterium. To perform optofluidic immersion refractometry, an integrated chaotic micromixer is used to vary the refractive index of the external medium from low to high.**[24]** Deionized (DI) water and Ficoll solution (Sigma-Aldrich, F4375) with a refractive index of 1.3326 and 1.4651, respectively, are used in the experiment. Hence, the refractive index of the external medium can be tuned from 1.333 up to 1.465 by changing the ratio of the flow rates of DI water and

Ficoll solution.

The sensitivity of the system depends on two criteria: (1) the phase shift detection limit of the phase-contrast microscopy, and (2) the resolution of the commercial liquid refractometer used to measure the refractive index of the medium. For a non-absorbing phase plate, it is possible to achieve a detectable phase change of less than $\lambda/100$ with ease. The wavelength used for the detection of bacteria is 514 nm, while the thickness is approximately 1 μm. Hence, the minimum detectable refractive index difference is approximately 0.005. The phase shift detection limit of the phase-contrast microscopy depends on the light absorption of the phase plate, in which $\lambda/1000$ can be achieved with heavily absorbing phase plates. The sensitivity can be theoretically improved to 0.0005.**[22]** The refractive index of the external medium is measured by using a refractometer (Atago, Pal RI) with a resolution of 10^{-4}, which is sufficiently precise such that it is not the limiting factor in the measurement.

Protocol of bacteria culture and sample preparation

In the experiments, three different species of bacteria are investigated, i.e. *E. coli*, *Shigella flexneri* and *Vibrio cholera*. All three bacterial species are supplied by DSO National Laboratories. For *E. coli*, Luria-Bertani (LB) broth medium is used as the culture medium. LB broth medium is prepared by mixing thoroughly 25g of broth powder (Sigma-Aldrich, L3522) in 1L DI water. On the other hand, nutrient broth medium is used for *Shigella flexneri* and *Vibrio cholera*. Nutrient broth medium is prepared by mixing thoroughly 13g of broth powder (Sigma-Aldrich, 70122) in 1L DI water. Both media are sterilized with a benchtop autoclave machine (Tuttnauer,

2340EKA) at 121°C for 15 min.

The vial with frozen bacteria is removed from the liquid nitrogen freezer and thawed by gentle agitation in a 37°C water bath (YIHDER, BT-150D). The bacteria are thawed until all ice crystals are melted. The vial is removed from the water bath and decontaminated with 70% ethanol solution. Then, the content is transferred to the prepared growth medium (10 mL). Once the bacteria have been fully cultured, 10% of the culture is used in the experiment while the remaining is frozen for future usage by cryopreservation. To freeze the bacteria, 10% glycerol (Invitrogen 15514011) is added to culture medium. Glycerol is prepared at twice the final concentration for freezing and mixed with an equal volume of cell suspension whereby 1 ml of bacterial suspension is aliquoted to each vial and sealed. The bacteria cells are equilibrated in the medium at room temperature for 40 mins. Next, the vials are placed in a temperature rate-controlled freezing chamber and stored in a -70°C freezer (SANYO, MDF-C8V1) for at least 24 hours before being transferred to liquid nitrogen tank (Thermo Scientific, 807). Since bacteria under different growth conditions have different lengths and widths, it is critical to control the environmental conditions before measurements. The bacteria to be used in the experiment are transferred from the culture medium to tap water and incubated for 24 hours before the biophysical measurements are performed to mimic the conditions of bacteria in drinking water.

Experimental setup and image processing analysis

The optofluidic chip is made of polydimethylsiloxane (PDMS) material based on standard soft-lithography techniques (Details in Supplementary Information).**[25]** The

optofluidic chip is placed on an inverted microscope (NIKON, Eclipse Ti) and a 60× objective lens is used to image the trapped bacteria. High precision syringe pumps (New Era Pump Systems Inc, NE-1000) are used to inject bacteria sample solutions, DI water and Ficoll solutions into the chip through the inlets. With the bacteria being trapped in the optofluidic chip, the phase contrast images are captured by using a digital CCD camera (NIKON, DS-Ri1) to measure the biophysical properties of the bacteria. For size and shape measurement, the captured phase contrast images are analyzed by using the image processing software, ImageJ, to measure the length, width and aspect ratio (length/width). For refractive index measurement, phase contrast images under different external media are captured. The intensity contrast between the intracellular and external medium are analyzed by intensity profiling using ImageJ. For better visualization, the phase contrast images (grayscale) are pseudo-colored by using ImageJ with “Phase” color tone.

Experimental results and discussion

In the experiments, a sample size of 250 for each bacteria is measured and statistically characterized as shown in Fig. 3. Both *E. coli* and *Shigella flexneri* have similar morphology whereby they are both rod shape bacteria. According to these results, *E. coli* has a mean length and width of 2.83 and 0.86 μm, respectively, and a mean aspect ratio of 3.87. On the other hand, *Shigella flexneri* has a mean length and width of 2.74 and 0.77 μm, respectively, and a mean aspect ratio of 3.48. *Vibrio cholera* is relatively smaller as compared to the two and has a shape of comma. *Vibrio cholera* has a mean length and width of 1.21 and 0.43 μm, respectively, and a mean aspect ratio of 2.84.

For the measurement of the effective refractive index of the bacteria, the external medium in the microchamber is varied by changing the ratio of flow rate of the DI water and the Ficoll solution. To characterize the mixing efficiency of the chaotic mixer, DI water and fluorescein-premixed Ficoll solution are used, such that the mixing efficiency can be measured by monitoring the fluorescence intensity at the end of the chaotic mixer. By changing the flow rate of the Ficoll solution from 20 to 40 μL/min, the measured fluorescence intensity is increased. Figure S1b shows the fluorescence intensity profiles across the microchannel (dashed line in Fig. S1a) under different flow rate conditions. The intensity profile shows that the liquids are well mixed with a variation lower than 1%, which verifies the accuracy and precision of the immersion refractometry. The refractive index of the external medium as a function of the flow rate ratio ($Q_{\text{ficoll}}/Q_{\text{water}}$) is illustrated in the graph of Fig. S2. When the microchannel is filled with DI water, the refractive index is 1.3326. Subsequently, the refractive index is increased at a rate of 0.029 RIU by increasing the flow rate of the Ficoll inlet. At the point where the flow rate ratio is 2.5 and the refractive index of the external medium is 1.4152, the increment rate is reduced to 0.004 RIU.

The optofluidic immersion refractometer is characterized by using a PDMS (Dow Corning, Sylgard 184) testing structure in a microfluidic chip and observing its phase contrast image when the external medium is changed from 1.410 to 1.414. For each phase-contrast microphoto, the intensity contrasts are determined by dividing the average pixel intensity of the PDMS testing structure with the average pixel intensity of the external medium. The intensity contrast measured under different external

media is illustrated in Fig. 4d. When the external medium has lower refractive index as compared to the one of PDMS, the PDMS testing structure appears to be brighter and protruding. On the other hand, when the external medium has higher refractive index as compared to the one of the PDMS, the PDMS testing structure appears to be darker and concave. There is a phase change between 1.410 and 1.414. Based on the interpolation of the measured data, the PDMS testing structure has a refractive index of 1.412, which matches with previous report.**[26]** It is confirmed by injecting external medium of the same refractive index and the PDMS testing structure has become invisible as shown in the inset of Fig. 4.

Figure 5a shows the pixel intensity analysis for single *E. coli* bacterium by varying the refractive index of the external medium. The intensity contrast is lower than 1 when the refractive index of the bacterium is higher than the one of the external medium, and vice versa. The matching refractive index of 1.388 occurs when the intensity contrast is equal to 1, which is also the effective refractive index of the *E. coli*. The refractive index of *E. coli* is comparable with the value obtained by using light scattering technique (1.390). [27] The measured results of *Shigella flexneri* are shown in Fig. 6b, which has an effective refractive index of 1.422. In addition, *Vibrio cholera* is measured and its effective refractive index is 1.365.

The statistical results of the morphological and effective refractive index measurements (mean ± standard deviation) of the three different species of bacteria are summarized as shown in Table 1. For morphology, *Vibrio cholera* has s relatively smaller size and is comma in shape as compared to *E. coli* and *Shigella flexneri* that are in rod shape. For effective refractive index, Vibrio cholera has the lowest effective

refractive index (1.365) as compared to *E. coli* (1.388) and *Shigella flexneri* (1.422). It is concluded that these three different species of bacteria can be easily differentiated based on their effective refractive index measured at least in the resolution of 5×10^{-3} RIU. Together with the parameters of size and shape, the biophysical signature of bacteria can be used to provide an alternative technique for waterborne bacteria identification in treated water source. Biophysical parameters may be unable to differentiate different strains of *E. coli*; however, it has high potential to identify different species of bacteria and provide a rapid detection of the presence of *E. coli* in water sample, which is an indicator of fecal pollution and possible presence of enteric pathogens, to avoid massive outbreak by quarantining the water source. **[8]** Further analysis can be performed to identify the *E. coli* strain without sacrificing population health.

Conclusions

In this paper, an on-chip optofluidic imaging system is designed, fabricated and experimented. The optofluidic imaging system uses a microfluidic chip for single bacterium trapping, and a phase contrast microscope to realize immersion refractometry. The effective refractive index of a single bacterium is measured by matching the refractive index of the external medium with its effective refractive index, using an on-chip micromixer to mix DI water and Ficoll solution. The size, shape and effective refractive index of *E. coli*, *Shigella flexneri* and *Vibrio cholera* are measured. The results show that *E. coli* and *Shigella flexneri* has similar size and same rod shape, but the effective refractive index of *E. coli* is lower as compared to

the one of *Shigella flexneri*. On the other hand, Vibrio cholera has a smaller size, distinctive comma shape and differentiable effective refractive index value as compared to *E. coli* and *Shigella flexneri*. The optofluidic imaging system based on immersion refractometry can be used not only in water quality monitoring, but it also has high potential applications for pathogenic bacteria detection in food and clinical industries.

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List of Captions

Fig. 1 (a) Schematic illustration of the optofluidic chip for biophysical measurement of single bacterium by using immersion refractometry. Inset shows the trapping structure with a gap of 500 nm. (b) Trapping of *E. coli* in the trapping site (Contrast enhanced and bacteria is colored by image processing).

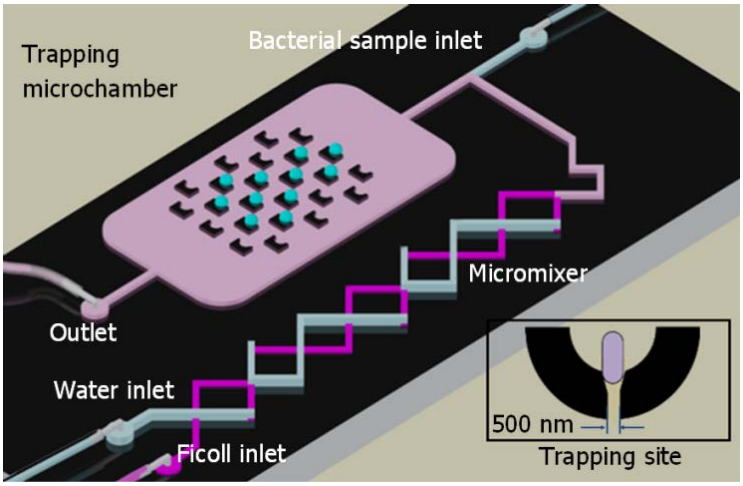
Fig. 2 Working principle of the null method in immersion refractometry: The phase transformation and the phase-contrast microphoto of a single bacterium being immersed in a medium with refractive index (a) lower than, (b) same as, and (c) higher than the one of the bacterium.

Fig. 3 Morphological measurements of (a) *E. coli*, (b) *Shigella flexneri*, and (c) *vibrio cholera*.

Fig. 4 **C**haracterization of the optofluidic immersion refractometer by measuring the refractive index of PDMS testing structure.

Fig. 5 Pixel intensity analysis of (a) *E. coli* and (b) *Shigella flexneri* when the external medium is tuned from low to high. E. coli and Shigella flexneri appear to be invisible when the refractive index of the external medium is 1.388 and 1.422, respectively.

Table 1 Biophysical measurements of three different bacteria (N = 250)



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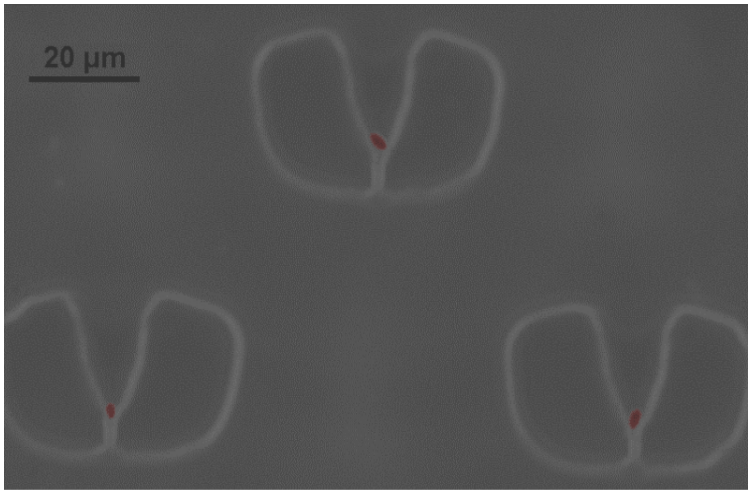


(b)

Fig. 1

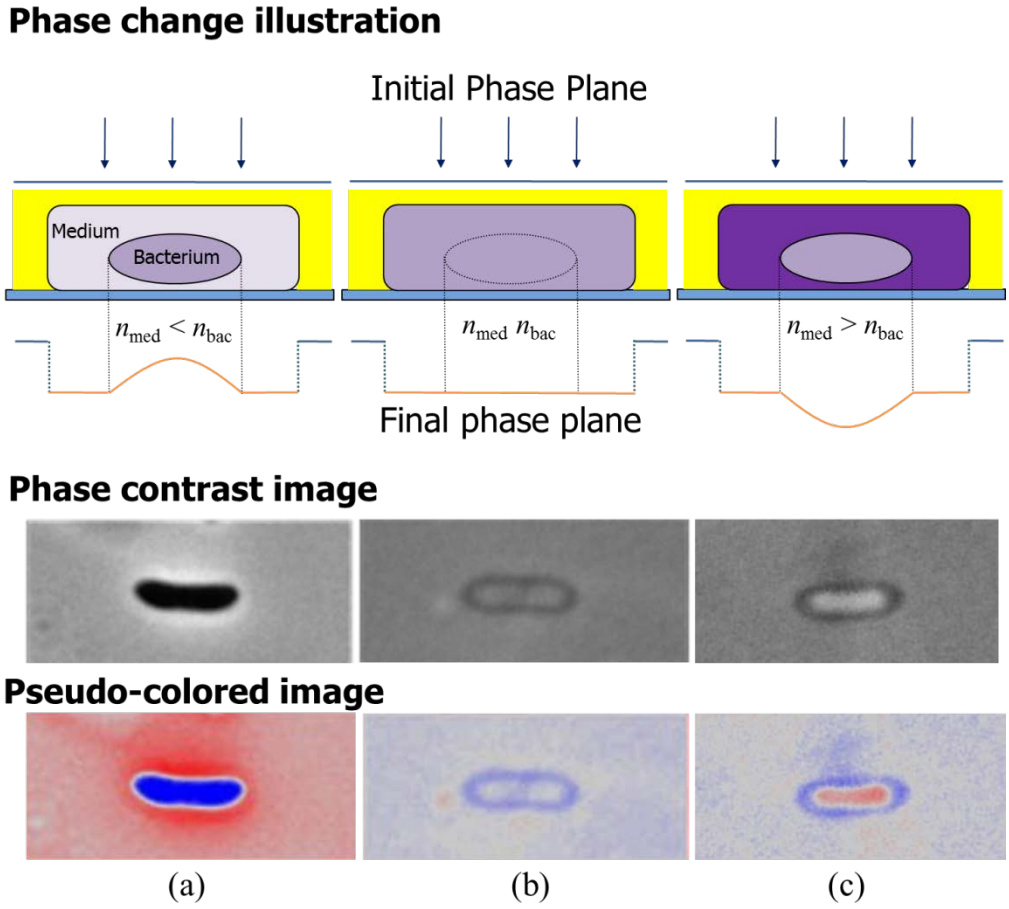


Fig. 2

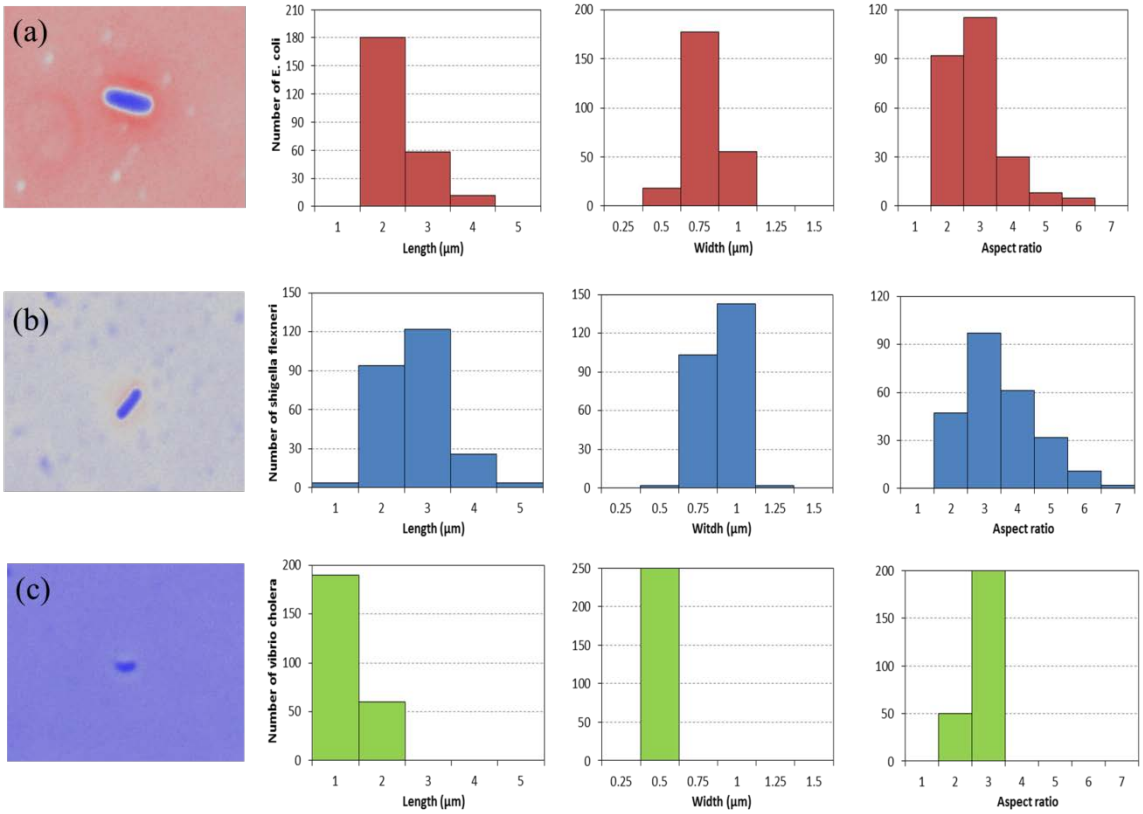


Fig. 3

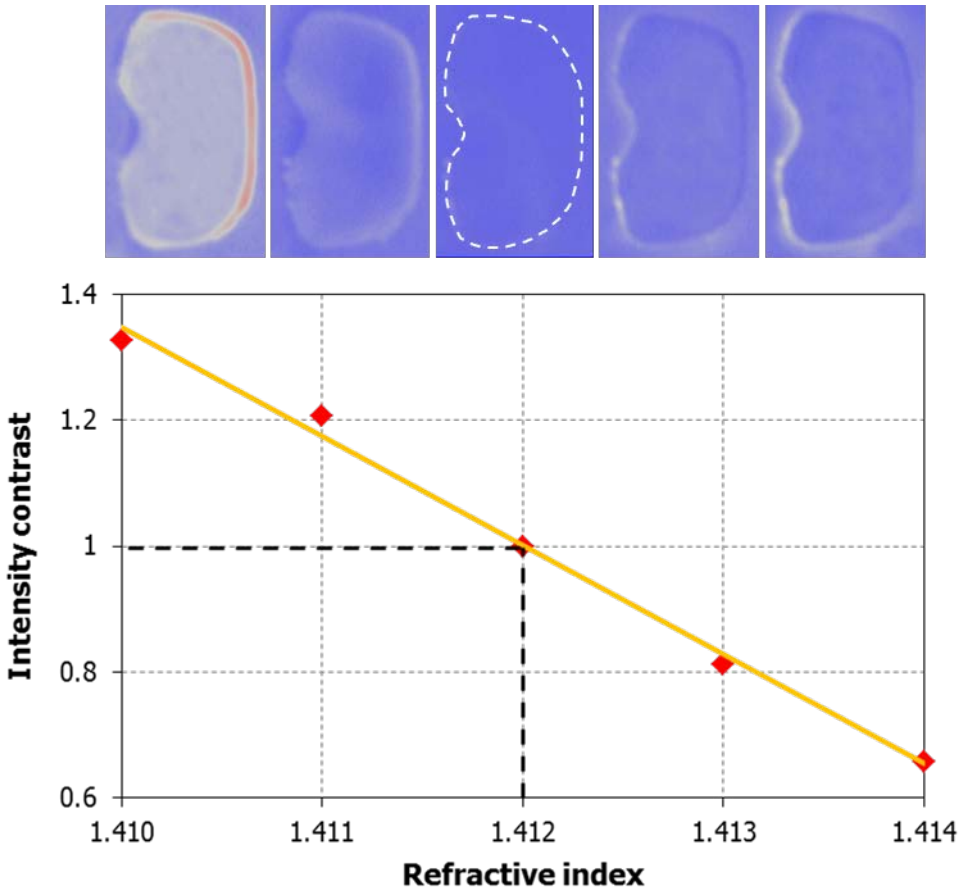
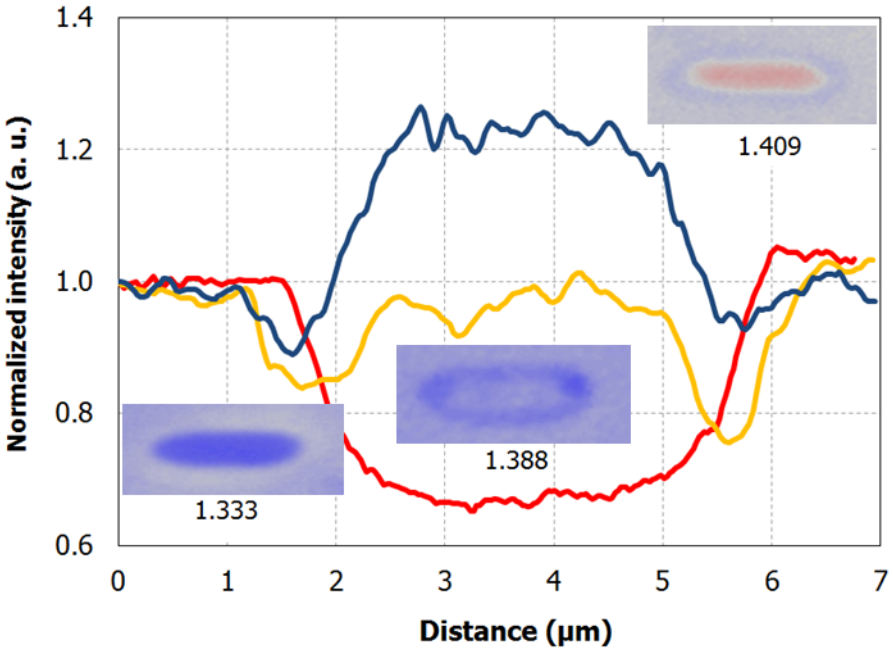


Fig. 4



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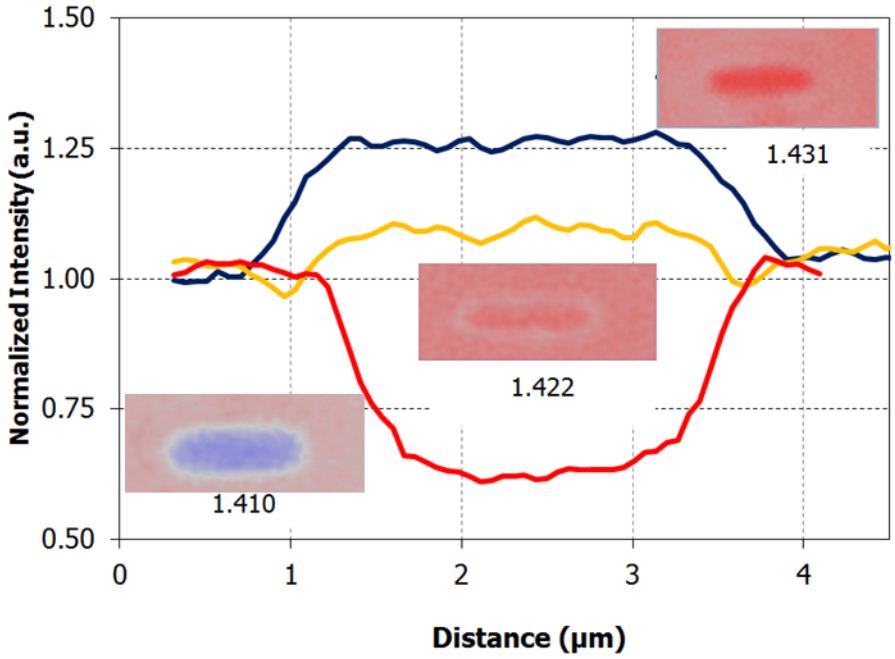

(b)

Fig. 5

Table 1

Bacteria	*E. coli*	*Shigella flexneri*	*Vibrio cholera*
Length (μm)	2.83±0.41	2.74±0.67	1.21±0.35
Diameter (μm)	0.86±0.10	0.77±0.10	0.43±0.09
Aspect Ratio	2.50±0.80	3.48±0.80	2.84±0.80
Refractive Index	1.388±0.005	1.422±0.005	1.365±0.005