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A real-time simultaneous measurement on a microfluidic device for individual bacteria discrimination

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ABSTRACT

Rapid detection of pathogenic bacteria is one of the important social issues for preventing and identifying cause of problems affecting human health. Ionic current sensing through pores has shown the ability to electrically measure bacteria. However, there is an inherent limitation to discriminate chemical characteristics of bacteria for existing ionic current sensing methods Here we propose a real-time simultaneous measurement method, which combines ionic current sensing and fluorescence observation on a microfluidic device. Our method can detect not only the size of individual bacteria passing through a micropore but also observe their stainability based on chemical properties of bacterial cell surface within 300 ms. We succeeded in discriminating each bacterium in a solution mixture including contaminant particles by combining highly accurate ionic current sensing which can detect a size difference of 70 nm, and fluorescence observation which can discriminate internal structures such as those that define bacteria as gram-positive or gram-negative. Our method can be applied to not only on-site bacteria detection but also to screening technology.

1. Introduction

Detection of pathogenic bacteria is one of the important procedures for maintaining health and safety, and therefore, many researchers have devoted their efforts to develop bacteria detection techniques [1–4]. However, since conventional methods still have a problem on analysis time, alternative method for rapid bacteria detection has been required. The ionic current sensing method enables bacteria identification based on its higher accuracy sample size detection capability compared to fluorescence microscopy, and it can be used as a rapid bacteria detection method [5,6]. However, it has remained as a problem that discrimination of bacteria from others having the same size and the different chemical properties is inherently difficult. In contrast, fluorescence microscopy provides information of rough size of bacteria, and stainability of bacterial internal structures which define chemical properties such as gram-positive or gram-negative [7,8]. However, it is difficult to optically distinguish bacteria whose size are slightly different; for example, although drug-resistant Staphylococcus aureus has a slightly thick peptidoglycan layer as compared with the normal one, their diameter difference is too small to be discriminated with a microscope [9]. And, fluorescence microscopy is not suitable for on-site bacteria detection because it requires a high-magnification lens, which is difficult to handle and expensive, for highly accurate identification.

In this study, we devised a way to discriminate bacteria based on the size and the chemical properties by a measurement method which performs ionic current sensing and fluorescence observation simultaneously [10]. In our previous research of ionic current sens-

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ing [11], we succeeded in detecting particles in a long micropore by using a microfluidic bridge circuit which offer a high signal-to-noise (S/N) ratio by increasing signal amplitude. Therefore, we can accept noise increasing due to connection of optical microscope during ionic current sensing, and observe the fluorescence of bacteria passing through the long micropore. We detected the bacterial size by ionic current sensing, and we detected the chemical properties based on the fluorescent stainability of the bacterial cell surface by using a microscope equipped with a low-magnification lens (20×) and a color CCD camera at the same time. As a result, we identified individual bacteria that could not be identified by using only a conventional ionic current sensing method or only a fluorescence observation method.

2. Experiment

2.1. Assembly of components for ionic current sensing and fluorescence observation

Ionic current sensing was performed by using a microfluidic bridge circuit which consists of a microfluidic chip and electric circuits (Fig. 1a). The microfluidic bridge circuit, which had been optimized in our previous study [11], has two types of circuit; the electrophoresis circuit (red line) is applied an electric field for electrophoresis of sample, and the sensing circuit (black line) detects ionic current signals by sample translocation (pink) in the micropore. In a microfluidic chip, channels for ionic current sensing and sample electrophoresis are connected to the micropore (bottom illustration of Fig. 1a). We applied the voltage and sensed the current using silver electrodes (FTVS-408, Oyaide), which were connected to reservoirs of microfluidic chip. Current flowing through the sensing circuit was monitored by an ammeter which consists of an amplifier (low noise current amplifier DLPCA-200, FEMTO), a signal converter (NI USB-6259, National Instruments), and hand-made software made by Lab view (National Instruments). Voltages were applied by batteries (6LR61YXJ/1S, Panasonic). The 1 kΩ resistance element (E-Globaledge Corporation) and variable resistance element (7270, BI Technologies) in sensing circuit were commercially available ones.

Fluorescence observation was done using a fluorescence microscope (Eclipse Ti, Nikon Corp.) which was equipped with a lens of 20×(0.45NA, Nikon Corp.) and a mercury lamp (USH-102D, Ushio Inc.). A EM-CCD camera (ImagEM, Hamamatsu photonics) was used for capturing 160 fps mono color video. A color CCD camera (HV-C20S, Hitachi, Ltd.) was used for capturing 30 fps RGB color video. The microfluidic bridge circuit was put on the microscope stage. A fluorescence filter block (B-2A, Nikon Corp.) which had an excitation filter of 450–490 nm and a fluorescence filter block (UV-1A, Nikon Corp.) which had an excitation filter of 360–370 nm were used for fluorescence observation.

2.2. Fabrication of a microfluidic chip

Using photolithography techniques, we fabricated microchannels on a PDMS plate (SILPOT 184 Dow Corning, Toray Co., Ltd.). The Microfluidic chip was fabricated by bonding a cover glass and the PDMS plate with the microchannels (Fig. 1b). The microchannels were filled with 5 × TBE buffer solution (0.45 M Tris, 0.45 M boric acid, 0.01 M EDTA) which had an electrical conductivity allowing current flow by the applied voltage.

2.3. Preparation of bacteria sample

Escherichia coli (JM109, Takara Bio Inc.) and *Bacillus subtilis* (ATCC6633, Eiken Chemical Co., Ltd.) were cultured in 2.5% LB medium (LB Broth (Miller), Sigma-Aldrich, Co.) for 48 h at 37 °C and diluted by phosphate buffer solution to 10^7 cells/mL. Bacteria were stained by 5 μg/mL FM1-43 (Life Technologies) for 1 h at 4 °C. Critical electric field of bacteria was higher than applied electric field in this paper.[12]

2.4. Preparation of polystyrene particle sample

As samples for confirmation of our method, we used polystyrene particles (E_x max = 441 nm/E_m max = 486 nm, Fluoresbrite Calibration Grade Microspheres, YG, Polysciences, Inc.) with a diameter of 3.10 μm and their concentration was 2×10^6 particles/mL. As samples for measurement of the four kinds of individual particles contained in the solution mixture, we used 0.93 μm and 1.00 μm blue fluorescent particles (E_x max = 360 nm/E_m max = 407 nm, Fluoresbrite Calibration Grade Microspheres, BB, Polysciences, Inc.), 1.00 μm green fluorescent particles (E_x max = 441 nm/E_m max = 486 nm, Fluoresbrite Calibration Grade Microspheres, YG), and 0.99 μm non-fluorescent particles (Polybead Polystyrene Microspheres, Polysciences, Inc.). According to the data sheet, standard deviation of diameter of the 1.00 μm green fluorescent particles was 20 nm. The concentration of each type particle was 4.55×10^7 particles/mL.

3. Results and discussions

3.1. Confirmation of simultaneous ionic current sensing and fluorescence observation

By the simultaneous measurement, we can detect the ionic current signals and the fluorescence by sample particles introduction, and obtain the correlation between the ionic current signal shape and the position of a sample particle around the micropore (Fig. 1c, d). By introducing a 3.10 μm diameter particle into the micropore, we obtained an ionic current signal which has two positive peaks and a negative peak. We defined the gap between the baseline and the minimum current value of the negative peak in the ionic current signal as I_{signal}. Based on our past research [11], the amplitude of I_{signal} reflects the volume of a sample particle. Size detection based on I_{signal} has equivalent accuracy compared with conventional particle sizing methods. Fluorescence observation was performed by using a fluorescence filter block which had an excitation filter of 450–490 nm. The height, width and length of the micropore were 7.5 μm, 4.0 μm and 80 μm, respectively. I_{signal} was 1.94 nA, which agreed with theoretical signal amplitude of 1.87 nA calculated by the following theoretical equation (see supporting information for derivation):

$$I_{\mathrm{signal}} = \frac{\Delta R_M}{2R_S + R_{1-k\Omega} + R_M} \times \frac{V_E}{2R_E + R_M} \quad (1)$$

where I_{signal} is signal amplitude, ΔR_M is resistance change of the micropore by sample introduction, V_E is applied voltage in the electrophoresis circuit, R_S is resistance of sensing channels, $R_{1-k\Omega}$ is resistance of the 1 kΩ resistance element, R_M is resistance of the micropore, and R_E is resistances of the electrophoresis channels. In the theoretical condition of Fig. 1c and d, ΔR_M was 65.8 kΩ, V_{Es} was 12 V, R_S was 8.0 MΩ, R_M was 6.8 MΩ, and R_E was 6.8 MΩ.

Simultaneous measurement revealed that the shape of the ionic current signal reflects the information of the particle around the micropore. To introduce sample particles smoothly, we made narrow guide structures, which are defined as guide-1 and guide-2, before and after the micropore (Upper photo image in Fig. 1c). Based on the series images of translocation of a fluorescent particle, it took 274.56 ms from entering into guid-1 to exiting from guid-2, and this time was almost consistent with interval of the positive signal peaks of 285 ms. As Siwy et al., said, it can be considered that these positive signal peaks are signals of ion desorption and adsorption

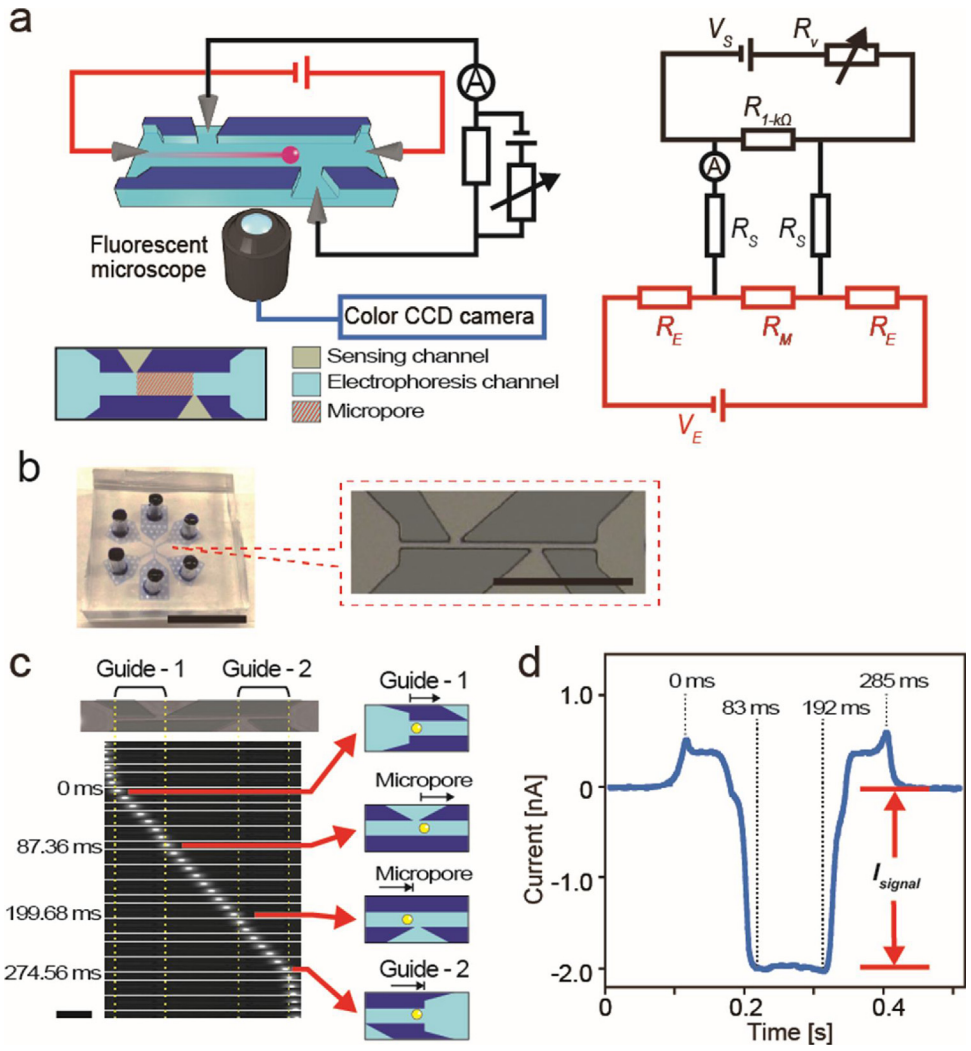

Fig. 1. Explanation of the simultaneous ionic current sensing and fluorescence observation method. (a) Schematic illustration and circuit diagram. The microfluidic bridge circuit for ionic current sensing has two circuits. The electrophoresis circuit (red line) applies an electric field for electrophoresis of sample. The sensing circuit (black line) detects ionic current signals of sample translocation (pink) in the micropore. The fluorescence microscope is set under the microfluidic bridge circuit. Bottom illustration shows roles of channels around the micropore. (b) Images of the microfluidic chip. The upper one shows the optically transparent microchip; scale bar, 1 cm. The lower one is an optical microscope image taken around the micropore; scale bar, 100 μm. (c) Fluorescence series images showing translocation of a 3.10 μm polystyrene particle in the micropore; scale bar, 30 μm. Frame rate was 160 fps. Four images with time marked represents entering and exiting of the fluorescent particle in the guide structure or the micropore. (d) Ionic current signal at the time of Fig. 1c. I_{signal} was defined as the difference between the baseline and the minimum current value of negative peak in the ionic current signal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

on the surface of the particles at the entrance and the exit of a narrow structure [13]. Thus, it was confirmed that the interval of the positive signal peaks means the particle's translocation time in the narrow structure. Similarly, translocation time of the particle passing through the micropore of 112.32 ms was almost consistent with the time width of the negative peak of 109 ms. The slight difference in time is due to moving of the particle within imaging time of one frame of 6.25 ms.

When the particles were clogged in guide-1 or guide-2, one of early or late positive signal peaks disappeared (Figure S-2, Supplementary Videos S-1 and S-2). Based on the result of simultaneous measurement, the particle gave a signal without the early positive signal peak when it was once clogged at the entrance of guide-1 and then passed through the micropore (Figure S-2a). Due to the clogging of particles at the entrance of guide-1, the time lag occurred between desorption of surface ions and the negative signal peak sensing. On the other hand, the particle gave a signal without the late positive signal peak when it passed through the micropore and then clogged in guide-2, (Figure S-2b). Due to the clogging of particles in guide-2, ion adsorption did not occur. Therefore, simultaneous measurement can ascertain what caused an irregular signal shape.

3.2. Discrimination of the four kinds of individual particles contained in the solution mixture

Using the simultaneous measurement method, we succeeded in obtaining information of the size and the fluorescent color of the four kinds of individual particles contained in the solution mixture (Fig. 2). Fluorescence observation was done using a fluorescence filter with an excitation filter of 360–370 nm. The height, width and length of the micropore were 7.5 μm, 4.0 μm and 80 μm, respectively. In ionic current sensing, we applied a voltage of 53 V to the micropore. In the case of only fluorescence observation, 70 nm diameter difference between 0.93 μm and 1.00 μm particles cannot be discriminated. In the case of only ionic current sensing, 1.00 μm blue fluorescent particles and 1.00 μm green fluorescent particles cannot be discriminated. The diameter of the particle was calculated based on the value of I_{signal} and the calibration curve obtained by detecting 1.00, 2.08, and 3.1 μm particles. Identifica-

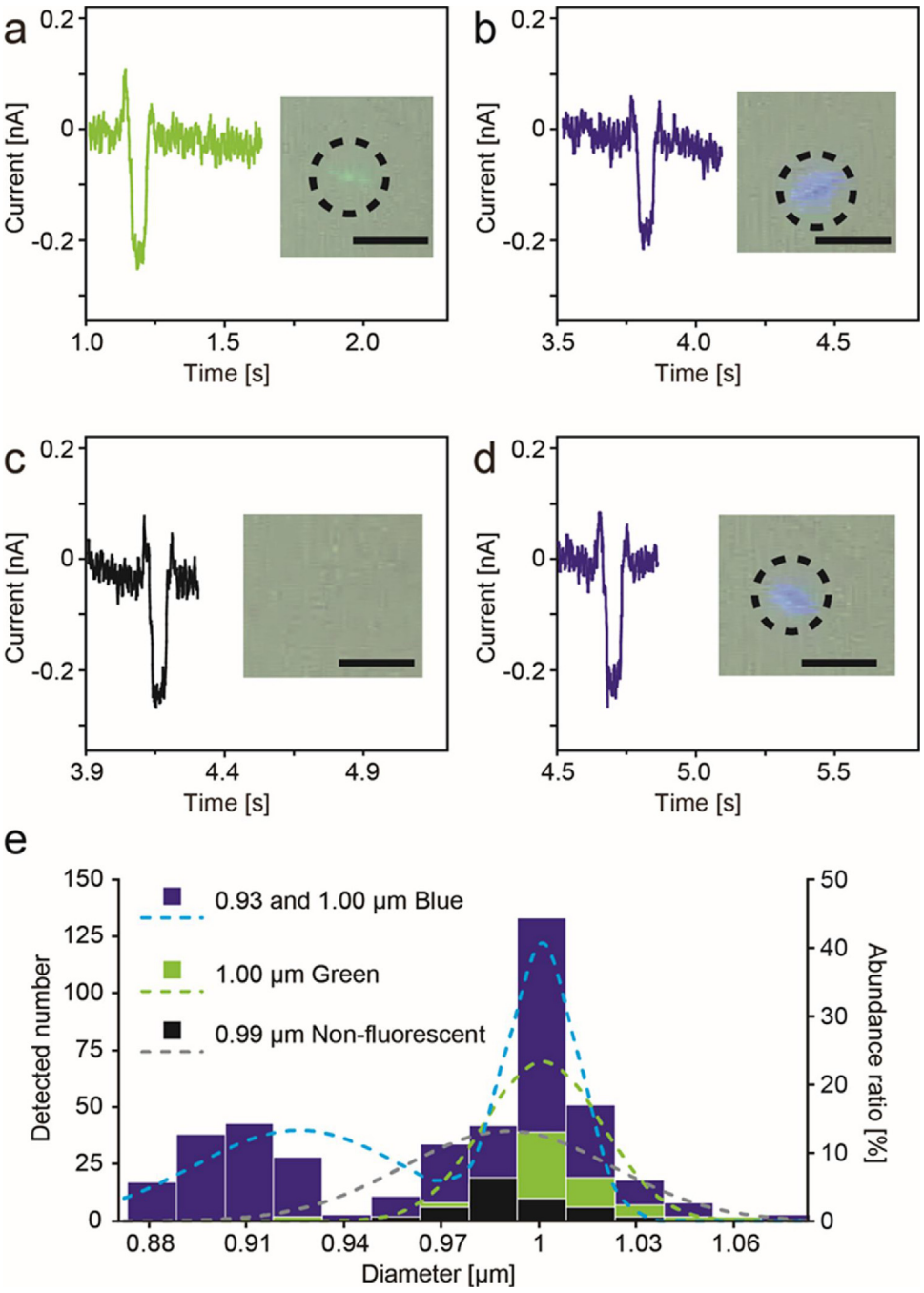

Fig. 2. Discrimination of solution mixture by the simultaneous ionic current sensing and fluorescence observation method. Four types of polystyrene particles were mixed together: 1.00 μm green fluorescent particles, 1.00 μm blue fluorescent particles, 0.93 μm blue fluorescent particles, and 0.99 μm non-fluorescent particles. Concentration of each type particle was 4.55×10^7 particles/mL. (a) Ionic current signal and fluorescence image of 1.00 μm green fluorescent particle; scale bar, 5 μm. The black dotted circle encloses a detected particle. (b) Ionic current signal and fluorescence image of 0.93 μm blue fluorescent particle. (c) Ionic current signal and fluorescence image of 0.99 μm non- fluorescent particle. Fluorescence was not observed. (d) Ionic current signal and fluorescence image of 1.00 μm blue fluorescent particle. (e) Histogram of calculated diameter from I_{signal} and fluorescent color of 572 particles which were detected in 6 min. Dots lines show expected abundance ratio of each type of polystyrene particles based on the data sheets. Histograms of 0.93 μm and 1.00 μm particles were clearly separated. Standard deviation of diameter of 1.00 μm green fluorescent particles was 20 nm which is same as that of product data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tion of each particle was done by synchronizing information about the diameter obtained by ionic current sensing and the fluorescent color obtained by fluorescence observation (Fig. 2a–d). The 1.00 μm green fluorescent and 1.00 μm blue fluorescent particles could be clearly discriminated because the fluorescent color values of the green fluorescent particles were R156, G129 and B167, and the those of the blue fluorescent particles were R94, G129 and B187 based on the numerical analysis of the fluorescence observation image. Furthermore, since the green particles with 1.0 μm diameter of our result and product data have the same standard deviation of 20 nm, our ionic current sensing method can detect the diameter of particles with high precision (Fig. 2e). In addition, significant difference was shown when the T-test was performed between calculated diameter of 1.0 μm green particles and 0.99 μm non-fluorescent particles (p-value=0.004 <0.05). On the other hand, the distribution of the histogram of 0.93 μm particles was slightly

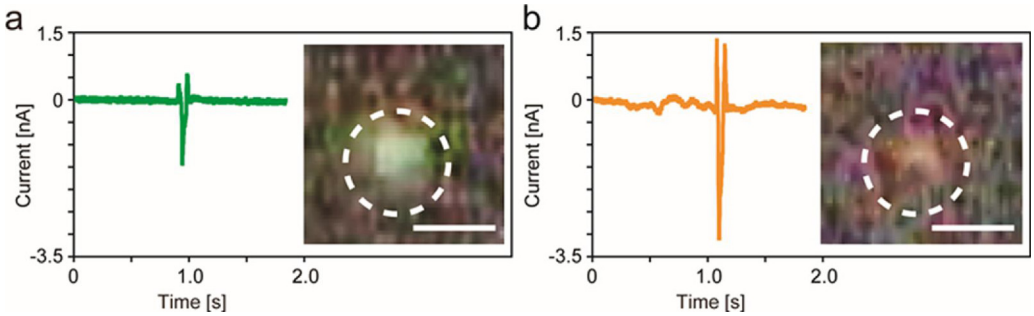

Fig. 3. Simultaneous ionic current sensing and fluorescence observation results of *E. coli* and *B. subtilis*. (a) Ionic current signal and fluorescence image of *E. coli* in the micropore; scale bar, 2 μm. Volume of cell was 2.2 fL which was calculated from I_{signal}. (b) Ionic current signal and fluorescence image of *B. subtilis* in the micropore. Volume of cell was 4.2 fL which was calculated from I_{signal}.

shifted toward the smaller particle diameter from accurate diameter. It depends on the accuracy of the calibration curve, which can be improved by increasing the numbers of sizes of the reference particles. Because there are regions where the particle size distribution overlaps, identification cannot be done using only the ionic current sensing, however, by performing the fluorescence observation at the same time, it was possible to discriminate the particles in solution mixture, which include 0.93 μm and 1.00 μm blue fluorescent particles, 1.00 μm green fluorescent particles, and 0.99 μm non-fluorescent particles.

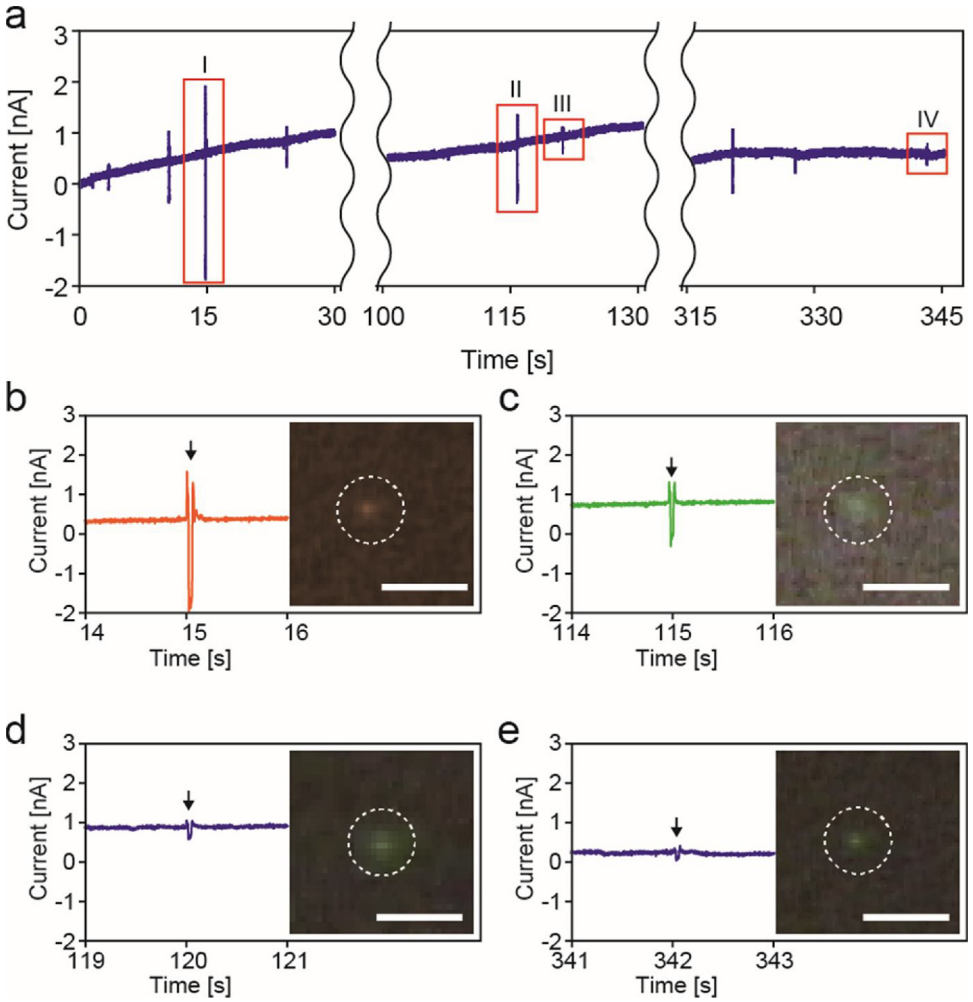

Fig. 4. Discrimination of solution mixture including bacteria sample and contaminants by the simultaneous ionic current sensing and fluorescence observation method. The solution mixture included *E. coli*, *B. subtilis*, 0.75 μm polystyrene particles, and 1.00 μm polystyrene particles. The solution mixture was introduced into the micropore. (a) Raw data of ionic current sensing. Signals I–IV were defined. (b) Expanded view and fluorescence image of signal I. Volume was calculated as 3.59 fL. Based on volume and fluorescent color, this signal was caused by introduction of *B. subtilis*. (c) Expanded view and fluorescence image of signal II. Volume was calculated as 1.79 fL. Based on volume and fluorescent color, this signal was caused by introduction of *E. coli*. (d) Expanded view and fluorescence image of signal III. Volume was calculated as 0.53 fL. Based on volume and fluorescent color, this signal was caused by introduction of 1.00 μm green fluorescent particle. (e) Expanded view and fluorescence image of signal IV. Volume was calculated as 0.23 fL. Based on volume and fluorescent color, this signal was caused by introduction of 0.75 μm green fluorescent particle. Scale bars of all images are 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Validating applicability of our method to the analysis of bacteria

We confirmed that our simultaneous measurement method could be used for the detection of bacteria and that the detected information of the size and the fluorescent stainability of individual bacteria coincided with the literature values (Fig. 3). In the application to the analysis of bacteria, we simultaneously measured *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*). *E. coli* are rod-like shaped bacteria, which have a volume of 1.5–4.4 fL [14–16]. *B. subtilis* are also rod-like shaped bacteria, which have a volume of 1.5–4.2 fL [14,17]. Due to the difference in thickness of the peptidoglycan layer, it was expected that gram-positive and −negative bacteria will emit different fluorescent colors when using cell membrane staining reagent. Fluorescence observation was performed using a fluorescence filter with an excitation filter of 450–490 nm. The height, width and length of the micropore were 8.2 μm, 3.8 μm and 80 μm, respectively. In ionic current sensing, we applied a voltage of 53 V to the micropore. The detected *E. coli* volume was 2.2 fL and that of *B. subtilis* was 4.2 fL; both corresponded to the literature values (Fig. 3a, b). Using cell membrane staining reagent, *E. coli* were stained as green and *B. subtilis* were stained as orange, which reflected the difference in internal structure of each bacterium. These results indicate that our measurement method can accurately obtain bacterial information. By simultaneous ionic current sensing and fluorescence observation, we can analyze the size and the fluorescent color of individual bacteria passing through the micropore. Therefore, two different bacteria with slight size difference and other two different bacteria with cell membrane difference can be discriminated in a same microfluidic chip.

3.4. Bacteria discrimination in a solution mixture containing contaminants

By simultaneous ionic current sensing and fluorescence observation, we succeeded in discriminating bacteria in a solution mixture containing contaminants (Fig. 4 and S-3). The solution mixture included *E. coli* and *B. subtilis* as bacteria, and 1.00 μm and 0.75 μm polystyrene particles as contaminants. 1.00 μm particles and 0.75 μm particles have volumes of 0.52 fL and 0.22 fL, respectively. Fluorescence observation was performed using a fluorescence filter with an excitation filter of 450–490 nm. The height, width and length of the micropore were 8.2 μm, 3.8 μm and 80 μm, respectively. In ionic current sensing, we applied a voltage of 53 V to the micropore. Four signals were designated as I–IV in a 345 s measurement, and the fluorescence observation images when each signal was detected were combined for discrimination (Fig. 4). Since fluorescence for signal I was orange and the volume estimated from the ionic current sensing was 3.59 fL, we identified it to be *B. subtilis* (Fig. 4b). Since fluorescence for signal II was green and the volume estimated from the ionic current sensing was 1.79 fL, we identified it to be *E. coli* (Fig. 4c). Since fluorescence for signal III was green and the volume estimated from the ionic current sensing was 0.53 fL, we identified it to be a 1.00 μm green fluorescent particle (Fig. 4d). Since fluorescence for signal IV was green and the volume estimated from the ionic current sensing was 0.23 fL, we identified it to be a 0.75 μm green fluorescent particle (Fig. 4e). In addition, instantaneous turbulence of the current baseline generated by clogging of the particles and a large signal generated by aggregates of green fluorescent particles were observed in another experiment shown in supplementary information (Figure S-3). When contaminates and *E. coli* have same fluorescence wavelength and size, they can be discriminated from the difference in fluorescence intensity (Figure S-4). We succeeded in identifying bacteria present in a solution mixture containing contaminants, which is possible only when ionic current sensing and fluorescence observation are performed at the same time.

4. Conclusion

By using our simultaneous ionic current sensing and fluorescence observation method, it becomes possible to simultaneously detect size and fluorescent stainability of individual bacteria within 300 ms, and as a result, it is possible to discriminate *E. coli* and *B. subtilis* which cannot be discriminated when fluorescence observation and ionic current sensing are independently performed. Since use of a lens with high-magnification is unnecessary in our method because fluorescent microscope isn't used for detecting sample size, users can apply the method to bacteria detection devices for on-site bacteria detection. Finally, since our method can obtain information of individual bacteria from multiple perspectives, it can be applied to screening technology.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.snb.2018.01.079.

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