Design of a large-scale femtoliter droplet array for single-cell analysis of drug-tolerant and drug-resistant bacteria

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Single-cell analysis is a powerful method to assess the heterogeneity among individual cells, enabling the identification of very rare cells with properties that differ from those of the majority. In this Methods Article, we describe the use of a large-scale femtoliter droplet array to enclose, isolate, and analyze individual bacterial cells. As a first example, we describe the single-cell detection of drug-tolerant persisters of Pseudomonas aeruginosa treated with the antibiotic carbenicillin. As a second example, this method was applied to the single-cell evaluation of drug efflux activity, which causes acquired antibiotic resistance of bacteria. The activity of the MexAB-OprM multidrug efflux pump system from Pseudomonas aeruginosa was expressed in Escherichia coli and the effect of an inhibitor D13-9001 were assessed at the single cell level.

Keywords: single-cell analysis, microdevice, drug tolerance, persister, drug resistance, drug efflux, transporter

INTRODUCTION
Opportunistic infection with bacteria resistant to multiple antibiotics is a continuing clinical challenge (Taube, 2008). The antibiotic resistance of bacteria can be classified into two categories, natural resistance (tolerance) and acquired resistance. In natural resistance, a very small proportion of the bacterial population is resistant to multiple antibiotics despite having the same genotype as the sensitive majority. These bacteria are often referred to as “persisters” (Lewis, 2010). However, the nature of these persisters is not fully understood because they occur at a very low frequency in a bacterial population (typically less than 1%), which makes systematic studies difficult. Therefore, the development of microdevices from the collection of cells from the device and their subsequent analysis can overcome the limitations associated with ensemble-averaged data from multiple cells, and enable the identification of very rare cells with properties that differ from those of the majority. Microfabricated devices have contributed greatly to the development of massively parallel and high-throughput single-cell analyses.

In this Methods Article, we describe a microdroplet-based method to identify and culture individual bacterial cells for efficient detection of persisters. In contrast, acquired antibiotic resistance is much smaller in size. Thus far, only a few studies have used microdevices for single bacterial cell analysis (Balaban et al., 2010; Lindstrom and Andersson-Svahn, 2010), because their size, a few millimeters to tens of micrometers, allows for easy handling compared to bacteria, which are much smaller in size. However, in most microdevices, the target cells are eukaryotic, such as mammalian cells and yeasts (Sims and Allbritton, 2007; Gupta et al., 2010, Lindstrom and Andersson-Svahn, 2010), because their size, a few micrometers to tens of micrometers, allows for easy handling compared to bacteria, which are much smaller in size.

In this Methods Article, we describe a microdroplet-based method for assessing the drug efflux activity of single bacterial cells.

ADVANTAGES OF SINGLE-CELL ANALYSIS USING A MICRODEVICE
Single-cell analysis is a powerful approach for detecting variations among the cells in a population, such as differences in the expression of proteins, the copy number of genes, and the concentration of metabolites (Li and Xie, 2011; Trouillon et al., 2013). Single-cell analysis can overcome the limitations associated with ensemble-averaged data from multiple cells, and enable the identification of very rare cells with properties that differ from those of the majority. Microfabricated devices have contributed greatly to the development of massively parallel and high-throughput single-cell analyses.

However, in most microdevices, the target cells are eukaryotic, such as mammalian cells and yeasts. Therefore, the development of microdevices from which individual bacterial cells can be recovered has been highly anticipated.
We recently developed a micron-sized femtoliter droplet array on a hydrophilic-in-hydrophobic micropatterned surface (Sakakihara et al., 2010). In our new microdevice, a large number of dome-shaped femtoliter droplets can be prepared that enclose individual bacterial cells (Iino et al., 2012a). One prominent feature of this array is that the individual droplets containing the enclosed cells can be accessed and collected with a micropipette. The array can also be used for mass culture and gene and protein analyses.

We prepared the hydrophilic-in-hydrophobic micropatterned surface through conventional microfabrication (Figure 1A). A hydrophobic polymer of carbon-fluorine (CYTOP, Asahi Glass, Japan) was deposited on a SiO2 cover glass, and photolithography was performed using a high-visibility photore sist. The resist-patterned substrate surface was dry-etched with O2 plasma by using a reactive ion-etching system to produce circular micropatterns on a hydrophilic SiO2 glass surface. The diameter of the exposed hydrophobic SiO2 surfaces was 10–30 μm, and they were surrounded by a hydrophobic polymer layer with a height of 1 μm. A fabricated micropatterned cover glass was attached to the bottom of a perforated petri dish (Figure 1B). The circular micropatterns were grouped into islands and were numbered 1–15 (Figure 1C).

To form a droplet array containing bacteria, we covered the micropatterned cover glass with medium containing a bacterial suspension (Figure 1C, left). Then, fluorinated oil (Fluorinert FC-40, Sigma-Aldrich, USA), which has a higher density than water, was flowed over the medium near the surface. The hydrophilic SiO2 glass surfaces retained the medium and the bacteria, whereas the hydrophobic surface was replaced with oil. As a result, many droplets containing one or more bacteria were formed (Figure 1C, right). More than 3 × 105 droplets could be simultaneously prepared in a 1-cm² area in a single device. Enclosure of the cells in the droplets was stochastic and was dependent on the cell density of the bacterial suspension. At an optical density (turbidity) of 600 nm (OD600) of 0.6, approximately 20–30% of the droplets contained single cells. Increasing the diameter of the hydrophilic surfaces to 20 or 30 μm increased the fraction of droplets containing multiple cells; however, the fraction of droplets containing single cells did not increase significantly (Figure 1D). In contrast, the number of droplets formed increased significantly when we used a device with hydrophilic surfaces of a smaller diameter. Therefore, to increase the total number of droplets containing single cells, we used a microdevice containing hydrophilic surfaces with a diameter of 10 μm.

**DETECTION OF PERSISTER BACTERIA IN A FEMTOLITER DROPLET ARRAY**

We generated a femtoliter droplet array of *Pseudomonas aeruginosa* PA01 using our microdevice to detect persisters under an optical microscope. In the control experiment without antibiotic treatment, most cells underwent multiple cell divisions after incubation overnight at 37°C (Figures 2A,B). The divided cells showed active flagellar motion, indicative of high metabolic activity. To detect persisters, an antibiotic, carbenicillin (at final concentration of 5 mg/mL, which is ∼100 times higher than the minimal inhibitory concentration), was added to the bacterial suspension that was grown to late exponential phase (OD600 ∼1.0) in trypticase soy broth. The suspension was further incubated at 37°C for 3 h, and then the cells were collected, washed, resuspended in fresh medium (OD600 ∼0.2). This suspension was enclosed in a droplet array. After enclosure, the whole device was placed in an incubator at 37°C, and the cells were cultured overnight. The persisters were easily identified under an optical microscope the overnight culture (Figure 2A). The divided cells were not cells that acquired resistance, but were actually persisters. This was confirmed by collecting the cells with a micropipette with an aperture diameter of 10–15 μm (Figures 2B,C), inoculating a culture in test tubes, and antibiotic susceptibility testing.

Bacterial cells that divided multiple times were counted, and the frequency of persisters was calculated. The frequency of persisters in the femtoliter droplet array (1.5 ± 0.72%, N = 4, Figure 3D) was quite unexpectedly much higher than that estimated by conventional agar plate assays (0.10 ± 0.03%, N = 4). In the plate assays, the carbenicillin-treated preculture sample was prepared as described above along with an untreated culture sample, and then the samples were serially diluted and cultured overnight at 37°C on agar plates. The number of colonies on the plates
from carbenicillin-treated and untreated preculture samples were counted and compared. It has been recently reported that quorum sensing autoinducer increased the frequency of persister appearance (Moker et al., 2010; Leung and Levesque, 2012, Vega et al., 2012), and that inhibiting the quorum signal restored antibiotic susceptibility (Pan et al., 2012, 2013). Furthermore, the quorum-sensing signal could be transduced even in single isolated cells when PAO1 was enclosed in picoliter-volume droplets (Boedicker et al., 2009). Therefore, enclosure of a single cell in a femtoliter droplet may enhance the quorum sensing signal and increase persister frequency. The effect of the quorum sensing signal on the frequency of persister appearance in the femtoliter droplet array can be more clearly confirmed by treating the cells with antibiotic after enclosure in the droplets by adding the antibiotic with a micropipette (Sakakihara et al., 2010).

**A SINGLE-CELL DRUG EFFLUX ASSAY IN A FEMTOLITER DROPLET ARRAY**

The AcrAB-ToLC multicomponent efflux pump system recognizes and expels a wide variety of compounds, including antibiotics, dyes, and detergents. In this system, AcrA is the membrane fusion protein that stabilizes the complex (Zgurskaya and Nikaido, 1999), AcrB is the inner membrane transporter protein that belongs to the resistance-nodulation-division (RND) family (Murakami et al., 2006; Nakashima et al., 2011, 2013), and TolC is the outer membrane channel protein (Koronakis et al., 2000). The AcrAB-ToLC efflux system is responsible for both intrinsic and acquired drug resistance of Gram-negative bacteria such as *Escherichia coli* and *Salmonella enterica* (Nishino and Yamaguchi, 2008, Nakaido and Takatsuka, 2009). Two systems *P. aeruginosa* that are homologous to the AcrAB-ToLC system, MexAB-OprM and MexXY-OprM, lead to multidrug resistance in clinical isolates (Morita et al., 2001; Livermore, 2002, Hocquet et al., 2006, 2007; Henrichfreise et al., 2007).

We have recently developed a single-cell drug efflux assay using the femtoliter droplet array (Figure 4; Iino et al., 2012a). In this assay, *E. coli* cultured in test tubes was mixed with a fluorogenic substrate, fluorescein-di-β-D-galactopyranoside (FDG), enclosed in a droplet array, and then cultured for 15–20 min at room temperature. Upon entering the cytoplasm of *E. coli*, FDG is hydrolyzed into the fluorescent dye fluorescein by β-galactosidase. Both FDG and fluorescein are substrates for the AcrAB-ToLC system (Matsumoto et al., 2011; Iino et al., 2012b). In wild-type cells, FDG was effectively pumped out before hydrolysis, and no fluorescence was detected (Figure 4A, left, and Figure 4B, top). In contrast, when FDG was imported into ΔacrB (ΔB) and ΔtolC (ΔC) strains it was hydrolyzed to fluorescein. In ΔB cells, not only the cells, but also the droplets themselves fluoresced (Figure 4A, center, and Figure 4B, middle) because the remaining minor RND efflux pumps slowly pumped out the fluorescein. Although only a small amount of the dye was pumped out, it could be easily detected because it was confined to the femtoliter droplet (Rondelez et al., 2005; Sakakihara et al., 2010, Kim et al., 2012). In ΔC cells, fluorescein accumulated in the cell (Figure 4A, right, and...
FIGURE 4 | Single-cell drug efflux assay. (A) Schematic of the principle of the single-cell drug efflux assay. (B) A representative assay. Phase-contrast (left) and fluorescence (right) images of the same field are shown.

Figure 4B, bottom) because TolC is a channel protein common to both the major and minor RND efflux pumps in *E. coli*.

With this method, the inhibitory effect of chemical compounds against the efflux pump can be easily assessed. The effect of an efflux pump inhibitor, D13-9001 (Yoshida et al., 2007), is shown in Figure 5. D13-9001 has been reported to enhance the antibacterial activities of several antibiotics by binding tightly to the drug binding pockets of AcrB and MexB (Nakashima et al., 2013). A ΔΔC double-knockout *E. coli* strain that stably expresses MexAB-OprM from *P. aeruginosa* was used for the experiment. This strain did not fluoresce in our assay, indicating that the exogenously expressed MexAB-OprM worked well in *E. coli*, and that the cells recovered drug efflux activity (Figure 5, top). Addition of D13-9001 increased the number of fluorescent cells (Figure 5, bottom). The fluorescence intensity of the cells increased as the concentration of D13-9001 increased, indicating a concentration-dependent inhibitory effect. D13-9001 is a specific inhibitor of MexB, a major efflux pump in *P. aeruginosa*. However, it does not inhibit MexY, which is another major efflux pump in *P. aeruginosa* (Yoshida et al., 2007). Our simple and rapid approach would be useful to screen for new inhibitors that are also effective against MexY and other efflux pumps.

FIGURE 5 | Effect of the pump inhibitor D13-9001 on the efflux activity of MexAB-OprM expressed in the ΔΔC *E. coli* strain. Phase-contrast (left) and fluorescence (right) images of untreated control cells (top) or cells treated with 16 μg/mL D13-9001 (bottom).

PHENOTYPIC CHANGE AFTER GENETIC TRANSFORMATION

As a demonstration of the rapid phenotypic change after genetic transformation, we introduced the *S. enterica* tolC gene into *E. coli* ΔC cells. After electroporation with the expression vector, the cells were incubated for different time durations in the presence of the selection marker kanamycin and drug efflux activity was assessed. The efflux-active phenotype was observed after 3 h (Figure 6, top), whereas no phenotypic change was observed in the control experiment (Figure 6, bottom).

A prominent feature of the femtoliter droplet array is the ability to access individual droplets. Using a micropipette, not only droplets but also the cells within the droplets can be collected (Figures 3B,C). Collected single cells divide multiple times after...
transfer to growth medium in a test tube. The plasmid in the divided cells can be extracted and used for subsequent gene analysis (Iino et al., 2011). Culture after cell collection can be omitted by amplifying the DNA by single-cell PCR (Ottesen et al., 2006). Considering the rapid detection (3 h) of the phenotypic change after genetic transformation, single-cell gene analysis would enable high-throughput screening.

**PERSPECTIVE**

As described above, the femtoliter droplet array is useful for single-bacterial-cell analysis. Single-cell analysis of persister bacteria could help elucidate the mechanism of persister appearance and the reversible switching dynamics between persister and sensitive cells. This single-cell drug efflux assay can be used to screen for pump inhibitors, which requires the testing of numerous compounds. Our method is a direct evaluation of efflux activity, it takes only 20-30 min, and its advantage over the conventional method, based on the shift in the minimal inhibitory concentration, is evident. Furthermore, with the advantage of individual droplet accessibility, single persister cells and cells exhibiting the efflux-active phenotype can be easily collected and used for subsequent analysis. It should be possible to screen for genes encoding functional efflux pump systems with a plasmid library of cloned genomic fragments. We believe that our approach will aid in addressing the challenge of infectious diseases caused by bacteria that are multihitdrug tolerant and resistant.

**REFERENCES**

Allison, K. R., Brynildsen, M. P., and Collett, J. L. (2011). Heterogeneous bacterial populations and engineering approaches to domesticate them. *Curr. Opin. Microbiol.* 14, 593–598. doi: 10.1016/j.mib.2011.09.002

Balaban, N. Q., Merrin, J., Chait, R., Kowalczyk, L., and Leibler, S. (2004). Bacterial persistence and toxin-antitoxin loci. *Nature* 434, 891–897. doi: 10.1038/nature03513

Balaban, N. Q., Merrin, J., Chait, R., Cai, L., Friedman, N., and Xie, X. S. (2006). Stochastic gene expression in individual cells at the single-molecule level. *Nature* 440, 576–580. doi: 10.1038/nature04599

Bardwell, J. C. V., Venkitaraman, R. K., and Limoges, R. F. (2009). Microfluidic confinement of single cells of bacteria in small volumes initiates high-density behavior of quorum sensing and growth and reveals its variability. *Angew. Chem. Int. Ed. Eng.* 48, 7980–7983. doi: 10.1002/anie.200901551

Cai, L., Friedman, N., and Xie, X. S. (2006). Stochastic protein expression in individual cells at the single molecule level. *Nature* 440, 368–372. doi: 10.1038/nature04949

Fischbach, M. A., and Walsh, C. T. (2009). Antibiotics for emerging pathogens. *Science* 325, 1088–1090. doi: 10.1126/science.1175867

Garcia, M. P., and Massey, F. (2012). Bacterial persistence and toxin-antitoxin loci. *Annu. Rev. Microbiol.* 66, 103–123. doi: 10.1146/annurev-micro-092810-181519

Gupta, R., Kim, D. H., Elkonin, D., Smith, C., Kanus, A., Yuan, J., et al. (2010). Lab-on-a-droplet devices as an emerging platform for stem cell biology. *Lab Chip* 10, 2019–2031. doi: 10.1039/b916069f

Henrichfreise, B., Wiegand, I., Pfister, W., and Weidmann, B. (2007). Resistance mechanisms of multiresistant pseudomonas aeruginosa strains from Germany and correlation with Hypertension dari...
in growing cyanobacteria require transcriptional feedback. Science 350, 737–740. doi: 10.1126/science.1342354
Toselli, P., Prasad, P., Wang, J., and Song, Y. (2013). A single-membrane enzymatic assay in a directly accessible femtoliter droplet array. Lab Chip 13, 1505–1512. doi: 10.1039/c3lc50270b
Toublanc, R., Passard, E., Zhang, C., Kong, H., and Yang, E. (2013). Chemical analysis of single cells. Anal Chem. 85, 522–542. doi: 10.1021/ac303290e
Torres, M. G., de Benito, E., and Albericio, F. (2013). Analysis of single mammalian cells on-chip. Lab Chip 13, 422–440. doi: 10.1039/c2lc30761b
Taubes, G. (2008). The bacteria fight back. Science 321, 356–361. doi: 10.1126/science.321.5887.356
Teng, S. W., Mudity, S., Mollitt, J. R., de Buyl, S., and O’Shea, E. K. (2013). Robust circadian oscillations in growing cyanobacteria require transcriptional feedback. Science 350, 2096–2099. doi: 10.1126/science.1229090
Truong, R., Passard, E., Zhang, C., Kong, H., and Yang, E. (2013). Chemical analysis of single cells. Anal Chem. 85, 522–542. doi: 10.1021/ac303290e
Vega, N. M., Allison, K. R., Khalil, A. S., and Collins, J. J. (2012). Signaling-mediated bacterial persister formation. Nat Chem Biol. 8, 431–435. doi: 10.1038/nchembio.915
Wakamoto, Y., Dhar, N., Chait, R., Schneider, K., Signorino-Gelo, F., Leibler, S., et al. (2013). Dynamic persistence of antibiotic-stressed mycobacteria. Science 339, 91–95. doi: 10.1126/science.1230996
Weng, B., Diluzio, W. R., and Whitesides, G. M. (2007). Microfabrication meets microbiology. Nat Rev Microbiol. 5, 209–218. doi: 10.1038/nrmicro1616
Yoshida, K., Nakamura, K., Ohnaka, M., Kuri, N., Yokomizo, Y., Sakamoto, A., et al. (2007). MexAB-OprM specific efflux pump inhibitors in Pseudomonas aeruginosa. Part 7: highly soluble and in vivo active quaternary ammonium analogues D15-9901, a potential preclinical candidate. Bioorg Med Chem. 15, 7087–7097.
Zgurskaya, H. I., and Nikaido, H. (1999). AcrA is a highly asymmetric protein capable of spanning the periplasm. J Mol Biol 285, 409–420. doi: 10.1006/jmbi.1998.2313
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