Surface Free Energy Activated High-Throughput Cell Sorting

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Supporting Information

ABSTRACT: Cell sorting is an important screening process in microbiology, biotechnology, and clinical research. Existing methods are mainly based on single-cell analysis as in flow cytometry and microfluidic cell sorters. Here we report a label-free bulk method for sorting cells by differentiating their characteristic surface free energies (SFEs). We demonstrated the feasibility of this method by sorting model binary cell mixtures of various bacterial species, including Pseudomonas putida KT2440, Enterococcus faecalis ATCC 29212, Salmonella Typhimurium ATCC 14028, and Escherichia coli DH5α. This method can effectively separate $10^{10}$ bacterial cells within 30 min. Individual bacterial species can be sorted with up to 96% efficiency, and the cell viability ratio can be as high as 99%. In addition to its capacity of sorting evenly mixed bacterial cells, we demonstrated the feasibility of this method in selecting and enriching cells of minor populations in the mixture (presenting at only 1% in quantity) to a purity as high as 99%. This SFE-activated method may be used as a stand-alone method for quickly sorting a large quantity of bacterial cells or as a prescreening tool for microbial discrimination. Given its advantages of label-free, high-throughput, low cost, and simplicity, this SFE-activated cell sorting method has potential in various applications of sorting cells and abiotic particles.

Cell sorting is an important screening process in microbiology and environmental science,¹,² biotechnology,³ and clinical research.⁴,⁵ To date, nearly all modern cell sorting methods rely on either flow cytometric or microfluidic platforms.⁶,⁷ Flow cytometry cell sorters commonly use the principle of fluorescence-activated cell sorting, which separates cells in a heterogeneous suspension by differentiating their fluorescence and light scattering properties on a single-cell basis.⁸−¹⁰ Although being a well-developed method, flow cytometry cell sorters are generally associated with high costs, need for skilled operators, and difficulties in selecting the most suitable fluorophores, especially in the existence of a high-level of autofluorescence background and nonspecific dye binding, such as in most aquatic microbial samples.¹¹,¹² Microfluidic cell sorters are capable of sorting cells without the use of fluorescence probes, i.e., label-free cell sorting.¹¹,¹² These devices sort cells on the basis of single-cell analysis of their intrinsic physical properties, such as size, deformability, dielectric, and optical properties.¹³−¹⁶ Although being a promising method, current microfluidic cell sorting platforms are generally limited in throughput.¹⁷

Here, we report an innovative cell sorting method based on differentiation of the intrinsic surface free energy (SFE) of heterogeneous cell populations in suspension. The SFE is an intrinsic physicochemical property of cells which measures the overall contribution of surface molecular structures on cellular surface properties and determines their self-aggregation and adhesion with solid surfaces.¹⁸−²¹ In comparison to single-cell screening methods such as flow cytometry and microfluidics, this is an easy-to-use, label-free, bulk method without apparent limitations on sorting throughput. We will show that this SFE-activated method can quickly separate a large number of bacterial cells (> $10^{10}$) with a high sorting efficiency and a high cell viability ratio. We will also show that this method can be used to select and enrich uncommon cells that present in a small quantity (1%) compared to other common cells in the mixture.

EXPERIMENTAL SECTION

Principles. Very recently, we have developed a novel spectrophotometric method for determining SFE of live cells and have demonstrated its feasibility in studying microalgal cells.²² Principles of this method are based on the analysis of colloidal stability of cell suspensions. According to the classical DLVO theory,²³ the colloidal stability of a particle suspension depends on the balance between van der Waals attraction and electrostatic repulsion. A minimum van der Waals attraction...
can be achieved by dispersing the particles in a liquid medium whose surface tension is close to the SFE of the particles, at which the particles are expected to disperse in the medium without significant aggregation and sedimentation. (Detailed thermodynamic model of particle interaction across a medium can be found elsewhere.22,24) Hence, when dispersing particles in a series of liquids with known surface tension values, surface tension of the liquid with the maximum level of particle dispersion should be equal to the SFE of the particles.22,24 The degree of particle dispersion in these liquids can be compared by measuring the optical density (OD) of the suspension. It is expected that there should be a maximum in OD that represents the highest degree of particle dispersion. Consequently, the surface tension of liquid at which the maximum OD appears should be close to the SFE of the particles.22 Accordingly, when a heterogeneous mixture of two cell populations is dispersed in the same series of liquids, two peaks in OD should be expected and each peak should correspond to the fingerprint SFE of one cell population. At each peak, the cell population whose SFE is equal to the surface tension of the suspending liquid should disperse uniformly in the supernatant, whereas the other cell population whose SFE differs from surface tension of the liquid (either higher or lower) should largely aggregate and sediment. Consequently, the two cell populations should be separated.

**Bacteria Culture.** We demonstrated the feasibility of the SFE-activated method mainly by sorting cells of two commonly studied bacterial species: *Pseudomonas putida* KT2440 and *Enterococcus faecalis* ATCC 29212. *P. putida* is a Gram-negative soil bacterium,27 while *E. faecalis* is a Gram-positive fecal bacterium.28 Additional sorting experiments were performed with *Salmonella Typhimurium* ATCC 14028 and *Escherichia coli* DH5α. Both *S. Typhimurium* and *E. coli* are Gram-negative bacteria.

All bacteria were cultured in tryptic soy broth and harvested at the stationary-stage (OD$_{600}$ approximately equal to 2). The harvested bacterial cells were centrifuged and washed three times using a phosphate buffered saline of pH 7.0, and then resuspended in working cell suspensions (approximately 10$^8$ cells mL$^{-1}$). The bacterial cell suspensions were vibrated for 1 min to produce homogeneous suspensions prior to use. Model bacteria mixtures were prepared by mixing two bacterial species on a 1:1 ratio (i.e., evenly mixed bacterial cells) or on a 1:100 ratio (i.e., unevenly mixed bacterial cells).

**Bacteria Sorting.** A series of liquid media with surface tensions ranging from 22 to 72 mJ m$^{-2}$, each at 0.5 mL, were prepared by binary mixtures of ethanol and water at predefined mixing ratios. Surface tensions of these liquids were quantified by a pendant drop method (data shown in Table S1 of the Supporting Information (SI)). Ten µL of bacterial mixture was added into each liquid medium, followed by vortex mixing and leaving undisturbed for 20 min. These liquid media were subsequently centrifuged at 100g for 6 min to fully separate supernatant from sediment. A 200-µL portion of supernatant of each liquid medium was transferred to a 96-well microplate. OD at the wavelength of 600 nm (OD$_{600}$) was measured by a microplate reader (Epoch, BioTek, Winooski, VT). (As shown in Figure S1 of the SI, the background OD$_{600}$ due to suspending liquids is negligible.) OD$_{600}$ was then plotted against surface tension of the liquid media and peaks were determined by polynomial fitting. All measurements were performed for at least three times. Results are presented in mean ± SD.

**Bacteria Analysis.** The bacterial cells after sorting were recovered from supernatant and sediment separately at peak OD values. Efficiency of bacteria sorting was determined by Gram staining followed by microscopic enumeration. Viability ratios of the sorted bacterial cells were determined by microscopic enumeration of live and dead cells based on cell membrane integrity using a bacterial viability kit (Live/Dead BacLight, Life Technologies, Carlsbad, CA).

**RESULTS AND DISCUSSION**

**Sorting Evenly Mixed Bacterial Cells: Sorting Efficiency and Cell Viability.** In this section, we used the SFE-activated method to sort a binary bacteria mixture with an even mixing ratio, i.e., the amount of one bacterial species is approximately on par with the other bacterial species. We focused on a model bacteria mixture of *P. putida* and *E. faecalis*.

We first determined the SFE of *P. putida* cells and *E. faecalis* cells separately. As shown in Figure 1a and 1b, OD$_{600}$ for *P. putida* and *E. faecalis* showed a single peak at the surface tension of 34.2 ± 0.3 and 64.1 ± 0.3 mJ m$^{-2}$, respectively, indicating the characteristic SFEs of these two bacterial cells. The SFE values measured by the spectrophotometric method are in good agreement with those reported in the literature.29,30 Figure 1c shows the OD measurement in the mixture of these two bacteria. It clearly shows that there are two peak OD$_{600}$ values, with each corresponding to the characteristic peaks of the two individual bacteria that are determined separately in Figure 1a and Figure 1b.
To provide direct evidence of bacterial cell sorting, Figure 2 shows the gram-stain images for the original *P. putida* and *E. faecalis* mixture (e), bacterial cells recovered from the supernatant (a) and sediment (b) at the low-surface-tension peak of Figure 1c; bacteria recovered from the supernatant (c) and sediment (d) at the high-surface-tension peak of Figure 1c. Because the Gram-negative *P. putida* is stained red and the Gram-positive *E. faecalis* is stained purple, these gram-stain images confirm separation of these two bacterial cell populations into supernatant and sediment at the two primary peaks of Figure 1c. (f) Quantified percentages, i.e., purities, of *P. putida* and *E. faecalis* in a–e.

*P. putida* cells are Gram-negative bacilli that appear red in gram-stain images, while *E. faecalis* cells are Gram-positive cocci that appear purple. It is clear that there was initially a well-mixed *P. putida* (60% ± 5%) and *E. faecalis* (40% ± 6%) (Figure 2e). When dispersing the mixture into a liquid of surface tension 34 mJ m\(^{-2}\) (i.e., at the low surface tension peak in Figure 1c), we found that the two bacterial cell populations were effectively separated into the supernatant and sediment, with 96% ± 3% *P. putida* in the supernatant (Figure 2a) and 75% ± 6% *E. faecalis* in the sediment (Figure 2b). Similarly, when the mixture was dispersed in a liquid with a surface tension of 65 mJ m\(^{-2}\) (i.e., at the high surface tension peak in Figure 1c), 88% ± 5% *E. faecalis* was found in the supernatant (Figure 2c) and 82% ± 8% *P. putida* was found in the sediment (Figure 2d).

We have determined the viability of recovered bacterial cells after sorting. Figure 3 shows the fluorescence microscopy images of recovered bacterial cells in which live cells appear green and dead cells appear yellow/red. Figure 3a and 3b show bacteria recovered from the supernatant (a) and sediment (b) at the low surface tension peak of Figure 1c. According to Figure 2a and 2b, the bacteria cells are mainly *P. putida* in the supernatant and *E. faecalis* in the sediment. It is quantified that *P. putida* (Figure 3a) and *E. faecalis* (Figure 3b) have a viability ratio of 35% ± 5% and 99% ± 1%, respectively. Similarly, Figure 3c and 3d show bacteria recovered from the supernatant (c) and sediment (d) at the high surface tension peak of Figure 1c. It is found that *E. faecalis* in the supernatant (Figure 3c) has a viability ratio of 99% ± 1% and *P. putida* in the sediment (Figure 3d) has a viability ratio of 97% ± 1%.

We found a relatively low viability for *P. putida* recovered from the supernatants at the low surface tension peak (Figure 3a). This has to be related to the high ethanol concentration (i.e., 30% v/v) used in preparing the low surface tension medium and the different ethanol tolerance levels of bacterial cells.\(^3\) In comparison, only less than 2% ethanol was used in the high surface tension medium. Except for this low viability, all other bacteria recovered from supernatant and sediment have a viability ratio higher than 97%. We are currently...
were determined to be 65.1\%. The characteristic SFEs of these two individual bacterial species that the sorting efficiencies of the cell populations to be separated differ sufficiently from each other. In this section, we applied the SFE-activated method to separate bacterial species with smaller differences in their characteristic SFEs than those presented in the first section, i.e., \textit{P. putida} and \textit{E. faecalis} which differ by 30 mJ m\(^{-2}\) in their characteristic SFEs (see Figure 1c). To avoid repetition in presentation, these results are shown in Figures S2–S5 of the SI.

We then attempted to sort evenly mixed \textit{E. coli} and \textit{E. faecalis}. The characteristic SFEs of these two individual bacterial species were determined to be 65.1 ± 0.5 and 64.1 ± 0.3 mJ m\(^{-2}\), respectively. Analysis with one-way ANOVA indicated that these two SFE measurements were not statistically significant. In other words, the characteristic SFEs of these two bacterial species can be considered identical or statistically indistinguishable with our spectrophotometric method. As shown in SI Figure S4, no bimodal features can be detected for OD measurements of the mixture. Accordingly, as shown in SI Figure S5, these two cell populations in the mixture cannot be separated with the present method.

Figure 4 summarizes the sorting efficiency of different model binary mixtures. It is clear that the sorting efficiency decreases with diminishing differences in the characteristic SFEs between cell populations in the mixture. It should be noted that the sorting efficiency presented here is actually the purity of recovered cells. Because the two cell populations were initially mixed at an approximate ratio of 1:1, the initial purity of each cell population in the mixture should be close to 50%. Hence a cell purity around 50% literally indicates failure of the method. Without strict definitions, as shown in Figure 4, when the cell purity falls within 85–100%, the sorting efficiency is considered to be high (green region), and within 70–85% is moderate (yellow region). When the cell purity is below 70%, the sorting efficiency is considered to be low (red region), indicating failure of the method in sorting cells.


Figure 3. Fluorescence microscopy images for measuring bacteria viability, in which live bacterial cells appear green and dead bacterial cells appear yellow/red. Images for bacteria in the supernatant (a) and sediment (b) at the low-surface-tension peak of Figure 1c demonstrate a viability ratio of 35% ± 5% and 99% ± 1%, respectively. Images for bacteria in the supernatant (c) and sediment (d) at the high-surface-tension peak of Figure 1c demonstrate a viability ratio of 99% ± 1% and 97% ± 1%, respectively.

Sorting Evenly Mixed Bacterial Cells: Sorting Efficiency for Different Bacteria Mixtures. It is obvious that the SFE-activated cell sorting method is only effective when the characteristic SFEs of the cell populations to be separated differ sufficiently from each other. In this section, we applied the SFE-activated method to separate bacterial species with smaller differences in their characteristic SFEs than those presented in the first section, i.e., \textit{P. putida} and \textit{E. faecalis} which differ by 30 mJ m\(^{-2}\) in their characteristic SFEs (see Figure 1c). To avoid repetition in presentation, these results are shown in Figures S2–S5 of the SI.

We first sorted evenly mixed \textit{S. Typhimurium} and \textit{E. faecalis} cells. As shown in SI Figure S2, SFEs of these bacterial species differ by about 15 mJ m\(^{-2}\). As shown in SI Figure S3, we found that the sorting efficiencies in supernatants at the two primary OD peaks are 90% and 80%, respectively; while in sediments at the two primary OD peaks are 70% and 75%, respectively.

We then attempted to sort evenly mixed \textit{E. coli} and \textit{E. faecalis}. The characteristic SFEs of these two individual bacterial species were determined to be 65.1 ± 0.5 and 64.1 ± 0.3 mJ m\(^{-2}\), respectively. Analysis with one-way ANOVA indicated that these two SFE measurements were not statistically significant. In other words, the characteristic SFEs of these two bacterial species can be considered identical or statistically indistinguishable with our spectrophotometric method. As shown in SI Figure S4, no bimodal features can be detected for OD measurements of the mixture. Accordingly, as shown in SI Figure S5, these two cell populations in the mixture cannot be separated with the present method.

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It is found that the sorting efficiency in the supernatant is always higher than that in the sediment. This may indicate cell coprecipitation (detailed in the next section), which decreases cell purity in the sediment. Hence, although theoretically speaking both supernatant and sediment at each primary OD peak may be used for sorting cells, recovering cells from supernatants at the two OD peaks provides a higher sorting efficiency.

It is also interesting to point out that the cell purity in the supernatant of the low surface tension suspending liquid (i.e., at the low OD peak) appears to be higher than that in the supernatant of the high surface tension suspending liquid (i.e., at the high OD peak). The cell purity in the sediment, however, shows opposite dependence on the surface tension of the suspending liquids. This may indicate a higher degree of cell coprecipitation when dispersing cell mixtures in the lower energy suspending liquid.

Sorting Unevenly Mixed Bacterial Cells: Cell Enrichment. In this section, we used the SFE-activated method to sort binary bacteria mixtures with uneven mixing ratios, i.e., one bacterial species (as the uncommon cells) presents at only 1% of the other bacterial species (as the common cells). This model system was used to test the hypothesis of detecting and...
enriching uncommon cells with the SFE-activated cell sorting method.

We first studied the model system of mixing only 1% E. faecalis with P. putida. As illustrated in Figure 5, OD₆₀₀ of the mixture shows only one primary peak at 34.5 mJ m⁻², corresponding to the characteristic SFE of P. putida; while only a shallow kink appears around the characteristic SFE of E. faecalis (i.e., 64.1 mJ m⁻²).

Figure 6 shows the gram-stain images for the original binary mixture of P. putida with 1% E. faecalis (e), bacterial cells recovered from the supernatant (a) and sediment (b) at the primary surface tension peak (i.e., 34.5 mJ m⁻²), and bacterial cells recovered from the supernatant (c) and sediment (d) at the characteristic SFE of E. faecalis (i.e., 64.1 mJ m⁻²). Figure 6f shows the quantitative results of bacteria ratios, i.e., purities, in the above five cases (a–e).

It is clear that at the primary OD peak, a significant amount of the common cells (i.e., P. putida) coprecipitated in the sediment with the uncommon cells (Figure 6b), indicating failure of cell sorting at this primary OD peak. However, in the supernatant of the suspending liquid of which surface tension is equal to the SFE of the uncommon cells (i.e., 64.1 mJ m⁻²), E. faecalis cells were effectively enriched to a purity of 99% (Figure 6c), indicating efficient cell enrichment.

To further verify our measurements, we have performed a concomitant experiment of switching the common and uncommon cells, i.e., sorting a binary mixture of 1% P. putida with E. faecalis. The experimental results are shown in Figures S6 and S7 of the SI. Again, we demonstrated a high efficiency...

Figure 5. Optical density (OD) at 600 nm as a function of surface tensions of suspending liquids for the binary mixture of P. putida KT2440 with 1% E. faecalis ATCC 29212.

Figure 6. Gram-stain images for the original mixture of P. putida KT2440 (red) with 1% E. faecalis ATCC 29212 (purple) (e); bacteria recovered from the supernatant (a) and sediment (b) at the primary OD peak of Figure 5; bacteria recovered from the supernatant (c) and sediment (d) in the suspending liquids of surface tension 64.1 mJ m⁻². (f) Quantified percentages, i.e., purities, of P. putida and E. faecalis in a–e. Enrichment of the uncommon cells (E. faecalis) is clearly demonstrated in (c), highlighted with a yellow square.
(98%) of enriching uncommon cells (i.e., *P. putida*) in the supernatant of the suspending liquid of which surface tension is equal to the SFE of the uncommon cells (i.e., 34.2 mJ m⁻²).

**CONCLUSIONS**

We have developed a novel label-free bulk method for sorting cells by differentiating their characteristic surface free energies (SFEs). Our method is implemented by conventional spectrophotometric means without the need for special equipment. This method can be used for quickly sorting a large quantity of cells, as commonly required in microbiology, biotechnology, and clinical research. It can be also used for effectively enriching uncommon cells that present in a small quantity compared to other common cells in the mixtures. When the sorting efficiency is of critical importance, this method may be used as a prescreening tool to improve efficiency and throughput of flow cytometry and/or microfluidic device. Given its advantages of label-free, high-throughput, low cost, and simplicity, this SFE-activated cell sorting method has potential in various applications of sorting cells and abiotic particles.

**ASSOCIATED CONTENT**

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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