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Specific Sorting of Single Bacterial Cells with Microfabricated Fluorescence-Activated Cell Sorting and Tyramide Signal Amplification Fluorescence in Situ Hybridization

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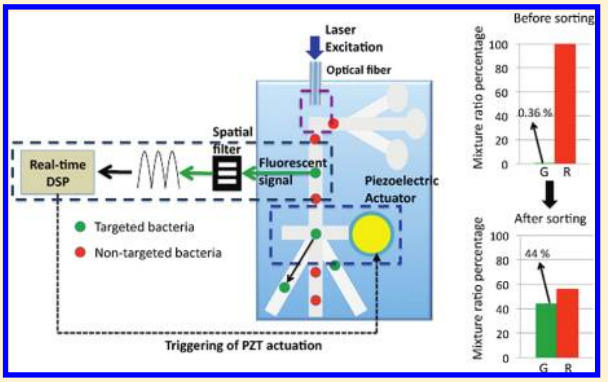
Supporting Information

ABSTRACT: When attempting to probe the genetic makeup of diverse bacterial communities that elude cell culturing, researchers face two primary challenges: isolation of rare bacteria from microbial samples and removal of contaminating cell-free DNA. We report a compact, low-cost, and high-performance microfabricated fluorescence-activated cell sorting (μFACS) technology in combination with a tyramide signal amplification fluorescence in situ hybridization (TSA-FISH) to address these two challenges. The TSA-FISH protocol that was adapted for flow cytometry yields a 10−30-fold enhancement in fluorescence intensity over standard FISH methods. The μFACS technology, capable of enhancing its sensitivity by ∼18 dB through signal processing, was able to enrich TSA-FISH-labeled *E. coli* cells by 223-fold. The μFACS technology was also used to remove contaminating cell-free DNA. After two rounds of sorting on *E. coli* mixed with λ-phage DNA (10 ng/μL), we demonstrated over 100 000-fold reduction in λ-DNA concentration. The integrated μFACS and TSA-FISH technologies provide a highly effective and low-cost solution for research on the genomic complexity of bacteria as well as single-cell genomic analysis of other sample types.



The Earth is populated by vast numbers of diverse microbial communities. A single human being hosts approximately 10^{14} microbial cells of many different species.

In order to understand the complex interactions and activities of microbes in our ecosystem, a large amount of research is devoted to studying their genetics. The amount of DNA available in a single common bacterial cell is only on the order of femtograms, which is insufficient for genomic sequencing. Therefore, cell culture has been considered a prerequisite for obtaining the genomic sequence of bacteria.

Owing to the difficulties in culturing many bacterial cells, it is estimated that less than 1% of bacterial species have been cultured and genotyped.[1] Metagenomics is a culture-independent approach that has provided the first insight into studying genomic diversities of bacteria that elude cell culturing.[2] However, as bacterial communities become highly complex, genomic assembly is rendered very challenging if not unfeasible. Enabled by the development of multiple displacement amplification (MDA) technique, an efficient whole-genome amplification (WGA) method for amplifying small quantities of DNA, single-cell genomics has provided an effective approach toward genomic analysis of highly heterogeneous bacterial samples by analyzing single cells one at a time.[3−8] The most commonly used strategies in single-cell genomics involve cell isolation using serial dilution,[3] micromanipulation,[9] and fluorescence-activated cell sorting (FACS)[4−7] followed by WGA using MDA.[3−7] Furthermore, isolation of targeted bacterial populations (e.g., low-abundance bacteria) for WGA has been achieved by using fluorescence in situ hybridization (FISH) labeling combined with FACS, thereby isolating taxon-specific microbes.[8,10]

Even though the methods discussed above have led to the sequencing of several individual bacterial genomes,[4,8,11] they suffer from several limitations. Two of the key limitations, which will be addressed by the proposed technologies in this manuscript, are (1) the separation of populations of cells for FACS sorting is often performed by target-specific 16S rRNA FISH. This technique often suffers from poor signal to noise due to the limited amount of bacterial ribosomal content,[12−14] thereby preventing the separation and isolation of multiple bacterial species by commercial FACS. (2) The presence of contaminating cell-free DNA from the environmental bacterial samples can be amplified along with the intended DNA template,[3,4] causing a high level of nonspecific amplification. The presence of these nontargeted exogenous DNA sequences can further cause complications during sequence assembly. After performing MDA from a single bacterial cell (*E. coli* K-12), Raghunathan et al. estimated only 30% of the total amplified DNA belonged to the bacteria.[7] Even though contamination of cell-free DNA is a serious issue in single-cell genome sequencing, only a few groups

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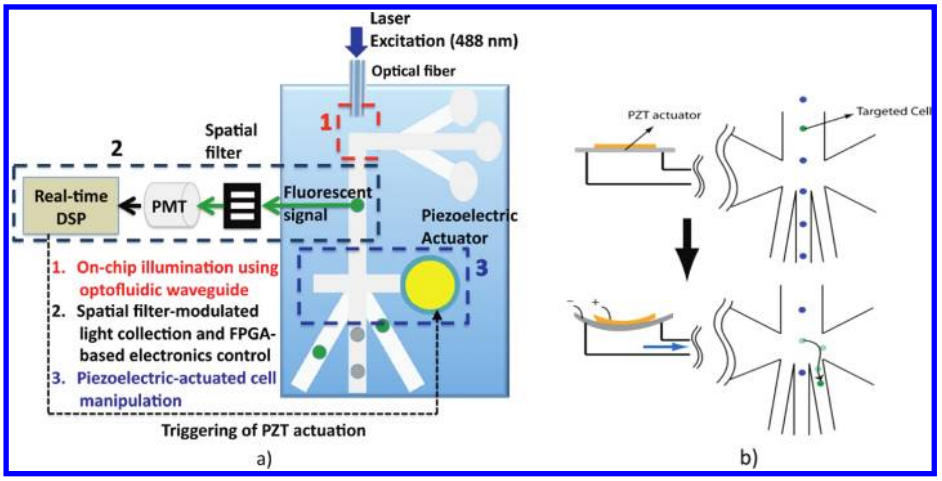

Figure 1. (a) μFACS platform using on-chip light illumination, on-chip piezoelectric actuation, and off-chip spatial filter-modulated light and electronics control system. (b) Schematic of the sorting mechanism: piezoelectric actuation alters the trajectory of the targeted particle from the waste channel to the collection channel.

have performed experiments to characterize the issue.[3,4] In addition to ultraclean handling,[3] two-round FACS flow sorting has been employed to significantly reduce cell-free DNA contamination in bacterial samples.[4] However, experiments that allow meticulous quantification of cell-free DNA removal after each round of sorting have yet been done.

In this paper, we apply a modified tyramide signal amplification FISH (TSA-FISH) process and an integrated μFACS technology as a cost-effective approach to address the aforementioned challenges by increasing the sensitivity limit of conventional FISH, demonstrating high cell-sorting performance, and quantifying the extent of cell-free DNA removal by the μFACS platform. In order to improve the fluorescence intensity of conventional FISH, researchers have applied TSA-FISH in immunohistochemistry studies[15−19] and biological samples fixed on glass slides.[20] However, the current μFACS/FACS system requires samples prepared in suspension, and therefore the standard TSA-FISH protocol must be modified for flow cytometric applications. The proposed TSA-FISH enables cells to be labeled directly in suspension (flow cytometry-compatible) while demonstrating significant fluorescence intensity enhancement over standard FISH.

As an alternative cell-sorting platform, we also report an integrated μFACS technology that provides a number of distinct technical features including high throughput (>1000 cells/s), high-speed cell manipulation (with integrated piezoelectric actuation), low-voltage operation (<10 V_{p-p}), and real-time signal processing for sensitivity enhancement (with an embedded real-time amplification algorithm). Even though commercial FACS have commonly been used to sort bacteria and reduce cell-free DNA contamination in bacterial samples,[4,5] they are bulky, expensive, require specially trained personnel for operation, and often a scarce resource due to these reasons. In comparison, the proposed μFACS technology can serve as a compact, low-cost cell-sorting platform. Furthermore, μFACS offers the unique benefits of elimination of cross-contamination through replacement of a disposable device and operation in a closed environment for minimal sample contamination. Other μFACS technologies that sort cells using optical,[21] electroosmotic,[22] dielectrophoretic,[23] and hydrodynamic switching[24,25] methods have also been demonstrated. However, to our knowledge, none of them have yet been used to address the issues of cell-free DNA contamination and sorting low-abundance bacteria and, therefore, have limited use for single-cell genomics.

We demonstrate the feasibility of this combined approach toward resolving the technical limitations in single-cell genomics by demonstrating detection and sorting of TSA-FISH-labeled *E. coli* cells. Then, the cell-free DNA removal capability of the integrated μFACS is quantified and compared to a state-of-the-art commercial FACS in a dual-round sorting experiment.

MATERIALS AND METHODS

Integrated μFACS. The integrated μFACS consists of three main components: (1) on-chip excitation using an optofluidic waveguide, (2) a spatial filter-modulated light collection system controlled by electronics, and (3) a piezoelectric-actuated cell sorting module (Figure 1a). The poly(dimethysiloxane) (PDMS) microfluidic device with integrated optofluidic waveguides has Teflon AF (transparent amorphous Teflon) coated microfluidic channels.[26] In order to confine the excitation laser light within the microfluidic channel for exciting fluorescent analytes in the optofluidic waveguide, it is essential to coat the PDMS channel, which has a refractive index of ~1.41, with a material having refractive index lower than PBS/water ($n \sim 1.33$). In this manner, PBS/water becomes the core of the waveguide and the low refractive index coating layer acts as a cladding layer for the optofluidic waveguide.

To enhance the detection sensitivity of fluorescent signals, spatial filters with specially designed slit patterns are formed on a mask plate (e.g., a photolithographic transparency mask) to transform the collected fluorescent signal into a temporarily encoded waveform (Figure 1a). As opposed to single-peak signals in conventional detection schemes, the temporarily encoded waveform shows multiple peaks for each cell traveling through the detection region. Upon detection by the photomultiplier tube (PMT), the temporally encoded waveform can be amplified digitally using a match filter algorithm. The method is called space−time coding because it converts a spatial pattern on the mask plate into a time-domain signal, thereby making the waveforms of the encoded signals distinctively different from the noise.[27,28]

The on-chip integrated piezoelectric (PZT) actuator sorts cells by displacing a certain amount (100 pL to 1 nL) of cell-containing fluid transversely at the sorting junction. The sorting mechanism is shown in Figure 1b. When a targeted cell enters the sorting junction, the PZT actuator is activated by a voltage pulse to deflect the cell-containing fluid from the center position toward the side collection channel. Without PZT actuation, cells travel straight down into the waste channel. Since the response time of the PZT actuator is between 0.1 and 1 ms, the μFACS system can operate at >1000 cells/s.

The sorting control system employs an embedded field-programmable gate array (FPGA) chip (CompactRIO, National Instruments Inc., U.S.A.) that allows precise timing control of PZT actuation to sort single cells. A detailed description of the control system can be found in Chen et al. and Cho et al.[27,28] Briefly, space−time-modulated signals are processed after being registered by the PMT and amplified digitally using a match filter algorithm (i.e., finite-impulse response filters). After signal-to-noise ratio enhancement, the improved signal is compared against a user-defined threshold for the cell sorting decision. Should the cell be chosen, a time delay equal to the cell travel time from the detection zone to the sorting junction is introduced before a voltage pulse (<10 V_{p-p}) is sent to trigger PZT actuation.

Fabrication of the Integrated μFACS Device. The integrated μFACS device was fabricated by using the PDMS replica molding technique[29] The Si mold was fabricated using the previously described cryogenic reactive ion etching (RIE) process.[28] For PDMS device fabrication, a mixture consisting of PDMS prepolymer and curing agent at a ratio of 1:10 was poured onto an SU-8 mold and baked for 4 h at 65 °C. After curing, the PDMS substrate was peeled off from the SU-8 mold and holes were punched to form the chamber under the PZT actuator as well as the inlet and outlet reservoirs. The device was then bonded to a PDMS-coated glass substrate after UV−ozone treatment (JeLight, U.S.A.) for 3 min. To couple laser light (488 nm wavelength) into the optofluidic waveguide, the end of a multimode optical fiber was cleaved, index-matched, and butt-coupled to the microfluidic device. To guide the illumination light, a 6% Teflon AF solution was introduced into the microfluidic channels to coat the surfaces of the channels to form the cladding layer of the waveguide. Excess Teflon AF solution was removed by applying vacuum (~20 kPa) at the fluidic outlets. The Teflon AF coated PDMS device was then heated to 155 °C for 20 min to evaporate the fluoro-inert solvent and then heated further to 175 °C for 20 min to form a smooth Teflon AF layer. To integrate a bimorph PZT actuator consisting of a PZT layer and a stainless steel layer with the microfluidic device, the stainless steel side of the PZT actuator was polished with alumina particles to enhance adhesion. The polished surface was then UV−ozone-treated to enable direct bonding between the PDMS substrate and the stainless steel surface of the actuator. The bonding process was carried out in an 85 °C oven for 4 h.

TSA-FISH for Flow Cytometric Applications. We used *E. coli* to demonstrate the enhancement of fluorescence intensity with the modified TSA-FISH technique. A sample containing 100 μL of *E. coli* at a concentration of 10^6 cells/μL was fixed in 100% EtOH for 1 h. The fixed cells were washed twice with PBS at room temperature (RT) and then labeled with biotinylated universal 16S rRNA probe (1000 ng/μL, 5′-GCT GCC TCC CGT AGG AGT-3′) in hybridization buffer (0.9 M NaCl, 0.01% SDS, 0.02 M Tris) for 1.5 h at 46 °C. Labeled cells were washed and incubated in 500 μL of wash buffer (0.01% SDS, 0.18 M NaCl, 0.02 M Tris, 5 mM EDTA) at 48 °C for 20 min. Following incubation, the cells were centrifuged (8000*g*, 3 min at RT) and the wash buffer was removed. The cells were then incubated in a diluted horseradish peroxidase (HRP) conjugation solution (1:50 in 1% blocking solution/PBS) (T-20932, Invitrogen) for 6 h at RT on a rocking plate. The cells were washed twice with 500 μL of PBS at RT, and the sample was incubated in a tyramide working solution (1:50 in amplification buffer) (T-20932, Invitrogen) for 2 h at RT on a rocking plate. After incubation, the cells were washed twice with PBS at RT.

To assess fluorescence intensity enhancement over standard FISH process, universal 16S rRNA FAM and Alexa-488 FISH probes (Invitrogen, Carlsbad, CA) were used. The FAM and Alexa-488 fluorophores are attached to the 16S rRNA probes through biotin−streptavidin interactions (e.g., 5′-biotin to streptavidin conjugated fluorophores). The FISH-labeling procedure followed the protocol mentioned above except for the steps involving TSA labeling. After completion of the labeling processes, both the TSA-FISH and FISH-labeled *E. coli* samples were analyzed by a commercial FACS (MoFlow; DakoCytomation Inc., San Diego, CA). Under identical forward and side scattering (FSC and SSC) gating conditions, the fluorescent intensity histograms for both samples were analyzed and compared.

***E. coli* Sorting Experiment.** Proof-of-concept demonstration of sorting of rare-abundance bacteria was performed using TSA-FISH-labeled *E. coli* A mixture consisting of Alexa-488 and Alexa-647-labeled *E. coli* at a total concentration of ~3×10^7 cells/mL was prepared by TSA-FISH labeling with tyramides coupled to Alexa-488 (495 nm: 519 nm) and Alexa-647 (650 nm: 668 nm) fluorophores (Invitrogen, Carlsbad, CA), respectively. The *E. coli* sample was introduced into the device through two syringe pumps with sample and sheath flow rates of 5 and 50 μL/min, respectively. The estimated average cell velocity was ~5 cm/s, resulting in a screening throughput of ~1500 cells/s. A sorting strategy was implemented to enrich Alexa-488-labeled *E. coli* from the mixture. After μFACS sorting, the relative population (e.g., ratio of Alexa-488-labeled to Alexa-647-labeled *E. coli*) was determined by using commercial FACS for both presort and postsort mixtures. The enrichment capability was quantitatively determined by comparing the mixture ratios.

Removal of λ-DNA through Two-Round Sorting. To characterize cell-free DNA removal capabilities, a dual-round sorting experiment was performed on TSA-FISH (Alexa-488 fluorophores) labeled *E. coli* cells spiked with λ-phage DNA (10 ng/μL). During each sorting operation, both the μFACS and the commercial FACS displaced targeted bacteria from the sample flow to the collection reservoir while the rest of the fluid enters the waste reservoir. Approximately 10^5 and 10^4 cells were sorted by the μFACS for first- and second-round sorting, respectively. The collected volume for each round was ~1 mL. For direct comparison, we also sorted ~10^5 and 10^4 cells for first and second round using a commercial FACS (MoFlo, DakoCytomation Inc., San Diego, CA). A more detailed depiction of the experimental process can be found in the Supporting Information.

Quantification of λ-DNA. The amount of spiked λ-genomic DNA in sorted (μFACS and FACS) and unsorted *E. coli* samples was measured by quantitative polymerase chain reaction (qPCR) using the Mastercycler ep realplex system (Eppendorf, Hauppauge, NY). The 20 μL reactions were assembled in 0.2 mL thin-well tubes containing 1 μL of *E. coli* sample suspension, 0.3 μM λ-specific oligonucleotide primers (5′-ACATTTTCGGTGCGAGTATC-3′

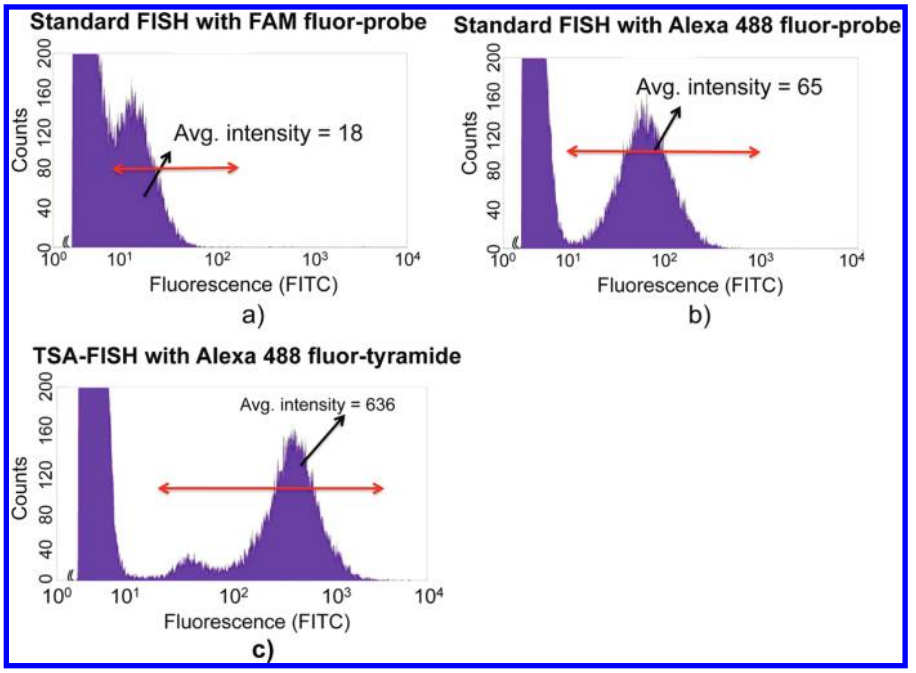

Figure 2. Comparison of standard FISH probe labeling with TSA-FISH on *E. coli* samples. Fluorescence intensity histograms in log (*x*-axis) linear (*y*-axis) scale of standard FISH with (a) FAM fluoroprobe and (b) Alexa-488 fluoroprobe show fluorescent intensities are close to the baseline noise. (c) TSA-FISH with Alexa-488 fluorotyramide shows intensities well above the baseline noise. The TSA-FISH technique enhances the signal by 10−30-fold over the standard FISH method with Alexa-488 fluoroprobes and FAM fluoroprobes, respectively.

(forward) and 5′-GCAGAGTCATAAAGCACCTC-3′ (reverse)), 0.25× SYBR I (Invitrogen, Carlsbad, CA), and 1× EconoTaq DNA polymerase (Lucigen, Middleton, WI). Reactions were incubated at 94 °C for 2 min, followed by 40 cycles at 94 °C for 40 s, 59 °C for 40 s, and 72 °C for 40 s. Dissociation curves were computed for each amplified product at the end of amplification. Each individual sample was run in triplicate, and the averaged critical threshold cycle (Ct) was used for data analysis. The Ct values obtained from sorted samples were compared to the Ct values of unsorted samples, where the difference in Ct values (ΔCt) was transformed into $2^{-\Delta Ct}$ to represent the estimated fold change of the λ-genomic DNA amount between successive rounds.

RESULTS AND DISCUSSION

Sensitivity Enhancement Using Modified TSA-FISH. To evaluate the effectiveness of the flow cytometric modified TSA-FISH labeling, *E. coli* samples (DH10B) were separately labeled with standard FISH fluoroprobes and TSA-FISH fluoroprobes. Figure 2 shows the results of the flow cytometric analyses on two standard FISH fluoroprobes (FAM and Alexa-488 fluoroprobes) and TSA-FISH with Alexa-488 fluorotyramide. A number of bacterial cells present in nutrient-limited conditions (e.g., soil, marine, and freshwater environments) often contain too little rRNA to yield sufficient fluorescence signal using standard FISH,[13,30−32] making detection and sorting of these bacteria very challenging. Although the Alexa-488 fluoroprobe-labeled sample has a peak that is separated from the baseline noise (Figure 2a), the FAM fluoroprobe labeled sample is partially embedded in the baseline noise (Figure 2b). The inability to fully separate signals from baseline noise could adversely affect the purity of sorted mixtures In contrast, the TSA-FISH method, which is generically applicable to flow cytometric-related applications, can provide significant enhancement in fluorescence intensity (10−30-fold over standard FISH with Alexa-488 fluoroprobes and FAM fluoroprobes, respectively) and clearly separate labeled bacteria from nonlabeled samples, thereby minimizing the interference of baseline noise during cell sorting. The signal enhancement capability of this technique also increases the chance of capturing microbes with low rRNA contents.

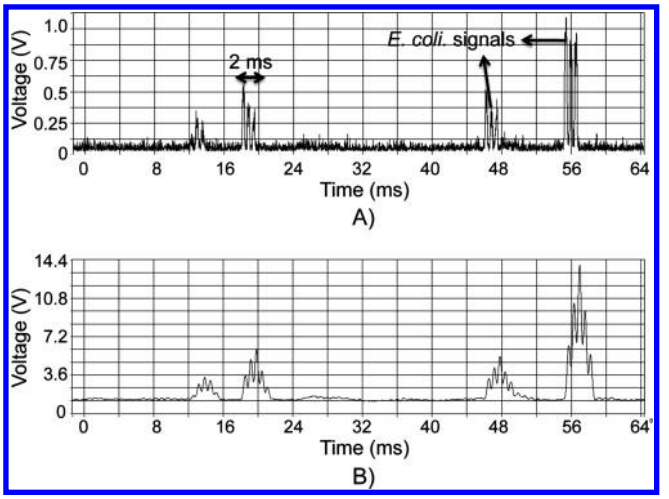

Figure 3. Detection of TSA-FISH-labeled *E. coli*. (A) Raw space−time coded signals detected from individual *E. coli*. The encoded waveform (i.e., a signal showing three peaks due to three slits in the optical spatial filter) of detected signals enables further amplification using a matched filter. The digitally processed signals are shown in panel B with an estimated signal-to-noise ratio enhancement of ∼18 dB.

Although other flow cytometry-compatible catalyzed reporter deposition (CARD) FISH[33−35] (also known as TSA-FISH[33−36]) processes have been reported, these procedures (e.g., filtering cells through a membrane and embedding cells into a gel) are

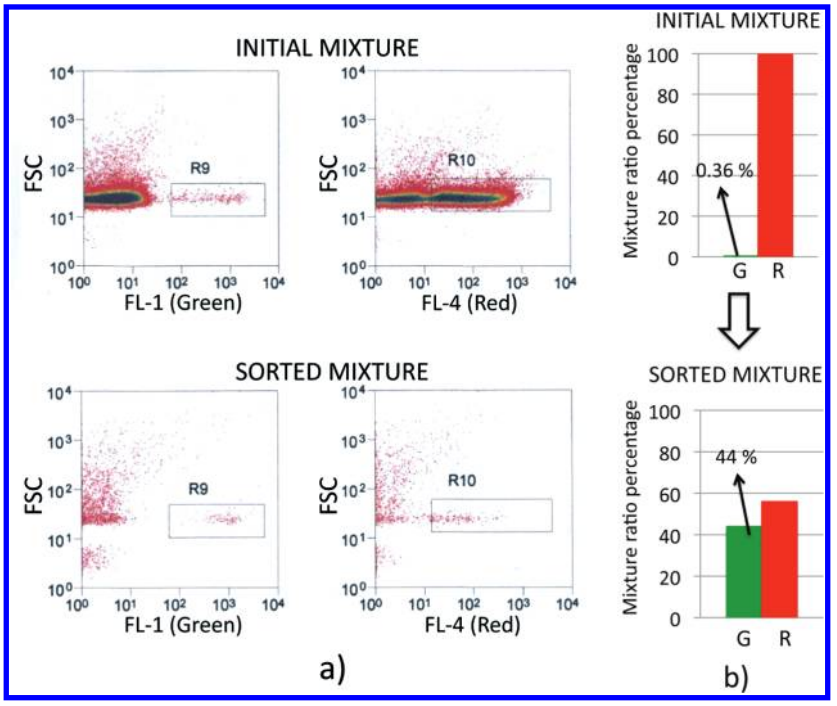

Figure 4. (a) Flow cytometric analysis plots (FSC to Fl-1 (green)/FL-4 (red) plots) with identical gating conditions for initial and sorted mixtures are shown. Purity was analyzed by totaling and analyzing the signals that are included within the gates. After one round of sorting, the initial mixture ratio (Alexa-488-labeled *E. coli*/Alexa-647-labeled *E. coli*) is increased from 0.0036 (1:280) to 0.79 (~4:5), resulting in an enrichment factor of 223-fold. To facilitate visualization of the result, bar charts (b) are used to show the mixture ratio percentage (G = Alexa-488-labeled *E. coli*: R = Alexa-647-labeled *E. coli*) prior to and after sorting.

complicated and laborious. In contrast, the proposed TSA-FISH, which makes use of commercial kits, labels cells directly in suspension, bypassing the cell filtering and embedding steps. This greatly simplifies the labeling process and shortens the sample preparation time (less steps).

Detection of *E. coli*. Prior to cell sorting, the integrated μFACS needs to detect single bacterial cells with sufficient sensitivity. The ability of the μFACS to convert slit patterns on the spatial mask into time-domain signals enables selective amplification of the modulated signals using a preprogrammed match filter algorithm. Figure 3A shows an example of temporarily encoded waveform of TSA-FISH-labeled *E. coli* cells. The signal from each *E. coli* cell has three distinct peaks corresponding to the three-slit pattern on the spatial mask. By means of the match filter algorithm, the quality of the signals is enhanced significantly from the raw data (by approximately 18 dB), as shown in Figure 3B. Even though unprocessed signals (Figure 3A) can be used to identify single *E. coli* cells in this experiment, the amplification enables the user to define a threshold significantly above noise, preventing false-positive detection.

Detecting *E. coli* cells using the μFACS instrument along with the proposed TSA-FISH technique can be seen as a proof-of-concept demonstration of how the combined approach can be implemented. However, compared to lab cultured bacteria, the labeled signal intensity can be highly variable for environmental bacterial samples (soil, water, and animals) due to large differences among cells (size, growth conditions, number of ribosomes, etc.).[37−39] The current μFACS system, which allows signal amplification, can tolerate approximately 10× reduction in signal intensity. Even though some bacteria can still elude detection, the detection capability of the μFACS can be improved in two ways. First, additional slit patterns can be added onto the spatial mask (e.g., four or five slits), further increasing its signal amplification power. Second, electronic noise can be minimized through using a low dark-count PMT and a low-noise PMT amplifier.

Sorting Performance. To make the μFACS technology useful for single-cell genomic studies, the μFACS system must sort bacteria at high throughput and with a high enrichment factor. The screening throughput, estimated using parameters such as flow rates, channel dimensions, and cell concentration, in the current experiment was approximately 1500 cells/s. The enriching capability of the μFACS system was determined by examining mixtures containing Alexa-488-labeled and Alexa-647-labeled *E. coli* cells before and after sorting. Flow cytometric analysis of the sorted result is shown in Figure 4a. By maintaining the same gating conditions for both initial and sorted mixtures, analysis of the FSC to FL-1/FL-4 plots showed that TSA-FISH-labeled green (Alexa-488 fluorophore) *E. coli* cells were enriched from an initial mixture ratio (Alexa-488-labeled cells/Alexa-647-labeled cells) of 0.0036 (1:280) to a sorted mixture ratio of 0.79 (~4:5), demonstrating an enrichment factor of 223. The bar charts (Figure 4b) show a clearer representation of sample enrichment. To the best of our knowledge, the achieved enrichment factor is the highest among μFACS systems in sorting bacteria. Despite significant sample enrichment, the sorted sample was still contaminated with an approximately equal amount (~50%) of Alexa-647-labeled *E. coli* cells due to unintentional cosorting of both targeted and nontargeted cells. Using a less concentrated sample and incorporating additional detection parameters can mitigate this issue. The length of the sorting junction of the μFACS is 100 μm, and the estimated average cell-to-cell spacing is ~33 μm in this experiment. Therefore, to minimize cosorting of cells, the sample concentration should be reduced by more

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than 3-fold to ensure an average cell-to-cell spacing of 100 μm. While reducing screening throughput, this approach can be used to enhance the purity of the sorted sample. Also, incorporation of additional detection parameters (e.g., forward or side scatter based gating)[40] could enable estimation of cell spacing and therefore allow sorting abortion when two or more cells are traveling too close together, thus further reducing coincidence errors due to sorting nontargeted cells with targeted cells.

Since isolation of rare-abundance species is of paramount importance for single-cell genomic studies, in addition to the high sample-enriching capability, robust operation becomes the key in obtaining a sufficient amount of targeted samples. Even though the *E. coli* concentration in our samples was relatively high compared to conventional FACS analysis ($\sim 10^6$ to 10^7 cells/mL), no cell clogging occurred over more than 2 h of continuous sorting operation, thus demonstrating robust operation for bacterial sorting. Unlike conventional FACS systems where individual cells are first confined to individual droplets and then sorted, our current μFACS system performs sorting under continuous fluid flow so that part of the sheath flow is constantly introduced to the sorted sample during the entire operation. This can lead to unwanted sample dilution. Nonetheless, the concentration (cells/μL) of the sorted sample in the current study is approximately 14 cells/μL, which is much higher than the concentration required ($\sim$1 cell in 10−50 μL, equivalent to 0.02−0.1 cells/μL) for single-cell MDA reactions.[3,4] After cell sorting, the sorted sample could be streamlined with other single-cell deposition technologies such as the microwell array technology to enable high-through single-cell WGA.

Cell-Free DNA Purification Performance. Contaminating cell-free DNA poses a great challenge to researchers in the field of single-cell genomics, because exogenous DNA can be amplified and sequenced simultaneously with the targeted bacterial DNA, causing complications during genomic data analysis. To address this challenge, the integrated μFACS technology was used to remove contaminating cell-free DNA from the bacterial samples by performing a two-round sorting experiment. In each sorting round, only the sample fluid volumes that contain targeted bacterial cells were sorted out of the contaminated sample fluid, resulting in a less contaminated sample (e.g., the ratio of amount of contaminated DNA to number of bacteria becomes lower). A control sorting experiment that collects the same number of cells in the same volume using commercial FACS was performed for direct performance comparison with our μFACS system. In the experiment, λ-phage DNA was artificially spiked (at a final concentration of 10 ng/μL) into the initial TSA-FISH-labeled bacterial sample to simulate cell-free DNA contamination because λ-phage DNA is not naturally present in cell culture and not affected by human DNA contamination during handling and sorting processes. A relatively high amount of λ-phage DNA (the ratio of λ-phage DNA content to cell-contained DNA content was approximately 20:1) was chosen to allow quantification of λ-phage DNA to fall within a reliable detection range (less than 35 amplification cycles) before and after two rounds of sorting using qPCR. qPCR curves for each initial and sorted mixture from both instruments can be found in the Supporting Information.

A summary of the results is shown in Table 1. By examining fold changes of contaminating DNA in successive rounds, the results give us insight into the amount of contaminating DNA that was removed after each round and the contaminating DNA removal capability of each instrument. After each round, the commercial FACS (MoFlow; DakoCytomation Inc., San Diego, CA) removed $\sim$2−2.5 times more than the integrated μFACS, and after two rounds of contaminating DNA removal, the difference was approximately 5-fold. This difference is small relative to the amount of purification for each round. Even though efforts have been invested toward removing contaminant DNA,[3,4] to our knowledge, this is the first meticulous quantification of cell-free DNA removal capability by a microfluidic μFACS device.

Table 1. Comparison of the Cell-Free DNA Removal Capability of the Microfluidic Lab-on-a-Chip μFACS and the Commercial FACS

	μFACS		commercial FACS	
rounds of sorting	λ-DNA (ng/μL)	fold reduction	λ-DNA (ng/μL)	fold reduction
initial amount	10		10	
first round	0.030	333	0.014	695
second round	8.5×10^{-5}	351	1.7×10^{-5}	843
total		1.17×10^5		5.86×10^5

Even though the state-of-the-art commercial FACS slightly out-performs the microfluidic μFACS in removing contaminating DNA, an important merit of the μFACS is closed-system operation (i.e., the sample is enclosed at all times during the sorting experiment), which minimizes airborne contamination, as opposed to open-system operation (i.e., sorted droplets are open to the surroundings during the sorting process) for commercial FACS. Additionally, cross-contamination between runs could present a major problem for today's commercial FACS due to equipment sharing. This problem is fundamentally eliminated for the microfluidic μFACS due to its disposable nature.

CONCLUSION

We have demonstrated a combined approach to address two specific challenges in single-cell genomics using a compact, low-cost, and high-performance integrated μFACS system and a modified TSA-FISH technique: (1) detection and sorting of single bacterial cells and (2) removal of contaminating cell-free DNA. In addition to the demonstrated 10−30-fold fluorescent intensity enhancement over conventional FISH using the modified TSA-FISH, the μFACS has demonstrated an approximately 223-fold enrichment factor per round of sorting and more than 10^5-fold reduction of contaminating DNA after two rounds of sorting.

In single-cell genomics, prior to performing single-cell whole genome amplification (WGA), single bacterial cells need to be sorted and deposited into individual wells (384-well or microwells). Even though the current μFACS can sort bacterial cells with high enrichment factor and throughput, to make the system useful for single-cell genomic applications, it is essential to streamline the μFACS technology with a viable cell deposition module. There are two ways we envision this can be achieved. First, after the μFACS enriches targeted bacteria and removes contaminating cell-free DNA, single-cell bacteria could be manually deposited on a microwell array device (currently under development in Dr. Kun Zhang's lab) with optimal cell dilution to allow high-throughput single-cell WGA. Second, with on-chip integration of single-cell deposition modules downstream of the μFACS, the integrated system could be automated to serve as a stand-alone instrument in preparing single cells for WGA. The latter approach, which can minimize contamination from surrounding

environment and provide savings in labor and reagents due to system automation, might be considerably more challenging to implement from a technical standpoint (e.g., single-cell trapping, system automation, removal of amplified DNA sequences for postgenomic sequencing, etc.). In contrast, even though the former approach is more labor-intensive and subjected to contamination from surrounding environment, the microwell array device can be readily streamlined with the current μFACS technology.

Compared to current commercial FACS, the μFACS could offer ~10−100 times cost and size reduction through the use of monolithic PDMS device structure and the incorporation of on-chip PZT actuator and optics. Considering the drastic cost and size reduction and the additional benefits of closed-system operation and disposability, the μFACS system, if combined with a viable single-cell deposition technology, offers a highly affordable and attractive technology that could significantly accelerate progress toward uncovering the genetic diversity of microbial communities at a single-cell level.

■ ASSOCIATED CONTENT

Supporting Information. Additional information and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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