Selective Single-Bacteria Extraction Based on Capture and Release of Microemulsion Droplets

Jiyu Li

City University of Hong Kong

Dinglong Hu

City University of Hong Kong

Chee Kent Lim

City University of Hong Kong

Jifeng Ren

City University of Hong Kong

Xin Yao

Nanyang Technological University

Chao Ma

New York University

Marcos .

Nanyang Technological University

Weiqliang Chen

New York University

Patrick K. H. Lee

City University of Hong Kong

Raymond H. W. Lam (✉ rhwlam@cityu.edu.hk)

City University of Hong Kong

Research Article

Keywords:

Posted Date: January 24th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1240019/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Human host-associated microbial communities in body sites can reflect health status based on the population distribution and specific microbial properties in the heterogeneous community. Bacteria identification at the single-cell level provides a reliable biomarker and also pathological information for clinical diagnosis. Nevertheless, biosamples extracted from some body sites cannot offer sufficient sample volume and number of target cells as required by most of the existing single-cell isolation methods such as flow cytometry. Herein we report a novel integrated microfluidic system, which consists of a microemulsion module for single-bacteria encapsulation and a sequential microdroplet capture and release module for selectively extract only the single-bacteria encapsulating microdroplets. We optimize the system for a success rate of the single-cell extraction to be >80%. We further verify applicability of the system with prepared cell mixtures (*Methylobacterium extorquens* AM1 and *Methylobacterium extorquens* BG8) and biosamples collected from human skin, in order to quantify the population distribution of multiple key species in a heterogeneous microbial community. Results indicate perfect viability of the single-cell extracts and compatibility with downstream analyses such as PCR. Together, this research demonstrates that the reported single-bacteria extraction system can be applied in microbiome and pathology research and clinical diagnosis as a clinical or point-of-care device.

Introduction

Human host-associated microbial communities in body sites such as gut, skin, mouth, and urogenital track largely determine the disease states of patients (Clemente, Ursell, Parfrey, & Knight, 2012). The presence of specific bacterial genera provides not only a reliable biomarker but also pathological information for clinical diagnosis (Chassaing, Aitken, Gewirtz, & Vijay-Kumar, 2012). For examples, primary skin infections, such as impetigo (Bowen, Tong, Chatfield, & Carapetis, 2014), folliculitis (Shu Wei et al., 2019), and boils (O'Gara, 2017), are caused by a type of β -hemolytic and coryneform streptococci, known as *Staphylococcus aureus*, under conditions of eczema or insect bites (Sarkiri, Fox, Fratila-Apachitei, & Zadpoor, 2019). Another example is skin rash, which is commonly examined via standard skin test, e.g. scarping (Hahler, 2006), biopsy (Tarantola et al., 2013), allergy test (Balato, Balato, Di Costanzo, & Ayala, 2011) and blood test (Caubet et al., 2011) to determine the skin bacteria compositions in the heterogeneous bacteria community and thus identify a narrow-spectrum of antibiotics for the effective treatment. Therefore, identification of the responsible organisms in bacterial infection is the key to achieve the corresponding efficient treatment (Mooney, Galloway, & Riley, 2019), yet identifying the specific species is technically challenging in terms of manipulation of single bacteria cell, particularly in infections caused by more persistent bacteria and sophisticated bacterial communities (Russo et al., 2016).

Conventional single bacterial cell isolation techniques include dilution-to-extinction (Giovannoni & Stingl, 2007), single-cell micromanipulation (Sato et al., 2009), and flow cytometry (Wang, Hammes, Boon, Chami, & Egli, 2009). However, most of the existing methods offer a limited isolation yield or require at least a few microliters of the biosample volume, which could be too much to be extracted from the

infection sites (Ishii, Tago, & Senoo, 2010). For example, dilution-to-extinction is hard to directly isolate rare cells and single-cell micromanipulation offers a very limited throughput. Though flow cytometry on the basis of fluorescence-activated cell sorting (FACS) can achieve a high-throughput single-cell isolation (Kim et al., 2013), it still requires a significant amount of the biosample volume (Burmølle, Hansen, Oregaard, & Sørensen, 2003) and indispensable procedures for cell staining which can alter cell properties and limit the more detailed live-cell analyses (Terekhov et al., 2018).

Microfluidics, owing to its capability of precise flow control and micro-particle manipulation, has garnered significant interests on cell isolation of the single cells. For examples, a deterministic ratchet for selective separation of particles with different sizes around 1 – 3 μm was reported by Loutharback *et al.* (Loutharback, Puchalla, Austin, & Sturm, 2009), yet its application for bacteria separation has not been demonstrated. By contrast, Chun *et al.* (C. H. Chen et al., 2011) reported a microfluidics-based FACS for isolating fluorescence-labeled *Escherichia coli* from a microbial sample. In this study single cells can be extracted by a piezoelectric actuator, however it can not separate the droplets for individual incubation and any downstream analysis afterward. In addition, microemulsion, which forms stable aqueous microdroplets in an immiscible buffer fluid, usually oil, has been demonstrated as a powerful tool for bacteria isolation and further analyses including incubation, DNA analysis, and drug screening (D. Chen et al., 2008; Dendukuri, Tsoi, Hatton, & Doyle, 2005; Edgar et al., 2009; Frenz et al., 2008; Koster et al., 2008; Shah, Kim, Agresti, Weitz, & Chu, 2008). For example, Eun *et al.* (Eun, Utada, Copeland, Takeuchi, & Weibel, 2011) presented the encapsulation of *Escherichia coli* in agarose particles and subsequently analysis by FACS, but the cell extraction for downstream inoculation and analysis was then technically difficult. In addition, Boedicker *et al.* (Boedicker, Li, Kline, & Ismagilov, 2008) presented a microemulsion system for rapid detection and drug susceptibility screening of bacteria in complex biological matrices. However, the isolated droplet cannot be extracted individually for single bacteria analysis, such as polymerase chain reaction (PCR) amplification and DNA sequencing. Moreover, Liu *et al.* (Liu, Kim, Lucchetta, Du, & Ismagilov, 2009) developed a microfluidic emulsion-based device for incubation of single bacteria isolated from a mixture of *P. curdlanolyticus* and *E. coli*. However, the operation configuration has not been optimized for the higher yield of single cell encapsulation and a microdroplet elimination scheme for the void and multi-bacteria droplets is required. Therefore, an integrated microfluidic system that achieves a high throughput yet efficient capture of bacteria at a single-cell level from a limited input of biosample and is highly compatible with various downstream analyses via controllable release is urgently needed yet largely unavailable.

Recently, we have developed a microfluidic strategy for sequential isolation of mammalian cells (10 – 20 μm) in aqueous biosamples, *e.g.* blood, in microfluidic based platform (Li et al., 2020; Tran, Kong, Hu, Marcos, & Lam, 2016) with a yield of >95%. Cells are sequentially captured in the micro-sieve structures, whose positions have been arranged carefully according to the deterministic lateral shift (Huang, Cox, Austin, & Sturm, 2004) for the maximum capture rate. Notably, on top of the additional functional capability offered by microemulsion, the cell-encapsulating microdroplets enlarge the physical scale (from sub-micron for bacteria to tens of micron for microdroplets) of the ‘particles’ to be manipulated. Hence, we hypothesized that integration of a microemulsion unit for encapsulation of bacteria in a

droplet of which the working scale is similar to our previous device will extend its application for single-bacteria manipulation and subsequent genetic and functional analyses.

In this work, we developed a micro-device integrated with microemulsion and sequential micro-sieves for single-bacteria isolation and selective cell extraction. We optimized the system dimensions and operation parameters by characterizing the microdroplet generation, the sequential microdroplet trapping, the single-bacteria encapsulation in droplets and the microdroplet extraction. As a proof-of-concept, we demonstrate applicability of the micro-device with prepared skin bacteria and human skin biosample and achieved single-bacteria extraction from multi-species bacterial communities and the following basic genetic analysis. We believe our integrated microfluidic system will demonstrate a useful and powerful tool for clinical diagnosis (Lei, 2012) of microbial infection and as well for screening potential drugs or antibiotics.

Results And Discussion

Single-bacteria extraction micro-device

We have designed and fabricated (as described in Methods) a microfluidic device for isolation and extraction of single-bacteria (Fig. 1a,b). The device consists of a microemulsion module for generating cell-encapsulating microdroplets, a series of micro-sieves for microdroplet isolation, and multiple pairs of side microchannels for selective droplet extraction. In the microemulsion module, a bacteria sample is inserted from the central inlet and buffer mineral oil is applied along the side channels (Fig. 1b, *left* inset). In the microemulsion module, we applied only the culture medium along the central biosample microchannel and mineral oil (M8410, Sigma) mixed with 5 % (volume ratio) surfactant (Span 80, S6760, Sigma) along the side buffer oil microchannels. The surfactant was added to increase the interfacial tension between the oil and generated droplets (3.1 mN/m) to maintain droplet stability (Evans, Mitchell, & Ninham, 1986).

The microemulsified droplets then entered the isolation microchannel region (width: 145 μm ; height: 30 μm) and were captured in the micro-sieves (Fig. 1b, *right* inset). We have adopted our previously reported micro-sieve placement scheme (Tran et al., 2016) for isolating the microdroplets. The micro-sieve arrangement can offer a high isolation yield ($\sim 95\%$), though the sequence of the trapped droplets is not the major concern. We considered a working droplet diameter of $20.35 \pm \text{SD } 1.3 \mu\text{m}$ and performed the simulation study (Fig. S1) to determine the alternating lateral displacements (5 μm to the *left/right* side) and dimensions (outer diameter: 45 μm ; inner diameter: 25 μm ; opening gap width: 10 μm) of the micro-sieves. Besides, the channel height (30 μm) and working flow rates ($\sim 0.25 \mu\text{l/min}$) of the buffer mineral oil (kinematic viscosity: 20.5 mm^2/s) implies a low *Reynolds* number ($\ll 1$) flow.

We supplied a sample volume of 10 nl for generating and isolating $\sim 87.7 \pm \text{SD } 3.15$ microdroplets (Fig. S2). The microdroplets containing only one stained bacterial cell can be identified under a fluorescence microscope. We then ungated the corresponding pair of side micro-valves (Fig. 1b, *right* inset) in

sequence along the extraction microchannels and apply a flow from the extraction inlet with a total volume of 2 μl in order to export the microdroplet to a PCR tube placed at the extraction outlet. By repeating such steps for each single-cell-encapsulated microdroplet, we extracted every individual bacterial cell in a PCR tube for various downstream processes, *e.g.* species identification, biochemical/molecular characterization and genetic modification.

Flow conditions for micro-droplet generation

We conducted experiments to characterize diameters of the generated micro-droplets as a function of the buffer oil pressure (0.5 – 8 psi), whereas the biosample pressure was maintained 0.5 psi. Diameters of the microemulsed droplets under the different pressure ratios were quantified from video snapshots (Fig. 2a) captured by a camera (Zyla 5.5, Andor) under a microscope (TE300, Nikon). Our results suggest that a higher driving pressure induced droplets with smaller diameters (Fig. 2b) at a higher generation rate (Fig. S2). Each constant pressure level of the oil buffer generated droplets with a range of diameters. For example, the buffer oil pressure of 1 psi can generate microdroplets with a diameter in of $\sim 20.35 \pm \text{SD } 1.3 \mu\text{m}$ (Fig. 2c and Fig. S3) and the corresponding rate of droplet generation is $\sim 2.13 \pm \text{SD } 0.46$ droplets/s.

Flow configuration for droplet isolation

We investigated the droplet deformation under different pressure levels across a micro-sieve region by both experiments and simulation analyses. The simulation model depicted a representative droplet-isolation microchannel section (length: 400 μm). It contained one micro-sieve with the lateral distance of 200 μm from the channel section center. Simulated results for the cases of an 'empty' micro-seive and (Fig. S1a) a micro-seive capturing a microdroplet (Fig. S1b) suggest that a separating distance of 400 μm between micro-sieves is sufficiently long for flow recovery that the deviation of flow direction could restore to a negligible amount ($< 5\%$).

In order to predict the maximum working buffer pressure level for successful encapsulation of a droplet with a defined diameter, we also conducted simulation by including the deformation and movement of a trapped droplet for different diameters. As illustrated in Fig. 3a (upper row), a droplet larger than the micro-sieve gap can still deform and pass through the gap under an essecive pressure level. The simulation results estimate the maximum pressure levels for trapping different droplet diameters as shown in Fig. 3b.

Further, we performed experiments to verify the simulation results. We applied the microemulsion at different different buffer pressure levels to generate droplets and examine whether the droplet can be trapped, or escape via the micro-sieve gap as shown in Fig. 3a (lower row). The diameter of generated droplets can be varied by tuning the sample inlet pressure in the range of 0.3 – 0.6 psi. We then recorded the success of droplet trapping for different droplet diameters and buffer pressure levels as plotted in Fig. 3b. The simulation and experimental results match reasonably for the buffer pressure pressure ≤ 1.3 psi

as the simulated cutoff could roughly separate the trapped and escaped droplet cases. Yet, for the higher buffer pressure, the simulation is no longer representative, possibly because the larger deformation of microdroplets under the higher pressure can no longer be considered as solid as adopted in our simulation.

To achieve the maximum droplet isolation rate, we selected the buffer pressure level to be able to trap for the entire diameter range of the generated droplets. Recalling Fig. 2c that the droplet diameter is $>17\text{ }\mu\text{m}$ for 1 psi buffer pressure, we adopted 1 psi for driving the oil buffer flow, as indicated in Fig. 3c (arrow). We have also quantified for the isolation rate of the generated droplets under different buffer pressures as shown in Fig. 3d, indicating that the ideal buffer pressure level for the maximum isolation rate ($\sim 95\%$) is indeed around 1 psi. As predicted, the isolation rate reduces for the pressure higher than 1 psi because the droplets can escape from the micro-sieve gaps. A lower isolation rate for the pressure below 1 psi could be explained by the exceeded lateral shift of the generated oversized droplets. Furthermore, we have verified that the configuration of microstructure dimensions and operation parameters can maintain sequential trapping of the generated microdroplets in micro-sieves as shown in Fig. 4 and Supplemental Video 1.

Single-bacteria capture and release

We performed experiments to characterize the yield of microdroplet isolation in the micro-sieves with different cell concentrations in biosamples. For each experiment run, we applied a sample volume of $11.3 \pm \text{SD } 0.594\text{ nl}$ for generating $\sim 95.75 \pm \text{SD } 0.96$ droplets. We counted the generated micro-droplets during the microemulsion process. Afterward we counted the microdroplets captured in the micro-sieves as shown in Fig. 5a. The rate of the microdroplet isolation is $94.54 \pm \text{SD } 0.322\%$, demonstrating a high capture efficiency of our design of the micro-sieve array.

We further tested different cell concentrations ($6.25 \times 10^6 - 4 \times 10^7\text{ cell/ml}$) of pre-stained *Methylobacteria extorquens* AM1 (Fig. 5b and Fig S4) to identify the range for a higher possibility of generating the single-cell-encapsulated micro-droplets. We found that the highest yield is $85.45 \pm \text{SD } 8.26\%$ with the cell concentration of $1 \times 10^7\text{ cell/ml}$ (Fig. 5c). Considering also the $\sim 95\%$ success rate of the microdroplet isolation, $>80\%$ of bacteria in the biosample can appear as a single-cell in a micro-sieve-captured microdroplet.

Beside capturing droplets containing single bacteria, extraction of target droplets encapsulating single-bacteria for further analysis such as clinical diagnosis is also critical (Evans et al., 1986). As demonstrated in Fig. 1, the micro-sieve is positioned between its corresponding pair of inlet and outlet channels with the lateral sieve location is further set along. As expected, the stimulation streamlines of the side-channel flow can easily release the droplet from the micro-sieve accordingly with an input flow (Fig. S5). In this case, the captured droplet can be individually extracted by opening the micro-valves of the side channels with the device inlet and outlet closed, followed by applying flow through the side

channels with a total volume of 2 μ l under a driving pressure of 0.5 psi (Fig. 6 and Supplemental Video 2), in order to transfer the cell-containing droplet to a syringe tube placed at the corresponding extraction outlet. Then, a culture medium was added in the syringe tube was vortex-shaked and centrifuged, followed by pipetting away the upper layer of mineral oil. Each isolated cell can then be transferred to another growth environment for further incubation or analysis.

We further investigated viability of the released cells. *M. extorquens* AM1 cells were collected from the extraction outlets after the cell isolation process. The isolated bacteria was transferred to a culture medium (500 μ l), which was amended with the antibiotic kanamycin (50 mg /ml) and methanol (v/v 1.3%) as the carbon source, and placed in in a shaking incubator (30 °C and 200 rpm). For this experiment, we placed 50 isolated single bacteria in the same syringe tube. Afterward, the bacteria culture was extracted after culture (between 12 h and 60 h), stained (LIVE/DEAD BacLight Bacterial Viability Kit, Thermo fisher, USA) and quantified for cell density and viability by the flow cytometry (FASCVerse, BD, USA). For comparison, we also applied the regular bacteria culture, whose desired cell density was prepared by dilution, without processed with the cell isolation steps as the control group. Our results (Fig. S6) indicate that the reported cell isolation technique can perfectly maintain the cell viability and growth as the regular culture.

Identification of single cells from multi-bacterial strain mixtures

As a proof-of-concept, we applied the device to extract single-bacteria from known-multistrain biosamples and unknown-multistrain biosamples and verified its bacteria identification by PCR tests. First, we prepared a known-strain sample by mixing *M. extorquens* AM1 (prelabeled with DAPI) and *M. album* BG8 (prelabeled with propidium iodide) with a 1:1 population ratio at a resultant cell density of 2×10^7 cell/ml. In this case, the color of extracted bacteria observed in microscope can reflect which species they belong to and be considered as the confirmation of the PCR results. In addition, we performed another experiments with only the mineral oil droplets without cells as the negative control. The PCR results (Table 1 and Fig. S7) indicate that 67 % of the isolated *M. extorquens* single cells and 88 % of the isolated *M. album* single cells could be amplified and correctly identified. There was no mis-identification of the type of bacteria and there was no signals for the control case, *i.e.* no false positive result. However, not all the sorted single cells could yield a positive PCR amplification from the isolated bacteria. This could be caused by factors that cells in the extracted droplets may fail to be transferred to the cell lysis reagent and genomic amplification with only one bacterium is a challenging task without a guaranteed success rate (Gawad, Koh, & Quake, 2016).

Table 1

PCR results of the sorted single cells from a defined two-species mixture

	Isolated sample	
	<i>M. extorquens</i> AM1 single cells	<i>M. album</i> BG8 single cells
PCR primers		
<i>M. extorquens</i> AM1-specific	16/24 ^a (67 %)	0/24 (0 %)
<i>M. album</i> BG8-specific	0/24 (0 %)	21/24 (88 %)

^a The number of positive PCR amplification over the number of single cells tested

To further validate the applicability for analyse of clinical biosample, we then adopted the same single-bacteria extraction procedures on human skin biosamples (collection protocol detailed in Methods) to conducted PCR for the gene sequences of the isolated single-cells. We have performed five repeated rounds of the cell extraction and identification experiments on different days from the same subject. The droplet isolation rate was $97.3 \% \pm \text{SE } 0.21 \%$ and the single-bacteria isolation rate was $84.1 \% \pm \text{SE } 2.4 \%$, agreeing with the values for other experiments described above. As demonstrated, we have captured all the micro-sieve regions of a representative run of experiments as shown in Fig. S8. The gene sequence analysis revealed that $\sim 16.1 \%$ of the isolated skin bacteria belonged to the bacterial genus *Cutibacterium* ($>95 \%$ similarity) (GenBank Accession no.: OM250408; OM250409; OM250410; OM266150), which is known to be a human skin commensal (Green & Ardley, 2018). The remaining 7.2% of bacteria include the *Variovorax guangxiensis* strain GXGD002 (Gao et al., 2015) ($>99 \%$ similarity) (GenBank Accession no.: OM250407) ($\sim 3.3 \%$ of totally remaining collected bacteria), which is present in the common environment, and other unknown strains ($\sim 3.9 \%$ of totally remaining collected bacteria). These results essentially demonstrates compatibility of the bacteria isolation technique with PCR, and probably with other downstream genetic analyses (Amann & Fuchs, 2008), such as rRNA sequencing (Klindworth et al., 2013) and metagenomics (Langille et al., 2013; Qin et al., 2012). Furthermore, this single-bacteria can also be applied to other human liquid biopsies as a minimally invasive method for disease diagnoses. For examples, meningococcal infection can be identified by examining bacteria in peripheral blood (Newcombe, Cartwright, Palmer, & McFadden, 1996) instead of directly obtaining the cerebrospinal fluid by surgery. Osteogenesis-associated microbial contamination in bone (Segura-Egea et al., 2012) can be predicted by obtaining the population distribution of different oeriapical periodontitis-related strains in saliva for the increased number of Epstein–Barr virus and some anaerobic bacteria (e.g. *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, or *Prevotella intermedia*) (Jakovljevic et al., 2018). On the other hand, the throughput of the single-cell extraction can be enhanced by the further system development. The device can be extended for including more micro-sieves. The single-bacteria encapsulation in droplets can be regonized by computer-assisted image analysis. The microfluidic operations can also be further automated.

Materials And Methods

Fabrication

The single-bacteria extraction micro-device is fabricated by multilayer soft lithography ([Unger, Chou, Thorsen, Scherer, & Quake, 2000](#)), as summarized in Fig. S9. Layers of AZ 50XT (AZ-50XT, AZ Electronic Materials) or/and SU-8 (SU-8 2010, Microchem) photoresists on 4-inch silicon wafers are micropatterned by photolithography to generate replica molds. In particular, the 'control layer' mold contains SU-8 (height: 10 μm) microstructures on a wafer. To fabricate the 'flow layer' mold, SU-8 (height: 30 μm) is micropatterned on a wafer, followed by micropatterning AZ 50XT (height: 25 μm) overhead. The reflow process (120 $^{\circ}\text{C}$, 1 min) is then applied to refine cross-sections of the AZ 50XT microstructures, for the complete closure of microvalves in the resultant micro-devices fabricated as described below. These two replica molds are treated by air plasma (energy: 5 kJ; PDC-002, Harrick Plasma) and deposited with trichloro (1H, 1H, 2H, 2H-perfluoro-octyl) silane (Sigma-Aldrich) for better release of the molded materials.

Polydimethylsiloxane (PDMS) monomer and the curing agent (Sylgard-184, Dow Corning) are mixed (10 % weight ratio), degassed. The PDMS pre-polymer is poured onto the flow layer mold with a thickness of 3 mm and spin-coated onto the control layer mold with a thickness of 40 μm . The PDMS pre-polymer samples are then cured at 80 $^{\circ}\text{C}$ for 2 h. The molded PDMS substrates are then cut and peeled off. Air plasma treatment (energy: 10 kJ) is applied to the PDMS surfaces, followed by aligning and bonding them under a dissection microscope. The bonded PDMS is cut, peeled off, punched at the inlets/outlets, treated with air plasma (energy: 10 kJ) and bonded on a glass slide.

Flow simulation

Computational studies are performed using COMSOL Multiphysics (COMSOL, Burlington, MA). Two three-dimensional models are constructed and analyzed for the flow characteristics with and without presence of a micro-droplet (diameter: 20 μm) captured in a micro-sieve. They are both a channel section containing a series of micro-sieves positioned along the channel with an alternating lateral offset along the consecutive sieves. The inlet pressure and outlet pressure of both models are defined as 0.2758 kPa and 0 kPa, respectively.

On the other hand, a three-dimensional model of a microchannel section containing a micro-sieve and a moving and deforming droplet is applied to determine the critical droplet diameter for the effective droplet capture. We consider the laminar two-phase flow for the droplet (water) and its surrounding liquid (mineral oil). The gage inlet pressure applied to the microchannel section should be $\sim 1\%$ of the inlet pressure of the device because each device contains a hundred of the micro-sieve sections consecutively. To reveal the dynamic droplet deformation during the encapsulation process, we adopt the moving mesh module for the time-lapsed computation. As the droplet may undergo a large deformation, we configure the hyperelastic mesh smoothing for the droplet body.

Stained bacteria mixture

Methylobacteria extorquens AM1 (ATCC 14718) (Green & Ardley, 2018) and *album* BG8 (ATCC 33003) (Whittenbury, Phillips, & Wilkinson, 1970) were purchased from the American Type Culture Collection. They were cultivated aerobically in 160-ml serum bottles in the modified *Methylococcus* medium (ATCC medium 1057), in which $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was replaced by $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ was replaced by $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, FeSO_4 was replaced by $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.5 % (v/v) methanol was added. The bottles were loosely capped and incubated at 30 °C with shaking (200 rpm). Stationary phase cells were collected by centrifugation (9600 ×g). 70 % isopropanol was used to disrupt cell membranes for staining. *M. album* BG8 were stained blue in color with 0.3 μM 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, USA) and *M. extorquens* AM1 red in color with 60 μM propidium iodide (Invitrogen, USA). The cell concentration was measured by a hemocytometer (Isolab, Germany). The two bacteria were mixed with an equal concentration (1×10^7 cell/ml) prior to experiments.

Bacteria extraction from human skin

Skin samples were collected from the forearm of a 28-year-old healthy Chinese male. As human subject is involved, the bacteria extraction procedures and the required ethical matters were approved by the Committee on the *Use and Care of Animals* of the City University of Hong Kong (study: A-0236). A written consent was also obtained from the volunteer. The collection of skin samples was approved by the City University of Hong Kong Human Subjects Ethics Sub-Committee (ref: H001553). Briefly, a sterile swab (Isohelix, UK) moistened in phosphate-buffered saline (Life Technologies, UK) was used to sample a 4-cm² area on the forearm in a back-and-forth motion for 40 times. Cells dislodged from a swab by vortexing. The cells were then stained by 20 mM SYTO™ 9 (Thermo Fisher Scientific, USA). The collected sample were then filtered by a syringe filter (0.2 μm, Sigma) to remove the skin cells. The cell concentration was measured by a hemocytometer (Isolab, Germany) and adjusted to 1×10^7 cells/ml before experiments. We confirm that all methods were performed in accordance with the relevant guidelines and regulations.

Polymerase chain reaction (PCR)

The isolated cells are trapped in microdroplets held within mineral oil with a volume of 2 μl, which were thermally lysed and mixed with PCR reagents. We identified the known species *M. extorquens* AM1 with the primer pair q_phaB-Mex [39], and *M. album* BG8 with the primer pair QpmoA-7 [40] using Premix Ex Taq Ver. 2.0 (Takara, Japan) PCR reagent. On the other hand, for unknown species in the human skin extracts, we applied the universal 16S rRNA gene primer set 63F and M1387R (Fredriksson, Hermansson, & Wilén, 2013) with Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs, USA). Sanger's sequencing was conducted on the positive PCR products. The resultant nucleotide sequences were searched with the NCBI nr database via BLASTn, in order to identify the corresponding stains.

Statistics

Error bars in plots represent standard errors with additionally specified. Two-tailed p -values are calculated by the Student's t-test. An asterisk shown in plots represents a significant difference ($p < 0.05$) between two data groups.

Conclusion

We successfully developed a single-bacteria extraction system based on microemulsion and deterministic lateral displacement of the generated micro-droplets sequentially flowing into micro-sieves. We demonstrated the single-cell isolation from heterogeneous microbial communities using our developed system can then be achieved with a high yield (>80% compared to 44.6% for the conventional techniques (Ishii et al., 2010; Rappe, Connon, Vergin, & Giovannoni, 2002)) and a small biosample volume of ~10 nL. Notably, the residual volume biosample can be recollected from the device outlet for the another round of single bacteria isolation process, implying that multiple rounds of the single-bacteria isolation process by recycling the biosample can achieve a higher overall cell isolation rate.

We have demonstrated the isolation of single-bacteria from a prepared bacteria mixture of *Methylobacterium) extorquens* AM1 and *Methylomicrobium album* BG8 and verified the unaffected viability. This demonstrates the compatible of our integrated microfluidic system with conventional downstream bioanalysis processes such as incubation, genetic detection and biochemical analysis. More importantly, our microfluidic single-bacteria extraction system was able to quantify the key bacteria population of human skin samples, highlighting its potential applications in other clinical and environmental heterogenous microbial samples. In summary, we envision that our microfluidic single-bacteria isolation system, with further system automation, will demonstrate a self-functioning medical device and achieve clinical and point-of-care diagnostic applications.

Declarations

Acknowledgments

We acknowledge financial supports from Hong Kong Research Grant Council (General Research Grant 11216220) and National Natural Science Foundation of China (NSFC Grant 31770920).

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

The DNA sequences adopted in this work have been submitted to INSDC member repository. The GenBank accession numbers are OM250407, OM250408, OM250409, and OM250410 for *Cutibacterium*, and OM266150 for *V. guangxiensis* GXGD002.

Author Contributions

JL conducted experiments and drafted the manuscript. DH designed the micro-device, and performed experiments and simulation. CKL helped PCR experiments. JR and XY helped simulation. CM, M and WC helped refine technical details and revise the manuscript. PKHL and RHWL initialed the research idea, supervised the project, verified data analysis and revised the manuscript.

References

1. Amann, Rudolf, & Fuchs, Bernhard M. (2008). Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. *Nature Reviews Microbiology*, 6(5), 339-348. doi: 10.1038/nrmicro1888
2. Balato, Anna, Balato, Nicola, Di Costanzo, Luisa, & Ayala, Fabio. (2011). Contact sensitization in the elderly. *Clinics in Dermatology*, 29(1), 24-30. doi: <https://doi.org/10.1016/j.clindermatol.2010.07.003>
3. Boedicker, James Q, Li, Liang, Kline, Timothy R, & Ismagilov, Rustem F. (2008). Detecting bacteria and determining their susceptibility to antibiotics by stochastic confinement in nanoliter droplets using plug-based microfluidics. *Lab on a Chip*, 8(8), 1265-1272.
4. Bowen, Asha C., Tong, Steven Y. C., Chatfield, Mark D., & Carapetis, Jonathan R. (2014). The microbiology of impetigo in indigenous children: associations between *Streptococcus pyogenes*, *Staphylococcus aureus*, scabies, and nasal carriage. *BMC Infectious Diseases*, 14, 727. doi: <http://dx.doi.org/10.1186/s12879-014-0727-5>
5. Burmølle, M., Hansen, L. H., Oregaard, G., & Sørensen, S. J. (2003). Presence of N-acyl homoserine lactones in soil detected by a whole-cell biosensor and flow cytometry. *Microbial Ecology*, 45(3), 226-236. doi: 10.1007/s00248-002-2028-6
6. Caubet, Jean-Christoph, Kaiser, Laurent, Lemaître, Barbara, Fellay, Benoît, Gervaix, Alain, & Eigenmann, Philippe A. (2011). The role of penicillin in benign skin rashes in childhood: a prospective study based on drug rechallenge. *Journal of Allergy and Clinical Immunology*, 127(1), 218-222. doi: <https://doi.org/10.1016/j.jaci.2010.08.025>
7. Chassaing, B., Aitken, J. D., Gewirtz, A. T., & Vijay-Kumar, M. (2012). Gut microbiota drives metabolic disease in immunologically altered mice. *Advanced Immunology*, 116, 93-112. doi: 10.1016/B978-0-12-394300-2.00003-X
8. Chen, Chun H., Cho, Sung H., Chiang, Hsin- I., Tsai, Frank, Zhang, Kun, & Lo, Yu-Hwa. (2011). Specific sorting of single bacterial cells with microfabricated fluorescence-activated cell sorting and tyramide signal amplification fluorescence in situ hybridization. *Analytical Chemistry*, 83(19), 7269-7275. doi: 10.1021/ac2013465

9. Chen, D., Du, W., Liu, Y., Liu, W., Kuznetsov, A., Mendez, F. E., . . . Ismagilov, R. F. (2008). The chemistrode: a droplet-based microfluidic device for stimulation and recording with high temporal, spatial, and chemical resolution. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(44), 16843-16848. doi: 10.1073/pnas.0807916105
10. Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell*, *148*(6), 1258-1270. doi: 10.1016/j.cell.2012.01.035
11. Dendukuri, D., Tsoi, K., Hatton, T. A., & Doyle, P. S. (2005). Controlled synthesis of nonspherical microparticles using microfluidics. *Langmuir*, *21*(6), 2113-2116. doi: 10.1021/la047368k
12. Edgar, J. S., Milne, G., Zhao, Y., Pabbati, C. P., Lim, D. S., & Chiu, D. T. (2009). Compartmentalization of chemically separated components into droplets. *Angewandte Chemie International Edition*, *48*(15), 2719-2722. doi: 10.1002/anie.200805396
13. Eun, Y. J., Utada, A. S., Copeland, M. F., Takeuchi, S., & Weibel, D. B. (2011). Encapsulating bacteria in agarose microparticles using microfluidics for high-throughput cell analysis and isolation. *ACS Chemical Biology*, *6*(3), 260-266. doi: 10.1021/cb100336p
14. Evans, D.F., Mitchell, D.J., & Ninham, B.W. (1986). Oil, water, and surfactant: properties and conjectured structure of simple microemulsions. *The Journal of Physical Chemistry*, *90*(13), 2817-2825.
15. Fredriksson, Nils Johan, Hermansson, Malte, & Wilén, Britt-Marie. (2013). The choice of PCR primers has great impact on assessments of bacterial community diversity and dynamics in a wastewater treatment plant. *PLoS ONE*, *8*(10), e76431.
16. Frenz, L., El Harrak, A., Pauly, M., Begin-Colin, S., Griffiths, A. D., & Baret, J. C. (2008). Droplet-based microreactors for the synthesis of magnetic iron oxide nanoparticles. *Angewandte Chemie International Edition*, *47*(36), 6817-6820. doi: 10.1002/anie.200801360
17. Gao, J. L., Yuan, M., Wang, X. M., Qiu, T. L., Li, J. W., Liu, H. C., . . . Sun, J. G. (2015). *Variovorax guangxiensis* sp. nov., an aerobic, 1-aminocyclopropane-1-carboxylate deaminase producing bacterium isolated from banana rhizosphere. *Antonie Van Leeuwenhoek*, *107*(1), 65-72. doi: 10.1007/s10482-014-0304-3
18. Gawad, Charles, Koh, Winston, & Quake, Stephen R. (2016). Single-cell genome sequencing: current state of the science. *Nature Reviews Genetics*, *17*(3), 175-188. doi: 10.1038/nrg.2015.16
19. Giovannoni, Stephen, & Stingl, Ulrich. (2007). The importance of culturing bacterioplankton in the 'omics' age. *Nature Reviews Microbiology*, *5*(10), 820-826. doi: 10.1038/nrmicro1752
20. Green, P. N., & Ardley, J. K. (2018). Review of the genus *Methylobacterium* and closely related organisms: a proposal that some *Methylobacterium* species be reclassified into a new genus, *Methylorubrum* gen. nov. *International Journal of Systematic and Evolutionary Microbiology*, *68*(9), 2727-2748. doi: 10.1099/ijsem.0.002856
21. Hahler, Barbara. (2006). An overview of dermatological conditions commonly associated with the obese patient. *Ostomy Wound management*, *52*, 34-40.
22. Huang, Lotien Richard, Cox, Edward C, Austin, Robert H, & Sturm, James C. (2004). Continuous particle separation through deterministic lateral displacement. *Science*, *304*(5673), 987-990.

23. Ishii, Satoshi, Tago, Kanako, & Senoo, Keishi. (2010). Single-cell analysis and isolation for microbiology and biotechnology: methods and applications. *Applied Microbiology and Biotechnology*, 86, 1281-1292. doi: 10.1007/s00253-010-2524-4
24. Jakovljevic, Aleksandar, Andric, Miroslav, Knezevic, Aleksandra, Miletic, Maja, Beljic-Ivanovic, Katarina, Milasin, Jelena, & Sabeti, Mohammad. (2018). Herpesviral infection in periapical periodontitis. *Current Oral Health Reports*, 5(4), 255-263.
25. Kim, S., Han, S. I., Park, M. J., Jeon, C. W., Joo, Y. D., Choi, I. H., & Han, K. H. (2013). Circulating tumor cell microseparator based on lateral magnetophoresis and immunomagnetic nanobeads. *Analytical Chemistry*, 85(5), 2779-2786. doi: 10.1021/ac303284u
26. Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, 41(1), e1. doi: 10.1093/nar/gks808
27. Koster, S., Angile, F. E., Duan, H., Agresti, J. J., Wintner, A., Schmitz, C., . . . Weitz, D. A. (2008). Drop-based microfluidic devices for encapsulation of single cells. *Lab on a Chip*, 8(7), 1110-1115. doi: 10.1039/b802941e
28. Langille, M. G., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., . . . Huttenhower, C. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology*, 31(9), 814-821. doi: 10.1038/nbt.2676
29. Lei, Kin Fong. (2012). Microfluidic systems for diagnostic applications: A review. *Journal of laboratory automation*, 17(5), 330-347.
30. Li, Jiyu, Liu, Ya, Ren, Jifeng, Tay, Benjamin, Luo, Tao, Fan, Lei, . . . Lam, Raymond. (2020). Antibody-coated microstructures for selective isolation of immune cells in blood. *Lab on a Chip*, 20(6), 1072-1082. doi: 10.1039/d0lc00078g
31. Liu, Weishan, Kim, Hyun Jung, Lucchetta, Elena M., Du, Wenbin, & Ismagilov, Rustem F. (2009). Isolation, incubation, and parallel functional testing and identification by FISH of rare microbial single-copy cells from multi-species mixtures using the combination of chemistode and stochastic confinement. *Lab on a Chip*, 9(15), 2153-2162. doi: 10.1039/B904958D
32. Loutherbach, K., Puchalla, J., Austin, R. H., & Sturm, J. C. (2009). Deterministic microfluidic ratchet. *Physical Review Letters*, 102(4), 045301. doi: 10.1103/PhysRevLett.102.045301
33. Mooney, Jason P., Galloway, Lauren J., & Riley, Eleanor M. (2019). Malaria, anemia, and invasive bacterial disease: a neutrophil problem? *Journal of Leukocyte Biology*, 105(4), 645-655. doi: <https://doi.org/10.1002/JLB.3R11018-400R>
34. Newcombe, Jane, Cartwright, Keith, Palmer, Walter H, & McFadden, Johnjoe. (1996). PCR of peripheral blood for diagnosis of meningococcal disease. *Journal of Clinical Microbiology*, 34(7), 1637-1640.
35. O'Gara, James P. (2017). Into the storm: chasing the opportunistic pathogen *Staphylococcus aureus* from skin colonisation to life-threatening infections. *Environmental Microbiology*, 19(10), 3823-3833. doi: <https://doi.org/10.1111/1462-2920.13833>

36. Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., . . . Wang, J. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*, *490*(7418), 55-60. doi: 10.1038/nature11450
37. Rappe, Michael, Connon, Stephanie, Vergin, Kevin, & Giovannoni, Stephen. (2002). Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature*, *418*, 630-633. doi: 10.1038/nature00917
38. Russo, A., Concia, E., Cristini, F., De Rosa, F. G., Esposito, S., Menichetti, F., . . . Bassetti, M. (2016). Current and future trends in antibiotic therapy of acute bacterial skin and skin-structure infections. *Clinical Microbiology and Infection*, *22*, S27-36. doi: 10.1016/s1198-743x(16)30095-7
39. Sarkiri, Maria, Fox, Stephan C., Fratila-Apachitei, Lidy E., & Zadpoor, Amir A. (2019). Bioengineered skin intended for skin disease modeling. *International Journal of Molecular Sciences*, *20*(6), 1407. doi: <http://dx.doi.org/10.3390/ijms20061407>
40. Sato, Tomoyuki, Hongoh, Yuichi, Noda, Satoko, Hattori, Satoshi, Ui, Sadaharu, & Ohkuma, Moriya. (2009). Candidatus *Desulfovibrio trichonymphae*, a novel intracellular symbiont of the flagellate *Trichonympha agilis* in termite gut. *Environmental Microbiology*, *11*, 1007-1015. doi: 10.1111/j.1462-2920.2008.01827.x
41. Segura-Egea, J. J., Castellanos-Cosano, L., Machuca, G., López-López, J., Martín-González, J., Velasco-Ortega, E., . . . López-Frías, F. J. (2012). Diabetes mellitus, periapical inflammation and endodontic treatment outcome. *Medicina Oral, Patología Oral y Cirugía Bucal*, *17*(2), e356-361. doi: 10.4317/medoral.17452
42. Shah, R. K., Kim, J. W., Agresti, J. J., Weitz, D. A., & Chu, L. Y. (2008). Fabrication of monodisperse thermosensitive microgels and gel capsules in microfluidic devices. *Soft Matter*, *4*(12), 2303-2309. doi: 10.1039/b808653m
43. Shu Wei, Teo, Yaqing Elena, Yong, Siew Mei Samantha, Ng, Ng, Fui Mee, Jeanette Woon Pei, Teo, Jureen, Roland, . . . Chia, C. S. Brian. (2019). Identification of an ultra-short peptide with potent *Pseudomonas aeruginosa* activity for development as a topical antibacterial agent. *International Journal of Peptide Research and Therapeutics*, *25*(1), 359-366. doi: <http://dx.doi.org/10.1007/s10989-018-9678-9>
44. Tarantola, T. I., Vallow, L. A., Halyard, M. Y., Weenig, R. H., Warschaw, K. E., Grotz, T. E., . . . Otley, C. C. (2013). Prognostic factors in Merkel cell carcinoma: analysis of 240 cases. *Journal of the American Academy of Dermatology*, *68*(3), 425-432. doi: 10.1016/j.jaad.2012.09.036
45. Terekhov, S. S., Smirnov, I. V., Malakhova, M. V., Samoilov, A. E., Manolov, A. I., Nazarov, A. S., . . . Altman, S. (2018). Ultrahigh-throughput functional profiling of microbiota communities. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(38), 9551-9556. doi: 10.1073/pnas.1811250115
46. Tran, Quang D, Kong, Tian Fook, Hu, Dinglong, Marcos, & Lam, Raymond HW. (2016). Deterministic sequential isolation of floating cancer cells under continuous flow. *Lab on a Chip*, *16*(15), 2813-2819.
47. Unger, Marc A, Chou, Hou-Pu, Thorsen, Todd, Scherer, Axel, & Quake, Stephen R. (2000). Monolithic microfabricated valves and pumps by multilayer soft lithography. *Science*, *288*(5463), 113-116.

48. Wang, Y., Hammes, F., Boon, N., Chami, M., & Egli, T. (2009). Isolation and characterization of low nucleic acid (LNA)-content bacteria. *ISME Journal*, 3(8), 889-902. doi: 10.1038/ismej.2009.46
49. Whittenbury, R., Phillips, K. C., & Wilkinson, J. F. (1970). Enrichment, isolation and some properties of methane-utilizing bacteria. *Microbiology*, 61(2), 205-218. doi: <https://doi.org/10.1099/00221287-61-2-205>

Figures

Figure 1

(a) A fabricated single-bacteria extraction micro-device. (b) Device design. Scale bar: 2 mm. The *left* inset shows the microchannel junction for microdroplet generation via microemulsion. The *right* inset is the microchannel section containing a series of micro-sieves and multiple pairs of microvalve-gated side channels for selectively extracting the target microdroplets.

Figure 2

(a) Micrograph of microfluidic emulsion. Scale bar: 100 μm . (b) Diameter of the generated microdroplets for different gage pressures of oil and a consistent biosample pressure of 0.5 psi. (c) Statistics of diameter of the generated micro-droplet under a 1 psi driving pressure of the sample flow.

Figure 3

(a) Cell shape and position at different reference time points obtained by simulation (*upper*) and experiments (*lower*), driven by a exceeding pressure of 2 psi such that the cell could deform and squeeze through the gap of the micro-sieve. Scale bar: 5 μm . (b) Counts of trapped and escaped droplets upon different diameters and oil buffer pressures. The *green* line specifies the simulated cutoff droplet diameter for different oil pressures, determining whether the droplet can be trapped (above the cutoff line).

- (c) Averages (points) and ranges (gray region) of generated droplet diameter for different oil pressures.
- (d) Isolation rate of micro-droplets under different oil pressures.

Figure 4

Trajectories of three droplets being captured sequentially in micro-sieves. Scale bar (*upperleft*): 30 μm .

Figure 5

(a) A micro-sieve contains a micro-droplet encapsulating a rod-shaped *M. extorquens* AM1 cell, labeled with SYTO™ 9. Scale bar: 10 μm . (b) Occurrence of different numbers of captured bacteria in a microdroplet upon cell concentration in the bio-sample. (c) Statistics of single-cell capture rate of microdroplets for different bacteria concentrations in sample medium.

Figure 6

Extraction of a target microdroplet (*white* arrow) containing single bacterial cell at different time points. Scale bar: 50 μm . Flow from the extraction inlet induces a backward flow along the micro-sieve region (*black* arrow) such that the microdroplet escape the micro-sieve and escape the device through the side channel outlet.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SRESI.docx](#)
- [Video1Capture.avi](#)
- [Video2Release.avi](#)