Trends in Analytical Chemistry 170 (2024) 117444

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

journal homepage: www.elsevier.com/locate/trac

Emerging single-cell microfluidic technology for microbiology

Yanqing Song [a,1], Jianan Yin [b,1], Wei E. Huang [c], Bei Li [d], Huabing Yin [a,*]

[a] Division of Biomedical Engineering, School of Engineering, University of Glasgow, Glasgow, G12 8LT, UK
[b] Key Laboratory of Systems Bioengineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin, China
[c] Department of Engineering Science, University of Oxford, Parks Road, OX1 3PJ, Oxford, UK
[d] State Key Laboratory of Applied Optics, Changchun Institute of Optics, Fine Mechanics and Physics, Chinese Academy of Sciences, Changchun, 130033, China

ARTICLE INFO

Keywords:
Single-cell microfluidics
Microbial communities
Raman spectroscopy
Antibiotic resistance
Droplet microfluidics

ABSTRACT

Cell-to-cell differences exist ubiquitously in microbial communities. This individual heterogeneity, often manifested in cell phenotypic function (e.g., antibiotic resistance), can be critical in determining the fate of a microbial community. However, studying such heterogeneity in microbial communities remains a significant challenge due to their enormous diversity and complex cell-cell interactions. Here we review recent advances in microfluidic technologies for detecting, manipulating, and sorting microbial populations at the single-cell level, which significantly advanced our understanding of microbial behaviour and their roles in the microbial ecosystem. We will highlight microfluidic systems with label-free detection methods, including optical imaging and Raman spectroscopy, due to their advantages in investigating real-world microbial communities. We will showcase these technologies in emerging applications, including rapid diagnosis of pathogens and antibiotic resistance, chemotaxis, and Raman-activated cell sorting to search for natural microbial cells of desirable phenotypic function.

1. Introduction

Microorganisms have enormous diversity and number on the planet, with an estimated 10^{30} microbial cells in the biosphere [1]. Understanding the function of microorganisms is fundamentally essential for microbial ecology, clinical microbiology and applied microbiology. However, this remains a significant challenge due to the diversity and complexity of interactions between community members. The central dogma to study microorganisms depends on colony culture to obtain pure isolates, which requires labour-intensive screening of suitable medium and a series of dilutions to reach a single cell. Most natural microorganisms have yet to be cultured in the lab and remain as "dark matter". Furthermore, even with a pure isolate, traditional functional assays measure the average response of a whole isolate population, which masks the difference between individual members. Such an individual difference can be critical to the function and fate of the whole population [2].

The advent of microfluidic technology has opened unprecedented opportunities for microbiology. Microfluidics has an extraordinary ability to control fluids at the microscale level and can precisely manipulate cells and their surrounding microenvironment. For example, laminar flow dominates in single-phase microfluidic devices and can generate precise gradients of solutions, pH or temperatures at the micrometre length scale [3]. Incorporating diffusion-based mixing (e.g., via hydrogel and porous membrane) offers additional flexibility to deliver reagents in controlled time and space. Similarly, geometric designs (e.g., barriers, wells, multi-layers) and active manipulation mean (dielectrophoresis, surface acoustic wave, optical tweezers etc.) allow precise manipulation of the trajectory of cells. Single-phase microfluidic systems offer significant flexibility to conduct multiple-step assays on the same cell or monitor the dynamic processes of cell responses (see a previous review on studying mammalian cells [4].

Droplet-based microfluidics first appeared in the early 21st century [5] and evolved rapidly into a promising, high throughput tool. Droplet microfluidics use immiscible liquid phases to generate microdroplets as picoliter or femtoliter microreactors. In recent years, droplet microfluidics have also been increasingly used for microbiology applications; there are several excellent reviews [6–8]. This review will showcase a single-cell approach via droplet microfluidic to tackle antibiotic resistance and explore natural microbiota.

Although investigations at the single-cell level open many opportunities, they also bring challenges in terms of detection. Microbial cells

* Corresponding author.
E-mail address: huabing.yin@glasgow.ac.uk (H. Yin).
[1] 1: Y.S and J.Y contributed equally to this work

https://doi.org/10.1016/j.trac.2023.117444
Received 19 February 2023; Received in revised form 12 November 2023; Accepted 13 November 2023
Available online 18 November 2023

are small (~1 μm), thus requiring advanced optical imaging technologies. Thanks to the small footprint of microfluidic devices, they can be easily integrated with modern optical microscopes. Conventional culture-based assays have been adapted on chip, including phase contrast imaging of cell growth, enzymatic reactions and fluorescence labelling [9–12]. Raman spectroscopy recently emerged as a label-free, non-invasive technology to explore the microbial world without prior knowledge [13–15]. The integration of microfluidic with Raman technology is a fast-developing field; it opens the opportunity for identifying and isolating functional microbial cells from the "dark matter" [16–18].

In this review, we describe recent developments in microfluidics to study microorganisms at the single-cell level. We start with the current main approaches for on-chip analysis of bacteria and then move to the emerging technologies for cell sorting. In light of increasing threats of antibiotic resistance to global health, we highlight systems designed for mechanistic investigations of antibiotic resistance and rapid antibiotic susceptibility tests (AST). We also illustrate another most prolific application of single-cell microfluidics in studying bacterial chemotaxis. While on-chip observations provide rich information for new biological insights, isolating target cells for downstream processes has become critical. This allows for harvesting microbial cells of a specific function and identifying their genetic makeup. Thus, our last section will present recent developments in bacterial sorting, especially on the emerging technology of Raman-activated cell sorting.

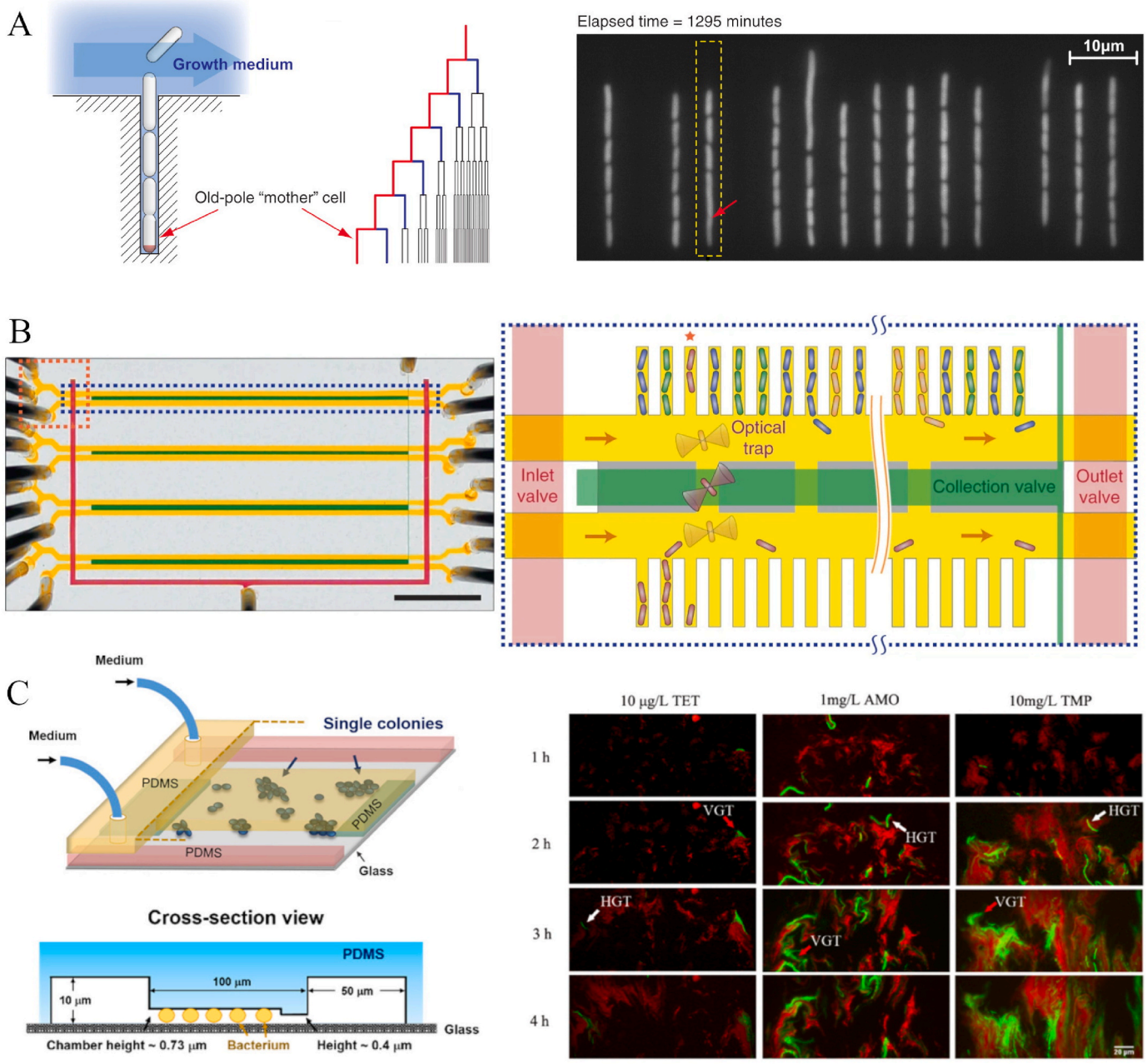

Fig. 1. Microfluidic devices for mechanisms investigations. (A) Schematic illustration of the mother machine device [22]. The old-pole mother cell is trapped at the end of a growth channel, and her progeny grows linearly along the channel. (B) Schematic representation of the single-cell retrieval principles of the SIFT platform [33]. Cells of interest are optically trapped and transported to designated collection or characterization zones by a series of collection valves. (C) Schematic illustration of the microchamber-based device to trap a monolayer of cells in low culture chambers (left) [9]. Mechanisms regarding the dynamic horizontal and vertical gene transfer processes under antibiotics treatment in activated sludge communities were analysed using this device (right) [12]. Figures adapted and reprinted with permissions from (A) Elsevier 2010, (B) Springer Nature 2020, (C) American Chemical Society 2017 and Elsevier 2019.

2. Single-cell microfluidics for on-chip analysis

Single-phase microfluidic devices integrated with microstructures and hydrogels have excellent flexibility for manipulating cells and fluid properties [19]. This offers unique advantages for cell culturing on chip, for example, maintaining a chemostatic culture to observe cell growth from individual cells over generations. The accurate control of growth conditions and fast exchange of reagents also permit monitoring physiological cell transitions from one metabolic state to another [20]. Coupled with automated time-lapse microscopic imaging and analysis software, microfluidic systems allow a phenotypic analysis of individual cells with high spatial and temporal resolutions. This provides rich information on the individual heterogeneity in a microbial population and opens avenues to quantitative microbiology. Below we describe the challenges and main approaches to designing microfluidic devices for studying microbial cells at the single-cell level, followed by their critical applications in investigating cell-to-cell interactions, tackling antibiotic resistance and studying chemotaxis.

2.1. Designs of microfluidic devices for single-cell analysis

Microbial cells are not only small but also motile. Thus, immobilisation of microbial cells on chip is often needed to track individual cells. Common approaches utilise narrow tracks (e.g. "Mother machine"), microchambers or agarose pads to trap or encapsulate cells in hydrogels [9,21–24]. Unconventional methods, such as dielectrophoretic and acoustic forces, have been used to trap cells [25–27]. Representative examples of these approaches and their distinct advantages for microbiology studies are described below.

"Mother Machine" devices: A microfluidic device named 'mother machine' consists of thousands of narrow growth channels with one end closed and the other end open to a trench, which has an excellent ability to control cell growth environments and acquire high-throughput single-cell data [22,28] (Fig. 1A). The flow in the trench supplies fresh medium by diffusion into the growth channels and removes daughter cells that emerge from the tracks into the trench. The cell lineage is easily identified, as the mother cell is trapped at the end of a growth channel while the daughter cells grow linearly along the track.

"Mother Machine" devices have found numerous usages in mechanism investigations and shed light on microbial strategies under specific environments, such as the physiology under nutrient fluctuations and the adaptation strategy of genetic modifications. For example, Kaiser et al. presented a novel integrated platform that consists of a dual-input Mother Machine (DIMM) chip and a Mother Machine Analyzer (MoMA) to monitor cell growth and gene regulation under continuously varying environments [29]. The DIMM chip contains two input ports and serpentine channels, allowing for mixing two media in an arbitrary time-dependent manner, whilst the MoMA software was used for cell segmentation and tracking. The author demonstrated the gene regulatory mechanisms involving the dynamic induction of the *lac* operon in response to alternating carbon sources between glucose and lactose. This work highlights the importance of temporal changes in bacterial growth regulation with different nutrient availability.

"Mother Machine" devices have been widely used in combination with auto-fluorescence, fluorescent probes, and genetically encoded fluorescent reporters for mechanistic study of the kinetics in microorganism-drug interactions [30–32]. For example, Cama et al. studied the accumulation dynamics of the fluoroquinolone antibiotic ofloxacin in hundreds of *E. coli* cells using the auto-fluorescence of the drug [32]. The auto-fluorescence property allows tracking drug dosage and cellular accumulation without labelling or affecting molecular transport. This platform was further used with synthesized fluorescent antibiotic probes to rapidly identify variants in the antibiotic accumulation [30]. The results showed that fast-growing cells could avoid macrolide accumulation and survive treatment compared to slow-growing variants - a contrary finding to the consensus that dormancy and slow growth lead to bacteria survival against antibiotics. More recently, optical tweezers were used with a "mother machine" device, which allows for isolating and retrieving individual cells of interest after tracking the growth of individual cells for multiple generations in growth lanes [33](Fig. 1B).

Although "mother machine" devices are powerful for long-term tracking of cell lineages, the need to trap individual cells in narrow tracks also imposes a high demand for microfabrication facilities to generate high-quality features $<\sim 1$ μm). This also restricts such devices from studying mixed species or natural microbial communities.

Microchambers or other physical barriers: Alternative designs, such as agarose pads and microchambers that randomly confine cells and force them into monolayer growth, have been presented [9,23,34,35]. The most straightforward format is an agarose pad/layer. A simple assembly of a thin agarose layer and PDMS chip can instantly create an antibiotic concentration gradient and perform assays on complex natural communities such as active sludge microbiota [36]. Similarly, microchamber-based devices usually have a lower culture chamber to trap cells for observation but higher side channels to facilitate reagent delivery and remove extra cells [9] (Fig. 1C). Compared to "one dimension" growing cells, monolayer growth in microchambers allows observation of cell morphological changes in X and Y dimensions. The growth dynamics, i.e., each cell's growth rate and lag time, were obtained. A 2D plot of these characteristics provides a measure of the heterogeneity of the population [9]. This facilitates the determination of cell growth characteristics and the discovery of new mechanisms [12,37,38].

Encapsulation of cells in hydrogel provides another simple method to immobilize cells and can be realised by mixing cells with agarose in a microfluidic culture chamber [39]. Due to the highly porous structure of hydrogels, molecular transport in hydrogels is driven by diffusion. This can be used to create gradients of drugs to test a wide range of conditions on the same chip within a short timeframe [39]. Various hydrogel microfluidic devices have been developed for testing bacteria responses to antibiotics via monitoring cell growth in a gel, as described in Section 2.3. Since tracking the growth of multilayer bacteria in a 3D gel is challenging, controlling gel thickness and specialised imaging analysis are essential.

2.2. On-chip analysis of microbial interaction

Microbial interactions are ubiquitous in the natural world. Elucidating the mechanisms of cell-cell interactions is fundamental in understanding the function and composition shifts of natural communities, which can be harvested to engineer synthetic communities. However, conventional approaches (such as "mating plates" and metagenomics) only give snapshots of the overall population, but the process of communications remains a "black box". The ability of single-cell microfluidic devices to track individual cells under purposely designed conditions opens new avenues to investigate underlying processes.

Many devices described in Section 2.1 have been used to investigate cell-cell communications via direct or indirect contact (such as through molecule diffusion) [40]. Coupled with fluorescence labelling techniques, single-cell microfluidic systems can monitor the growth dynamics and allow the dissection of complex and diverse intraspecies and interspecies interactions inside microbial communities. These applications include studies on genetic oscillations, quorum sensing, horizontal gene transfer, syntrophic interactions, community stability and functions, as illustrated below.

2.2.1. Direct cell-cell contact

Microfluidic systems for investigating direct cell-cell contact often use cultivation microchambers where multiple strains or species with possible interactions are mixed and confined to grow into monolayers in close proximity [41–44]. A synthetic microbial consortium that consists of an activator strain and a repressor strain was cultivated in a

microfluidic device with trapping chambers [42] (Fig. 2A). The robust and synchronized oscillations in the two-strain gene circuit were generated only when the two strains were co-cultivated in growth chambers, showing their programmed cellular signalling interactions. The competition for space was demonstrated in a synthetic community of two *E. coli* strains within cell trapping regions on chip [41], revealing the spatiotemporal growth dynamics of the two strains.

Visualising direct cell-cell contact over a time course allows for investigating horizontal gene transfer (HGT) processes in microbial communities. Cooper et al. demonstrated cross-species HGT in the predator strain *Acinetobacter baylyi* and its prey *E. coli* in microfluidic traps [43]. It was discovered that the predator cells conferred resistance to antibiotics by taking up resistance genes released from their adjacent lysed prey. An example of natural microbial consortia is the plasmid-mediated HGT between *E. coli* and an activated sludge bacteria community in a microfluidic agarose device [44]. The dynamic transfer process showed that the gene transfer routes strongly depend on the bacterial community compositions. The authors exposed the sludge community to three antibiotics and dissected the contributions of horizontal (HGT) and vertical gene transfer (VGT) to the transmission of antibiotic resistance genes (ARG) [12] (Fig. 1C). It was found that different inhibitory mechanisms of the antibiotics and their targets affected HGT. VGT significantly contributed to transconjugants formation and subsequent ARG spreading.

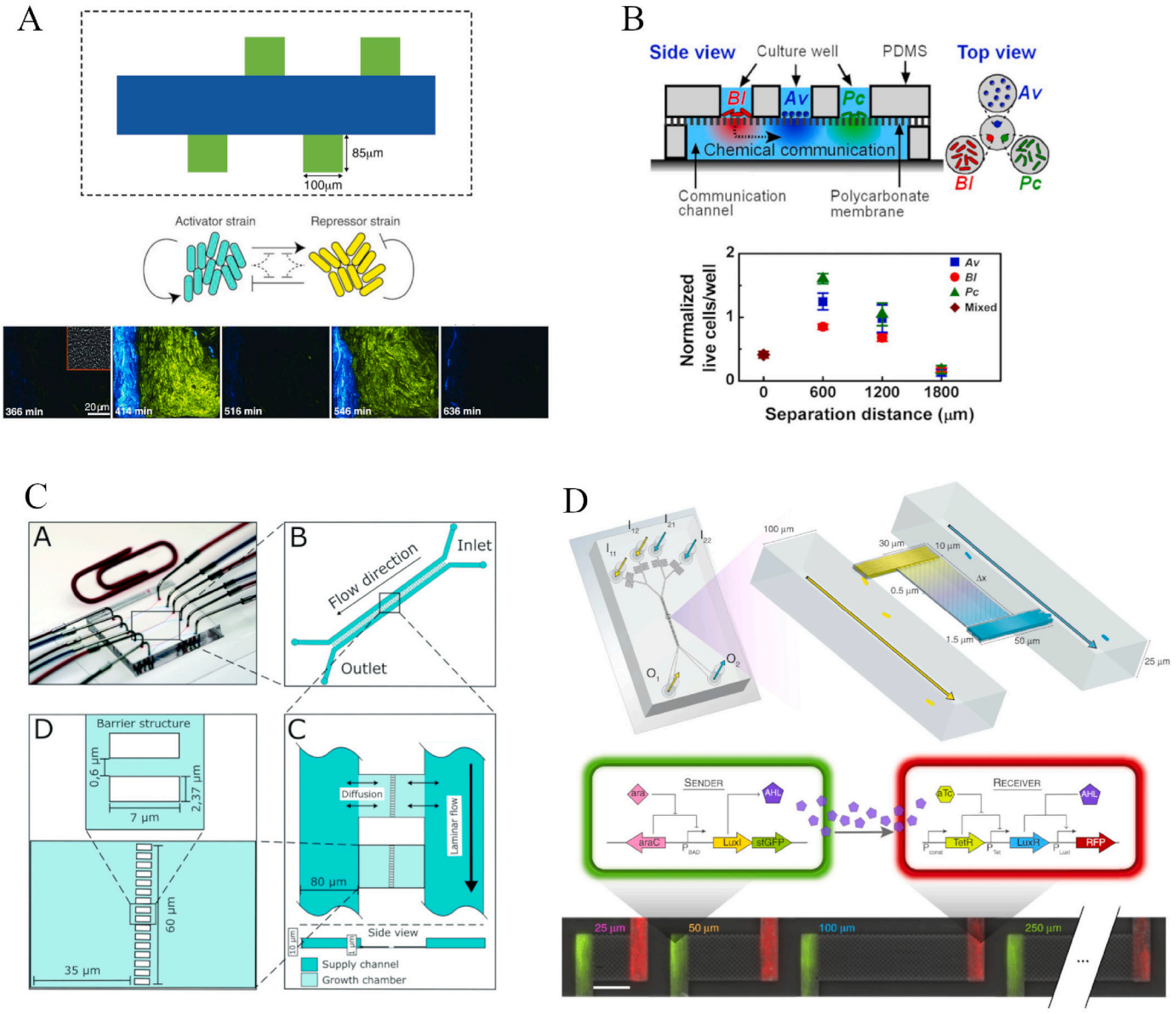

Fig. 2. Microfluidic systems for the investigation of cell-cell interactions. (A) Direct cell-cell contact allows synchronized oscillations in a synthetic microbial consortium that consists of an activator strain and a repressor strain co-cultured in microfluidic traps [42]. (B) Non-contact cell-cell communication. Schematic of the porous membrane integrated microfluidic device used to co-culture a three-species bacterial community by imposing spatial structure with three culture wells and a communication channel [46]. (C) Microfluidic co-cultivation device with a barrier structure to separate growth chambers [47]. Metabolite exchange between adjacent growth chambers occurs via diffusion through nanochannels. (D) Schematic of the MISTiC microfluidic platform to quantify the role of the defined spatial structure of microbial populations and the periodic changes of the environmental signals [48]. Pairs of growth chambers are separated by interaction channels with different lengths. Figures adapted and reprinted with permissions from (A) American Association for the Advancement of Science 2015, (B) the National Academy of Sciences 2008, (C) the Royal Society of Chemistry 2019, and (D) Springer Nature 2020.

2.2.2. Indirect cell-cell contact

Microbial interactions without cell-cell contact often involve the diffusion of metabolites between species, which can be realised using patterned agarose [24], porous membranes [45], and nanochannels. The microfluidic structures can be designed to control molecular diffusion processes and understand how they contribute to the structure and function of microbial ecosystems. For example, Moffitt et al. developed patterned agarose with submicron linear tracks to constrain bacteria and direct their growth into linear microcolonies [24]. The colonies separated by different distances were monitored to examine proximity-dependent communications.

Porous membranes have been used to control molecular diffusion between species. An early example involves exploring how the spatial organization balances the competition and syntropic interactions in a three-species bacterial community [46] (Fig. 2B). A porous polycarbonate membrane was assembled between culture wells and a fluid channel, allowing molecular diffusion between wells through the fluid channel while localising each species in an individual well. The coexistence was highly unstable in well-mixed conditions in a test tube. In contrast, a stable community could be maintained in the microfluidic device when each population was separated at an intermediate distance.

For co-culture microfluidics, a barrier structure is often incorporated on chip to separate adjacent microchambers. For example, Burmeister et al. used barriers to create an array of nanochannels that allow metabolite diffusion but prevent cross-chamber flow [47] (Fig. 2C). In a similar setup, pairs of growth chambers are connected by 0.5 μm high separation channels with a series of separation distances [48] (Fig. 2D). A lattice of pillars supports the separation channels, serves as a physical barrier for the cells and permits biomolecular diffusion. The authors investigated the inter-strain interactions of three pairs of synthetic microbial consortia. They quantified the role of the defined spatial structure of microbial populations and the periodic changes of the environmental signals [48]. Their results elucidated the critical parameters for gene expression patterns in gene circuit oscillators, sender-receiver quorum-sensing and metabolite cross-feeding.

3D printing has emerged as a flexible means to create tailored ecosystems where multiple populations of bacteria can be organised within essentially any geometry. A 3D printing method was used to develop a core-shell arrangement of selected bacteria (two human pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*) in gelatin to study microbial interaction [49]. The results show that the *S. aureus* core was protected by the *P. aeruginosa* shell, which elucidates the significantly enhanced survival rate of *S. aureus* during treatment with a β-lactam antibiotic. These examples illustrate the importance of controlling spatial distances between microbial cells for the mechanistic understanding of the dynamics of multispecies interactions and community stability.

2.3. Single-cell microfluidics offers a powerful tool to tackle antibiotic resistance

Antimicrobial resistance (AMR) has seriously threatened global health and the ecosystem. However, detecting AMR still relies on 150 years old culture methods to observe colony growth under antibiotic treatments (such as disk diffusion and E-test) [50]. This requires intensive labour and a long time to receive results (>2 days). For critically ill patients, such as those with sepsis, every hour delay without appropriate treatments could be the difference between life and death. In recent years, substantial efforts have been put into developing technologies for rapidly diagnosing infectious pathogens and antibiotic resistance [51, 52]. Single-cell technology has emerged as the most promising approach for rapid antibiotic susceptibility tests (AST) since the direct analysis of single cells removes the need for time-consuming culture and thus reduces assay time. Moreover, minimal consumption of bacteria samples in microfluidic systems has an unparallel advantage when the number of microorganisms is very limited in clinical samples [52].

Furthermore, antibiotic resistance is an inherent biological evolution. It usually arises from a small subset of cells (even within a genetically identical isolate) that survive antibiotic treatments. Single-cell microfluidics provides a powerful tool to identify these cells, speed up AST tests and facilitate the investigation of diverse mechanisms, as illustrated below with examples of recent critical development.

2.3.1. Single cell-based methods for rapid AST

Qualitatively, based on cell phenotypic changes: Most microfluidic-based ASTs depend on monitoring cell morphological changes in the presence of antibiotics (e.g., cell area, length, number, shape, filamentation). To enable this time-course analysis, immobilisation or trapping of cells on chip (using the microfluidic devices described in Section 2.1) is needed for optical imaging. An early study of AST on chip using GFP-modified *E Coli* to facilitate single-cell detection showed the possibility of completing AST within 4 h [53].

However, label-free and easy operation are essential for clinical samples. In this context, most on-chip AST assays opt for bright-field or phase contrast imaging of bacterial cells. For example, Baltekin et al. designed a chip with narrow cell traps and obtained the diagnostic readout within 30 min after loading a sample [54] (Fig. 3A). However, the need for matching the dimensions between the trap and cells limits its use to single species/strain. To overcome this problem, recently, Li et al. proposed a height-adaptable cell trap system [55] (Fig. 3B). The microfluidic system used tunable on-chip valves to exert different pressures onto the parallel chambers below and thus trapping cells of various sizes. This allows for rapid ASTs and performs pathogen classification based on cell shapes and sizes. The authors did a pilot study using clinical urine samples and obtained a specificity of 83.33 % for pathogen classification.

Forces such as dielectrophoretic force (DEP) have been used to improve the loading efficiency of bacteria on-chip and to facilitate the observation of bacterial growth in defined locations. For instance, Lu et al. reported using AC electrokinetics to facilitate the dynamic loading of *E. coli* cells into narrow microchannels and improve the loading efficiency from 30 % to 75 % [56]. Peitz et al. used DEP force generated from microfluidic electrode structures to immobilize *E. coli* cells in the imaging focal plane [57]. They performed the antibiotic susceptibility of polymyxin B by detecting individual cell growth under an optical microscope. Image analysis algorithms were developed to identify and count cell numbers automatically over time.

Recently, the movements of cells have been used as a signature of viability in the diagnosis of antimicrobial resistance. This enables rapid ASTs to be based on multiparameter characteristics and machine learning. Pitruzzello et al. developed a microfluidic device that simultaneously monitors the evolution of cell morphology and motility to assess their responses to antibiotics [58]. This device confines individual *E. coli* cells in cup-shaped trap arrays. By evaluating the length and the amplitude of movement of each cell, susceptible and resistant strains could be identified within an hour. This work highlights that a combined detection method provides insights that single-parameter and traditional tests cannot offer.

It should be noted that the approaches mentioned above were normally designed for testing one antibiotic concentration at a time on chip. Since parallel measurement imposes the complexity of delivering different concentrations and instrumentation to scan large areas, the number of antibiotic concentrations that can be analysed simultaneously is limited.

Quantitative AST with minimum inhibition concentration: The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that prevents the visible growth of a microorganism under defined conditions. In clinical practice, MIC is used to classify a microorganism as either susceptible, intermediate, or resistant to the tested antibiotics. MIC determinations require testing cell growth against a series of increasing antibiotic concentrations. To realise rapid AST with MIC values, various microfluidic systems employ porous

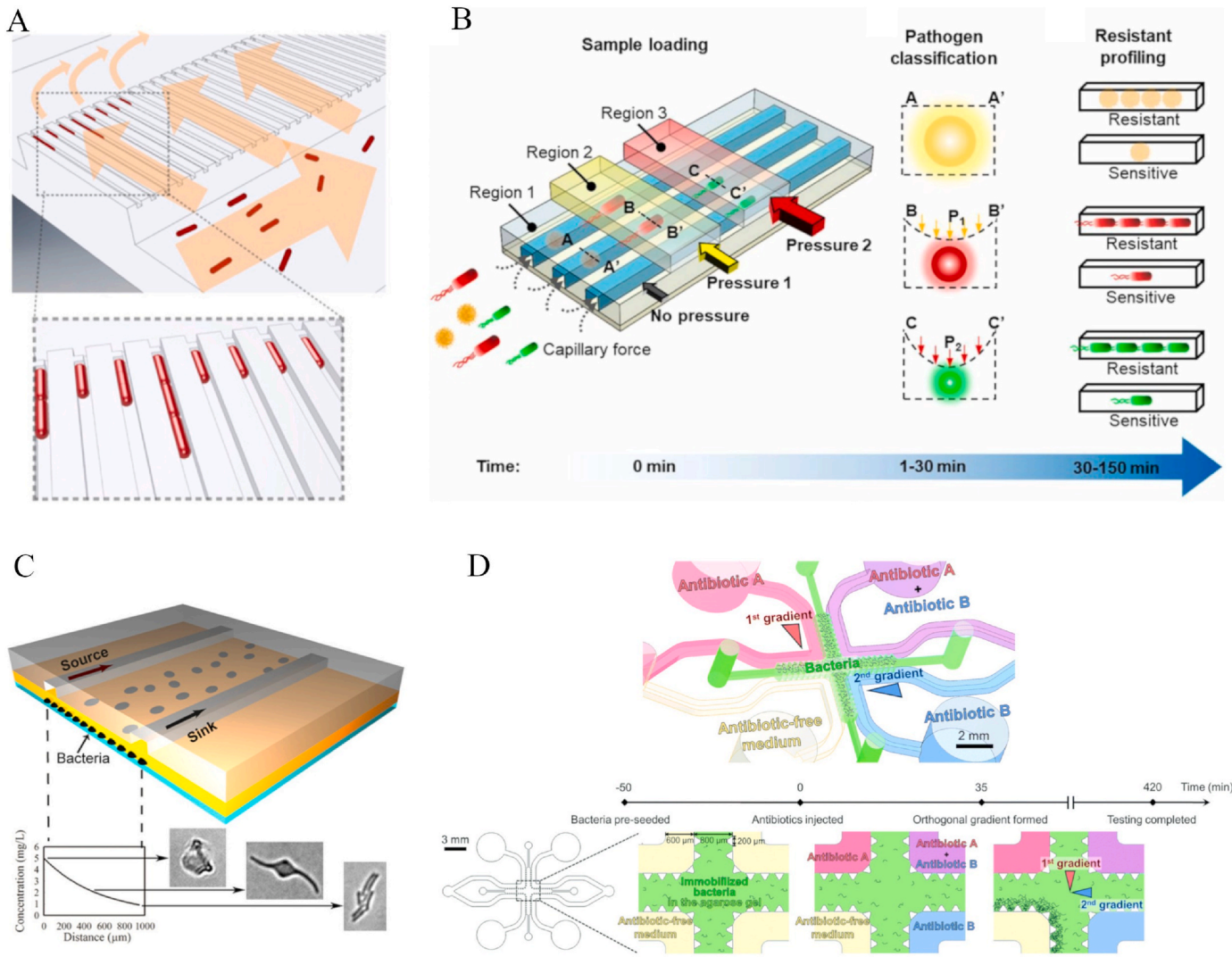

Fig. 3. Single-cell microfluidic devices for rapid antimicrobial susceptibility tests. (A) The microfluidic chip with cell traps consists of narrow channels with a constriction at the end to load bacteria [54]. It can obtain a readout within 30 min of loading a sample. (B) Schematic of the adaptable microfluidic device for pathogen classification and AST based on cell shape and size under different pneumatic pressures [55]. (C) The gradient device with agarose gel assembled within. It enables rapid quantification of MIC within a single experiment [36]. (D) Representative schematics of the chip that generates two orthogonal concentration gradients in bacteria-mixed agarose gel and tests combinatory antibiotic effects [74]. Figures adapted and reprinted with permissions from (A) and (B) the National Academy of Sciences 2017 and 2019, (C) American Chemical Society 2014, and (D) the Royal Society of Chemistry 2019.

hydrogel to form stable antibiotic concentration gradients. One of the most straightforward gradient microfluidic devices was developed by Li et al. illustrating rapid quantification of MIC within a single experiment (<5 h for *E. coli*) [36] (Fig. 3C). Moreover, the authors showed that the platform benefits the cultivation and antibiotic inhibition assessment of slow-growing ammonia-oxidizing bacteria *Nitrosomonas europaea*. The time required was significantly reduced to within four days using the chip-based gradient system, compared with weeks of culturing in flasks.

Encapsulation of bacteria cells in a microfluidic agarose channel (MAC) has also been shown to reduce the AST assay time to ~4 h [39]. Various antibiotics were supplied from side-branch channels to cells via diffusion through agarose. The growth rates of individual cells were processed and calculated from time-lapse optical images. The MAC device was fully integrated with a 96-well plate and tested against four standard bacterial strains and 189 clinical samples using a single-cell morphological analysis approach [59]. The morphological changes in cell dividing, filamentation and swelling of gram-negative and gram-positive cells were tracked, analysed, and categorized by an automated program to determine their antimicrobial susceptibility.

Despite the majority of approaches using cell immobilisation, recently, Yu et al. demonstrated the ability to quantify MIC values from non-immobilised cells via deep learning video microscopy-enabled AST (DLVM-AST) [60]. Using traditional 2D image processing techniques, it is difficult to define and extract specific features such as tumbling and filament formation. To differentiate inhibited cells from uninhibited ones, the authors applied a deep learning model to train against thousands of images by analysing multiple phenotypic features from videos of cell movements. DLVM-AST can accurately determine the MIC to inhibit *E. coli* within 30 min. It should be noted that an extensive training data set is needed for model training.

2.3.2. *Single-cell droplet approach for rapid AST*

Single-cell droplet microfluidics is a potential high throughput technology for rapid AST. This approach identifies antibiotic resistance by monitoring the growth of encapsulated single cells in droplets in the presence of antibiotics. It can perform rapid, parallel testing of many antibiotic concentrations as demonstrated by Boedicker et al. [61] (Fig. 4A). However, detecting unlabelled single bacteria cells in a moving droplet is challenging, so fluorescence indicators, such as resazurin (or AlamarBule), were used as a surrogate in the AST assays.

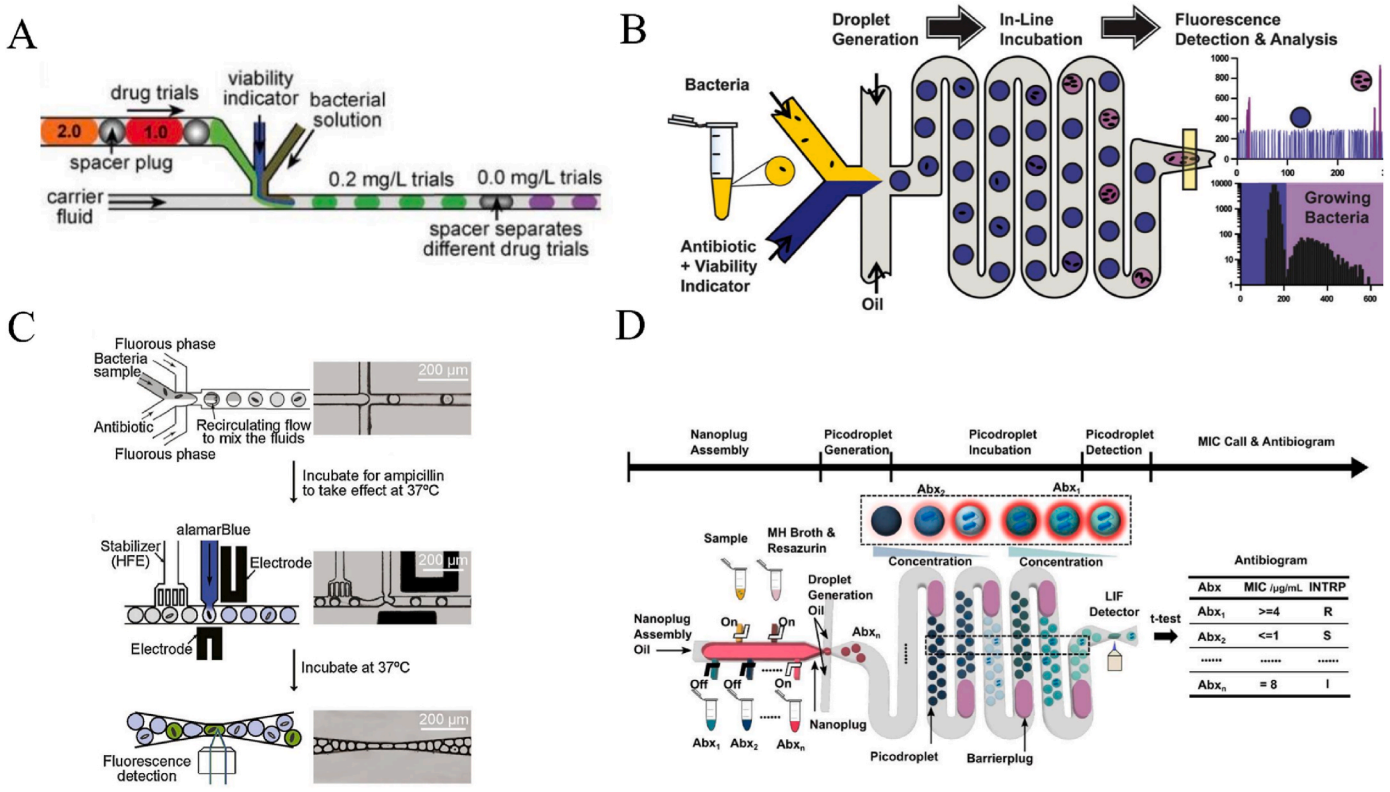

Fig. 4. Single-cell droplet microfluidics for rapid antimicrobial susceptibility tests. (A) Droplet plugs for fast screening of many antibiotics, consisting of bacteria, viability indicator, and antibiotics from a pre-formed array of plugs of different antibiotics [61]. (B) DropFAST system consisting of multiple steps online with a fluorescence detection [62]. (C) A single-cell droplet platform for quantitative phenotyping of heteroresistance [63]. (D) SCALe-AST: a droplet microfluidic platform that integrates programmable microvalve-controlled reaction assembly and single-cell AST in picolitre-scale droplets [64]. With permissions from (A) the Royal Society of Chemistry 2008, (B) Elsevier 2017, (C) Elsevier 2018, and (D) Wiley 2021.

Metabolically active bacteria cells reduce resazurin to fluorescent resorufin, which can be easily detected. This approach can quantify MIC values and significantly reduces the detection time to ~7 h [61]. It was suggested that the detection time was proportional to the volume of the plugs containing a single bacterium. This was later demonstrated in the dropFAST (droplet-based Fluorescent Antimicrobial Susceptibility Test) platform that integrated single-cell encapsulation, incubation, and fluorescence detection online [62] (Fig. 4B). They showed bacterial growth detection in 1 h using resazurin as an indicator in 20 pL droplets. Similarly, Lyu et al. encapsulated Alamar Bule with individual bacteria cells from a microbial community in ~75 pL droplets and could identify resistant bacteria within 4 h [63] (Fig. 4C).

To test many different antibiotic concentrations required in clinical settings, Zhang et al. developed a novel Single-Cell Assembly Line AST (SCALe-AST) system that can control each step in AST assay in a programmable manner [64] (Fig. 4D). Multiple antibiotic conditions can be measured at one time. The authors successfully produced clinically useful antibiograms with MIC values in 90 min for the first antibiotic and then 2 min for every subsequent antibiotic condition. In addition, the AST results from three clinical isolates, and eight urine samples against four antibiotics agreed well (100 % and 93.8 %, respectively) with conventional AST assays (>48 h), showing its potential for both high accuracy and throughput. In addition to rapid AST tests, Wang's group has also demonstrated a highly sensitive, amplification-free classification of single bacterial cells by confining them in picoliter droplets and detection with fluorogenic peptide nucleic acid probes that target their 16S rRNA [65]. The high throughput of this droplet approach enables the detection of heteroresistance in homologous populations.

It is worth noting that a recent study of a combined phenotypic AST with subsequent species identification showed the possibility of single-cell approach for rapid AST testing of mixed-species samples [66]. However, to make single-cell AST useful in a clinical setting, parallel testing of multiplex antibiotics concentration and automation need to be achieved [67]. Nevertheless, the existing evidence shows the promise of single-cell microfluidic technology for rapid diagnosis of infection and antimicrobial susceptibility tests, which will allow timely and precise treatment for patients, especially, those suffering from life-threatening infections. This may dramatically improve clinical outcome. It will also reduce mis-and unnecessary use of broad-spectrum antibiotics thus delaying the emergence of antibiotic resistance.

2.3.3. *Single-cell approach enabling advanced mechanistic investigations*

Microorganisms within a genetically identical culture have distinct phenotypic responses to antibiotic treatment that may eventually lead to the failure of antibiotic therapy. Single-cell microfluidics facilitates the investigation of diverse mechanisms, such as persistence, resistance and tolerance, by which bacteria survive antibiotic exposure [30,68,69]. Time-resolved direct observation of individual cells and their progenies enables quantitative and high-throughput analysis of antibiotic resistance and kinetics of microorganism-drug interactions. For example, early pioneer work by Balaban et al. [69] discovered pre-existing heterogeneity in bacterial populations. By tracing the growth history of individual cells before antibiotic exposure and their regrowth in fresh media thereafter, phenotypic switching was discovered between normally growing cells and persister cells with slow growth. In another study, the microfluidic mother-machine device made it possible to analyse the growth dynamics of thousands of single cells subjected to strong and acute stresses, which resulted in triggered antibiotic persistence [70]. Broad cell heterogeneity of normal growth, growth arrest and death at the single-cell level were observed. It was discovered that, unlike a regulated state that prepares cells for fast recovery under gradual stress exposure, a disrupted cellular state due to acute stress

reveals slow and heterogeneous recovery dynamics, which may be described by the general properties of large random networks.

The combination of antibiotics or drugs offers a productive strategy to address the widespread emergence of multidrug resistance bacteria [71]. Hydrogel-based microfluidic systems have been developed to test microorganism-antibiotic combinations on a single device within a short timeframe. Sun et al. demonstrated a whole-hydrogel microfluidic chip that generates a two-dimensional gradient to investigate the synergistic effect of two drugs [72]. Cells on the gel surface are exposed to stable linear gradients of drugs diffused from the channels inside the gel. An improved design of the hydrogel device was fabricated by using multi-layer gels with two overlaying channels [73]. This design extends the coverage of the gradient zone and enables the testing of different drug combinations.

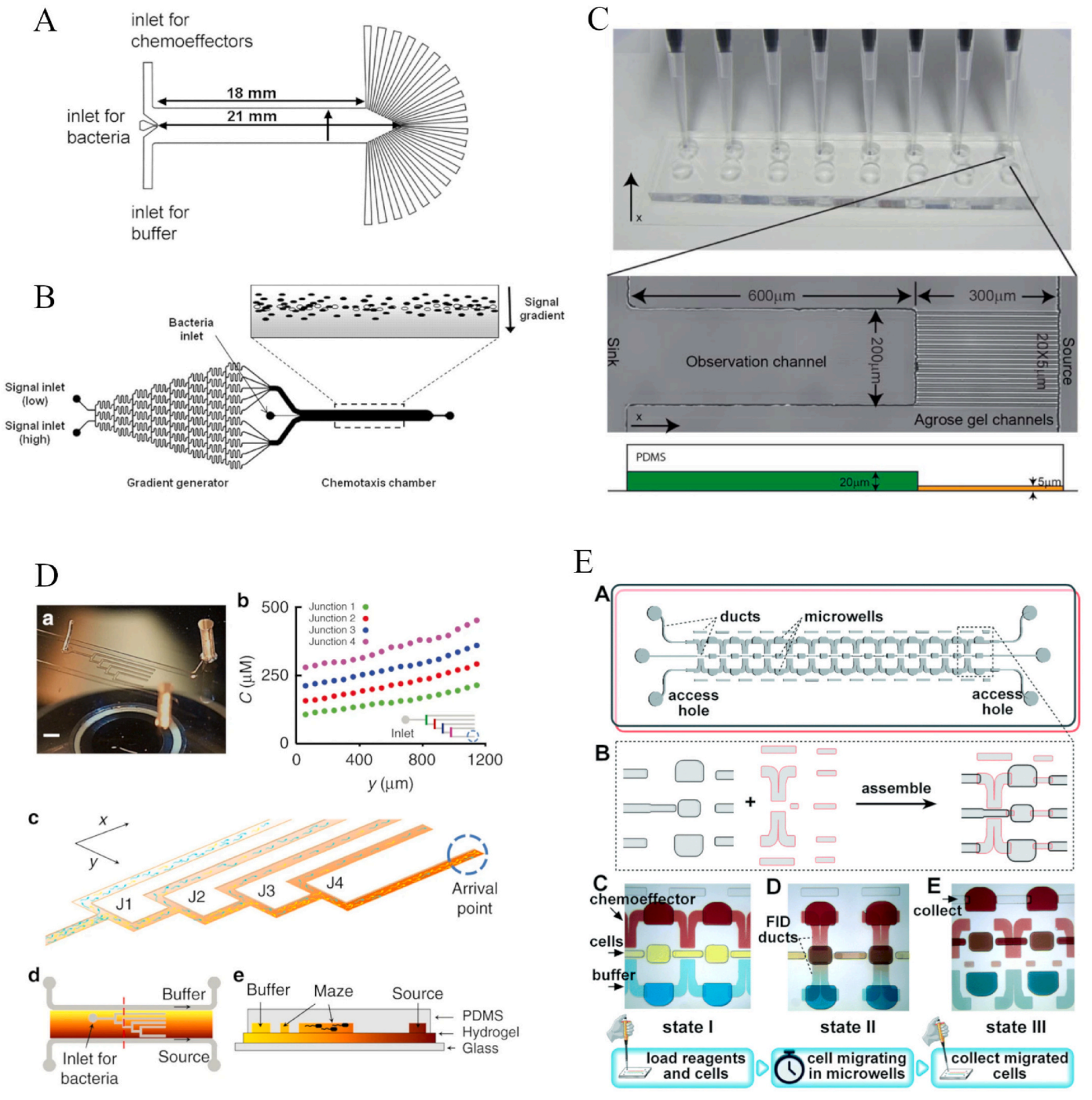

Fig. 5. Microfluidic-based microbial chemotaxis assays. (A) The flow-based T-junction device [81]. Molecular diffusion between the chemoeffector and buffer creates an evolving concentration gradient perpendicular to the flow direction. (B) Schematics of the device with a network of channels for gradient-mixing and a chemotaxis observation chamber [82]. (C) The multi-channel diffusion-based device [89]. Agarose gel-filled channels prevent fluid flow and generate a concentration gradient via diffusion. (D) Schematics of the microfluidic T-maze [94]. Bacterial cells swim through the maze under a steady chemoattractant gradient, making multiple chemotactic decisions through the consecutive junctions. (E) Schematics of the SlipChip microfluidic device [96]. The chemotaxis assay and bacterial collection are operated by slipping the glass plates. Figures adapted and reprinted with permissions from (A) National Academy of Sciences 2003, (B) American Society for Microbiology 2009, (D) Springer Nature 2019, and (C) and (E) the Royal Society of Chemistry 2012 and 2014.

Similarly, Kim et al. presented a microfluidic screening chip to investigate the effects of two antibiotic combinations [74] (Fig. 3D). The chip generates two orthogonal concentration gradients in bacteria-mixed agarose gel to determine whether synergic or antagonistic interactions occur between the antibiotic pairs. 121 concentration combinations can be tested on the chip simultaneously.

2.4. Single-cell analysis for chemotaxis

Chemotaxis is the ability of microorganisms to sense chemical gradients and direct their movement in a biased manner towards favourable (attraction) or away from unfavourable (repulsion) environments [75,76]. As part of a complex network of signalling pathways, chemotaxis plays an essential role in symbiotic associations, pathogenicity, biofilm formation, and development [76,77]. Conventional methods such as capillary and swarm plate assays are endpoint and qualitative, limiting the measurement resolution to the population scale [78]. Microfluidic-based chemotaxis assays are advantageous in controlling the precise formation of chemical gradients due to the laminar flow present in microchannels [78–80]. Besides mathematical prediction and experimental quantification using fluorescence microscopy, microfluidic systems provide high spatial and temporal resolution for analysing microbial responses toward chemical gradients at the single-cell level. Based on how chemical gradients generate in microchannels, microfluidic systems can be broadly categorized as flow systems and diffusion systems, as detailed below.

2.4.1. Flow-based chemotaxis system

In the case of microbial chemotaxis studies, the first microfluidic system was a flow-based T-junction device [81] (Fig. 5A). The device comprises three inlets for chemoeffector, buffer and bacteria and 22 outlets for collecting migrated cells. Mixing between chemoeffector and buffer occurs only by molecular diffusion. As a result, an evolving concentration gradient develops perpendicular to the flow direction. The detection limit of this microfluidic assay was three orders of magnitude lower than that of the standard capillary assay. Englert et al. developed a μFlow device that consists of a gradient-mixing module and a chemotaxis observation module to study the effects of the individual and combined chemoeffector gradients on *E. coli* chemotaxis [82,83] (Fig. 5B). A nearly linear concentration gradient is generated in the observation channel via diffusive mixing from two flow inputs through a 'Christmas tree' network of channels. Roggo et al. devised a chemotaxis chip, where the observation channel is linked to the buffer and attractant channels by shallow pores that cells cannot pass [84]. A stable concentration gradient across the middle observation channel is created via diffusion from the side buffer and attractant channels. The authors proposed an interesting application of this chip for biosensing environmental toxicants by quantifying the chemotactic movement of *Cupriavidus pinatubonensis* JMP134 towards the herbicide 2, 4-dichlorophenoxyacetate.

Another microfluidic chemotaxis device creates an ephemeral, unsteady nutrient gradient using a microinjector tip [85]. The authors studied the responses of the marine bacterium *Pseudoalteromonas haloplanktis* to nutrient pulses and plume [86]. It was found that the chemotactic behaviour of *P. haloplanktis* was an order of magnitude faster than the classic model *E. coli*. Bacterial hot spots formed within tens of seconds upon exposure to the nutrient patches. In another study, they observed the chemoattraction of several motile strains of marine organisms towards microscale pulses of dimethyl sulfoniopropionate [87]. The results illustrated how adaptations to microscale chemical gradients shape microbial food webs in the ocean.

2.4.2. Diffusion-based chemotaxis system

In flow-based chemotaxis devices, chemical gradients are produced from laminar flows in microfluidic channels, which requires dedicated flow control. This could be problematic for motility analysis as bacterial cells experience shear stress in the flows. Moreover, cell movements are confounded with fluid flow variations along microfluidic channels [78]. In addition, the concentration gradient profiles are complex and inconsistent as diffusion-mixing evolves along the bacterial chemotaxis channel. Diffusion-based chemotaxis devices, on the other hand, provide a flow-free microenvironment for microbial chemotaxis assays. The devices are comprised of source and sink channels or reservoirs. Porous membranes or hydrogels are often integrated into the device to enable chemical diffusion and the resulting steady gradient in the observation channel in the middle.

An early example of diffusion-based devices is a gradient generator chip consisting of three channels on porous nitrocellulose membranes [88]. Hydrostatic solution deliveries were maintained in the sink and source channels. The swimming behaviours of bacterial cells were observed under a linear gradient produced by molecular diffusion through the porous membrane walls. Si et al. developed a multi-channel microfluidic device, which features agarose gel-filled channels between two large pores [89] (Fig. 5C). Agarose serves as a semi-permeable membrane that prevents fluid flow and permits the diffusion of small molecules for concentration gradient generation. This setup does not require syringe pumps or other equipment to maintain fluid flows. Besides, it allows for parallel operation and fast result readout, which is convenient for most biology labs. Recently, Hu et al. incorporated natural biopolymer membranes into a three-channel microfluidic device [90]. The membrane formation leverages the pH-dependent solubility of chitosan, which dissolves in water in acidic environments and transitions to hydrogel near pH 6.5. Two parallel arrays of chitosan membranes were assembled in situ for the generation of a static linear gradient. Chemotactic migration of *E. coli* and *Pseudomonas aeruginosa* under glucose gradients was demonstrated using this approach.

An alternative method to design a diffusion-based chemotaxis device without the need for membranes or hydrogels was demonstrated by Jeong et al. [91]. They incorporated air ventilation channels, junction microchannels and spacers into the device to manipulate fluids by wetting and capillary action. Chemical gradients are generated by the rapid diffusion of chemoeffectors using a liquid–liquid interface. Using this microfluidic device, the authors showed an interesting phenomenon: bacteria switched from swimming towards to away from certain chemoeffectors in a concentration-dependent manner.

2.4.3. Complex decision-making via chemotaxis

In natural habitats, microorganisms often confront multiple and complex chemical gradients that emerge from various sources. Single-cell microfluidics has been employed to mimic the natural living environment of microorganisms and probe their chemotactic responses and decision-making processes under several conflicting chemical sources. For example, Kalinin et al. used an agarose three-channel device to explore the behaviours of *E. coli* cells under two opposing gradients of chemoattractants, methyl-aspartate and serine [92]. They found that chemotactic decisions could correlate with the corresponding chemoreceptors Tar/Tsr expression levels. Using a different agarose gradient device, Zhang et al. investigated *E. coli* chemotaxis behaviour in the presence of a strong chemoattractant gradient and an opposing gradient of tryptone broth growth medium [93]. They discovered that cells initially accumulated near the chemoattractant source but later formed an escaping band that moved toward the metabolically richer nutrient source. Using various mutant strains and experimental conditions, this phenomenon could be explained by the competition between Tap and Tar chemoreceptors of *E. coli* cells.

More complicated microfluidic geometries, such as branching mazes or channel networks, have been implemented to provide important biological insights into the decision-making of chemotactic bacteria under environmental stimuli. Salek et al. adopted an iterative microfluidic T-maze that consists of 4 consecutive T-junctions, where bacteria are exposed to a series of chemoattractant gradients [94] (Fig. 5D). The authors quantified the bacterial behaviours of migrating up or down the

concentration gradient at each junction. Coupled with mathematical modelling, they demonstrated that strong heterogeneity in chemotactic sensitivity exists even within clonal populations of bacteria. Their results suggest the functional role of heterogeneity in migratory bet-hedging strategies. Another design proposed by Borer et al. consists of a network of micropores with varying pore connectivity [95]. Two intermixed species, which can disperse and colonize via chemotactic motility, were exposed to opposing gradients of oxygen and carbon. This microfluidic system mimics the architecture and the nutrient and oxygen gradients postulated in a soil aggregate. The visualization and modelling results of this work develop a mechanistic representation of bacterial community organization in soil pores.

2.4.4. Cell isolation via chemotaxis

Microbial chemotactic behaviours can be applied for selective sorting and isolation of functional microorganisms in addition to understanding their ecological roles in the natural environment. Microfluidic devices capable of harvesting chemotactic microorganisms of interest for subsequent omics-based analysis have demonstrated great value in this regard.

One example is a reusable microfluidic SlipChip device that consists of two glass plates with reconfigurable microwells and ducts [96] (Fig. 5E). Solutions and bacterial suspensions were first loaded in the aligned fluidic paths. Then the top plate was moved relative to the bottom plate to align the chemical diffusion paths, where the bacteria microwells were exposed to chemoeffector gradients generated by free interface diffusion. Lastly, the two plates were slipped again to connect the collection paths, so that migrated cells could be collected by pipetting for further analysis. The SlipChip has been used as the first step in the pipeline for chemotactic screening and isolation of effective pollutant degraders from imidazolinone-contaminated soil [97]. The microbial consortium isolated via chemotaxis achieved approximately 10 % higher degradation efficiency of imidazolinone than those derived directly from soil samples.

Another recent example is an in situ chemotaxis assay (ISCA), which comprises an array of microwells connected to the outside solution by a port [98]. To use this device, the microwells are filled with a chemoattractant, which diffuses out of the port and into the surrounding solution forming a chemical plume. Microorganisms that respond to specific chemical cues by swimming into the well via chemotaxis can be collected using syringes and needles. The ISCA device was deployed at a coastal field site to retrieve marine bacteria and archaea with chemotactic responses toward phytoplankton-derived dissolved organic matter [99].

3. Signle cell droplet apporach for microbial discovery

In comparison to the single-cell microfluidic approach, as described in Section 2, single cell droplet microfluidics offers significant advantages in high throughput screening of many conditions. Each droplet is an isolated microreactor, which can be manipulated by various means for multi-step assays or sorting. Encapsulating individual microbial cells in microdroplets can precisely control cell microenvironment, which is particularly valuable for studying microorganisms that are challenging to cultivate conventionally [100]. This section will highlights recent developments in using single-cell droplet cultivation to explore natural microbiomes and functional microbial producers for novel metabolites.

3.1. Single-cell droplet cultivation to discover rare microorganisms from natural microbiota

The phenotypic characterization of new microorganisms still relies on the availability of pure isolates obtained via traditional culture. However, for the natural microbiome, little is known about interspecies interactions; consequently, most natural microorganisms (>95 %) have yet to be cultured in the lab [101,102]. Studies using single-cell droplet cultivation to explore natural microbiota and search for new functional microorganisms have emerged rapidly. By isolating individual cells in microdroplets and screening a library of culture medium for cultivation, rare and previously uncultured microorganisms can be discovered and enriched [103,104]. In addition, each population in a droplet is a pure strain since it starts from a single cell; thus, droplet-based cultivation can eliminate the laborious manual selection of individual colonies [105, 106].

A distinct advantage of single-cell droplet cultivation is that it eliminates the competition from fast-growing populations, thus it can significantly enhance the diversity and richness of obtained microorganisms from complex communities, facilitating the discovery of new species. For example, Hu et al. developed a high throughput droplet microfluidic streak plate platform (MSP) for isolating microorganisms from deep-sea sediments collected from the Southwest Indian Ridge and discovered 15 potential novel species [107,108] (Fig. 6A). However, picking individual droplets and scale-up cultivation for downstream phylogenetic identification can be labour intensive.

Considering single-cell droplet approach generates either empty droplets or single cell colonies, Villa et al. successfully combined droplet cultivation with high throughput DNA sequencing without droplet picking or sorting [109] (Fig. 6B). There, 16*S* rRNA genes were used as intrinsic DNA barcodes to measure growth of taxa in droplets. They showed that droplet approach cultivated 2.8 times more gut bacterial taxa than traditional batch culture methods. Similarly, Meng et al. combined droplet-based high throughput culture with shotgun metagenomic sequencing, which improved strain level diversity analysis and revealed potential novel honeybee gut bacterial species [110] (Fig. 6C).

While most droplet cultivation methods employs single emulsion droplets, recently, Spormann et al. developed a high throughput platform based on single cell encapsulation and growth within double emulsions (GrowMiDE) the GrowMiDE system for rare microorganism cultivation [111]. They showed that not only the GrowMiDE system can enrich underrepresented taxa (e.g. slower-growing *Negativicutes* and *Methanobacteria* from stool samples) but it is also compatible with commercial fluorescence-activated cell sorting (FACS). The combination of GrowMiDE and FACS sorting provides a promising new high-throughput enrichment platform that can be easily applied to diverse microbial enrichment.

3.2. High throughput screening for functional microbial producers

Natural microbiotas provide enormous, untapped source for the discovery of functional microbes, bioactive products (e.g., enzymes), new antibiotics and other drugs. However, since 99 % of natural microorganisms are yet to be cultured, using traditional culture approaches for the search would be labour intensive and ineffective. Single cell droplet microfluidics enables high throughput processing of a large number of individual cells in a microbiota, thus offering a powerful tool to search for functional microbes. Moreover, this technology can be integrated with other cutting-edge techniques such as fluorescence microscopy and metagenomics [112,113], to develop microbial activity assays to identify functional cells and genes. Here, we will highlights representative examples to demonstrate the significance of single-cell droplet microfluidics for the discovery of functional microbial strains and their natural products.

To identify functional microbes from a complex community, an assay for rapid and quantitative measuring of microbial activities is essential. Fluorescence detection is highly desirable due to its strong signal. One fluorescence assay using enzyme-based reactions has been used for high throughput screening of polyethylene terephthalate (PET)-degrading microorganisms or enzymes (PETases) from wastewater microbiome [114] (Fig. 7A). The microfluidic platform consists of a unit that injects substrates into each droplet and applies fluorescence-Activated Droplet Sorting (FADS) to sort and collect the droplets containing target cells. Another fluorescence-based novel transcription-related FADS system

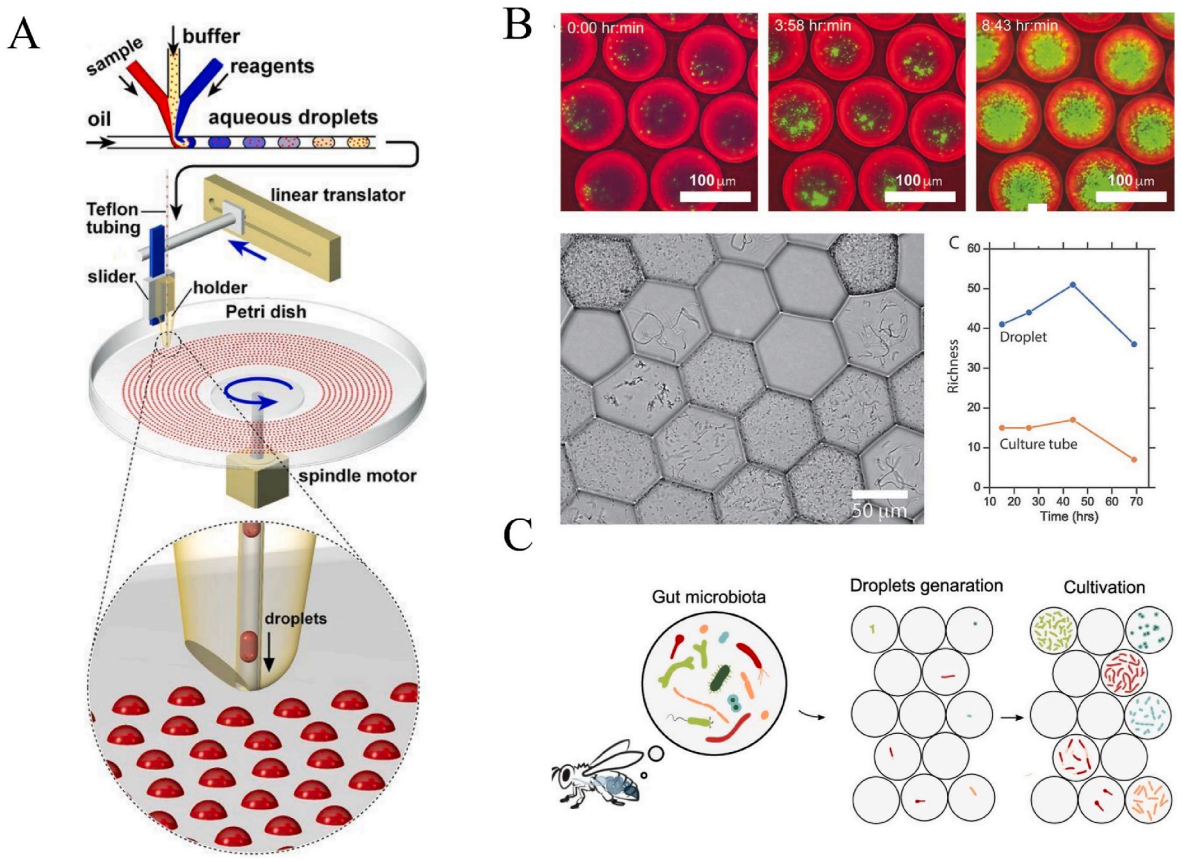

Fig. 6. Microbial cultivation by single cell droplet microfluidics. (A) Microfluidic streak plate (MSP) for single microorganism isolation and cultivation, the diversity was enhanced using this system [108]. (B) MicDrop platform for separating and culturing gut bacteria, enabling real-time monitoring of bacterial growth conditions [109]. (C) Single-cell droplet microfluidics combines with metagenomics revealed potential novel honeybee gut bacterial species [110]. With permissions from (A) American Society for Microbiology 2016, (B) American Society for Microbiology 2020, and (C) Springer Nature 2022.

has been developed recently to search hyperproducers for di- Rhamnolipids from the crude oil microbial community [115] (Fig. 7B). The assay links the phenotype function and genotype through monitoring gene expression at the transcriptional level and shows that intracellular transcription-associated GFP intensity can be used to measure the yield of di- Rhamnolipids between populations of droplets.

Since most conventional microbiology labs don't have turnkey microfluidic sorting systems, recent development has focused on adapting droplet culture with commonly accessible practices in microbiology labs. One interesting approach is to incorporate fluorescent reporters into double emulsion droplets as a way of indirectly assessing natural cells, which are then sorted using standard fluorescence-activated cell sorting (FACS) instrumentation. A notable example of this approach is the search for novel antibiotic producers in the oral microbial community of Siberian bears [116] (Fig. 7C). By co-encapsulating and co-culturing genetically modified fluorescent bacteria with individual cells from the microbial community in a bilayer emulsion, the team discovered a strain of Bacillus subtilis that produced the antibiotic transaminase A, which is effective against *S. aureus*.

Another simpler approach is to directly deposit droplets (either single cells or empty droplets) onto conventional agar plates or 96-well plates, resulting in individual bacterial colonies [105]. This method also allows for functional analysis of cell-cell interactions by introducing metabolites from a specific strain into the agar plates [105,117]. With no need for sophisticated instruments, it is easy to conduct the process in conventional anaerobic chambers, thus facilitating microfluidic technology for underexplored anaerobic microorganisms.

It should be noted that the examples in this section only included limited representative studies of exploiting single-cell droplet microfluidic technology for investigating natural microbiota. The potential for this approach to explore the untapped natural resource for functional microorganisms in drug discovery, search for new antibiotics, microbial therapies etc., is significant.

4. Microfluidics with single-cell Raman spectroscopy: culture-independent approach to explore dark matter

Bringing unknown microorganisms into culture remains a formidable challenge. To investigate the function of a vast diversity of natural microorganisms, Raman spectroscopy emerges as a promising culture-independent technology with a single-cell resolution. Single-cell Raman spectroscopy (SCRS) provides fingerprint information on a microbial cell's components, giving insights into cell gene expression, biosynthesis of specific compounds and metabolic profiles [14,118,119]. With stable-isotope (SIP) labelling techniques (e.g., using ^{13}C, ^{15}N and D-labelled substrates), SCRS-SIP can reveal specific substrate metabolisms [13]. For example, Berry first demonstrated that in the presence of D_2O, metabolically active cells could incorporate the deuterium from D_2O into the cells via NADH/NADPH regeneration and form C-D band (2040 and 2300 cm^{-1}) in the Raman silent region [120]. This C-D band can be a generic Raman biomarker to probe the general metabolic activity of cells, and D_2O-SCRS has since been rapidly taken up in investigating microbiome function [121,122], and clinical antibiotic susceptibility tests [123].

Similar to fluorescence activated cell sorting, Raman activated cell sorting (RACS) based on single-cell Raman spectra is one of the most promising technologies for isolating functional microbial cells from nature. Since the first demonstration of Raman tweezers sorting of single

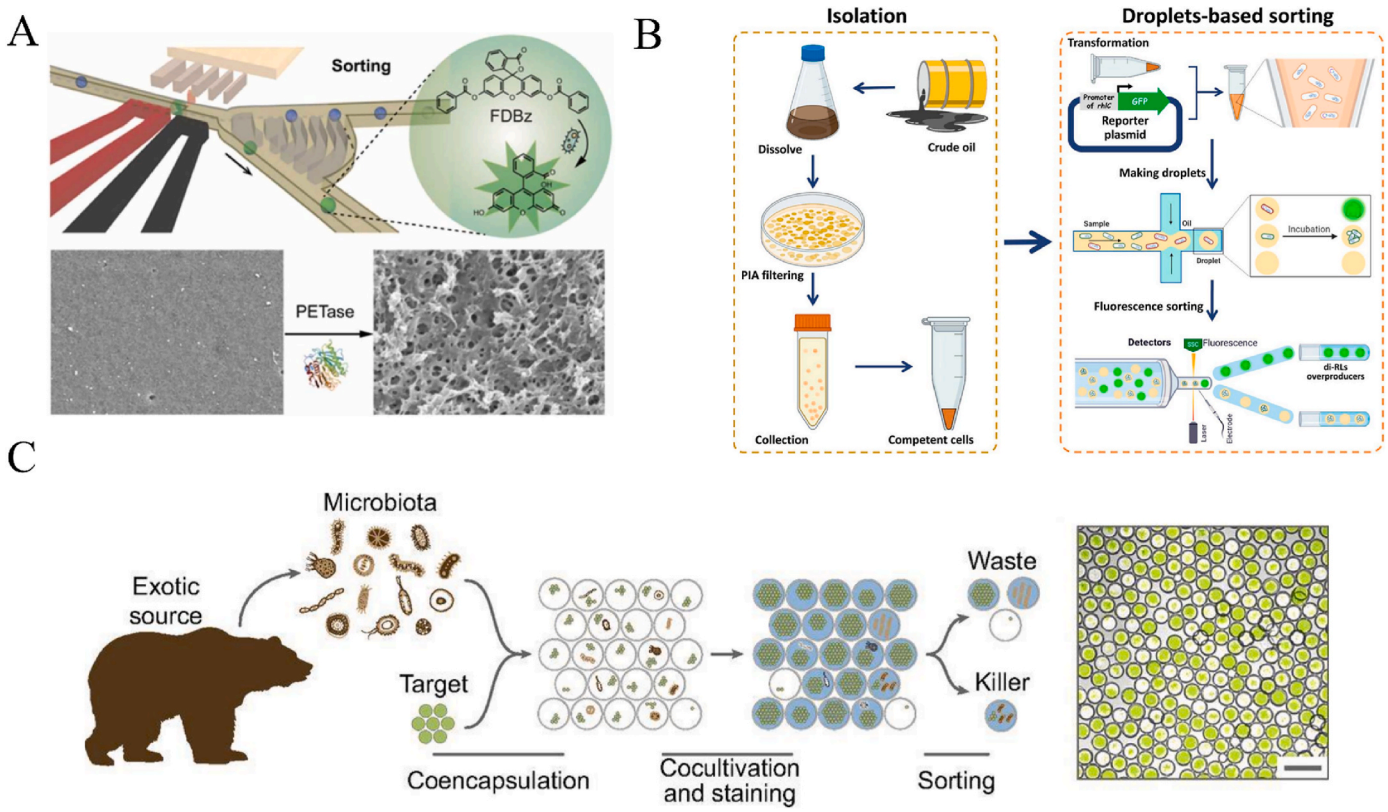

Fig. 7. Single-cell droplet microfluidics with analytical techniques for functional microorganisms screening. (A) FADS (Fluorescence-Activated Droplet Sorting) for the targeted cultivation of PET degradable bacteria [114]. (B) Overview of the transcriptionally relevant FADS pipeline for detecting diRL overproducers [115]. (C) Double emulsion-based droplet microfluidic for the ultrahigh-throughput isolation and cultivation of probiotics with antimicrobial activity [116] with permissions from (A) Elsevier 2022, (B) American Chemical Society 2022, and (C) National Academy of Science 2018.

microbial by Huang et al., in 2009 [124], significant advancements have been made in terms of throughput and automation [122,125–128]. Considering there are a few reviews on the development of Ramanactivated cell isolation and sorting [17,18], here, we highlight the advances in the past five years and key examples of their applications in exploring environmental and human microbiome systems.

4.1. *Ramanactivated cell ejection (RACE)*

RACE uses laser-induced forward transfer to eject cells of interest, where the light-absorbing layer on the substrate disintegrates upon irradiation by a pulsed laser and provides forward momentum that ablates selected cells of the substrate [129]. This approach offers simplicity and can deal with complex natural microbiome systems. By developing an integrated all-in-one RACE system [130] (Fig. 8A), the first RACE coupled with single-cell genomics was demonstrated to isolate unknown carotenoid-containing bacteria from actual environmental samples. It is worth noting that RACE has been translated into commercially available systems (PRECI SCS, Hooke Instrument Ltd), enabling the semi-automation of the whole process and making the technology accessible to microbiology communities. In combination with SIP-SRCS, this system has been used to isolate single cells of specific functional properties (such as antibiotic resistance) from complex microbial samples such as biofilms, soils, and human/animal faeces [131–133].

4.2. *Raman activated cell sorting in flow*

Sorting cells in flow offers significant advantages in maintaining cell viability and throughput. RACS in flow can operate with cell trapping, for example, using optical tweezers (OT) [124] or positive dielectrophoresis (pDEP) [134], or "trap-free" mode [127]. Significant advancements have been made in terms of throughput and automation. For example, a recent OT-RACS platform developed by Lee et al. increased the throughput of Raman tweezers sorting to 3.3–8.8 cells/-min and has been applied to discover functional cells from natural gut microbial communities for downstream culture and genomics profiling [122] (Fig. 8B). The pDEP trap-release system was combined with a downstream droplet sorting module, realising an accuracy of 98.3 % and a higher throughput of ~260 cells/min [135] (Fig. 8C). At the same time, cell isolation in droplets improved the recovery rate of individual cells with no significant difference observed in averaged survival rate between the sorted and unsorted cells.

A novel trap-free 3D-RACS strategy has also been developed to improve sorting accuracy, purity, and throughput [125,136]. An automated 3D-RACS system consists of a 3D printed detection chamber, a sorting unit, optical sensors and an in-line collection module to allow automated operation [136] (Fig. 8D). The independently controlled sheath flow-focusing system offers the flexibility of precise cell flow control and provides excellent advantages in acquiring weak Raman signals of individual cells. Continuous RACS demonstrated a purity level of 92.0 % at a throughput of 310 cells/min when sorting mixtures of *Chlorella vulgaris* and *E. coli*. The system enables sorting a wide range of cell sizes and mixtures without blockage for long-term stable operation. It is envisaged as a versatile tool for function-based sorting applications in microbiology, biotechnology, life science and diagnostics.

As a non-invasive and label-free technology, Raman microscopy is heralded as a promising next-generation physiology approach to study microbiome function at single cell level [2]. Ideally, cells can be physically separated solely on its phenotypic properties in high throughput (10 [3] −10 [7] cells per hour) [2]. Current state-of-the-art systems based on spontaneous Raman spectroscopy [126,136] have already reached 10^4 cells per hour and those based on coherence Raman

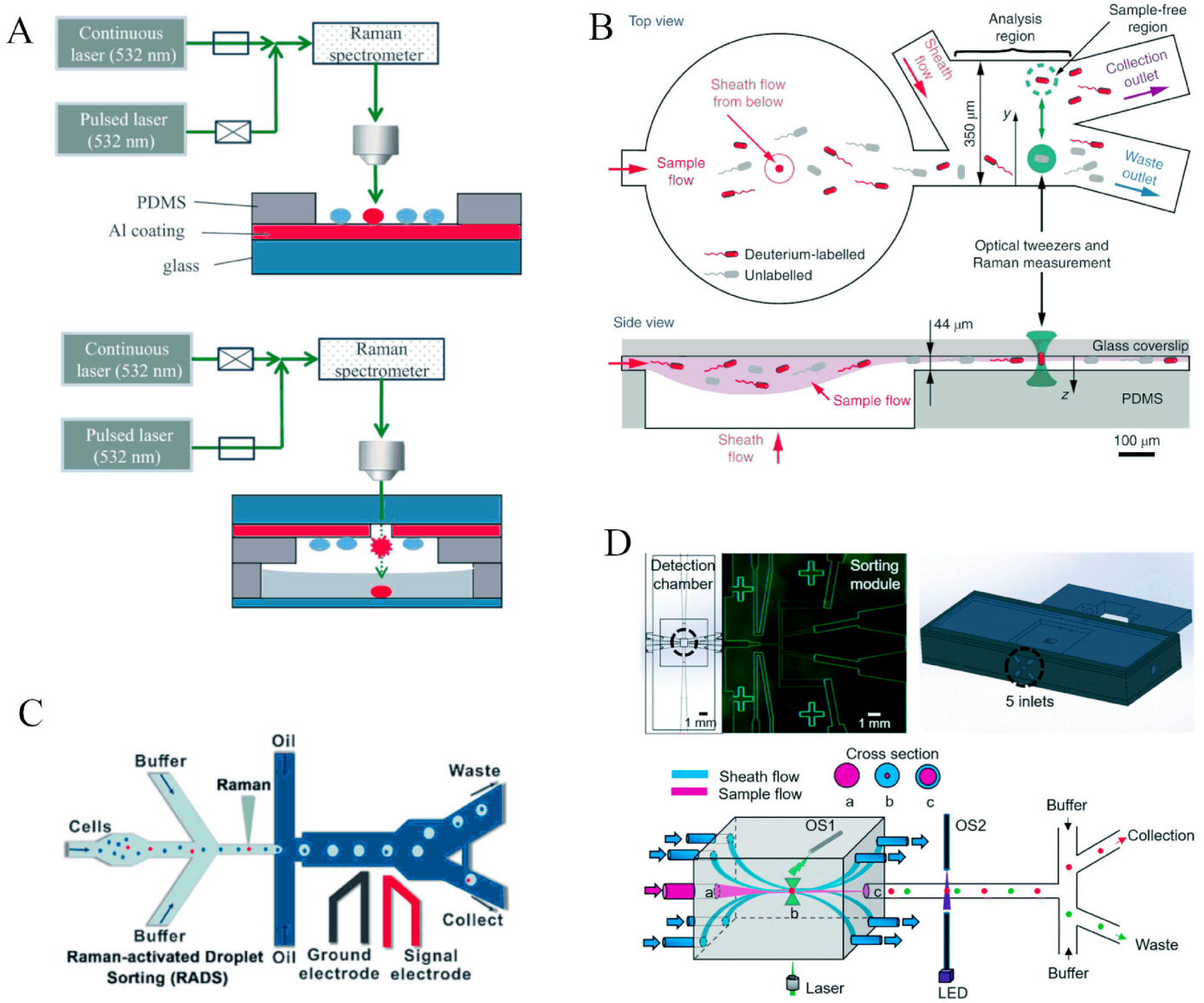

Fig. 8. Microfluidics with single-cell Raman spectroscopy for Raman activated cell sorting (RACS). (A) All-in-one Raman activated cell ejection (RACE) assisted by laser-induced forward transfer (LIFT) [130]. (B) Microfluidic platform with vertical and horizontal sheath flows coupled with Raman tweezers for single-cell trapping and sorting [122]. (C) DEP-based Raman activated droplet sorting system [135]. (D) Modular 3D hydrodynamic focusing Raman activated cell sorting platform (3D-RACS) in continuous flow [136]. With permissions from (A) John Wiley and Sons 2017, (B) Springer Nature 2019, (C) American Chemical Society 2017, and (D) the Royal Society of Chemistry 2020.

spectroscopy demonstrated higher throughput (2×10^5 cells per hour) [137] – all using laboratory strains. However, to reach such throughput for the natural microbiome, challenges associated with the complex microbial composition and sizes, as well as limited prior knowledge of the microbiome must be addressed. Nevertheless, despite low throughput [122], the potential of using RACS to identify and isolate gut microorganisms utilizing key nutrients for rationally designing probiotics has been demonstrated [138]. Future development to enhance robustness, reliability and signal collection power will facilitate the implementation of the technologies in the microbiology fields in general.

5. Limitations and oppotunities for single cell technology

Although single-cell microfluidic technology opens many opportunities to investigate microorganisms, it presents its own limitations and challenges. Designing microfluidic platforms must consider challenges associated with the inherent features of microorganisms. This section highlights notable limitations and discusses areas for future improvements.

5.1. Microbe size and complexity

A natural microbiome comprises a complex community of microbes and extracellular polymeric substances (EPS). Pre-sample treatment is indispensable for separating the microbial communities from the complex matrix (e.g., using filters). The diverse sizes of the microbes and their associated EPS could easily block microfluidic channels. Thus, the design of microfluidic devices should consider the trade-off between clog-free operation and the efficiency of single-cell handling. Unlike single-cell microfluidic devices (as described in section 2), droplet microfluidics is more resistant to clogging since each cell is encapsulated in droplets. In addition, droplet microfluidic devices with large channel dimensions can incorporate active means (such as optical tweezers and acoustic focusing) to manipulate cells and enhance the efficiency of forming single-cell droplets.

Even for laboratory microbial strains, their small dimensions and motility impose significant constraints on imaging cells on a chip. Physically trapping cells can be a practical approach for high-resolution and long-term imaging of cells. However, fabrication of the required submicron structures normally requires costly e-beam lithography. Such small dimensions are prone to clogging. Commonly cultivable strains (e.g. *E. coli*) grow fast and fill microfluidic devices rapidly, so long-term imaging of single cells should consider means to remove excess cells off-chip as well as preventing the formation of biofilms (unless biofilm itself is the subject of study).

5.2. Detection and throughput

Label-free, non-destructive approaches, such as Raman spectroscopy and optical imaging, measure the intrinsic properties of a cell and provide dynamic information about cell activities. However, these signals are often weak, requiring high-end instrumentation and sophisticated data/image analysis but limiting the throughput of an assay. For instance, a high NA objective lens can resolve the morphology of individual cells but can only measure a small area (100s of microns by 100s of microns). This restricts the number of cells and conditions to be tested. Single-cell droplet microfluidic offers high throughput capability, but label-free detection of single bacterial cells in droplets remains an unmet challenge due to the undefined location of a cell inside the droplet.

Recent studies of combining a label-free approach with downstream fluorescence in situ hybridisation (FISH) [66] or Omics' methods [139] can link phenotypic function with genetic information. These open new avenues to study various ecosystems and explore untapped microbiome resources for drug discovery and the biotechnology industry.

5.3. Translation

However, significant hurdles remain to be overcome for microbiologists to adopt microfluidic approaches widely. The lack of available hardware and software tools still impedes its widespread use outside a few laboratories with high-level expertise in the technology. Although simple microfluidic devices are commercially available (e.g., microfluidic ChipShop, Dolomite Microfluidics), detecting single bacterial cells on a chip still requires sophisticated instrumentation and specialist skills. In the case of single-cell AST testing, existing platforms are often complex and expensive, jeopardising their clinical utility. Also, it is essential to design a device that integrates core functions with a user-friendly interface and requires little to no training to fit in clinical settings.

6. Conclusions

Microfluidics has been rapidly implemented in microbiology in recent decades as a result of its excellent ability to control and study microbial communities at the single-cell level. Numerous discoveries at the microscale level have been made, which were not possible via conventional methods. These findings have had profound impacts on our understanding of the evolution and transmission of antibiotic resistance in microbial ecosystems. The high throughput ability of single-cell microfluidics and droplet microfluidic opens avenues to screen for untapped novel microorganisms with desirable functions to tackle global challenges in healthcare and sustainability, such as those that can produce new antibiotics or degrade plastic waste. The convergence of Raman spectroscopy and microfluidics provide a powerful tool for discovering microbial "dark matter".

However, significant hurdles remain to be overcome for the wide adoption of microfluidic approaches by microbiologists. In order to address these challenges and enable high-throughput analysis of natural microbiota, innovation must be fostered. Effective integration of these breakthroughs into traditional microbiological practice requires the translation of microfluidics into ready-to-use integrated systems. This will not only increase the efficiency of microbiological research, but also ensure its seamless integration into existing workflows, thus advancing our understanding of the natural microbiota. In summation, while microfluidic technologies offer promising avenues for single-cell bacterial analysis, the path to their widespread adoption is filled with challenges that need collaborative efforts to address.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

Acknowledgements

We acknowledge the support from the EPSRC IAA (EP/X5257161/1 and EP/R511705/1) and InnovateUK (104984). WEH thank EPSRC (EP/M02833X/1) for instrumentation support, as well as finance support from EPSRC (EP/M002403/1) and international collaboration project between University of Oxford and Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences.

References

[1] H.C. Flemming, S. Wuertz, Bacteria and archaea on Earth and their abundance in biofilms, Nat. Rev. Microbiol. 17 (4) (2019) 247.
[2] R. Hatzenpichler, V. Krukenberg, R.L. Spietz, Z.J. Jay, Next-generation physiology approaches to study microbiome function at single cell level, Nat. Rev. Microbiol. 18 (4) (2020) 241.
[3] D.B. Weibel, W.R. DiLuzio, G.M. Whitesides, Microfabrication meets microbiology, Nat. Rev. Microbiol. 5 (3) (2007) 209.
[4] H.B. Yin, D. Marshall, Microfluidics for single cell analysis, Curr. Opin. Biotechnol. 23 (1) (2012) 110.
[5] N. Convery, N. Gadegaard, 30 years of microfluidics, Micro Nano Eng. 2 (2019) 76.
[6] B. Hu, P. Xu, L. Ma, D. Chen, J. Wang, X. Dai, L. Huang, W. Du, One cell at a time: droplet-based microbial cultivation, screening and sequencing, Mar. Life Sci. Technol. 3 (2) (2021) 169.
[7] T.S. Kaminski, O. Scheler, P. Garstecki, Droplet microfluidics for microbiology: techniques, applications and challenges, Lab Chip 16 (12) (2016) 2168.
[8] O. Scheler, W. Postek, P. Garstecki, Recent developments of microfluidics as a tool for biotechnology and microbiology, Curr. Opin. Biotechnol. 55 (2019) 60.
[9] X.F. Yuan, J.M. Couto, A. Glidle, Y.Q. Song, W. Sloan, H.B. Yin, Single-cell microfluidics to study the effects of genome deletion on bacterial growth behavior, ACS Synth. Biol. 6 (12) (2017) 2219.
[10] O. Gefen, O. Fridman, I. Ronin, N.Q. Balaban, Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity, Proc. Natl. Acad. Sci. USA 111 (1) (2014) 556.
[11] C.S. Karamitros, M. Moryan, A. Vigne, J. Lim, P. Gruner, T. Beneyton, J. Vrignon, J.C. Baret, Bacterial expression systems for enzymatic activity in droplet-based microfluidics, Anal. Chem. 92 (7) (2020) 4908.
[12] B. Li, Y. Qiu, Y.Q. Song, H. Lin, H.B. Yin, Dissecting horizontal and vertical gene transfer of antibiotic resistance plasmid in bacterial community using microfluidics, Environ. Int. (2019) 131.
[13] Y. Wang, W.E. Huang, L. Cui, M. Wagner, Single cell stable isotope probing in microbiology using Raman microspectroscopy, Curr. Opin. Biotechnol. 41 (2016) 34.
[14] A. Harz, P. Rosch, J. Popp, Vibrational spectroscopy-A powerful tool for the rapid identification of microbial cells at the single-cell level, Cytom. Part A 75A (2) (2009) 104.
[15] K.S. Lee, Z. Landry, F.C. Pereira, M. Wagner, D. Berry, W.E. Huang, G.T. Taylor, J. Kneipp, J. Popp, M. Zhang, et al., Raman microspectroscopy for microbiology, Nat. Rev. Methods Primers 1 (1) (2021).
[16] S. Pahlow, S. Meisel, D. Cialla-May, K. Weber, P. Rosch, J. Popp, Isolation and identification of bacteria by means of Raman spectroscopy, Adv. Drug Deliv. Rev. 89 (2015) 105.
[17] Y.Z. Song, H.B. Yin, W.E. Huang, Raman activated cell sorting, Curr. Opin. Chem. Biol. 33 (2016) 1.
[18] S.S. Yan, J.X. Qiu, L. Guo, D.Z. Li, D.P. Xu, Q. Liu, Development overview of Raman-activated cell sorting devoted to bacterial detection at single-cell level, Appl. Microbiol. Biotechnol. 105 (4) (2021) 1315.

[19] F. Wu, C. Dekker, Nanofabricated structures and microfluidic devices for bacteria: from techniques to biology, Chem. Soc. Rev. 45 (2) (2016) 268.
[20] S. Taeuber, E. von Lieres, A. Gruenberger, Dynamic environmental control in microfluidic single-cell cultivations: from concepts to applications, Small 16 (16) (2020).
[21] Z. Long, E. Nugent, A. Javer, P. Cicuta, B. Sclavi, M. Cosentino Lagomarsino, K. D. Dorfman, Microfluidic chemostat for measuring single cell dynamics in bacteria, Lab Chip 13 (5) (2013) 947.
[22] P. Wang, L. Robert, J. Pelletier, W.L. Dang, F. Taddei, A. Wright, S. Jun, Robust growth of Escherichia coli, Curr. Biol. 20 (12) (2010) 1099.
[23] A. Grünberger, N. Paczia, C. Probst, G. Schendzielorz, L. Eggeling, S. Noack, W. Wiechert, D. Kohlheyer, A disposable picolitre bioreactor for cultivation and investigation of industrially relevant bacteria on the single cell level, Lab Chip 12 (11) (2012) 2060.
[24] J.R. Moffitt, J.B. Lee, P. Cluzel, The single-cell chemostat: an agarose-based, microfluidic device for high-throughput, single-cell studies of bacteria and bacterial communities, Lab Chip 12 (8) (2012) 1487.
[25] Q. Luan, C. Macaraniag, J. Zhou, I. Papautsky, Microfluidic systems for hydrodynamic trapping of cells and clusters, Biomicrofluidics 14 (3) (2020), 031502.
[26] C. Dusny, A. Grünberger, C. Probst, W. Wiechert, D. Kohlheyer, A. Schmid, Technical bias of microcultivation environments on single-cell physiology, Lab Chip 15 (8) (2015) 1822.
[27] K. Kolesnik, M.X. Xu, P.V.S. Lee, V. Rajagopal, D.J. Collins, Unconventional acoustic approaches for localized and designed micromanipulation, Lab Chip 21 (15) (2021) 2837.
[28] Z. Long, E. Nugent, A. Javer, P. Cicuta, B. Sclavi, M.C. Lagomarsino, K. D. Dorfman, Microfluidic chemostat for measuring single cell dynamics in bacteria, Lab Chip 13 (5) (2013) 947.
[29] M. Kaiser, F. Jug, T. Julou, S. Deshpande, T. Pfohl, O.K. Silander, G. Myers, E. Van Nimwegen, Monitoring single-cell gene regulation under dynamically controllable conditions with integrated microfluidics and software, Nat. Commun. 9 (1) (2018).
[30] U. Lapinska, M. Voliotis, K.K. Lee, A. Campey, M.R.L. Stone, B. Tuck, W. Phetsang, B. Zhang, K. Tsaneva-Atanasova, M.A. Blaskovich, et al., Fast bacterial growth reduces antibiotic accumulation and efficacy, Elife (2022) 11.
[31] Y. Koganezawa, M. Umetani, M. Sato, Y. Wakamoto, History-dependent physiological adaptation to lethal genetic modification under antibiotic exposure, Elife (2022) 11.
[32] J. Cama, M. Voliotis, J. Metz, A. Smith, J. Iannucci, U.F. Keyser, K. Tsaneva-Atanasova, S. Pagliara, Single-cell microfluidics facilitates the rapid quantification of antibiotic accumulation in Gram-negative bacteria, Lab Chip 20 (15) (2020) 2765.
[33] S. Luro, L. Potvin-Trottier, B. Okumus, J. Paulsson, Isolating live cells after high-throughput, long-term, time-lapse microscopy, Nat. Methods 17 (1) (2020) 93.
[34] A. Groisman, C. Lobo, H. Cho, J.K. Campbell, Y.S. Dufour, A.M. Stevens, A. Levchenko, A microfluidic chemostat for experiments with bacterial and yeast cells, Nat. Methods 2 (9) (2005) 685.
[35] H. Wang, G.M. Conover, S.-I. Han, J.C. Sacchettini, A. Han, Development of single-cell-level microfluidic technology for long-term growth visualization of living cultures of Mycobacterium smegmatis, Microsyst. Nanoeng. 7 (1) (2021).
[36] B. Li, Y. Qiu, A. Glidle, D. McIlvenna, Q. Luo, J. Cooper, H.C. Shi, H.B. Yin, Gradient microfluidics enables rapid bacterial growth inhibition testing, Anal. Chem. 86 (6) (2014) 3131.
[37] B. Li, Y. Qiu, A. Glidle, J. Cooper, H.C. Shi, H.B. Yin, Single cell growth rate and morphological dynamics revealing an "opportunistic" persistence, Analyst 139 (13) (2014) 3305.
[38] B. Li, Y. Qiu, H.C. Shi, H.B. Yin, The importance of lag time extension in determining bacterial resistance to antibiotics, Analyst 141 (10) (2016) 3059.
[39] J. Choi, Y.-G. Jung, J. Kim, S. Kim, Y. Jung, H. Na, S. Kwon, Rapid antibiotic susceptibility testing by tracking single cell growth in a microfluidic agarose channel system, Lab Chip 13 (2) (2013) 280.
[40] A. Burmeister, A. Grunberger, Microfluidic cultivation and analysis tools for interaction studies of microbial co-cultures, Curr. Opin. Biotechnol. 62 (2020) 106.
[41] R.N. Alnahhas, J.J. Winkle, A.J. Hirning, B. Karamched, W. Ott, K. Josić, M. R. Bennett, Spatiotemporal dynamics of synthetic microbial consortia in microfluidic devices, ACS Synth. Biol. 8 (9) (2019) 2051.
[42] Y. Chen, J.K. Kim, A.J. Hirning, K. Josić, M.R. Bennett, Emergent genetic oscillations in a synthetic microbial consortium, Science 349 (6251) (2015) 986.
[43] R.M. Cooper, L. Tsimring, J. Hasty, Inter-species population dynamics enhance microbial horizontal gene transfer and spread of antibiotic resistance, Elife 6 (2017), e25950.
[44] B. Li, Y. Qiu, J. Zhang, X. Huang, H.C. Shi, H.B. Yin, Real-time study of rapid spread of antibiotic resistance plasmid in biofilm using microfluidics, Environ. Sci. Technol. 52 (19) (2018), 11132.
[45] H.J. Kim, J.Q. Boedicker, J.W. Choi, R.F. Ismagilov, Defined spatial structure stabilizes a synthetic multispecies bacterial community, Proc. Natl. Acad. Sci. USA 105 (47) (2008), 18188.
[46] H.J. Kim, J.Q. Boedicker, J.W. Choi, R.F. Ismagilov, Defined spatial structure stabilizes a synthetic multispecies bacterial community, Proc. Natl. Acad. Sci. USA 105 (47) (2008), 18188.
[47] A. Burmeister, F. Hilgers, A. Langner, C. Westerwalbesloh, Y. Kerkhoff, N. Tenhaef, T. Drepper, D. Kohlheyer, E. Von Lieres, S. Noack, et al., A microfluidic co-cultivation platform to investigate microbial interactions at defined microenvironments, Lab Chip 19 (1) (2019) 98.
[48] S. Gupta, T.D. Ross, M.M. Gomez, J.L. Grant, P.A. Romero, O.S. Venturelli, Investigating the dynamics of microbial consortia in spatially structured environments, Nat. Commun. 11 (1) (2020).
[49] J.L. Connell, E.T. Ritschdorff, M. Whiteley, J.B. Shear, 3D printing of microscopic bacterial communities, Proc. Natl. Acad. Sci. USA 110 (46) (2013), 18380.
[50] J. Dietvorst, L. Vilaplana, N. Uria, M.-P. Marco, X. Muñoz-Berbel, Current and near-future technologies for antibiotic susceptibility testing and resistant bacteria detection, TrAC Trends Anal. Chem. 127 (2020), 115891.
[51] B. Behera, G.K.A. Vishnu, S. Chatterjee, V. Sitaramgupta, N. Sreekumar, A. Nagabhushan, N. Rajendran, B.H. Prathik, H.J. Pandya, Emerging technologies for antibiotic susceptibility testing, Biosens. Bioelectron. 142 (2019).
[52] A. van Belkum, C.A.D. Burnham, J.W.A. Rossen, F. Mallard, O. Rochas, W. M. Dunne, Innovative and rapid antimicrobial susceptibility testing systems, Nat. Rev. Microbiol. 18 (5) (2020) 299.
[53] R. Mohan, A. Mukherjee, S.E. Sevgen, C. Sanpitakseree, J. Lee, C.M. Schroeder, P. J.A. Kenis, A multiplexed microfluidic platform for rapid antibiotic susceptibility testing, Biosens. Bioelectron. 49 (2013) 118.
[54] O. Baltekin, A. Boucharin, E. Tano, D.I. Andersson, J. Elf, Antibiotic susceptibility testing in less than 30 min using direct single-cell imaging, Proc. Natl. Acad. Sci. USA 114 (34) (2017) 9170.
[55] H. Li, P. Torab, K.E. Mach, C. Surrette, M.R. England, D.W. Craft, N.J. Thomas, J. C. Liao, C. Puleo, P.K. Wong, Adaptable microfluidic system for single-cell pathogen classification and antimicrobial susceptibility testing, Proc. Natl. Acad. Sci. USA 116 (21) (2019), 10270.
[56] Y. Lu, J. Gao, D.D. Zhang, V. Gau, J.C. Liao, P.K. Wong, Single cell antimicrobial susceptibility testing by confined microchannels and electrokinetic loading, Anal. Chem. 85 (8) (2013) 3971.
[57] I. Peitz, R. van Leeuwen, Single-cell bacteria growth monitoring by automated DEP-facilitated image analysis, Lab Chip 10 (21) (2010) 2944.
[58] G. Pitruzzello, S. Thorpe, S. Johnson, A. Evans, H. Gadêlha, T.F. Krauss, Multiparameter antibiotic resistance detection based on hydrodynamic trapping of individual E. coli, Lab Chip 19 (8) (2019) 1417.
[59] J. Choi, J. Yoo, M. Lee, E.G. Kim, J.S. Lee, S. Lee, S. Joo, S.H. Song, E.C. Kim, J. C. Lee, et al., A rapid antimicrobial susceptibility test based on single-cell morphological analysis, Sci. Transl. Med. 6 (267) (2014) 13.
[60] H. Yu, W. Jing, R. Iriya, Y. Yang, K. Syal, M. Mo, T.E. Grys, S.E. Haydel, S. Wang, N. Tao, Phenotypic antimicrobial susceptibility testing with deep learning video microscopy, Anal. Chem. 90 (10) (2018) 6314.
[61] J.Q. Boedicker, L. Li, T.R. Kline, R.F. Ismagilov, Detecting bacteria and determining their susceptibility to antibiotics by stochastic confinement in nanoliter droplets using plug-based microfluidics, Lab Chip 8 (8) (2008) 1265.
[62] A.M. Kaushik, K.W. Hsieh, L.B. Chen, D.J. Shin, J.C. Liao, T.H. Wang, Accelerating bacterial growth detection and antimicrobial susceptibility assessment in integrated picoliter droplet platform, Biosens. Bioelectron. 97 (2017) 260.
[63] F. Lyu, M. Pan, S. Patil, J.-H. Wang, A.C. Matin, J.R. Andrews, S.K.Y. Tang, Phenotyping antibiotic resistance with single-cell resolution for the detection of heteroresistance, Sensor. Actuator. B Chem. 270 (2018) 396.
[64] P. Zhang, A.M. Kaushik, K. Hsieh, S. Li, S. Lewis, K.E. Mach, J.C. Liao, K. C. Carroll, T.-H. Wang, A cascaded droplet microfluidic platform enables high-throughput single cell antibiotic susceptibility testing at scale, Small Methods 6 (1) (2022).
[65] K. Hsieh, K.E. Mach, P. Zhang, J.C. Liao, T.-H. Wang, Combating antimicrobial resistance via single-cell diagnostic technologies powered by droplet microfluidics, Acc. Chem. Res. 55 (2) (2022) 123.
[66] V. Kandavalli, P. Karempudi, J. Larsson, J. Elf, Rapid antibiotic susceptibility testing and species identification for mixed samples, Nat. Commun. 13 (1) (2022).
[67] K. Zhang, S. Qin, S. Wu, Y. Liang, J. Li, Microfluidic systems for rapid antibiotic susceptibility tests (ASTs) at the single-cell level, Chem. Sci. 11 (25) (2020) 6352.
[68] N.Q. Balaban, S. Helaine, K. Lewis, M. Ackermann, B. Aldridge, D.I. Andersson, M.P. Brynildsen, D. Bumann, A. Camilli, J.J. Collins, et al., Definitions and guidelines for research on antibiotic persistence, Nat. Rev. Microbiol. 17 (7) (2019) 441.
[69] N.Q. Balaban, J. Merrin, R. Chait, L. Kowalik, S. Leibler, Bacterial persistence as a phenotypic switch, Science 305 (5690) (2004) 1622.
[70] Y. Kaplan, S. Reich, E. Oster, S. Maoz, I. Levin-Reisman, I. Ronin, O. Gefen, O. Agam, N.Q. Balaban, Observation of universal ageing dynamics in antibiotic persistence, Nature 600 (7888) (2021) 290.
[71] M. Tyers, G.D. Wright, Drug combinations: a strategy to extend the life of antibiotics in the 21st century, Nat. Rev. Microbiol. 17 (3) (2019) 141.
[72] H. Sun, Z. Liu, C. Hu, K. Ren, Cell-on-hydrogel platform made of agar and alginate for rapid, low-cost, multidimensional test of antimicrobial susceptibility, Lab Chip 16 (16) (2016) 3130.
[73] Z. Liu, H. Sun, K. Ren, A multiplexed, gradient-based, full-hydrogel microfluidic platform for rapid, high-throughput antimicrobial susceptibility testing, ChemPlusChem 82 (5) (2017) 792.
[74] S. Kim, F. Masum, J.-K. Kim, H.J. Chung, J.S. Jeon, On-chip phenotypic investigation of combinatory antibiotic effects by generating orthogonal concentration gradients, Lab Chip 19 (6) (2019) 959.
[75] S.M. Butler, A. Camilli, Going against the grain: chemotaxis and infection in Vibrio cholerae, Nat. Rev. Microbiol. 3 (8) (2005) 611.
[76] J.-B. Raina, V. Fernandez, B. Lambert, R. Stocker, J.R. Seymour, The role of microbial motility and chemotaxis in symbiosis, Nat. Rev. Microbiol. 17 (5) (2019) 284.
[77] G.H. Wadhams, J.P. Armitage, Making sense of it all: bacterial chemotaxis, Nat. Rev. Mol. Cell Biol. 5 (12) (2004) 1024.

[78] T. Ahmed, T.S. Shimizu, R. Stocker, Microfluidics for bacterial chemotaxis, Integr. Biol. 2 (11–12) (2010) 604.
[79] H. Somaweera, A. Ibraguimov, D. Pappas, A review of chemical gradient systems for cell analysis, Anal. Chim. Acta 907 (2016) 7.
[80] J. Wu, X. Wu, F. Lin, Recent developments in microfluidics-based chemotaxis studies, Lab Chip 13 (13) (2013) 2484.
[81] H. Mao, P.S. Cremer, M.D. Manson, A sensitive, versatile microfluidic assay for bacterial chemotaxis, Proc. Natl. Acad. Sci. USA 100 (9) (2003) 5449.
[82] D.L. Englert, M.D. Manson, A. Jayaraman, Flow-based microfluidic device for quantifying bacterial chemotaxis in stable, competing gradients, Appl. Environ. Microbiol. 75 (13) (2009) 4557.
[83] D.L. Englert, M.D. Manson, A. Jayaraman, Investigation of bacterial chemotaxis in flow-based microfluidic devices, Nat. Protoc. 5 (5) (2010) 864.
[84] C. Roggo, C. Picioreanu, X. Richard, C. Mazza, H. van Lintel, J.R. van der Meer, Quantitative chemical biosensing by bacterial chemotaxis in microfluidic chips, Environ. Microbiol. 20 (1) (2018) 241.
[85] J.R. Seymour, T. Ahmed, Marcos, R. Stocker, A microfluidic chemotaxis assay to study microbial behavior in diffusing nutrient patches, Limnol Oceanogr. Methods 6 (9) (2008) 477.
[86] R. Stocker, J.R. Seymour, A. Samadani, D.E. Hunt, M.F. Polz, Rapid chemotactic response enables marine bacteria to exploit ephemeral microscale nutrient patches, Proc. Natl. Acad. Sci. USA 105 (11) (2008) 4209.
[87] J.R. Seymour, R. Simó, T. Ahmed, R. Stocker, Chemoattraction to dimethylsulfoniopropionate throughout the marine microbial food web, Science 329 (5989) (2010) 342.
[88] J. Diao, L. Young, S. Kim, E.A. Fogarty, S.M. Heilman, P. Zhou, M.L. Shuler, M. Wu, M.P. DeLisa, A three-channel microfluidic device for generating static linear gradients and its application to the quantitative analysis of bacterial chemotaxis, Lab Chip 6 (3) (2006) 381.
[89] G. Si, W. Yang, S. Bi, C. Luo, Q. Ouyang, A parallel diffusion-based microfluidic device for bacterial chemotaxis analysis, Lab Chip 12 (7) (2012) 1389.
[90] P. Hu, K.L. Ly, L.P.H. Pham, A.E. Pottash, K. Sheridan, H.-C. Wu, C.-Y. Tsao, D. Quan, W.E. Bentley, G.W. Rubloff, et al., Bacterial chemotaxis in static gradients quantified in a biopolymer membrane-integrated microfluidic platform, Lab Chip (2022), https://doi.org/10.1039/D2LC00481J.
[91] H.-H. Jeong, S.-H. Lee, C.-S. Lee, Pump-less static microfluidic device for analysis of chemotaxis of Pseudomonas aeruginosa using wetting and capillary action, Biosens. Bioelectron. 47 (2013) 278.
[92] Y. Kalinin, S. Neumann, V. Sourjik, M. Wu, Responses of *Escherichia coli* bacteria to two opposing chemoattractant gradients depend on the chemoreceptor ratio, J. Bacteriol. 192 (7) (2010) 1796.
[93] X. Zhang, G. Si, Y. Dong, K. Chen, Q. Ouyang, C. Luo, Y. Tu, Escape band in Escherichia coli chemotaxis in opposing attractant and nutrient gradients, Proc. Natl. Acad. Sci. USA 116 (6) (2019) 2253.
[94] M.M. Salek, F. Carrara, V. Fernandez, J.S. Guasto, R. Stocker, Bacterial chemotaxis in a microfluidic T-maze reveals strong phenotypic heterogeneity in chemotactic sensitivity, Nat. Commun. 10 (2019).
[95] B. Borer, R. Tecon, D. Or, Spatial organization of bacterial populations in response to oxygen and carbon counter-gradients in pore networks, Nat. Commun. 9 (1) (2018) 769.
[96] C. Shen, P. Xu, Z. Huang, D. Cai, S.-J. Liu, W. Du, Bacterial chemotaxis on SlipChip, Lab Chip 14 (16) (2014) 3074.
[97] D. Chen, S.-J. Liu, W. Du, Chemotactic screening of imidazolinone-degrading bacteria by microfluidic SlipChip, J. Hazard Mater. 366 (2019) 512.
[98] B.S. Lambert, J.-B. Raina, V.I. Fernandez, C. Rinke, N. Siboni, F. Rubino, P. Hugenholtz, G.W. Tyson, J.R. Seymour, R. Stocker, A microfluidics-based in situ chemotaxis assay to study the behaviour of aquatic microbial communities, Nat. Microbiol. 2 (10) (2017) 1344.
[99] J.-B. Raina, B.S. Lambert, D.H. Parks, C. Rinke, N. Siboni, A. Bramucci, M. Ostrowski, B. Signal, A. Lutz, H. Mendis, et al., Chemotaxis shapes the microscale organization of the ocean's microbiome, Nature 605 (7908) (2022) 132.
[100] C.M.B. Ho, Q. Sun, A.J.T. Teo, D. Wibowo, Y. Gao, J. Zhou, Y. Huang, S.H. Tan, C.-X. Zhao, Development of a microfluidic droplet-based microbioreactor for microbial cultivation, ACS Biomater. Sci. Eng. 6 (6) (2020) 3630.
[101] P.D. Schloss, R.A. Girard, T. Martin, J. Edwards, J.C. Thrash, Status of the archaeal and bacterial census: an update, mBio 7 (3) (2016), e00201.
[102] A.D. Steen, A. Crits-Christoph, P. Carini, K.M. DeAngelis, N. Fierer, K.G. Lloyd, J. Cameron Thrash, High proportions of bacteria and archaea across most biomes remain uncultured, ISME J. 13 (12) (2019) 3126.
[103] Y. Liu, M. Walther-Antonio, Microfluidics: a new tool for microbial single cell analyses in human microbiome studies, Biomicrofluidics 11 (6) (2017), 061501.
[104] L. Mahler, S.P. Niehs, K. Martin, T. Weber, K. Scherlach, C. Hertweck, M. Roth, M. A. Rosenbaum, Highly parallelized droplet cultivation and prioritization of antibiotic producers from natural microbial communities, Elife (2021) 10.
[105] J. Yin, X. Chen, X. Li, G. Kang, P. Wang, Y. Song, U.Z. Ijaz, H. Yin, H. Huang, A droplet-based microfluidic approach to isolating functional bacteria from gut microbiota, Front. Cell. Infect. Microbiol. (2022) 12.
[106] L. Boitard, D. Cottinet, N. Bremond, J. Baudry, J. Bibette, Growing microbes in millifluidic droplets, Eng. Life Sci. 15 (3) (2015) 318.
[107] B. Hu, B. Xu, J. Yun, J. Wang, B. Xie, C. Li, Y. Yu, Y. Lan, Y. Zhu, X. Dai, et al., High-throughput single-cell cultivation reveals the underexplored rare biosphere in deep-sea sediments along the Southwest Indian Ridge, Lab Chip 20 (2) (2020) 363.
[108] C.Y. Jiang, L.B. Dong, J.K. Zhao, X.F. Hu, C.H. Shen, Y.X. Qiao, X.Y. Zhang, Y. P. Wang, R.F. Ismagilov, S.J. Liu, et al., High-throughput single-cell cultivation on microfluidic streak plates, Appl. Environ. Microbiol. 82 (7) (2016) 2210.
[109] M.M. Villa, R.J. Bloom, J.D. Silverman, H.K. Durand, S. Jiang, A. Wu, E.P. Dallow, S. Huang, L. You, L.A. David, Interindividual variation in dietary carbohydrate metabolism by gut bacteria revealed with droplet microfluidic culture, mSystems 5 (3) (2020), e00864.
[110] Y. Meng, S. Li, C. Zhang, H. Zheng, Strain-level profiling with picodroplet microfluidic cultivation reveals host-specific adaption of honeybee gut symbionts, Microbiome 10 (1) (2022) 140.
[111] A.L. McCully, M. Loop Yao, K.K. Brower, P.M. Fordyce, A.M. Spormann, Double emulsions as a high-throughput enrichment and isolation platform for slower-growing microbes, ISME Commun. 3 (1) (2023) 47.
[112] F. Ma, T. Guo, Y. Zhang, X. Bai, C. Li, Z. Lu, X. Deng, D. Li, K. Kurabayashi, G. y. Yang, An ultrahigh-throughput screening platform based on flow cytometric droplet sorting for mining novel enzymes from metagenomic libraries, Environ. Microbiol. 23 (2) (2021) 996.
[113] Z. Zhang, Q. Guo, Y. Wang, H. Huang, High-throughput screening of microbial strains in large-scale microfluidic droplets, Front. Bioeng. Biotechnol. 11 (2023), 1105277.
[114] Y. Qiao, R. Hu, D. Chen, L. Wang, Z. Wang, H. Yu, Y. Fu, C. Li, Z. Dong, Y.-X. Weng, Fluorescence-activated droplet sorting of PET degrading microorganisms, J. Hazard Mater. 424 (2022), 127417.
[115] A. Xu, X. Zhang, S. Cao, X. Zhou, Z. Yu, X. Qian, J. Zhou, W. Dong, M. Jiang, Transcription-associated fluorescence-activated droplet sorting for di-rhamnolipid hyperproducers, ACS Synth. Biol. 11 (6) (2022) 1992.
[116] S.S. Terekhov, I.V. Smirnov, M.V. Malakhova, A.E. Samoilov, A.I. Manolov, A. S. Nazarov, D.V. Danilov, S.A. Dubiley, I.A. Osterman, M.P. Rubtsova, et al., Ultrahigh-throughput functional profiling of microbiota communities, Proc. Natl. Acad. Sci. USA 115 (38) (2018) 9551.
[117] J. Riba, T. Gleichmann, S. Zimmermann, R. Zengerle, P. Koltay, Label-free isolation and deposition of single bacterial cells from heterogeneous samples for clonal culturing, Sci. Rep. 6 (2016).
[118] D.I. Ellis, R. Goodacre, Metabolic fingerprinting in disease diagnosis: biomedical applications of infrared and Raman spectroscopy, Analyst 131 (8) (2006) 875.
[119] H. Jayan, H.B. Pu, D.W. Sun, Recent developments in Raman spectral analysis of microbial single cells: techniques and applications, Crit. Rev. Food Sci. Nutr. 62 (16) (2022) 4294.
[120] D. Berry, E. Mader, T.K. Lee, D. Woebken, Y. Wang, D. Zhu, M. Palatinszky, A. Schintlmeister, M.C. Schmid, B.T. Hanson, et al., Tracking heavy water (D_2O) incorporation for identifying and sorting active microbial cells, Proc. Natl. Acad. Sci. USA 112 (2) (2015) E194.
[121] F.C. Pereira, K. Wasmund, I. Cobankovic, N. Jehmlich, C.W. Herbold, K.S. Lee, B. Sziranyi, C. Vesely, T. Decker, R. Stocker, et al., Rational design of a microbial consortium of mucosal sugar utilizers reduces Clostridiodes difficile colonization, Nat. Commun. 11 (1) (2020).
[122] K.S. Lee, M. Palatinszky, F.C. Pereira, J. Nguyen, V.I. Fernandez, A.J. Mueller, F. Menolascina, H. Daims, D. Berry, M. Wagner, et al., An automated Raman-based platform for the sorting of live cells by functional properties, Nat. Microbiol 4 (6) (2019) 1035.
[123] X.F. Yi, Y.Z. Song, X.G. Xu, D. Peng, J.K. Wang, X.W. Qie, K.C. Lin, M. Yu, M.F. Ge, Y. Wang, et al., Development of a fast Raman-assisted antibiotic susceptibility test (FRAST) for the antibiotic resistance analysis of clinical urine and blood samples, Anal. Chem. 93 (12) (2021) 5098.
[124] W.E. Huang, A.D. Ward, A.S. Whiteley, Raman tweezers sorting of single microbial cells, Environ. Microbiol. Rep. 1 (1) (2009) 44.
[125] X.F. Yuan, A. Glidle, H. Furusho, H.B. Yin, A 3D hydrodynamic flow-focusing device for cell sorting, Microfluid. Nanofluidics 25 (3) (2021).
[126] X.X. Wang, Y. Xin, L.H. Ren, Z. Sun, P.F. Zhu, Y.T. Ji, C.Y. Li, J. Xu, B. Ma, Positive dielectrophoresis-based Raman-activated droplet sorting for culture-free and label-free screening of enzyme function in vivo, Sci. Adv. 6 (32) (2020).
[127] D. McIlvenna, W.E. Huang, P. Davison, A. Glidle, J. Cooper, H.B. Yin, Continuous cell sorting in a flow based on single cell resonance Raman spectra, Lab Chip 16 (8) (2016) 1420.
[128] K.S. Lee, F.C. Pereira, M. Palatinszky, L. Behrendt, U. Alcolombri, D. Berry, M. Wagner, R. Stocker, Optofluidic Raman-activated cell sorting for targeted genome retrieval or cultivation of microbial cells with specific functions, Nat. Protoc. (2020), https://doi.org/10.1038/s41596-020-00427-8.
[129] Y. Wang, Y. Ji, E.S. Wharfe, R.S. Meadows, P. March, R. Goodacre, J. Xu, W. E. Huang, Raman activated cell ejection for isolation of single cells, Anal. Chem. 85 (22) (2013), 10697.
[130] Y. Song, A.-K. Kaster, J. Vollmers, Y. Song, P.A. Davison, M. Frentrup, G. M. Preston, I.P. Thompson, J.C. Murrell, H. Yin, et al., Single-cell genomics based on Raman sorting reveals novel carotenoid-containing bacteria in the Red Sea, Microb. Biotechnol. 10 (1) (2017) 125.
[131] Y. Wang, J.B. Xu, L.C. Kong, B. Li, H. Li, W.E. Huang, C.M. Zheng, Raman-activated sorting of antibiotic-resistant bacteria in human gut microbiota, Environ. Microbiol. 22 (7) (2020) 2613.
[132] P. Liang, B. Liu, Y. Wang, K.X. Liu, Y.P. Zhao, W.E. Huang, B. Li, Isolation and culture of single microbial cells by laser ejection sorting technology, Appl. Environ. Microbiol. 88 (3) (2022).
[133] H.-Z. Li, K. Yang, H. Liao, S.B. Lassen, J.-Q. Su, X. Zhang, L. Cui, Y.-G. Zhu, Active antibiotic resistome in soils unraveled by single-cell isotope probing and targeted metagenomics, Proc. Natl. Acad. Sci. USA 119 (40) (2022), e2201473119.

[134] P.R. Zhang, L.H. Ren, X. Zhang, Y.F. Shan, Y. Wang, Y.T. Ji, H.B. Yin, W.E. Huang, J. Xu, B. Ma, Raman-activated cell sorting based on dielectrophoretic single-cell trap and release, Anal. Chem. 87 (4) (2015) 2282.
[135] X. Wang, L. Ren, Y. Su, Y. Ji, Y. Liu, C. Li, X. Li, Y. Zhang, W. Wang, Q. Hu, et al., Raman-activated droplet sorting (RADS) for label-free high-throughput screening of microalgal single-cells, Anal. Chem. 89 (22) (2017), 12569.
[136] Y.K. Lyu, X.F. Yuan, A. Glidle, Y.C. Fu, H. Furusho, T.X. Yang, H.B. Yin, Automated Raman based cell sorting with 3D microfluidics, Lab Chip 20 (22) (2020) 4235.
[137] M. Lindley, J.G. de Pablo, J.W. Peterson, A. Isozaki, K. Hiramatsu, K. Goda, High-throughput Raman-activated cell sorting in the fingerprint region, Adv. Mater. Technol. 7 (10) (2022).
[138] F.C. Pereira, K. Wasmund, I. Cobankovic, N. Jehmlich, C.W. Herbold, K.S. Lee, B. Sziranyi, C. Vesely, T. Decker, R. Stocker, et al., Rational design of a microbial consortium of mucosal sugar utilizers reduces *Clostridiodes difficile* colonization, Nat. Commun. 11 (1) (2020).
[139] Y.G. Xiaoyan Jing, Huihui Pan, Yu Meng, Yishang Ren, Zhidian Diao, Runzhi Mu, Teng Xu, Jia Zhang, Yuetong Ji, Yuandong Li, Chen Wang, Lingyun Qu, Li Cui, Bo Ma, Jian Xu, Single-cell Raman-activated sorting and cultivation (scRACS-Culture) for assessing and mining in situ phosphate-solubilizing microbes from nature, ISME Commun. 2 (2022) 106.