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REVIEW

Microfluidic hotspots in bacteria research: A review of soil and related advances

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HIGHLIGHTS

- Microfluidic technology promotes the development of soil bacteria research.
- Microfluidics can achieve real time observation and analysis of microorganisms in controlled environments.
- Microfluidics generally use optical and electrochemical methods to detect single cells combined with polymerase chain reaction (PCR) to realize high throughput gene detection on chips.
- Microfluidics is mainly applied in chemotaxis, biofilm, antibiotic and horizontal gene transfer research of soil bacteria.

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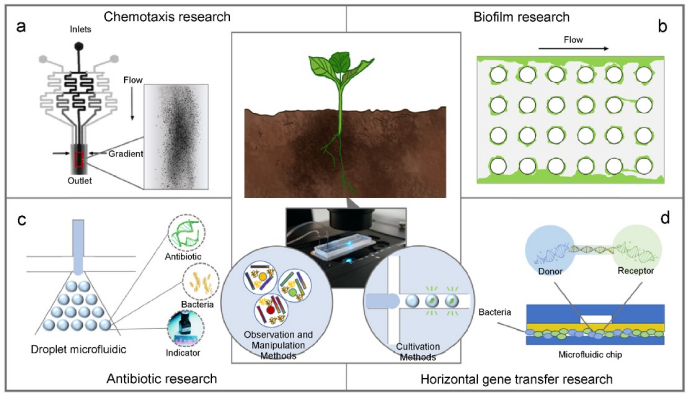

ABSTRACT

Soil science is an inherently diverse and multidisciplinary subject that cannot develop further without the continuous introduction and promotion of emerging technologies. One such technology that is widely used in biomedicine and similar research fields, microfluidics, poses significant benefits for soil research; however, this technology is still underutilized in the field. Microfluidics offers unparalleled opportunities in soil bacterial cultivation, observation, and manipulation when compared to conventional approaches to these tasks. This review focuses on the use of microfluidics for bacteria research and, where possible, pulls from examples in the literature where the technologies were used for soil related research. The review also provides commentary on the use of microfluidics for soil bacteria research and discusses the key challenges researchers face when implementing this technology. We believe that microfluidic chips and their associated auxiliary technologies provide a prime inroad into the future of soil science research.

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1 Introduction

Soil is an environment wherein there is a continuous exchange of material and energy and can be considered as one of the most active parts of the Earth ecosystem. The unique physical structures created by complex peds and pores combined with soil's rich chemical and biological elements continuously promote evolution within this environment (Eisenhauer et al., 2017; Rillig et al., 2017). Microorganisms are the core actors in the pedosphere, primarily through their role in biogeochemical cycling (Pronk et al., 2017). Expanding our understanding of soil bacteria is pivotal to unravelling soil functional mechanisms, thereby establishing a theoretical grounding for soil nutrient regulation, microbial community control, and contaminant degradation (Zhu et al., 2017). Advanced harnessing of the soil system is an economic and environmental imperative which will benefit all aspects of human productivity and life. However, cultivation of and observation of soil microbes have long posed a substantial challenge – a challenge which has had new possibilities opened using microfluidics.

Microfluidics is known as *laboratory-on-a-chip*. The technology arose from the development of integrated circuits, promoting the miniaturization of analytical instruments (Aleklett et al., 2018). The first concept of a microfluidic chip as a miniaturized analysis instrument that integrates sample pretreatment, separation, and detection was presented by Manz and colleagues in 1990 (Manz et al., 1990). In the following three decades, the processing methods, material development and testing methods related to microfluidic chips have rapidly developed (Sackmann et al., 2014).

Since its inception, the technology has been widely applied in chemical synthesis and biomedicine. It integrates a range of instrumental analyses with the chips themselves and its use in biochemical analyses has several advantages over traditional methods (Aleklett et al., 2018). Firstly, far smaller quantities of sample and reagents are required for experiments than is conventionally needed. Mixing and reacting are quickly completed and this greatly decreases material consumption and time costs. Secondly, chip structures can be modified according to different functional requirements: analysis equipment such as sensors can be miniaturized and light transmittance can be altered via the chip materials, allowing high-throughput parallel experiments (Volpatti and Yetisen, 2014). Furthermore, fluid control within the chip allows for the precise regulation of physicochemical conditions in the microenvironment, broadening the toolkit for microorganism cultivation and interaction mechanism studies.

Microfluidics integrates sample preparation, reaction, separation, detection, etc., and provides a rich functional platform for microbial research (Aleklett et al., 2018). Soil microbiomes are complex living systems that include bacteria, archaea, fungi, viruses, protists and other micro-fauna (Zhu et al., 2017). However, previous research into soil microbiomes has been challenged by technical limitations; these limitations are structural and observational. Structural limitations include our inability to accurately simulate the soil environment on the appropriate scale due to the strong physical and chemical heterogeneity of the soil environment. Related to this limitation is the difficulty of observing or sensing interactions between microorganisms and their environment. The soil system is a perfect candidate for microfluidic research as this provides highly controllable microscale simulations combined with multitudinous analysis methods for physical structure and chemical components. Recent works show microfluidics being used to simulate soil physical pores, to culture and screen unknown microbial strains, and to investigate interactions between microbes and between microbes and roots (Aleklett et al., 2018).

In this review, we explore the use of microfluidics in microbial research from the perspective of technical requirements and applications. We further reflect on potential methodological or technical defects and discuss possible challenges and key issues to be addressed in the future.

2 Technology and methods

2.1 Observation and manipulation methods

Microorganism analyses, requiring high throughputs and automated sorting that normally use multi-well plates, are currently transitioning to microfluidic chips. As a result, the development of microfluidic chips for high throughput observation and manipulation is rapidly expanding, particularly in the use of photoelectric equipment and emerging methods that combine polymerase chain reaction (PCR) methods with microfluidics.

2.1.1 Optical detection

Optical methods currently used for microfluidic chip analyses include laser-induced fluorescence (LIF), immunofluorescence technology (IFT), fluorescence *in situ* hybridization (FISH) and surface enhanced Raman spectroscopy (SERS) (Table 1). Fluorescence based detection methods are commonly used as they allow the objects of interest to be easily identified against background noise.

In contrast to direct observation of objects under bright light, fluorescence allows clear and accurate detection of cells with reduced background interference. While most substances can absorb light of a specific wavelength, those which have a high fluorescence efficiency are best for LIF detection (Mazouffre, 2012). When combined with LIF, microchip electrophoresis (MCE) can quickly separate bacteria such as *Bifidobacterium, Lactobacillus casei, Lactobacillus acidophilus,* and *Enterococccus faecalis* (Cheng et al., 2012). In the experiment outlined by Cheng et al. (2012), bacteria were separated within 150 s using a running buffer of pH 8.5. When used with LIF, microfluidic chip analyses generally include a laser confocal microscope or similar confocal optical equipment. Microarray chips can be further combined with laser-induced fluorescence microscopy (LIFM) to reduce sample

Table 1 Comparation of optical and electrochemical detection methods.

Detection method	Advantage	Disadvantage	Reference
Laser-induced fluorescence (LIF)	a) Rapid analysis speed; b) Noninvasive	a) Mostly need fluorescence labeling or derivatization b) Interfered by other fluorescent materials that can be excited by laser, insufficient accuracy	Liu and Zhu, 2020
Immunofluorescence technology (IFT)	a) High specificity; b) Rapid analysis speed	a) Non-specific background interference; b) Single test item	Khemthongcharoen et al., 2021
Fluorescence *in situ* hybridization (FISH)	a) Spatial multiplexing of different probes on a single tissue; b) Cellular and histological morphology is unaffected; c) Real-time monitoring of the hybridization kinetics of probes	a) Multiplexing is limited to the number of color channels of fluorescence microscopes.	Huber et al., 2018; Rodriguez-Mateos et al., 2020
Surface enhanced Raman spectroscopy (SERS)	a) Label free; b) Noninvasive; c) Online analysis	a) Low Raman scattering yield; b) Sensitivity and signal-to-noise ratio are limited by choice of nanoparticle in SERS	Sun et al., 2020; Fu et al., 2021
Electrochemical detection	a) Derivatization of analytes free; b) Insusceptible	a) Low throughput b) Few detectable indicators, and additional electrodes need to be developed	Lu and Kutter, 2020

volume and detection time and has been shown to reproducibly and quantifiably detect DNA damage in the presence of lead (Pb) and cadmium (Cd) ions (Koo et al., 2011). Although fluorescence is a highly sensitive technique, the effect of fluorescent labeling reagents on cells and a lack of native fluorescence limits its analytic value; the creation and identification of analytes with a fluorophore prior to analysis is therefore a research priority (Jokerst et al., 2012).

Immunofluorescence technology, also known as fluorescent antibody technology, is one of the earliest developments in labeled immunosurgery. By combining fluorescent tracers with antibodies, IFT uses antigen-antibody reactions to locate antigens within tissues or cells. In soil research, IFT has been used for detection and quantification of soil-associated microbes such as *Mycobacterium bovis* and *Pseudomonas* (Dazzo et al., 2007; Sweeney et al., 2007). This fluorescence technique has been used in microfluidic chips with microbeads to achieve rapid detection of bacteria and to test bacterial antibiotic sensitivity (He et al., 2014). They used a structure with four compartments, where one was used for bacterial identification and the other three were used for drug susceptibility testing. During their experiment, microbeads were coated with antibodies specific to the target pathogen which then formed a bead-bacterial complex after which a fluorescence microscope was used to measure the fluorescence intensity.

Fluorescence techniques focusing on the morphology and dynamic distribution of labeled organelles or DNA fragments and RNA molecules, such as FISH, can also be paired with gene specific methodologies to retain spatial information. This technology uses specific nucleic acid probes with fluorescent labels to hybridize with the corresponding target DNA fragment or RNA molecule in the cell. After hybridization, the fluorescent signal is captured under a fluorescence microscope to determine the morphology and distribution of organelles, or the location of DNA fragments and RNA molecules; the signal may also be analyzed combined with other fluorescent probe signals in chromosomes or other organelles (Valm et al., 2012). This method has been refined with single-molecule fluorescence *in situ* hybridization (smFISH), which is based on tracking single targets and can be combined with live-imaging of fluorescent transcriptional reporters to quantify multi-level gene expression information with single-cell resolution (Fig. 1A; Wan et al., 2020). Chips used with smFISH and live-imaging can be applied to the exploration of other biological models after optimization.

Following a similar path to fluorescence detection, chemiluminescence detection is also widely used; surface enhanced Raman spectroscopy (SERS) is the most common of these methods. This method is compatible with microfluidic chips and allows high-throughput bacterial detection. Raman spectroscopy originally worked by monitoring energy changes

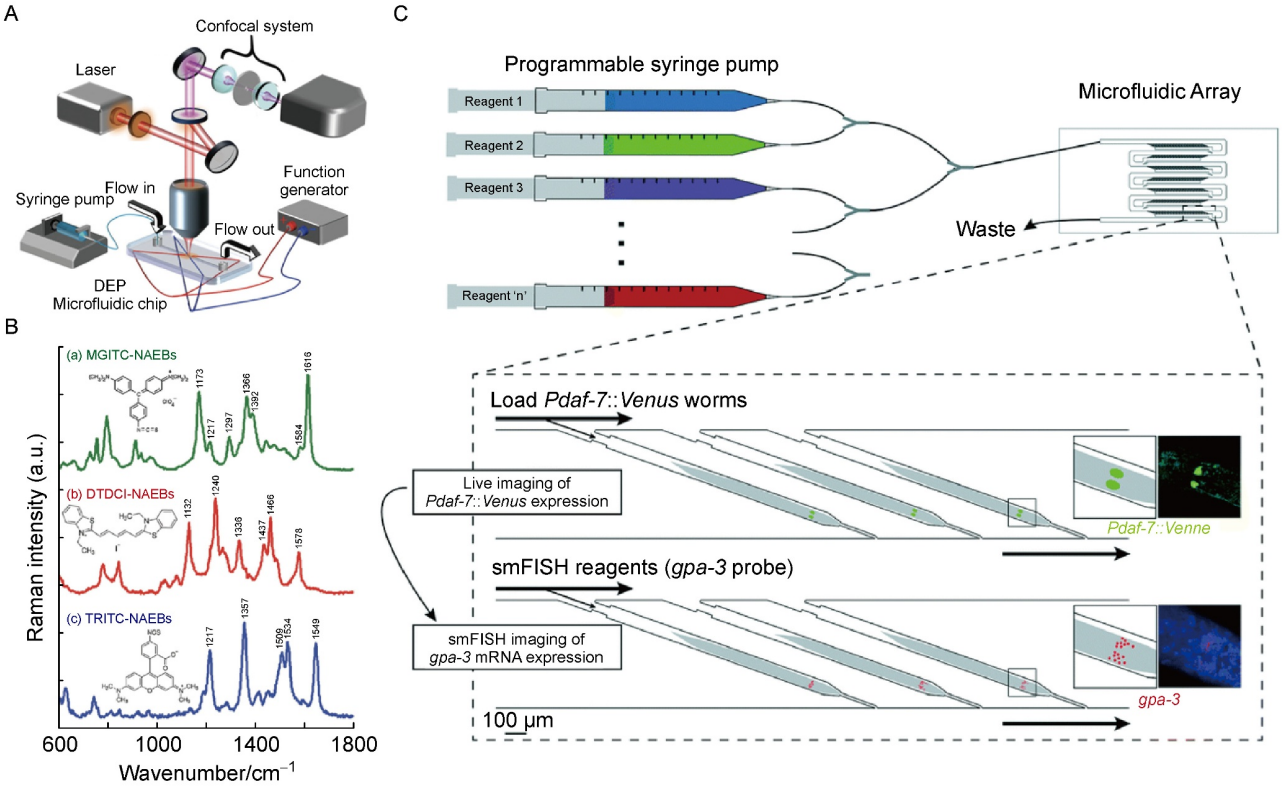

Fig. 1 Microfluidic devices combined with optical detection. (A) A microfluidic dielectrophoresis (DEP) device integrated with a SERS nanoprobe (from Lin et al., 2014 Small, 10(22), 4700–4710). (B) SERS spectra of (a) MGITC-, (b) DTDCI-, and (c) TRITC-labeled NAEBs (from Lin et al., 2014 Small, 10(22), 4700–4710). (C)Microfluidic chip using smFISH. A programmable syringe pump can consistently deliver multiple reagents over multiple cycles. The microfluidic device has individual, trackable traps that can efficiently load animals and consistently deliver reagents. *Pdaf-7::Venus* worms are imaged for *daf-7* expression and then smFISH is performed to quantify *gpa-3* mRNA counts (from Wan et al., 2020 Laboratory on a Chip, 20(2), 266–273).

in scattered light relating to the molecule of interest's rotational vibration state and can therefore identify molecular level analytes (Fleischmann et al., 1974). In its modified form, SERS greatly enhances the Raman signal on the surface of analytes and metal nanostructures, significantly improving sensitivity (Negri et al., 2013).

Detection methods using SERS have been further improved through the development of a SERS nanoprobe that, when integrated with a microfluidic dielectrophoresis (DEP) device, can be used for rapid detection of *Salmonella enterica* serotype Choleraesuis and *Neisseria lactamica* (Lin et al., 2014). The SERS nanoprobes are fabricated by immobilization of specific antibodies onto the surface of nanoaggregate-embedded beads (NAEBs), which are silica-coated, dye-induced aggregates of a small number of gold nanoparticles (AuNPs). The combination of NAEB-DEP-SERS has achieved online SERS detection and accurate identification of suspended bacteria, with detection precision up to a single bacterium. The practical detection limit for this method, when used with a measurement time of 10 min, was estimated to be 70 CFU mL^{-1} (Fig. 1B; Lin et al., 2014).

The SERS method has also been modified by including a disposable low-cost microfluidic chip device that uses a nanoporous membrane (Krafft et al., 2021). The device connects two stacked perpendicular microfluidic channels, one providing the sample and the other providing the driving force to attract the sample. Silver nanoparticles are added to the sample for surface enhancement and the membrane pores act as a filter to capture microbes and nanoparticle clusters, thereby creating suitable conditions for sensitive SERS detection. The device can be used to detect common pathogens such as *Escherichia coli* DH5α and *Pseudomonas taiwanensis* VLB120 from spiked tap water (Krafft et al., 2021).

2.1.2 Electrochemical detection

Electrochemical detection is a label-free quantitative detection method with high sensitivity that detects microorganisms and cells adsorbed on the surface of the functionally modified electrode, thereby allowing the development of portable devices (Table 1). Electrochemical methods include linear scan voltammetry (LSV) and electrical impedance technology (EIT), which can monitor cell size and shape changes. When these methods are integrated into microfluidic devices, they can achieve high-throughput single-cell detection and can distinguish differences between cells.

When combined with microfluidic technology, electroche-

mical detection can capture and count low concentration cells with high sensitivity and short reaction times. Loop-mediated isothermal amplification (LAMP), which amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions (Notomi et al., 2000), and linear scan voltammetry (LSV) has been combined to achieve electrochemical detection of DNA amplification, allowing the rapid detection of *E. coli* in a single chamber (Safavieh et al., 2012). During method testing, their chip could detect 48 CFU mL^{-1} bacteria within 35 min and was unresponsive when used with non-target bacterial DNA (*Listeria* and *Staphylococcus aureus*). Another widely used method is EIT, which uses a small alternating current to respond to weak changes in the target; using EIT with microfluidic technology greatly improves its sensing performance. By combining microfluidic dielectrophoresis with EIT, an integrated microfluidic analysis system has been developed for bacterial enrichment and *in situ* impedance detection (Liu et al., 2017a). Using this system with optimized detection conditions (voltage, 10 V; frequency, 500 kHz), the detection limit of *E. coli* was 5×10^4 CFU mL^{-1} within 6 min. Electrochemical-based microfluidic detection methods enable rapid real-time *in situ* detection of bacteria and make possible the real-time detection and sorting of environmental pathogens in the field, thereby increasing research convenience.

2.1.3 Gene amplification measurement

The measurement of gene amplification is a key to studying the function and mechanisms of microorganisms and cells. To facilitate this, nucleic acid amplification methods such as PCR have been combined with microfluidic technologies to obtain a high-throughput DNA sequencing method using microfluidic chips. The current expansion of microfluidic PCR methods focuses on improving simultaneous gene detection on chips, increasing the sensitivity of single cell analysis and sample response to perturbations. High-throughput PCR chips integrated with biological sample processing have already been developed; for example, a microfluidic chip has been developed to identify bacteria in saliva by integrating DNA extraction, amplification and detection (Oblath et al., 2013). This chip was designed with a monolithic alumina membrane (AOM) for DNA extraction and contained seven parallel reaction wells for real-time polymerase chain reactions (rtPCR). By changing the well primers and probes, multiple target species and strains could be simultaneously identified in samples (Oblath et al., 2013).

Droplet microfluidics has already begun to be applied to PCR chips; droplet digital PCR (ddPCR) is based on the amplification of a single target DNA molecule in many separated droplets, providing a new method for accurately quantifying DNA copy number. Within this analysis, target DNA molecules follow the Poisson distribution, meaning that most droplets contain one or none of the target DNA molecule (Vogelstein and Kinzler, 1999; Zhong et al., 2011). Therefore, the total number of PCR positive reactions is effectively equal to the number of template molecules initially present. Furthermore, unlike qPCR, ddPCR does not rely on the cycle threshold (C_T), nor does it require a calibration curve (Hindson et al., 2011; Pinheiro et al., 2012). The method also has high sensitivity and precision for low copy number target nucleic acids (Hindson et al., 2011; Hayden et al., 2013). Droplet digital PCR can be combined with fluorescent probes to improve the detection of microbial strains. Recently, ddPCR was used to culture *E. coli* and quantify gene targets from soils (Capone et al., 2021). Moreover, ddPCR has also been optimized to quantify soil total bacteria and archaea as well as the nitrification (bacterial and archaeal *amoA*) and denitrification (*nirS, nirK, nosZI, nosZII*) genes involved in the generation or reduction of the greenhouse gas nitrous oxide (Voegel et al., 2021).

The application of micro-droplet encapsulation and on-chip PCR amplification also makes high-resolution microbial community research possible. Current microbiome analysis methods (such as metagenomic sequencing) require homogenization of input samples, which means that basic spatial information is lost during processing. Although microscopic imaging can reveal spatial information, it relies on diversified spectra and limited DNA probes, resulting in output data with low biological classification resolution and usually requires extensive empirical optimization. Bacterial aggregation in natural systems restricts the use of imaging methods only to synthetic communities with simple compositions. However, spatial patterns in microbial communities can be captured by metagenomic plot sampling by sequencing (MaPS-seq) (Sheth et al., 2019). In this method, microbiome samples are immobilized in a gel matrix and then cryofractured into particles. The particles are then encapsulated in microdroplets for barcoded 16S rRNA amplification and deep sequencing to identify adjacent microbial taxa in the particles. This method has been used to determine the local spatial organization of a gut microbiome, through which a robust association between Bacteroidales taxa was found (Fig. 2; Sheth et al., 2019).

In addition to PCR-based amplification, LAMP and other technologies can also be used in the rapid detection of bacteria. Of these alternative methods, LAMP is fast and easy to operate and therefore has great potential. When combined with carbon nanotube (CNT) multilayer biosensors and microfluidic chip-based LAMP, *E. coli* O157: H7 and its associated toxicity genes can be detected at concentrations as low as 1 CFU mL^{-1} without use of complicated instruments (Li et al., 2017). This visual method is both sensitive and can be conducted at point-of-care.

2.1.4 Future applications in soil bacteria research and limitation

Soil microorganisms and their metabolites can be used as indicators of soil quality, with measures such as soil enzyme activity, microbial community diversity, genetic diversity, microbial biomass, pathogenic bacteria and soil biological network complexity used as proxies for soil health (Alkorta et al., 2003; de Vries and Wallenstein, 2017; Hermans et al.,

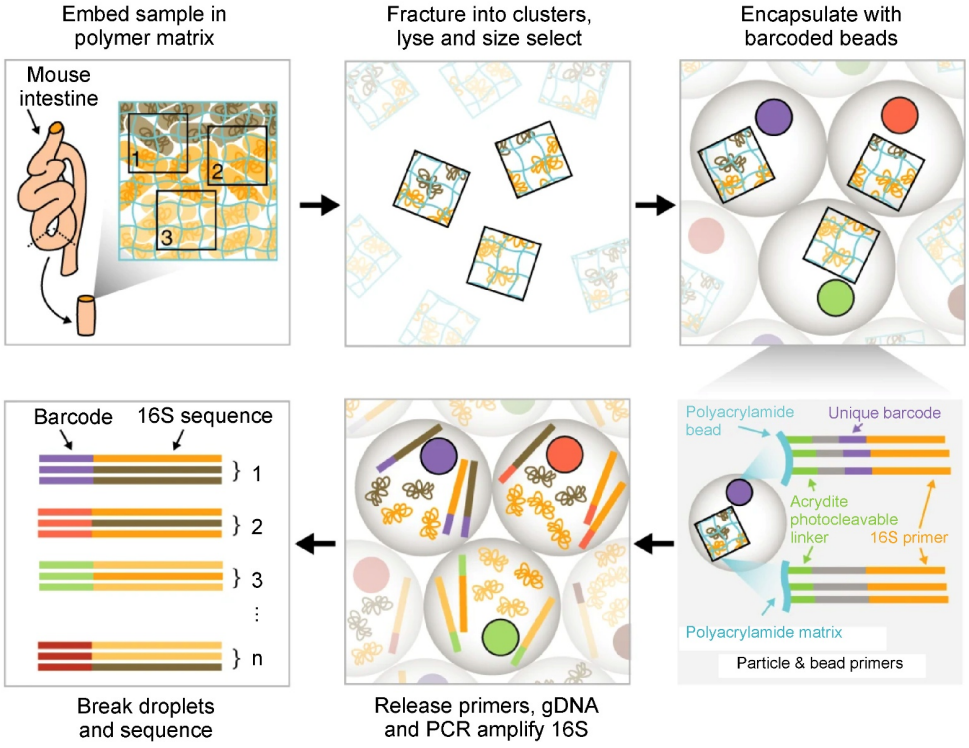

Fig. 2 A method based on microfluidics that keeps spatial information to study microbiome: MaPS-seq. Intact microbiome samples are immobilized in a gel matrix and cryofractured into particles. Neighboring microbial taxa in the particles are then identified by droplet-based encapsulation, barcoded 16S rRNA amplification, and deep sequencing (from Sheth et al., 2019. Nature biotechnology, 37(8), 877–883).

2020). A key member of microbial communities is bacteria which, as single celled organisms, are highly heterogenous at both the community and individual level. However, the previous lack of single-cell analysis methods means that results have often been averaged across populations, obscuring individual information and hindering mechanistic study of soil microorganisms. The expansion of single celled analyses using microfluidic chips and cross-disciplinary detection methods is therefore significant to the study of soil bacteria.

Fluorescent labeling allows the study of bacterial growth and interaction in microfluidic chip simulated soil environments, moving to address the methodological issue of directly studying bacteria in the soil environment. However, as there are many soil bacterial species in soil that cannot be directly distinguished with this method, future studies may benefit from chip-based metabolite fluorescent labeling methods. Furthermore, microfluidic chips combined with SERS and EIT also would provide a reliable means for screening and separating functional microorganisms and their active compounds from soil as can be seen from works concentrating on discovering new antibiotics from natural products (Harvey et al., 2015; Zhu et al., 2017). Single cell sorting combined with microfluidics has already allowed the screening of a variety of new antibiotics from soil microorganisms (Hamamoto et al., 2015; Ling et al., 2015) and further advances will undoubtedly improve the screening efficiency of related substances from soil microorganisms.

Thanks to the maturity of droplet microfluidics and on-chip PCR, methods such as MaPS-seq have been able to provide high taxonomic resolution microbiome information. Acquiring genomic distribution information with high spatiotemporal and taxonomic resolution is significant for constructing dynamic microbial interaction networks and exploring function mechanisms of core network nodes. The new methods outlined above provide feasible ways to reconstruct the spatial structures of soil microbiomes, systematically study the functions of soil microbiome, determine soil process mechanisms, regulate ecological environments, and also provide new ideas for more effective regulation of soil microbiomes.

However, while the application of microfluidics to soil bacterial research is inspiring, there are challenges that cannot be avoided. Firstly, microfluidic chips are unable to simulate the soil environment completely and accurately. Materials such as PDMS, PMMA, and glass, which are normally used in chips, have substantially different surface properties than soil mineral surfaces. Although there have been attempts to modify chip surfaces through the attachment of soil particles to microchannel surfaces (Huang et al., 2017), such surfaces are still noticeably different to soil aggregates. Simultaneously, soil environment heterogeneity is also a challenge to full simulate with the relatively simple tools available for microenvironment regulation. Secondly, microfluidics is technologically complex and materials and equipment can be difficult to obtain for soil scholars as the in-laboratory production and application of microfluidic devices

require highly advanced facilities. While some commercial microfluidic products are available, they are inadequate for the needs of research.

The challenges facing microfluidics research in soil science are not insurmountable, however, targeted collaboration is key to further progress. For example, further advances in electrochemical and nucleic acid amplification through interdisciplinary collaboration will improve the detection of microorganisms within fluidic systems. Similarly, the detection of further fluorescent labels, and development of new sensors, are simple if expensive pathways to future technological advances. Regardless of the challenges inherent in microfluidic research, technological breakthroughs in soil bacterial research are possible with appropriate collaboration.

2.2 Cultivation methods

Due to the difficulty of obtaining an appropriate culture environment for soil microorganisms, about 95% of environmental microbes cannot be cultivated (Martiny, 2019, 2020; Steen et al., 2019), and therefore cannot be easily developed as genetic resources. At present, technologies such as iChip can carry out microbial culture *in situ*; however, limitations remain as related technologies can only provide *in situ* physical and chemical environments and cannot guarantee the transmission and interaction of signal factors between different bacterial species (Alkayyali et al., 2021). Regardless, the development of microfluidic and microdroplet technologies in recent years has highlighted the possibility of maintaining symbiosis and the transmission of signal factors during the culture process.

2.2.1 Building complex physical structures

Physical structures have important impacts on the morphology and attachment of microorganisms during the growth process (Wang et al., 2015) and building physical structures that better simulate the original environment improves microbe cultivation outcomes (Wang et al., 2015; Shao et al., 2019). Microdroplets, for example, provide a cavity structured environment for microbial growth and can also provide a physical channel for signal molecule transmission between different single bacteria (Shao et al., 2019). Water/oil/water (W/O/W) double emulsions with multiple encapsulated inner droplets composed from UV curable materials can be used for porous microcarrier fabrication, with the cells within the microcarrier protected from flow shear forces (Fig. 3A; Wang et al., 2015). In a similar vein, complex physical environments using microfluidics have been constructed by stacking emulsion droplets and filling the gaps between droplets with GO hydrogel (Shao et al., 2019). The emulsion droplets are then washed off after curing to form a structure that creates tiny channels between the cavities that can carry out the transportation and exchange of materials (Shao et al., 2019). In the aforementioned study, cells and microorganisms were enriched and cocultured in the spherical cavities and, since signal substance transmission was

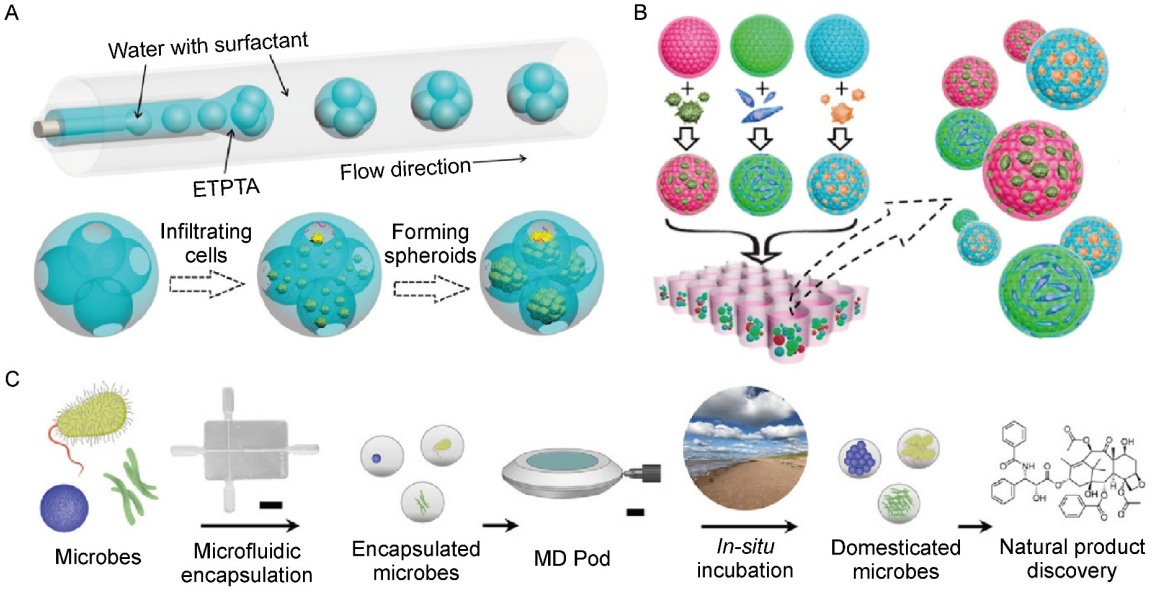

Fig. 3 Microfluidics with cultivation methods. (A) Use of droplets microfluidics to build complex physical structures. Cell and microbes could be cultivated in the cavity, which can reduce the influence of flow shearing force on growth (from Wang et al., 2015. ACS Applied Materials & Interfaces, 7, 27035–27039). (B) Use of droplets microfluidics combined with fluorescent particles to achieve co-cultivation. Different cells and microorganisms could be identified based on fluorescent label (from Fu et al., 2016. ACS Applied Materials & Interfaces, 8, 13840–13848). (C) *In situ* culture technologies, Microbe Domestication Pod (MD pod). The single bacteria isolated from the environment were encapsulated with hydrogel droplets, suspended in the culture medium, and packaged with a device and permeable membrane, and placed *in situ* for cultivation (from Alkayyali et al., 2021. Biotechnology and Bioengineering, 118, 1166–1176).

enabled, cell and microorganism co-cultivation was successfully achieved (Shao et al., 2019).

2.2.2 Building biochemical microenvironments

Environmental bacteria cannot exist independently of the growth environment's biochemical factors (Zhao et al., 2017). Droplet microfluidic technology provides a reliable biochemical microenvironment for the co-cultivation of microorganisms to more accurately represent natural synergistic and competitive effects that rely on the biochemical environment (Park et al., 2011; Fu et al., 2016). The constructed environments use various materials, microdroplets, and bacterial packaging methods to address a wide variety of research needs. For example, using porous hydrogel in addition to various cells and fluorescent labels allows the testing of cytotoxity of drugs in a system where metabolites and signal factors are free to enter and exit the test cavities, ensuring that the synergy among different cells can be preserved (Fig. 3B; Fu et al., 2016). Co-cultivation can also be achieved by packing multiple strains into one droplet. Droplets can be generated rapidly using microfluidics, allowing many parallel experiments to be performed simultaneously. The growth status and interaction of multiple bacteria can be relatively simply assayed using such experiments (Park et al., 2011).

2.2.3 *In situ* cultivation

Thanks to the mature development of microdroplets for microbial cultivation, many of the shortcomings of *in situ* culture technologies such as iChip have been solved (Alkayyali et al., 2021). The iChip platform has been recently improved by Alkayyali et al. (2021) by using hydrogel microdroplets to allow *in situ* cultivation while ensuring bacterial interconnection in the Microbe Domestication Pod (MD pod); this process has successfully cultivated microorganisms from marine sediments. In their research, bacteria isolated from the environment were encapsulated within hydrogel droplets, suspended in the culture medium, and packaged with a device and permeable membrane, and placed in situ for cultivation. The temperatures reached during encapsulation may pose a limitation for some bacteria; however most mesophilic bacteria should not be adversely affected by the process, and encapsulation may be additionally beneficial for the metabolic activity of the species (Fig. 3C; Alkayyali et al., 2021).

2.2.4 Future applications in soil bacteria research and limitation

Droplet microfluidics has unique advantages in the simulation of the physical and biochemical environment for microbial cultivation, and has long been applied in the fields of marine microbial culture and medicine. In soil bacteria cultivation, further methodological development could include encapsulating soil micro-aggregates, plant protoplasts, and microorganisms in microdroplets to construct a micro-environment for co-cultivation research. Soil researchers can also take advantage of the rapid generation of a large number of droplets to conduct high-throughput parallel experiments to explore changes in communities, find resistance genes, and determine pollutant repair mechanisms. By taking advantage of the simulated physical environments, microdroplets with different structures could be placed in the soil for *in situ* microbial enrichment and co-cultivation. Platforms such as the MD pod can also be used for *in situ* soil cultivation with benefits such as retained signal molecule transmission leading to improved cultivation range and success rate.

However, the limitations of using microdroplets for microbial cultivation are obvious. Not only do microdroplets require generation and control of extremely precise mechanisms, but the materials used in microdroplets and their carrier fluids also need a host of experimentation for testing and adjustment. Environmental conditions during droplet sealing (such as temperature and the composition of droplets) would impact microorganism growth (Alkayyali et al., 2021), and subsequent detection of microdroplet cultures similarly requires highly precise flow control. Regardless of these limitations, microdroplet culture methods are already an important complement to traditional microbial culture methods and will continue to be so.

3 Research applications

3.1 Chemotaxis research

Chemotaxis is a basic behavior of microorganisms that enables microbes to sense chemical gradients indicating favorable or harmful directions, so that they can selectively colonize beneficial environments (Smriga et al., 2016; Salek et al., 2019). Chemotaxis research has been broadly divided into the quantitative analysis of microorganism chemotaxis in simple chemical gradients and complex environments.

Microfluidic chips used for chemotaxis research require a stable chemical concentration gradient, a task which is commonly achieved using flow-based or diffusive-based microdevices. To generate gradients in flow-based microdevices, laminar mixing of solutes is conducted in microchannels with the gradient controlled through microchannel design and flow control (Murugesan et al., 2016). Flow rates of 0.1–1.0 mm s^{-1} do not necessarily affect the chemotactic movement of bacteria, but do affect the gradient stability and may cause actual concentration to deviate from the anticipated concentration (Lanning et al., 2008). In contrast to flow-based, diffusive-based microdevices have less effect on microorganism movements as they do not use external equipment to exert pressure on the fluid. This method often uses agarose gel or other porous media to achieve the diffusion gradient (Cheng et al., 2007). Simple linear chemical gradients are often used to study the chemotaxis mechanism of single substances, such as showing wild type *E. coli* chemotactic behavior concerning aspartic acid (Cheng et al., 2007).

The generation of chemical gradients has developed from simple linear gradients to gradients in two and three dimensions (2D and 3D, respectively) and now provides powerful tools for observing chemotactic mechanisms under multiple attractants. Microfluidics has more flexibility and controllability than conventional tools for studying microbial chemotaxis as microfluidic chips can use geometric microchannels and various materials to create complex chemical environments. These chips can be constructed directly using patterned microfluidics, in which maze patterns are normally used to study chemotaxis selection processes, for example by exposing microorganisms to a series of optional concentration gradients using a T-maze (Salek et al., 2019). By using such a maze, heterogeneity of chemotaxis in *E. coli* has been observed in clonal populations and was determined to be caused by pathway gain, which might have a functional effect (Fig. 4; Salek et al., 2019). However, this strategy has physical limitations due to the microchannel size impacting cell morphology and reducing migration velocity (Tong et al., 2012). Complex environments can also be simulated by coculturing cells, allowing quantitative chemotactic migration studies to take place in the physiological environment of cell-to-cell interactions. An example of this strategy developed a chip integrated chemokine gradient generator with a spatial distribution of different cell types to investigate the effect for chemotaxis of breast cancer cells on CXCR7 expressing cells in the CXCL12 gradient (Torisawa et al., 2010). The broad use of microfluidics in chemotaxis research indicates that it is likely to replace traditional methods as the primary tool to construct complex and realistic physio-biochemical environments *in vitro* for cell migration and chemotaxis studies.

Spatial heterogeneity in the distribution of soil moisture, nutrients, and pollutants has led to the evolution of complex chemotaxis mechanisms, affecting colony spatiotemporal distribution and compositional diversity, which thereafter alters soil physicochemical properties. Bacterial chemotaxis promotes nutrient cycling (Pedler et al., 2014) and the degradation of soil organic pollutants (Krell et al., 2013; Gupta et al., 2015; Adadevoh et al., 2016, 2018); however, the mechanisms involved are still a mystery. To simulate the physical structures of soil and soil pores, microfluidic platforms have moved from linear concentration gradients to multidimensional systems with fluid variables such as concentration, velocity and temperature, and scaffolding variables such as surface roughness, hardness and wettability (Cruz et al., 2017). Microfluidic chips, through their controllable physicochemical environment, provide the ability to explore chemotaxis mechanisms *in situ* and at the micron scale (Lambert et al., 2017; Zhu et al., 2019).

3.2 Biofilm research

Bacteria grow in porous media and on porous surfaces; on surfaces, they can secrete extracellular polymeric substances under certain moisture and nutrient conditions to protect themselves from the environment. These natural structures, may contain a single species or complex communities. The conventional method of studying biofilms in soil is to construct an artificial porous structure in a soil column and then scan the column with X-ray computed tomography (CT) to obtain 3D grayscale images of the pore, liquid phase, solid phase and biofilm phase distributions (Peszynska et al., 2016). However, this method is laborious, uneconomical, and difficult to control the initial porosity and flow environment. In contrast, the material commonly used in microfluidics, polydimethylsiloxane (PDMS), is permeable to light and can be used to simulate soil structures and spatiotemporal heterogeneity (Aleklett et al., 2018). We therefore believe that microfluidics has potential for practical applications in accurately and precisely simulating soil pores, increasing our capacity to study soil processes at the pore scale.

Simple microfluidic biochips have been used to study biofilms since 2007 at least, with real-time observation of fungal biofilm growth kinetics indicating that this structure could dynamically monitor shear stress and respond to antimicrobial agents (Richter et al., 2007). However, the

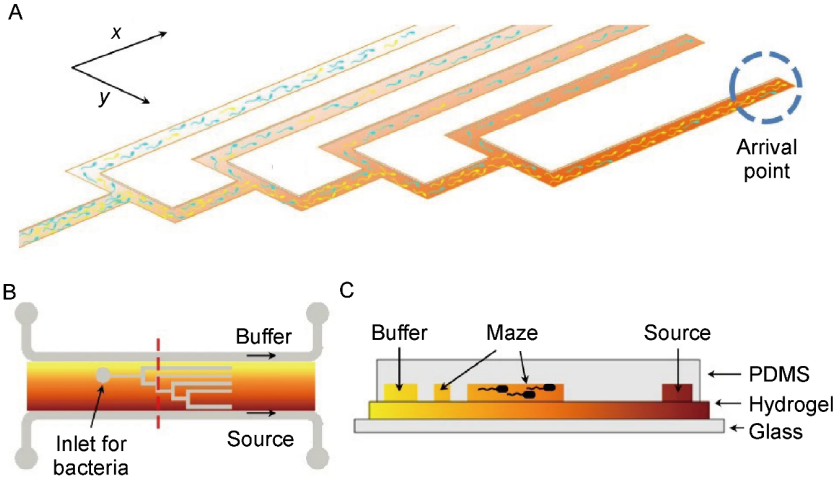

Fig. 4 Microfluidic chips use special structures for simulating complex environments. Chip design using a T-maze to study chemotactic heterogeneity (from Salek et al., 2019. Nature communications, 10(1), 1–11).

original culture chamber was a cavity and did not contain complex topological structures. More complex chips followed, with curved microchannels used to simulate the fluid flow path in porous media and observe biofilm streamers (Rusconi et al., 2010). Microfluidics has further been used in combination with FISH to determine the mechanism of rapid clogging caused by biofilm streamers, and proved that streamers are ubiquitous in both natural and artificial porous media (Drescher et al., 2013). Even though it is obvious that soil environments are more complex than microchannels, simple structures are still useful for soil research. Pillar arrays, for example, have been successfully used to capture streamer formation in artificial pores (Valiei et al., 2012). Such methods can be combined with confocal imaging and numerical simulations to determine, for example, how hydrodynamic conditions alter the quantity variance of streamers along the porous media depth (Fig. 5; Valiei et al., 2012).

A challenge of biofilm study is that, in porous media, biofilms are significantly affected by the flow field while also changing it. Karimifard et al. (2021) illustrated that biofilm permeability influenced the flow field of porous media with constructed porous structures by using barium titanate solid glass microspheres that were inoculated with *E. coli* under continuous flow. Changes in the flow field caused by biofilms also lead to competition between bacterial genotypes. The effect of biofilms on chip flow fields has also been observed by combining microfluidic experiments with pore-scale flow field models. Local growths could alter the overall flow field and increase shear stress on the adjacent biofilms; it is possible that biofilms may gain a competitive advantage by growing slowly as determined by fluid dynamics and game theory (Coyte et al., 2017). Most studies to date use a single microorganism when studying biofilms. An exception is the study by Scheidweiler et al. (2019) in which filtered stream water was combined with Luria-Bertani (LB) broth and perfused through a porous media chip at a constant flow. The sequencing results revealed that community compositions of both base and stream biofilm architectures were highly similar, indicating an underlying architectural plasticity of biofilm systems in porous media (Scheidweiler et al., 2019).

Biofilms can strengthen the interspecific and intraspecific interactions of microorganisms and change the physical and chemical properties of soil–including surface roughness, chemical composition and hydrophilicity (Cai et al., 2019). However, soil and other naturally occurring porous media are usually opaque and it is difficult to capture the dynamic process of pore blockage caused by biofilm growth *in situ.* Furthermore, soil physical and chemical environments are highly variable through space and time and it is difficult to manipulate soil temporal and spatial heterogeneity through

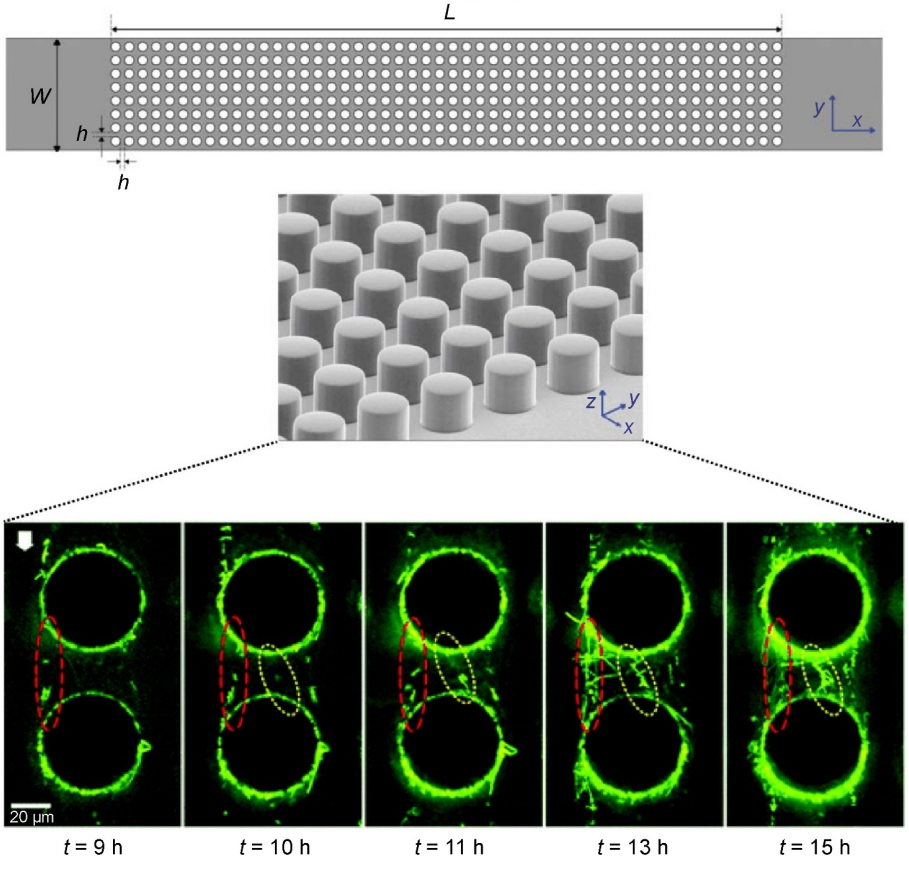

Fig. 5 Microfluidics for biofilm study. The figure depicts the temporal evolution of a streamer located between two pillars in microfluidic chips. Dashed ellipses show the position of a parallel and a transverse streamer (modified from Valiei et al., 2012. Laboratory on a Chip 12 (24), 5133–5137).

artificial means (Ettema and Wardle, 2002). As such, microfluidics is a potential pathway to better understanding of biofilms in porous environments, as has been shown by studies into soil bacteria-mineral interactions (Roh et al., 2019; Zhang et al., 2019), pollutant degradation (Tang et al., 2015), horizontal gene transfer (Li et al., 2018; Qiu et al., 2018) and the relationship between biofilm populations (Hong et al., 2012; Humphries et al., 2017).

3.3 Antibiotic research

Bacterial antibiotic susceptibility tests (ASTs) can be divided into phenotypic and genotypic analyses. Conventional phenotypic ASTs use methods such as disk diffusion, broth dilution and E-test impregnated strips with agarose plates. However, such tests may take several days or more to determine if bacterial growth is present when identified visually (Liu et al., 2017b). In contrast, by using microfluidics, bacterial ASTs can be conducted rapidly and accurately, reducing the turnaround time by 4–24 times (Parsley et al., 2020).

The most extensive microfluidic designs for ASTs generally use gradient generators or droplet microfluidics to determine the number of infected bacteria in antibiotic gradients and thereby establish the minimum inhibitory concentration (MIC) (Reshef et al., 2011; Zhou et al., 2019) and minimum biofilm eradication concentration (MBEC) (Olson et al., 2002; Park et al., 2012). For microfluidic gradient generators, the MIC of *E. coli* in response to ampicillin and streptomycin has been tested using an adapted microchannel network structure (Hattori et al., 2009; Kim et al., 2015); MIC was ascertained within 3 h. A drawback of the 2D structure, however, was that it could not produce symmetric fluidic gradients between three or more inputs. This limitation was addressed by creating a 3D concentration gradient generator (3D *μ-CGG*; Fig. 6A), which can generate symmetric 3D gradients within 5 h (Sweet et al., 2020).

In contrast to gradient generators, droplet microfluidics eliminates the requirement for bacterial culture media and concentration generators. Generally, each separated droplet contains bacteria, antibiotics and fluorescence substrates, and when the antibiotics inhibit bacterial growth and metabolism, the MIC can be obtained by detecting the fluorescence density through a fluorescence microscope. This strategy was used to design the nanoliter-sized droplets encapsulating a single *Staphylococcus aureus* bacterium, cefoxitin and alamarBlue (an activity indicator; Fig. 6B) to complete a detailed functional characterization of the sample in less than 7 h (Boedicker et al., 2008). The results are therefore an indirect count of the number of bacterial infections. In the cases where precise numbers are required rather than fluorescence density, Integrated Comprehensive Droplet Digital Detection (IC 3D) (Fig. 6C) can be used. This method has been used to detect bacteria resistant to *β*-lactam with reliable counting in 10 min after collecting the samples of cultured bacteria (Li et al., 2020).

Genotypic ASTs are used to detect known and unknown antibiotic resistance genes (ARGs), even in those bacteria in which the resistance genes or related mutations cannot be transformed into phenotypic resistance (Cirz et al., 2005). There is a wide variety of strategies employed for microfluidic chip detection of ARGs, many of which rely on isothermal amplification technology (ITA) as it does not need heat cycling (Giuffrida and Spoto, 2017). *β*-lactam and carbapenem ARGs

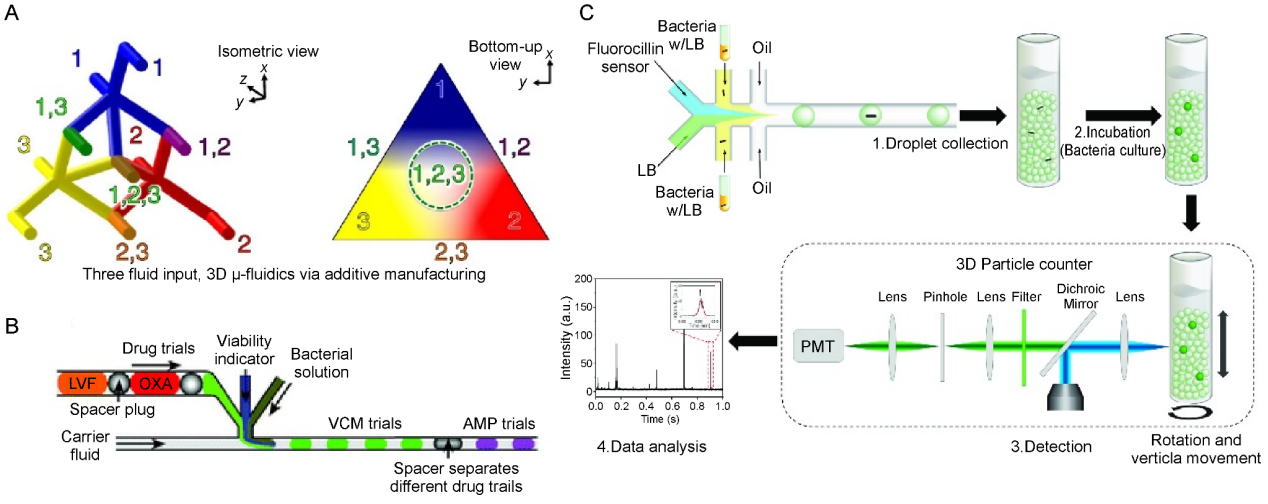

Fig. 6 Microfluidics for antibiotic research: (A) 3D microchannel network can generate symmetric 3D gradients of three or more input fluids (i.e., inputs 1 and 2, 2 and 3, 1 and 3, and 1, 2, and 3) (modified from Sweet et al., 2020. Microsystems & Nanoengineering 6(1), 1–14); (B) Each droplet comprises bacteria solution, viability indicator, and antibiotic (LVF and OXA) from a pre-formed array of plugs of different antibiotics which was used to screen antibiotics against the same bacterial sample (from Boedicker et al., 2008. Laboratory on a Chip 8(8), 1265–1272); (C) Bacteria were encapsulated within droplets with antibiotics and a fluorescence-producing *β*-lactamase sensor. Detectable fluorescent droplets when excited by a laser indicate β-lactamase production from grown bacteria. The IC 3D droplet counter quantifies the positive spikes and reports the bacteria counts in the sample (from Li et al., 2020. Sensors 20(17), 4667).

CTX-M-15, KPC and NDM 1 have been detected in gram-negative bacteria using ITA in combination with a programmable digital microfluidic platform (DMF) to allow recombinase polymerase amplification (RPA) (Kalsi et al., 2017). RPA has also been used in foil box microfluidics, which can process >30 samples in parallel, detecting and amplifying the ARG mecA of *Staphylococcus aureus* within 20 min (Lutz et al., 2010). Another method involved in genotypic ASTs includes paper analytical devices (PADs), which have been used in isothermal DNA assays, including LAMP, helicase-dependent DNA isothermal amplification (HDA), and rolling cycle amplification (RCA) (Shin et al., 2019).

Most exogenous antibiotics in soil come from organic fertilizers made from livestock manure. Antibiotic feed additives used for animal husbandry are excreted by livestock (Zhan and Xiao, 2017) with residual antibiotics affecting soil fertility and biological health. The excessive use of antibiotics as a growth promoter thereby spreads antibiotic resistant bacteria (ARB) and ARGs in the soil, threatening public safety (Zhu et al., 2019). However, the study of exogenous antibiotics in soil has progressed slowly or stalled, mainly due to the time constraints imposed by conventional AST experiments. The integration and automation of microfluidics therefore pave the way for further timely research into antibiotic-contaminated soil and its microbial ecology, accelerating the development of antibiotic pollution standards for soils. Furthermore, soil is a significant source pool for antibiotics, with many antibiotics originally identified in soil microorganisms based on intrinsic resistance. There is, however, a discovery bottleneck for new antibiotics as a result of the difficulties encountered while trying to culture soil microorganisms. The discovery of teixobactin in 2015 using iChip culturing and screening (Ling et al., 2015) heralded a new era of antibiotic discovery using high-throughput microfluidics.

3.4 Horizontal gene transfer (HGT) research

Horizontal gene transfer is a pivotal mechanism for the evolution and adaptation of soil bacteria (Arber, 2000; Ou et al., 2003). This mechanism is commonly studied by co-culturing donor and recipient microorganisms and observing the response of recipient organisms to toxic compounds. Frequency of HGT is therefore quantified by the number of recipient colonies growing on the selective medium. However, as previously established, observational culturing of soil microorganisms is slow and has a low success rate.

There has been little emphasis given to the identification of gene migration in unculturable microorganisms due to the difficulties encountered with conventional methods. HGT, for example, cannot be tracked between single cells cultured on a medium. However, near real-time transformations of *Streptococcus pneumoniae* have been observed at the single-cell level by using time-delayed fluorescence microscopy and injecting the cells with exogenous DNA and competence-stimulating peptide (CSP), thereby promoting cell absorption of DNA (Mortier-Barrière et al., 2020). There are, of course, several pathways that can transfer DNA between cells. While exogenous DNA can be directly transferred into bacteria in soil, genetic material may be moved via phage transduction, fimbriae conjugation (Berthold et al., 2016) and by adhesin promoting plasmids (Von Wintersdorff et al., 2016). Bacteria can also engage in HGT through unorthodox methods, such as was seen using microfluidics when *Acinetobacter baylyi* took advantage of the Type VI secretion system (TS66), lysing *E. coli* and thereafter horizontally transferring *E. coli* genes (Cooper et al., 2017, 2018).

Unlike transfers occurring within the same species such as that observed by Cooper, above, natural environments such as soil or wastewater are multispecies systems; biofilms comprised of densely mixed species are considered a critical pathway for ARG transmission (Flemming et al., 2016). Ease of transfer may be determined by genus level variables, with donor ARG organisms cocultured with an activated sludge biofilm indicating that *Proteobacteria* and *Firmicutes* were more like to transfer than *Bacteroides* when analyzed by fluorescence activated cell sorting (FACS) and high-throughput sequencing (Qiu et al., 2018). Similarly, when observing the transfer of ARG-encoding plasmid RP4 in *Pseudomonas putida* KT2240 to *E. coli* or activated sludge biofilms, the results indicate a greater HGT frequency in pure strains compared to the mixed. This shows that the secondary conjugation by transconjugants may exist in a dense single-strain recipient biofilm, with transduction efficiency depending on biofilm structure and composition (Li et al., 2018).

Exogenic soil ARGs pose a significant threat to human health though their effects on natural pathogens in soils, plants, and animals. A major pathway of ARG diffusion in soil is HGT; these genes can survive in the soil for extended periods of time and can spread between indigenous microorganisms through mobile genetic elements (MGEs) (Zhang et al., 2018). To control the health threat caused by ARGs, it is necessary to thoroughly understand soil HGT mechanisms. The development of microfluidic methods to monitor or simulate transduction, transfection and conjugation in soil bacteria within the same species or between different species is therefore an urgent priority.

4 Research prospects and challenges

Over recent years, microfluidics has matured from infancy of the technology to extensive practical implementation and has subsequently substantially boosted the development of bacterial research. This is manifested through processes such as droplet microfluidics, isothermal amplification, and paper-based microfluidics, and through methodological means such as label substitution, high throughput analyses, and cell sorting. Important advances in microfluidic ASTs (Boedicker et al., 2008; Churski et al., 2012), pathogen

detection and analysis, and synthetic biology (Boedicker et al., 2009; Agresti et al., 2010) will rapidly change the landscape of bacteria research.

This review shows clearly that microfluidics techniques have played an active role in the study of single cell screening and sorting, chemotaxis, biofilm, antibiotic detection and resistance, and HGT. Soil is perhaps one of the most difficult environments in which to conduct microbial research and the introduction of microfluidics will certainly advance research on soil bacteria.

Although microfluidics offers unprecedented opportunities for soil microbial research, there are still four key challenges to consider. (1) Simulated chemical microenvironments are still too simple to accurately portray the spatial heterogeneity of soil. Furthermore, increasing the spatial complexity of chips is unlikely to occur in the short-term as additional studies are required to focus on the control of key environmental factors and then to explore the interactions and mechanisms of soil microorganisms. (2) Community hub microbiota need to be discovered to understand both environmental function and species interactions. Current methodologies and chip technologies can only capture a single bacterium when using high-throughput analysis and sorting; it is therefore difficult to determine the key functional bacteria in the community. With the incorporation of methods such as genomics, microfluidics may allow the development of more problem-solving methods for interdisciplinary research. (3) Microfluidic chips are generally made of PDMS, a hydrophobic material, which makes it difficult to simulate soil aggregate texture and soil's natural hydrophilic surfaces. While there are strategies available to change surface-water interactions, it is still difficult to construct structures that accurately simulate a heterogeneous soil's physical and chemical environment without breakthrough discoveries in chip technology and materials. And finally, (4) soil pore structure simulations continue to be overly simple as they are limited by lithography machine precision. Currently, chip pore design cannot cover the natural distribution of soil pore sizes, which significantly limits both biofilm studies and the isolation and culture of difficult-to-cultivate bacteria. Regardless of these limitations, we believe the interdisciplinary cooperation physics, biology, and soil science along with advancement of microfluidic technologies will play a vital role in future soil bacteria research.

Abbreviations

AOM, alumina membrane
ARB, antibiotic resistant bacteria
ARGs, antibiotic resistance genes
ASTs, antibiotic susceptibility tests
AuNPs, gold nanoparticles
C_T, threshold
CT, X-ray computed tomography
CNT, carbon nanotube
CSP, competence-stimulating peptide
ddPCR, droplet digital polymerase chain reaction
DEP, microfluidic dielectrophoresis
DMF, digital microfluidic platform
EIT, electrical impedance technology
FACS, fluorescence activated cell sorting
FISH, fluorescence *in situ* hybridization
HDA, helicase-dependent DNA isothermal amplification
HGT, horizontal gene transfer
IFT, immunofluorescence technology
ITA, isothermal amplification technology
LAMP, loop-mediated isothermal amplification
LB, Luria–Bertani
LIF, laser-induced fluorescence
LIFM, laser-induced fluorescence microscopy
LSV, linear scan voltammetry
MaPS-seq, metagenomic plot sampling by sequencing
MBEC, minimum biofilm eradication concentration
MCE, microchip electrophoresis
MD, pod microbe domestication pod
MGEs, mobile genetic elements
MIC, minimum inhibitory concentration
NAEBs, nanoaggregate-embedded beads
PADs, paper analytical devices
PCR, polymerase chain reaction
PDMS, polydimethylsiloxane
qPCR, quantitative polymerase chain reaction
RCA, rolling cycle amplification
RPA, recombinase polymerase amplification
rtPCR, real-time polymerase chain reaction
SERS, surface enhanced Raman spectroscopy
smFISH, single-molecule fluorescence *in situ* hybridization

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