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Going local: technologies for exploring bacterial microenvironments

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Abstract

Microorganisms lead social lives and use coordinated chemical and physical interactions to establish complex communities. Mechanistic insights into these interactions have revealed that there are remarkably intricate systems for coordinating microbial behaviour, but little is known about how these interactions proceed in the spatially organized communities that are found in nature. This Review describes the technologies available for spatially organizing small microbial communities and the analytical methods for characterizing the chemical environment surrounding these communities. Together, these complementary technologies have provided novel insights into the impact of spatial organization on both microbial behaviour and the development of phenotypic heterogeneity within microbial communities.

Microorganisms are social and engage in complex behaviours in response to other organisms and the extracellular environment. Historically, the study of these behaviours in the laboratory has been limited to pure-culture populations grown in shaken liquid. These studies have been informative, providing insights into how microorganisms respond to stimuli at the population level. However, most microbial ecosystems, including commensal and pathogenic populations in humans, are composed of diverse microbial species growing in high-density microcolonies^{1–4}. Moreover, like most ecosystems, microbial communities are spatially organized, and the physical location of individuals within the community has profound effects on intercellular interactions. For example, the localization of a bacterium at a defined position within a chemical gradient or at a defined distance from another cell will affect its behaviour. Although spatial organization is of clear importance in most ecosystems, few studies have examined the fundamental question of how spatial organization affects microbial behaviour in natural populations.

Technological advances in two areas have recently been applied to study the impact of spatial organization on microbial behaviour. The first main breakthrough was the development of microscale techniques that facilitate the confinement of small bacterial populations in defined locations, such as microfluidic devices and protein traps (TABLE 1). The second area of advancement was in analytical techniques such as scanning

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electrochemical microscopy (SECM) and imaging mass spectrometry (IMS), which allow real-time measurement of the signals and environmental cues that are present in the microenvironments surrounding microbial communities.

Just as enhanced magnification and resolution in light microscopy advanced our understanding of bacterial cell biology, these new technologies are improving our ability to probe the behaviour of individual cells in microbial communities. Although the tools and techniques presented here are useful for examining diverse microbial behaviours, this Review focuses on their use to study how chemical gradients and microbial interactions affect behaviour and phenotypic heterogeneity. The complete array of approaches available to study microbial behaviour is extensive and beyond the scope of this Review. Here, we focus on confinement strategies and analytical techniques for probing small populations, including single cells. We also discuss how the combination of these technologies has the potential to revolutionize our understanding of microbial community dynamics.

Confinement using microfluidic devices

The use of microfluidic devices is a common strategy for confining small microbial populations and assessing their response to environmental signals and chemical gradients. Similar to flow cells^{5–8}, microfluidic devices offer a means to experimentally probe the impact of spatial structure and chemical gradients on microbial interactions. Microfluidic devices can be patterned topographically with precise, complex microscale or sub-microscale features to produce channels⁹ and mazes^{10,11} (FIG. 1). Microfluidic channels often have volumes of $\sim 10 \mu\text{l}$ ¹², although they have been engineered to use considerably smaller volumes^{13,14}. In microbiology, these devices are generally made of transparent polydimethyl-siloxane (PDMS) using soft lithography techniques^{9,15}, although other polymers have also been used¹⁶. Microfluidic devices can be combined with non-invasive imaging techniques^{15,17–19}, such as video microscopy or confocal laser scanning microscopy (CLSM), to study individual cells in heterogeneous microbial populations^{9,12}. In addition, they can be used to precisely organize small microbial communities in three dimensions^{13,20} and to separate individual cells for the demonstration of phenotypic heterogeneity in genetically identical populations¹³. As flow within microfluidic channels is generally laminar, and mixing between adjacent laminar fluid streams occurs by diffusion, precise chemical gradients can be established to study the behaviour of spatially organized populations in defined chemical environments^{9,21}.

Monitoring chemotaxis

Microfluidic flow cells have the advantage of allowing researchers to spatially confine or position cells in a way that cannot be achieved with traditional flow cells. For example, microfluidic flow cell devices have been used to probe the chemotactic behaviour of *Escherichia coli*²². In this study, a chemoattractant and a buffer were injected into the device, and a parallel flow of these two liquids results in diffusion and mixing during transit through the chamber, producing a concentration gradient of chemoattractant perpendicular to the direction of flow (FIG. 1a). A narrow stream of bacteria was injected between the buffer and chemoattractant stream, and the distribution of bacteria within the chemoattractant gradient was measured at several outlets at the end of the chamber. This system provides a high degree of resolution, allowing individual bacteria to be imaged and counted directly. Other researchers have improved the sensitivity of microfluidic flow cell-based chemotaxis assays by integrating arrays of ‘concentrator’ elements into the main channel of the devices²³. In these assays, rather than cells being counted as they exit the device, they are collected at several locations along the main channel (between the source of the chemo-attractant and the channel exit). The cells accumulate as long as there is flow through the channel, which allows the assay to be carried out over many hours.

Microfluidic devices have also been used to model natural environments²⁴ such as nutrient patches and nutrient plumes, which serve as carbon and nutrient sources and act as chemoattractants for microorganisms in natural aquatic environments¹². Microfluidic stopped-flow assays are one type of technique that has been used to investigate the chemotactic response of marine bacteria to nutrient patches in the ocean^{18,25}. To produce a chemoattractant gradient for a stopped-flow assay, chemoattractant and bacteria are simultaneously injected into the microfluidic device via two separate ports. The chemoattractant is injected downstream of the bacterial injection port, at the centre of the device channel. A stream of chemoattractant moves through the centre of the channel, producing a tight band of chemoattractant. When flow stops, the tight band of chemoattractant diffuses outwards, producing a gradient. This allows chemotaxis to be observed under flow-free conditions. The spatiotemporal dynamics of bacteria can be recorded by video microscopy, which allows the response of individual cells to be monitored in addition to population-level behaviour. With this approach, investigators have monitored chemotaxis towards phytoplankton culture filtrates^{18,25} and the phytoplankton-produced solute dimethylsulphoniopropionate, which is degraded by multiple bacterial species in the ocean to produce dimethylsulphide, a molecule that forms cloud condensation nuclei in the atmosphere²⁶.

An alternative microfluidic approach for studying chemotaxis involves the construction of microfabricated 'mazes' (FIG. 1b). These devices differ from other micro-fluidic devices in that they are not linearly organized, but instead contain partitions that allow bacteria to auto-aggregate by accumulating in pockets within the mazes. These devices have been used to demonstrate that *E. coli* and *Vibrio harveyi* move towards self-produced chemo-attractants and develop local aggregates within the topographical features of the device¹¹. This work has elucidated a previously unknown role for chemotaxis in promoting bacterial cell aggregation (so-called self-attractive behaviour)¹¹ and has provided insight into the mechanisms that bacteria might use to form aggregates in natural environments.

Other microfluidic devices have revealed details about the self-organizing behaviour of bacteria¹⁴ and the mechanisms that bacteria use to aggregate in heterogeneous landscapes^{10,11}. For studies over long timescales, miniaturized chemostats have been constructed using microfluidic techniques. These devices operate according to the same basic principle of traditional chemostats, providing a homogeneous environment and promoting synchronized growth of planktonic cells²⁷. Modified microfluidic chemostats have been developed that incorporate defined topographical features, providing microorganisms with the opportunity to spatially organize. In one study, a microfluidic chemostat with growth chambers of distinct sizes and shapes was used to show that bacterial colonies gradually self-organize, resulting in increased movement of nutrients into and evacuation of waste out of the colonies¹⁴.

Studying bacterial persistence

In addition to their application to the study of chemotaxis, microfluidic devices have been instrumental in the study of bacterial persistence, a phenomenon that was first recognized nearly 70 years ago^{17,28,29}. The molecular mechanisms controlling bacterial persistence are poorly understood and are probably species specific and influenced by growth conditions. In one study, a microfluidic device was developed to investigate the relationship between metabolic activity and the persistence phenotype. Using this device, individual bacteria from a genetically identical (isogenic) population were monitored over time as they grew within fine, linear channels (dimensions 1.5µm×0.5µm)¹⁷. As the cells multiplied, the channel became progressively occupied, and the length of channel occupied correlated directly with growth rate. Using this system, the investigators demonstrated that individual cells derived

from an isogenic population varied in growth rate. Interestingly, slow-growing cells exhibited higher survival rates following treatment with the β -lactam antibiotic ampicillin, suggesting that growth rate heterogeneity within an isogenic population is linked to the persister phenotype. This study also contributes to a growing appreciation for the importance of phenotypic heterogeneity within isogenic populations during growth in fluctuating environments^{30,31}.

Confinement using hydrogels

In combination with microfluidic devices, hydrogels have been instrumental as a means of confining small microbial populations. Hydrogels have a long history in microbiology, beginning with the work of Robert Koch's laboratory, in which molten agar was mixed with nutrients to produce a hydrogel that served as a solid growth medium³². Koch used an agar medium to culture *Mycobacterium tuberculosis* in 1882 (REFS 33,34), and agar remains a vital tool in virtually all microbiology laboratories today.

A key advantage of hydrogels is that they can be used to quickly and precisely create environments with defined mass transfer properties. Such environments can be devised to mimic bacteria growing in natural biofilm populations, wherein cells are encased in a complex extracellular polymeric matrix that changes the mass transfer properties of the microenvironment. To mimic these microenvironments, hydrogels with various mass transfer properties are used to immobilize cells in semi-solid media. In contrast to solid, polymerized PDMS, hydrogels are permeable to small hydrophilic molecules, allowing diffusion of these molecules between cells and the surrounding medium.

Optical trapping techniques have been used to precisely arrange bacteria in a three-dimensional array within a hydrogel³⁵ (FIG. 1c–f). Using this system, it has been demonstrated that the induction of quorum sensing genes is dependent not only on cell density, but also on the mass transfer rate of the fluid surrounding the hydrogel 'biofilm' (FIG. 1c–f). Complementary results were obtained from a microfluidics-based experiment, which led to the conclusion that the induction of quorum sensing is suppressed by hydrodynamic flow in the surrounding environment³⁶. Another study combined microfluidic chambers with hydrogels to examine the diffusion of acyl-homoserine lactone (AHL) quorum sensing signals³⁷. This study showed that *Pseudomonas aeruginosa* can detect AHLs originating 8 mm away, suggesting that AHL signals produced by a microcolony are capable of influencing the behaviour of other micro-colonies more than 1,000 cell lengths away. Similar results were obtained from an experiment using millimetre-sized agar lanes³⁸. Collectively, these studies demonstrate the power of using microfluidic devices with hydrogels as a platform for studying intercellular interactions.

Multiphoton lithography

An additional method for producing hydrogels to study mass transfer effects on microbial communities involves the confinement of bacteria in microchambers with walls of various porosities. One such technique, multiphoton lithography (MPL) (FIG. 2a), is capable of producing picolitre-sized cavities (referred to as bacterial lobster traps³⁹) with walls composed of crosslinked protein (FIG. 2b), wherein a single cell can be confined. Importantly, the permeability of the walls can be manipulated to allow diffusion of nutrients, waste products and other small molecules. These microfabricated biomaterials are responsive to external stimuli, and the geometry and size of the microchambers can be altered in real time by modulating environmental conditions, including pH, temperature, osmolarity and light intensity^{39,40}. Many proteins, including bovine serum albumin (BSA), avidin, lysozyme and cytochrome *c*^{41,42}, can be used to fabricate confinement walls with various porosities and unique chemical characteristics. Moreover, because these protein-

based structures can be fabricated on the coverslip of a traditional flow cell⁷, the rate of mass transport through the system can also be tuned, and the population can be observed microscopically in real time³⁹.

Importantly, studies of *P. aeruginosa* have shown that bacteria growing within the traps display generation times that are indistinguishable from those observed for *in vitro* flask-grown bacteria (~40–45 minutes)³⁹ and somewhat faster than those seen for *P. aeruginosa* growing in a chronic human lung infection (~130 minutes)⁴³. MPL-constructed traps have also provided insights into bacterial communication and antibiotic resistance. Indeed, similar to the studies using microfluidic chambers and hydrogels described above, traps constructed using MPL have been used to show that the ability of *P. aeruginosa* to communicate via quorum sensing is influenced not only by cell density but also by population size and mass transfer³⁹ (FIG. 2c,d). In addition, high-level resistance of *P. aeruginosa* to a clinically relevant antibiotic was shown to occur in as few as 150 cells confined within 2 pl traps³⁹, whereas the same concentration of antibiotic eradicated 10⁷ bacteria growing in a test tube. Interestingly, bacteria within the trap were growing at the same rate as the test tube-grown bacteria, indicating that different growth rates could not explain the high level of antibiotic resistance observed for trapped bacteria.

Low-volume confinement

As described for persister cells above, phenotypic heterogeneity exists within a clonal population, and we are only just beginning to understand the mechanisms controlling this phenomenon. Elucidation of these mechanisms requires the development of high-throughput techniques for confining and studying single cells. Recently developed techniques provide methodologies for confining a single bacterium in volumes as small as ~100 fl¹³.

Lipid–silica structures

One of the primary methods for confining single cells involves trapping micro-organisms in lipid–silica containers with volumes on the picolitre scale^{44,45} (FIG. 3a). However, recent advances have allowed confinement of single cells in significantly smaller volumes. One technique involves a refinement of an evaporation-induced self-assembly approach⁴⁶ in which cell surfaces organize lipids (such as diacyl-phosphatidylcholines) to direct the assembly of a silica structure. Other alterations and advancements to basic confinement techniques include an aerosol-assisted approach⁴⁶ using dihexanoylphosphatidylcholine⁴⁵ and the use of pre-formed lipid-templated silica films⁴⁷. These approaches produce porous lipid–silica structures formed around individual cells or small groups of cells at densities of 10⁹–10¹² cells per ml. Because the culture volume remains physically and chemically isolated within a lipid–silica structure, the diffusion of molecules through the structure is limited. This methodology has been used to study the effects of confinement on quorum sensing in *Staphylococcus aureus*⁴⁵ (FIG. 3b). The quorum sensing signals were found to accumulate over time within diffusion-limited lipid–silica environments, and a single *S. aureus* cell displayed quorum sensing initiation after 10 hours of isolation⁴⁵.

Multiple studies have used PDMS-based microfluidic techniques to produce arrays of droplets composed of culture media and cells. One group produced ~100 fl droplets¹³ by passing low-density bacterial cultures through a PDMS channel on top of biocompatible resin wells^{48,49} (FIG. 3c). Introduction of an air bubble on top of the wells allowed individual droplets to form, producing extremely low-volume bacterial cultures surrounded by air. Within these droplets and confined at extremely high cell densities (~10¹⁰–10¹¹ cells per ml), *P. aeruginosa* initiated quorum sensing with as few as one to three cells (FIG. 3d). However, a limitation to these droplet-based and lipid–silica structures is that cells do not grow at any appreciable rate over an extended period, perhaps owing to waste accumulation

or insufficient nutrients. This lack of growth influences the metabolic activity of the cells and thus calls into question the physiological relevance of the findings from such experiments.

High-throughput applications

Advances in droplet microfluidics⁵⁰, such as the ability to fuse or split droplets^{51,52}, along with computer-controlled automation⁵³, have enabled high-throughput studies of cells within low-volume droplets confined in microfluidic chambers. Droplets within microfluidic systems are generally produced using a T-junction⁵⁰ or a flow-focusing microfluidic device^{54,55} (FIG. 4a), in which the droplet size can be tuned by varying the flow of immiscible liquids⁵⁶. Microbiological studies using such devices often detect and sort droplets on the basis of a fluorescent marker, such as fluorogenic substrate turnover or the expression of a fluorescent protein. A recent study isolated bacteria on the basis of their ability to metabolize a fluorogenic β -galactosidase substrate⁵⁵. Droplets were sorted into different channels by a fluorescence-activated droplet sorter (FADS) according to the presence or absence of fluorescence. FADS uses dielectrophoresis⁵⁷, a technique in which a high-voltage alternating current is applied across electrodes to produce an electric field that deflects droplets in a specified direction within the microfluidic device (FIG. 4b). Other studies have produced droplets using a flow-focusing microfluidic device and then sorted cells outside of the microfluidic device using fluorescence-activated cell sorting (FACS). For example, FACS has been used to analyse antibiotic resistance of single GFP-expressing *E. coli* cells encapsulated in agarose microparticles of 1–50 μ l and suspended in mineral oil⁵⁶. The microparticles were extracted from the oil phase and analysed using FACS to determine growth rates and to isolate spontaneous mutants displaying increased resistance to the antibiotic rifampicin. The mutations conferring rifampicin resistance were then mapped by sequencing the genomes of the resistant mutants.

High-throughput droplet microfluidic systems have also been used to study the effects of a combination of multiple antibiotics on bacterial survival. For example, one group investigated the metabolic activity of *E. coli* exposed to predefined concentrations of three antimicrobials⁵⁸ (FIG. 4c). The researchers included the non-fluorescent dye resazurin in the droplets, which is converted to the fluorescent compound resorufin when metabolized⁵⁸. The use of the dye allowed for the distinction between metabolically active cells (high fluorescence) and those cells which were inhibited by the drug and therefore demonstrated low metabolic activity (low or no fluorescence). The high-throughput nature of the screen not only allowed elucidation of the minimum inhibitory concentrations of the three antibiotics against *E. coli*, but also revealed pairwise drug interactions. For example, when chloramphenicol and tetracycline were used in combination, their effects were additive (that is, the drugs do not interfere with each other), whereas the combination of ampicillin and chloramphenicol was antagonistic. Compared with classic techniques that rely on the screening of large volumes and large numbers of cells, these techniques reduce both the amount of reagents and the time required for experiments.

Cell confinement using droplet microfluidics has also provided a platform for comparative genomics using single cells and small bacterial populations. Importantly, these techniques can be used to isolate single cells from natural populations⁵⁹. This strategy involves capturing individual cells in nanolitre- to picolitre-sized droplets, followed by separation of the droplets into individual chambers. The genomes of individual cells contained within the droplets can then be sequenced using a PCR-based whole-genome amplification protocol (such as Picoplex from Rubicon Genomics)⁵⁹.

Detecting the molecular environment

The ability to confine small numbers of microorganisms is an important step towards understanding the impact of spatial structure on microbial behaviour. However, it is equally important to develop techniques for measuring the small-molecule signals and cues that are responsible for modulating behaviour. Importantly, these techniques must be applicable to small bacterial numbers and, ultimately, to single cells. Below, we describe technologies that have been used to quantitatively assess the chemical environment surrounding bacterial communities, down to the microcolony level (TABLE 2). These studies have provided new insights into the chemical world that microorganisms inhabit.

Scanning electrochemical microscopy

SECM is an analytical technique that was originally used to chemically characterize inanimate surfaces, but has recently been used to quantify redox-active molecules surrounding bacterial colonies. To monitor a specific redox-active molecule using SECM, the potential of an ultramicroelectrode (UME) is held at the standard reduction potential of the molecule of interest, such that only the target molecule is either reduced or oxidized at the electrode tip. SECM can measure the presence of a single redox species and provides real-time spatial data on the concentration of a molecule of interest.

In SECM, the UME is positioned in close proximity (nanometres to micrometres) to a group of cells using a feedback approach curve⁶⁰, followed by scanning of the colony in three dimensions (*x*, *y* and *z*) (FIG. 5a). As the UME oxidizes (or reduces) nearby molecules, the electrochemical response of the tip is altered, which provides quantitative measurements of the molecule of interest. A calibration curve is generated using known amounts of a redox-active small molecule, thereby allowing real-time quantification during an experiment. As the distance between the tip and the substrate can be fixed, the spatial map produced by scanning in the *x* and *y* dimensions can be used to form a three-dimensional view of the concentration and redox state of a single electroactive molecule surrounding a microbial population^{61,62} (FIG. 5b).

SECM has been used to measure both the concentration and redox state of pyocyanin⁶², a multifunctional signalling molecule produced by *P. aeruginosa*. Using a *P. aeruginosa* biofilm model, it was discovered that pyocyanin exists in the reduced form proximal to the biofilm surface, with an electrocline extending >400 µm from the surface of the biofilm (FIG. 5b). By maintaining a gradient of pyocyanin in the reduced state near the surface of the cells, the bacteria established a chemical micro-environment in which insoluble Fe³⁺ was actively converted to soluble Fe²⁺, making iron more bioavailable. This behaviour is likely to be beneficial in iron-limited environments, including inside the human body. SECM has also been used to study small-molecule-mediated interactions between bacterial species⁶¹, allowing for the fine-scale measurement of metabolic cues such as hydrogen peroxide in multispecies biofilms.

Imaging mass spectrometry

SECM generally measures one molecule at a time, but other techniques have the capacity to monitor multiple molecules simultaneously. Imaging mass spectrometry (IMS) is one such technique and enables researchers to define the spatial distribution of hundreds of biomolecules, with the goal of linking chemical distributions to observed phenotypes (FIG. 5c). The basic IMS approach requires an ionization probe capable of producing ions from a sample surface (a process known as sputtering), a computer-controlled sample stage to manipulate the orientation of the sample in the *x*–*y* plane and a mass analyser to detect sputtered ions. IMS is a flexible analytical tool that takes advantage of a variety of sample

preparation techniques, ionization sources and mass spectrometers⁶³. It is well suited for analysis of the spatial heterogeneity of natural bacterial populations (for example, microbial consortia in sediments⁶⁴ and microbial mats⁶⁵) because it can detect endogenous biomolecules; that is, the detection of biomolecules is not dependent on the introduction of non-native labels. Several excellent reviews detailing the techniques, applications and challenges of IMS have been published elsewhere^{63,66,67}. A key requirement of most IMS techniques (with the exception of desorption electrospray ionization (DESI)) is that samples are ionized under vacuum; therefore, biological samples must be dried and, in some cases, coated with a chemical matrix before analysis.

Secondary-ion mass spectrometry

Secondary-ion mass spectrometry (SIMS) is based on the bombardment of a sample with a beam of energetic primary ions, which releases secondary analyte ions that can be detected by a mass spectrometer^{63,66}. SIMS achieves the greatest spatial resolution of all IMS techniques (TABLE 2) and, thus, has been very popular as a means of imaging single bacterial cells and colonies. SIMS can be carried out in two modes, static and dynamic, of which the former has a greater analytical mass range and the latter has greater spatial resolution and depth-profiling capacity. The two techniques vary with respect to the primary ion beam and the ionization mass analyser used (see REFS 63,66 for a complete discussion of their unique advantages). NanoSIMS is an ultra-sensitive version of the dynamic SIMS technique, with improved ion optics that result in even greater analyte sensitivity and spatial resolution (sub-50 nm resolution). In a landmark study, fluorescence *in situ* hybridization (FISH) was coupled to SIMS in order to map naturally occurring carbon isotope ratios to specific microorganisms within a community⁶⁴ (FIG. 5d). Using this technique, researchers visualized the syntrophic metabolism of methanogenic archaea and sulphur-reducing bacteria within consortia engaging in the aerobic oxidation of methane^{64,68}. As a follow-up to this study, FISH–nanoSIMS was used in combination with isotope-labelling experiments to demonstrate that methanogenic archaea within this consortia were responsible for N₂ fixation⁶⁹.

SIMS and nanoSIMS have been used extensively to track nitrogen cycling within microbial populations. Using a nanoSIMS technique known as multi-isotope IMS (MIMS), the incorporation of N₂ into bacterial symbionts of wood-eating shipworms was investigated^{70,71}. In addition, nanoSIMS has been used to track N₂ fixation by open-ocean diazotrophic cyanobacteria⁷². In this study, N₂ fixation rates of the symbiotic cyanobacteria were measured against a background community that contained other diazotrophic species. The cyanobacteria fixed a greater amount of N₂ when they were associated with diatoms, implying that N₂ fixation is dependent on a physical association of the cyanobacteria with their diatom symbionts. This finding, which was made possible by nanoSIMS, suggests that the spatial organization of microbial populations, even in the open ocean, affects bacterial metabolism and global nutrient cycling.

Although SIMS and nanoSIMS have been popular in microbial ecology studies, IMS techniques including matrix-assisted laser desorption–ionization (MALDI) and DESI are particularly well suited to, and frequently used for, studying the production of natural products by bacterial colonies in the laboratory. The primary difference between SIMS, MALDI and DESI is the nature of the ionization probe. Whereas SIMS uses a primary ion beam, MALDI uses an ultraviolet or infra-red laser to ionize the sample surface, and DESI uses a pneumatically controlled stream of charged organic solvent. MALDI requires fixed samples to be coated in an organic matrix, whereas DESI can be used to analyse wet samples at normal atmospheric pressure. Currently, DESI samples must be transferred to a non-conductive surface for analysis, but as the ionization sources become softer (through the

use of gentle ionization that minimizes fragmentation of the analyte) and the sensitivity of measurements made at atmospheric pressure improves, it might be possible in the future to analyse cells without killing them⁷³. In fact, nanoDESI is already being used to image bacterial samples on hydrated agar surfaces⁶³.

Together, IMS and SECM provide novel means to spatially characterize the chemical environments surrounding small populations of cells. Using these tools, investigators can characterize molecular gradients, which will help to improve our understanding of the role of these gradients in modulating microbial behaviour. In addition, UME tips have recently been engineered in the nanometre range^{74–77}, and these smaller tips can provide higher spatial resolution in SECM imaging. Investigators are already using nanometre-sized probes to determine the positions and concentration of substrates produced by eukaryotic cells, with subcellular resolution⁷⁸. In the near future, it is likely that this technology will be further enhanced to enable researchers to map and quantify the production of substrates at the surface of individual bacterial cells.

Conclusions and future directions

The microbial world is fascinating and is defined by mutualistic and antagonistic interactions that occur on the micrometre scale. Although this has been appreciated by microbiologists for some time, recent technological advances have provided the tools to begin to understand how these interactions affect bacterial behaviour, both in the laboratory and in nature. However, there is still a need for higher-resolution analytical techniques to probe bacterial microenvironments, as well as for a means of studying microbial spatial structure in natural populations. As microbiology continues to develop at the confluence of interdisciplinary science, the integration of ecological and evolutionary principles with the technologies described in this Review will be sure to provide a more comprehensive understanding of microbial community dynamics.

A distinct driving force in interdisciplinary microbiology is the need to study heterogeneity in microbial systems. Whether this involves studying phenotypic or genotypic heterogeneity in the same species or studying inter- or intraspecies interactions in structured systems, microbiologists have been coaxed out of their comfort zone. The traditional microbiology approach was largely founded on pure-culture techniques that are limited in their usefulness for some of the questions now being asked. Thus, we are in an exciting time when microbiologists are being forced to creatively adapt or develop experimental systems to test questions of fundamental importance. One apparent truth that is emerging from these studies is that, in general, bacteria are much more complex than most of us had imagined. We are now grappling with the implications of heterogeneity in microbial populations that were traditionally thought to be uniform. A classic example of this is the presence of persister subpopulations in pure-culture communities and the implications that this has for antimicrobial tolerance. These studies have revealed that bacteria are hardwired to ensure the generation of heterogeneity in their populations. This widespread heterogeneity might also force us to re-examine and, in some cases, re-interpret data generated from shaken liquid cultures.

The future is bright for collaborative, interdisciplinary research. Engineers, mathematicians, physicists, chemists and materials scientists are reaching out to biologists for experimental questions or contexts for which their expertise or methodologies are applicable, and *vice versa*. We predict that many of the surprises that microbiology still has in store for us will be discovered through these collaborative efforts using these unconventional methodologies.

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Glossary

Microcolonies	Small aggregates of bacteria. The number of cells within an aggregate is not officially defined, but in this Review this term refers to aggregates of less than 100 cells
Microfluidic devices	Devices that rely on micrometre-scale features to move, mix and trap fluids
Microenvironments	Small, defined regions of the environment. In microbial communities, a microenvironment refers to the area immediately surrounding a single cell or small group of cells and is generally distinct from its environs on the basis of characteristics such as nutrient availability and mass transfer
Flow cells	50–250 μ l channels bored out of polycarbonate, mounted on a glass coverslip and used for cultivating large numbers of cells (10^6 – 10^8) under continuous-flow conditions. In combination with microscopy techniques, flow cells allow for non-invasive, real-time observations of biofilms in three dimensions. Some flow cells can establish reproducible two-dimensional environmental gradients
Polydimethylsiloxane	(PDMS). An optically clear silicone-based organic polymer with elastic properties. PDMS can be topographically patterned to isolate individual cells, or can be sealed against flat surfaces to create microfluidic systems with diverse applications. PDMS is a particularly popular material in microbiological devices because it is biocompatible, non-reactive, transparent, gas permeable and inexpensive
Soft lithography	A set of techniques used to pattern soft materials, such as polydimethylsiloxane, with topographical features on the order of micrometres to nanometres
Video microscopy	A technique that relies on a charge-coupled device (CCD) camera paired with a light microscope. The CCD camera records a series of high-speed images (10–30 frames per second) that can be played back in the form of a movie
Confocal laser scanning microscopy	A technique that enables the detection of emitted light at specific <i>x</i> , <i>y</i> and <i>z</i> coordinates. Emitted light is detected by a photomultiplier tube or other detector. Using this technique, a three-dimensional image can be acquired either through an attached camera or through a computer that detects electrical signals generated by photomultiplier tubes
Nutrient patches	Microscale ephemeral nutrient point sources that can contain biologically labile organic compounds at concentrations two to three orders of magnitude higher than in the surrounding bulk environment

Nutrient plumes	Microscale regions of elevated nutrient levels. These plumes often form in the wake of a sinking point source of nutrients (for example, sinking detritus or faecal pellets) in an aqueous environment
Cloud condensation nuclei	Submicrometre-scale particles (aerosols) around which cloud droplets condense from water vapour in the atmosphere
Chemostats	Bioreactors that are used for continuous culture of microorganisms. Fresh medium is continuously added to the bioreactor as equal volumes of culture liquid are removed to maintain a constant culture volume. By changing the rate with which medium is added to the bioreactor, microbial growth rate is easily controlled
Bacterial persistence	A phenomenon in which a small number of phenotypic variants within an isogenic population display tolerance to antibiotic treatment but produce antibiotic-sensitive progeny
Hydrogels	Hydrophilic networks of biocompatible crosslinked polymers that can be used to create environments with defined mass transfer properties
Optical trapping	A technique that uses a tightly focused laser beam to manipulate the physical location of individual cells. Cells (and other nanometre- to micrometre-sized dielectric particles) are attracted along an electric field gradient towards the location of the strongest electric field, which is at the centre of the narrowest point of the focused beam
Quorum sensing	An intercellular communication system that coordinates microbial group behaviour via the production and sensing of small signalling molecules
Multiphoton lithography	(MPL). A technique in which a highly focused laser beam initiates a nearly simultaneous absorption of multiple photons at a single focal point ($<1\ \mu\text{m}$ voxel), forming crosslinks between the photo-oxidizable side chain residues of protein molecules. The laser beam is raster scanned and sequentially focused deeper into a protein solution, enabling the fabrication of three-dimensional structures with submicrometre-sized features. The photo-crosslinked protein structure can have a mechanical stiffness similar to polydimethylsiloxane
Ultramicroelectrode	An extremely small electrode that can be used to quantify changes in current. The tip of such an electrode has a radius of $\sim 10\ \text{nm}$ to $25\ \mu\text{m}$, depending on the tip material
Feedback approach curve	A method used in scanning electrochemical microscopy to determine the location of an animate or inanimate surface. The curve is a plot of current detected by the ultramicro-electrode as a function of distance above a given substrate. Plotting these variables enables investigators to calculate both the positional location and concentration of a given redox-active small molecule

Electrocline	A gradient of redox potential
Desorption electrospray ionization	An ionization technique that uses a stream of high-pressure charged solvent to desorb molecules from a solid sample
Syntrophic metabolism	The cooperation of multiple species to catabolize a substrate that, on their own, the individual species could not catabolize
Matrix-assisted laser desorption–ionization	A soft ionization technique that is appropriate for the ionization of fragile biomolecules and large organic molecules before their introduction into a mass spectrometer. This technique produces molecular ions (providing data about the molecular weight of the ion precursor) of 300–5,000 Da, although it can be used to analyse mass fragments of molecules with a molecular mass of up to 50,000 Da

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Figure 1. Microfluidic devices, hydrogels and optical trapping for the study of bacterial interactions in spatially organized communities

a | An example of a microfluidic device that was used to probe the chemotactic behaviour of *Escherichia coli*²². The device is constructed from polydimethylsiloxane and contains three separate inlets, one each for the chemoeffector, bacteria and buffer. Fluid flow is left to right along the 21 mm central chamber, and fluid exits the device on the right-hand side through an array of outlets. The red vertical arrow indicates the chemoeffector gradient in the direction of highest concentration. In this example, the bacteria are moving towards a higher concentration of a chemoattractant. **b** | *Vibrio harveyi* accumulation in a microfabricated maze¹¹. The narrowest passages are 100 μm wide. *V. harveyi* accumulation is the result of self-attractive behaviour and results in increased population density. The dark-field image (left) displays autoaggregation of cells at dead ends and cul-de-sacs, which results in the quorum sensing-dependent production of luminescence (right), as detected by a photon-counting charge-coupled device camera. **c** | A microfluidic device wherein signal-producing ‘transmitter cells’ are flowed in from the left channel and signal-perceiving ‘receiver cells’ are flowed in from the right channel. Optical trapping is used to spatially organize transmitter and receiver cells in a photo-polymerized hydrogel (dashed box)³⁵. **d–f** | Within this hydrogel, at 380 minutes, transmitter cells (expressing RFP; part **e**) are induced to secrete acyl-homoserine lactone signalling molecules. These molecules are sensed by receiver cells at 675 minutes, resulting in receiver cell GFP expression (parts **d,f**; above and below transmitter cells, respectively). Part **a** is modified, with permission, from REF. 22 © (2003) US National Academy of Sciences. Part **b** is reproduced, with permission, from REF. 11 © (2003) American Association for the Advancement of Science. Parts **c–f** are reproduced, with permission, from REF. 35 © (2009) UK Royal Society of Chemistry.

Figure 2. Bacterial lobster traps

a | A simplified optical schematic of mask-based multiphoton lithography. A pulsed laser beam is scanned and focused on the face of an electronic reflectance mask (a digital micromirror device (DMD)). The DMD is positioned in a plane conjugate to the focal plane of a high-numerical-aperture objective, and the binary images are displayed on the DMD to direct protein fabrication of the mask pattern with a one-to-one spatial correspondence in the specimen plane (dashed line). Complex, three-dimensional microstructures can be fabricated within minutes in a layer-by-layer process by moving the multiphoton fabrication voxel in defined vertical steps along the optical (z) axis between each fabrication plane³⁹. **b** | Scanning electron microscopy (SEM) image of a trap filled with *Pseudomonas aeruginosa*. The tear in the roof occurred during SEM preparation. The bacteria are false-coloured green. **c–d** | Initiation of *P. aeruginosa* quorum sensing is dependent on population size. A *P. aeruginosa* strain that produces GFP on initiation of quorum sensing was captured inside 2 pl (part **c**) and 6 pl (part **d**) traps and exposed to a flow rate of 250 μ l per minute. When filled to near capacity, little GFP is observed in the 2 pl traps, whereas significant GFP expression is observed in the 6 pl traps. The scale bar represents 5 μ m. Part **a** is modified and parts **b–d** are reproduced, with permission, from REF. 39 © (2010) American Society for Microbiology.

Figure 3. Small-volume confinement

a | Lipid silica structures (not drawn to scale) provide picolitre-volume chambers and are used to confine small bacterial populations at high densities⁴⁵. **b** | An individual *Staphylococcus aureus* cell (red) induces quorum sensing within a droplet (false-coloured blue). In this instance, GFP production serves as an indicator of quorum sensing-mediated gene expression⁴⁵. **c** | Confinement of small bacterial populations in ~100 fl droplets on biocompatible resin wells surrounded by an air bubble¹³. **d** | Some cells (indicated by green arrows) within the ~100 fl volume initiate quorum sensing (as indicated by GFP expression in the bottom panel) after 8 hours of incubation, whereas other cells (indicated by white arrows) do not turn on quorum sensing¹³. Parts **a,b** are modified and reproduced, respectively, with permission, from REF. 45 © (2010) Macmillan Publisher Ltd. All rights reserved. Parts **c,d** are modified and reproduced, respectively, with permission, from REF. 13 © (2009) Wiley.

Figure 4. Production of droplets for confining, sorting and spatially arranging bacteria

a | A polydimethylsiloxane flow-focusing microfluidic device. The flow of mineral oil is perpendicular to the flow of cells (which are admixed with agarose) and results in the production of cell-containing droplets (agarose microparticles) suspended in mineral oil⁵⁶. **b** | An example of a droplet-sorting mechanism in which aqueous droplets are sorted by the presence (red) or absence (inset) of an electric field⁵⁵. The scale bars represent 100 μm. **c** | A microfluidic system capable of generating droplets containing cells in environments with defined volumes and chemical compositions⁵⁸. Droplets of defined chemical composition (packets of known volume) are created and then combined in the merging chamber to produce diverse chemical environments within microdroplets. Bacterial cells within these droplets can then be incubated off the microfluidic chip, and metabolic activity can be assessed using a fluorogenic substrate, thus allowing a rapid assessment of the impact of the growth environment on metabolic activity. This methodology has been used to examine interactions between multiple antibiotics by creating droplets containing combinations of different antimicrobials. Part **a** is modified, with permission, from REF. 56 © (2011) American Chemical Society. Part **b** is reproduced, with permission, from REF. 55 © (2009) UK Royal Society of Chemistry. Part **c** is modified, with permission, from REF. 58 © (2012) UK Royal Society of Chemistry.

Figure 5. Detecting metabolic activity in spatially organized populations

a | The scanning electrochemical microscopy (SECM) set-up that was used for carrying out a three-dimensional quantification of molecules surrounding a biofilm⁶². A biofilm is grown on top of a membrane surrounded by a polydimethylsiloxane (PDMS) stencil within a petri dish. A water bath and a copper heating plate serve to heat the culture. In this set of experiments, a 10 µm platinum ultra-microelectrode (UME) was used to detect the microbial production of pyocyanin with a 0.5 mm tungsten wire as a counter-electrode and Hg/Hg₂SO₄ (Radiometer) as a reference electrode. **b** | A SECM-generated reactive image of pyocyanin reduction by a *Pseudomonas aeruginosa* biofilm. The UME tip was held at a constant height (20–30 µm) above a 1 mm diameter *P. aeruginosa* biofilm (dotted outline), and a two-dimensional scan was acquired by moving the SECM tip in the *x* and *y* axes. The UME was held at 0.3 V to oxidize pyocyanin, and the rate at which the biofilm reduced pyocyanin was measured. Colour represents the current measured by SECM and correlates to the rate of pyocyanin reduction by the biofilm, from low (blue) to high (orange) current. **c** | A nano-scale secondary-ion mass spectrometry (nanoSIMS) instrument⁶⁶. A Cs⁺ primary-ion beam is used to sputter a bacterial cell, and the secondary ions that are emitted are directed into a mass spectrometer, where secondary-ion images for various masses (here, ¹²C, ¹³C, ¹²C¹⁴N and ¹²C¹⁵N) are generated. Secondary electrons, which are emitted during the sputtering process, can also be collected by the nanoSIMS secondary-ion

collector and used to generate a topographical image of the sample surface. Isotope ratio images can be used to visualize the relative enrichment or depletion of specific chemical species within the sample. **d** | NanoSIMS has been used to show that deep-sea anaerobic methane-oxidizing archaea fix N_2 within specialized anaerobic, methane-oxidizing archaea–bacteria consortia⁶⁹. Consortia were incubated with $^{15}N_2$, labelled with fluorescence *in situ* hybridization (FISH) probes and imaged by fluorescence microscopy and nanoSIMS. FISH probes targeting methanogenic archaea are red, and those targeting sulphate-reducing bacteria (from the family Desulfobacteriaceae) are green. A nanoSIMS image of the same sample displays data as ratios of $^{12}C^{15}N$ to $^{12}C^{14}N$. Higher ratios indicate incorporation of the labelled $^{15}N_2$. Regions of the consortia where archaea are localized have higher ratios, indicating that these organisms are fixing N_2 . Part **a** is modified and part **b** is reproduced, with permission, from REF. 62 © (2011) US National Academy of Sciences. Part **c** is modified, with permission, from REF. 66 © (2012) Wiley. Part **d** is reproduced, with permission, from REF. 69 © (2009) American Association for the Advancement of Science.

Table 1

Cell confinement technologies

Technology	Description	Applications	Key advantages	Key limitations	Accessibility and cost
Microfluidics ^{9,24}	Soft lithography is used to produce devices with microscale (generally ~5–500 µm) feature sizes and volumes of <10 µl	<ul style="list-style-type: none"> Spatial organization of small populations and single cells Production of physical and chemical gradients to study particular phenotypes 	<ul style="list-style-type: none"> Device shapes are customizable for precise control of the physical and chemical environment Generally optically transparent and compatible with CLSM or video microscopy 	<ul style="list-style-type: none"> Device material (typically PDMS) is generally non-porous Specialized equipment is required to produce custom devices 	<ul style="list-style-type: none"> Several universities have established public foundries for microfabrication, but rates and fees vary Moulds can be produced for <US\$200 Materials and equipment for casting and curing PDMS might cost ~\$1250
Droplet microfluidics ^{53–55,58,59}	A T-junction or flow-focusing microfluidic device is used to form low-volume (picolitre to nanolitre) droplets ^{54,79}	<ul style="list-style-type: none"> Screening of mutant libraries and chemical libraries for phenotypes of interest 	<ul style="list-style-type: none"> Small amounts of reagents are sufficient Low volumes and process automation at high speeds enables ultra-high-throughput studies Droplets can be split, merged, sorted and stored for later analysis 	<ul style="list-style-type: none"> Proper surfactant is required to maintain a stable and inert oil–water interface Washing cells or removing liquid reactants within droplets can be difficult 	<ul style="list-style-type: none"> Requires access to a FACS sorter or the FADS technique⁵⁵ All platforms for high-throughput techniques require computer-controlled execution
Hydrogels ^{33–35,80}	A hydrated network of crosslinked polymers that is permeable to small molecules	<ul style="list-style-type: none"> Spatial organization of cells Generation of chemical gradients 	<ul style="list-style-type: none"> Defined mass transfer properties Various hydrogel polymers are available Some hydrogels can be synthesized at specific three-dimensional coordinates by photopolymerization³⁵ 	<ul style="list-style-type: none"> Surface properties can be inconsistent with natural growth environments Constituent polymers can be heterogeneous 	<ul style="list-style-type: none"> Readily available from commercial suppliers Photopolymerization requires the proper pre-polymer and an excitation source, such as ultraviolet light
Protein hydrogels ^{39,40}	Three-dimensional structures with submicron-sized features can be fabricated using protein as the primary constituent	<ul style="list-style-type: none"> Spatial organization of cells in chambers with varying topographies 	<ul style="list-style-type: none"> Does not require access to clean rooms Templates of any desired shape can be designed <i>in silico</i> and patterned rapidly MPL hydrogels are porous to nutrients and waste products, and cells grow at 	<ul style="list-style-type: none"> MPL set-ups can be cost prohibitive Free radicals generated during fabrication can be toxic to cells 	<ul style="list-style-type: none"> A complete MPL setup can range from ~\$70,000 to \$500,000

Technology	Description	Applications	Key advantages	Key limitations	Accessibility and cost
	appreciable rates within chambers				
Lipid-silica structures ^{44,47}	Lipid-silica structures are templated around bacteria, sealing cells in containers with low-picolitre to ~100 fl volumes	<ul style="list-style-type: none">• Confinement of single cells or small groups of cells in low volumes	<ul style="list-style-type: none">• Low numbers of cells are isolated in small volumes• Secreted metabolites accumulate quickly	<ul style="list-style-type: none">• Cellular growth is restricted• No free exchange of solutes• pH inside structures is low	<ul style="list-style-type: none">• Spin coaters cost from \$3,000 to \$7,000, and dip coaters are generally \$10,000

CLSM, confocal laser scanning microscopy; FACS, fluorescence-activated cell sorting; FADS, fluorescence-activated droplet sorter; MPL, multiphoton lithography; PDMS, polydimethylsiloxane.

Table 2

Analytical techniques for studying microenvironments

Technology	Description	Applications	Key advantages	Key limitations	Accessibility and cost
Scanning electrochemical microscopy (SECM) ^{60,61}	Specific redox-active molecules are quantified on the micrometre scale using a UME	<ul style="list-style-type: none"> Generation of a real-time spatial map of the concentration of a redox-active molecule proximal to a population of cells 	<ul style="list-style-type: none"> Highly sensitive Does not require fixing of cells 	<ul style="list-style-type: none"> UME tips must be fabricated and are sensitive to damage Measurement of a single molecule only 	<ul style="list-style-type: none"> Complete SECM set-up is ~US \$44,000–\$80,000 Less expensive set-ups can be constructed in-house
Imaging mass spectrometry (IMS) ⁶³	The spatial distribution of biomolecules is assessed	<ul style="list-style-type: none"> Detection of a diverse range of biomolecules in an environment 	<ul style="list-style-type: none"> Determines the spatial distribution of multiple elements or biomolecules surrounding cells 	<ul style="list-style-type: none"> Instrumentation is expensive Samples must often be dried and fixed prior to analysis 	<ul style="list-style-type: none"> Requires purchase of specialized equipment or use of a pay-per-use facility Instrument costs vary widely*
Secondary-ion mass spectrometry (SIMS) ^{63,67}	Cells are bombarded with a continuous (dynamic SIMS) or pulsed (static SIMS) primary-ion beam that releases secondary analyte ions, which are detected by a mass spectrometer	<ul style="list-style-type: none"> Imaging of single cells or bacterial colonies Detection of low-molecular-mass compounds (<1,500 Da, with a lower mass range detected by dynamic SIMS and a higher mass range detected by static SIMS⁶⁷) 	<ul style="list-style-type: none"> Highest spatial resolution of currently available IMS techniques 	<ul style="list-style-type: none"> Yields limited molecular information Detects the lowest mass range of all the IMS techniques listed here 	<ul style="list-style-type: none"> Cost varies*
NanoSIMS ^{63,66,67}	An ultrasensitive dynamic SIMS technique	<ul style="list-style-type: none"> Imaging of individual cells Obtaining carbon and nitrogen isotope ratios at subcellular resolution 	<ul style="list-style-type: none"> High analyte sensitivity Highest spatial resolution of currently available SIMS techniques (sub-50 nm resolution)⁶⁷ 	<ul style="list-style-type: none"> Does not detect a wide mass range; mass detection is limited to seven discrete masses detected at very high resolution Does not yield molecular information⁶⁷ 	<ul style="list-style-type: none"> Cost varies*
Desorption electrospray ionization (DESI) mass spectrometry ^{63,67}	Analyte molecules are desorbed from the sample surface by a pneumatically assisted stream of organic	<ul style="list-style-type: none"> Imaging larger microbial communities such as colonies growing on agar plates 	<ul style="list-style-type: none"> Operates at atmospheric pressure, so samples need not be dried 	<ul style="list-style-type: none"> Low spatial resolution Samples must be mounted on an insulating surface, so hydrated colonies on agar 	<ul style="list-style-type: none"> Cost varies*

Technology	Description	Applications	Key advantages	Key limitations	Accessibility and cost
	solvent, and ions are then detected by the mass spectrometer		<ul style="list-style-type: none"> Detects intact molecules Possible to bias the suite of desorbed compounds Can be paired with reactants for reactive imaging 	<ul style="list-style-type: none"> surfaces cannot be imaged unless nanoDESI is used⁶³ 	
Matrix-assisted laser desorption-ionization (MALDI) mass spectrometry ^{63,67}	An ultraviolet or infrared laser ionizes the sample surface, and ions are detected by the mass spectrometer	<ul style="list-style-type: none"> Imaging microbial colonies and communities 	<ul style="list-style-type: none"> Detects the widest mass range of all currently available IMS techniques (300 50,000 Da) Good for analysing proteins and peptides 	<ul style="list-style-type: none"> Samples must be dried and coated with an organic matrix, which obscures compounds with molecular masses <300 Da 	<ul style="list-style-type: none"> Cost varies*

UME, ultramicroelectrode.

* Costs of the IMS techniques listed here range from \$40,000 to \$2,500,000, depending on the specific technique used^{63,67}.