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Abstract

The ability afforded by microfluidics to observe the behaviors of microbes in highly controlled and confined microenvironments, across scales from a single cell to mixed communities, has significantly contributed to expand the frontiers of microbial ecology over the last decade. Spatially and temporally varying distributions of organisms and chemical cues that mimic natural microbial habitats can now be established by exploiting physics at the micrometer scale and by incorporating structures with specific geometries and materials. Here we review applications of microfluidics that have resulted in highly insightful discoveries on fundamental aspects of microbial life, ranging from growth and sensing to cell-cell interactions and population dynamics. We anticipate that this flexible, multidisciplinary technology will continue to facilitate discoveries regarding the ecology of microorganisms and help uncover strategies to control phenomena such as biofilm formation and antibiotic resistance.

Keywords

Microenvironment; Gradients; Surface interactions; Single-cell analysis; Population dynamics; Antibiotics

1 INTRODUCTION

Microfluidics is the technology of driving and controlling fluids at the micrometer scale. Initially stemming from microanalytical methods and microelectronic circuits (Whitesides 2006) in the early 1990s, the field has expanded dramatically in the last decade, largely due to the introduction of an easily accessible fabrication technique soft-lithography for creating microdevices based on patterned elastomeric polymers. The most commonly used material to date is polydimethylsiloxane (PDMS), owing to its transparency, flexibility, gas permeability, chemical inertness, and biocompatibility (Weibel et al. 2007). With these developments, microfluidics has revolutionized fundamental and applied research in many fields, from soft-matter physics to chemical engineering, disease diagnostics and biomedicine. The introduction of microfluidic platforms into the study of microbial ecology is very recent and in many cases still in its infancy, yet existing studies already reveal the great potential of this technology to extend the boundaries of the field (Wessel et al. 2013).

A number of features make microfluidics an appealing technology for microbial studies. As illustrated in Figure 1, the ability and flexibility of controlling the microbial environment at the scale of single cells or small populations sets microfluidics apart from traditional tools.

One can capitalize on the laminar nature of fluid flows at these scales to generate precise spatial gradients of solutes, gases, or temperature. Incorporating porous materials (e.g., hydrogels, membranes) or nanoscale channels allows for the supply of dissolved nutrients and the removal of soluble waste by diffusion, while impeding the passage of microorganisms, thus achieving selective physical confinement without chemically isolating cells. Funnels, obstacles, and barriers can be seamlessly fabricated and used to study the interaction of microbes with boundaries, which leads to novel ways of concentrating motile cells. More generally, boundaries can be used to confine populations or individual cells in minuscule liquid volumes, thus allowing a wide range of investigations, from short-term competition studies to multi-generation experiments on adaptation and evolution.

In this review we describe the most recent developments in microfluidics that have opened new doors in microbial ecology, focusing on those studies that combined technical advances with new microbial insights. For reasons of focus, we will not review studies performed using traditional flow chambers, nor microfluidic platforms for single-cell screening, for which we direct the reader to excellent recent reviews (Bennett & Hasty 2009, Zare & Kim 2010). We begin with a discussion of bacterial chemotaxis and, more generally, microbial navigation, likely the earliest and most prolific application of microfluidics to the study of bacterial behavior. We then review microfluidic examples of how the external environment, such as fluid flow, can modify microbial motility, and how motility, in turn, can modify the transport properties of chemicals in the surrounding fluid. Next we discuss transient and permanent interactions of microbes with solid boundaries, a pervasive feature in the life of many microbe and exceedingly fertile ground for microfluidic investigations. After an excursion into new microfluidic tools developed to explore and control cell growth, we focus on the microfluidic confinement of bacterial populations, which has allowed original studies on the interactions within and among microbial communities. We conclude by presenting key applications of microfluidics in the field of antibiotic resistance.

2 MICROBIAL NAVIGATION

Microfluidics has changed the way we study the motility of microbes and their response to cues from the environment. Chemotaxis, the best-studied form of microbial navigation, has received considerable attention in the microfluidic literature, and its study offers a blueprint for the rich gamut of microbial navigation strategies, including aerotaxis, thermotaxis, galvanotaxis, and magnetotaxis, to name but a few. Microfluidics is ideally suited for such studies because it affords accuracy, flexibility and control in creating gradients steady or unsteady, linear or nonlinear, single or multiple at scales relevant to microbes (hundreds of micrometers). These gradients, owing to the precision in the design and fabrication of microfluidic systems and the laminar nature of flow in microchannels, are straightforward to predict mathematically and easy to quantify experimentally, for example using fluorescent dyes. The transparency of microchannels further allows for direct, quantitative observation of microbial responses to gradients at the level of single cells using video microscopy, automated image analysis and cell tracking. These advantages offered by microfluidics over prior methods have already been extensively reviewed (Ahmed et al. 2010b). Here we focus on the impact that microfluidics has had on the study of microbial navigation and highlight the most recent developments.

2.1 Chemical Attraction

Bacterial chemotaxis is one of the best studied biological sensory systems since it is highly tractable at the molecular level and plays a fundamental role in a broad range of ecological and environmental processes, including trophic interactions, chemical cycling in natural environments, and the onset of disease. The earliest microfluidic gradient generator for bacterial chemotaxis was proposed by Mao et al. (2003). Their three-inlet parallel-flow device (see also Englert et al. (2010)) was based on the confluence of three streams, joining into a single microchannel in a T-shaped configuration. Bacteria flowing within the middle stream had the possibility of swimming towards either one of the two flanking streams, one containing a chemoeffector and the other containing buffer. Chemotaxis was assessed as the preferential movement of cells towards the chemoeffector stream. Using this approach, Mao et al. (2003) demonstrated that the chemotactic sensitivity of *Escherichia coli* was considerably higher (1000-fold) than measured by traditional capillary-based chemotaxis assays, and the cells responded to amino acid concentrations as small as a few nanomolar. Kim et al. (2011) recently proposed an interesting variant of this method by including arrowhead-shaped nooks along the microchannel sidewalls to concentrate bacteria, and thus magnifying the readout of their chemotactic response.

When gradients evolve by diffusion in the absence of flow, single cells are considerably easier to follow, allowing one to probe fundamental properties of chemotaxis. By tracking *E. coli* within an unsteady gradient of *α*-methylaspartate, Ahmed & Stocker (2008) obtained a detailed map of its chemotactic velocity. The gradient, quantified using a fluorescent dye, was established by filling a side channel with a chemoattractant solution that gradually diffused out into a perpendicular main channel containing a flowing suspension of bacteria. The resulting unsteady gradient was used to assess the chemotactic velocity as a function of the local, instantaneous concentration and gradient of chemoattractant. This work revealed that *E. coli* can achieve chemotactic velocities as high as 35% of their swimming speed (more than double the typical literature values of 15%) and verified continuum models of bacterial transport based on single-cell swimming information. Unsteady gradients can also be created by the bacteria themselves through consumption of nutrients in the medium. Saragosti et al. (2011) explored this case by confining bacteria in a narrow region at one extremity of a microfluidic channel using centrifugation, and then observing dense waves of bacteria migrating along the channel (Fig. 2c). Tracking of individual, fluorescently labeled cells within the migrating front confirmed that their mean run length was longer in the direction of propagation, as is typical in *E. coli* chemotaxis, but also revealed that the tumbling direction was skewed in the direction of the gradient. This previously unknown modulation of reorientations, due to the different number of flagellar motors undergoing the change in rotation direction that triggers tumbling, provides an additional, moderate increase in the chemotactic velocity.

While self-generated or self-evolving gradients afford numerous possibilities to learn about chemotaxis, it is the ability to directly control gradients that affords the most robust microfluidic approaches to chemotaxis. A simple way to achieve a steady gradient is to connect two large reservoirs through a small test channel, resulting in a linear concentration profile within that channel. This method has been recently adopted by Masson et al. (2012)

to track swimming *E. coli* cells and infer their impulse response function (the response to a pulse of chemoeffector, which can be used to infer responses to more complex gradients), without the need for tethering cells to surfaces as was traditionally done. One downside of this approach is the potential for fluid flow through the test section, as precise pressure equalization is challenging. The same principle can be applied with considerably more control by incorporating in the fabrication porous materials permeable to molecular diffusion but not to fluid flow to separate the test channel from flanking microchannels used as the chemoattractant reservoirs (source and sink). Regulating the concentrations in the reservoirs affords complete control over the gradient that microbes experience in the test channel, allowing for long-term observations in steady gradients or for the temporal modulation of gradients without any confounding effects due to flow. This concept was proposed by Diao et al. (2006), who used a nitrocellulose membrane, later replaced with the hydrogel agarose by Cheng et al. (2007). A detailed characterization of the gradients by confocal microscopy was provided by Ahmed et al. (2010a). Agarose can be accurately molded, improving the fabrication of the diffusion-permeable barriers, and its transparency is ideal for microscopy. Kalinin et al. (2009) adopted the device from Cheng et al. (2007) to quantify the steady-state distribution of *E. coli* in linear chemoattractant concentration profiles. Exploiting the devices ability to independently control the mean concentration and the gradient, they demonstrated that *E. coli* senses the logarithm of the concentration, a property known as log-sensing. Suitable microchannel arrangement further allows one to establish concentration profiles of arbitrary shape (Ahmed et al. 2010a, Wu et al. 2006) and to overcome the otherwise long timescale for gradient establishment by exploiting fluid flow in a compartment immediately adjacent to the test channel (Kim et al. 2009).

Perfusion from reservoirs through permeable substrates can also be used to establish multiple, simultaneous chemoeffector gradients or modulating gradients over time. Kalinin et al. (2010) exposed *E. coli* to opposing gradients of two amino acids, revealing that *E. coli*s chemotactic preference depends on the ratio of expression between the two receptors, which in turn varies with growth conditions. By varying the frequency of an alternated release of chemoattractant, Zhu et al. (2012) demonstrated that *E. coli* could follow the gradient and oscillate in synchrony with the environment at low driving frequencies, but lost the tune and began oscillating out of phase in fast-changing environments due to their finite adaptation time.

2.2 Aerotaxis and Thermotaxis

A special case of chemotaxis, from the point of view of gradient generation, is aerotaxis (taxis towards oxygen). In this case, one can take advantage of the gas permeability of PDMS to create steady oxygen gradients without the need for hydrogels or membranes (Adler et al. 2010). This principle used by Adler et al. (2012), who studied aerotaxis in *E. coli* under steady, linear profiles of oxygen ranging from microaerobic to aerobic conditions and showed that it does not obey log-sensing. Furthermore, the irreversible bonding that can be achieved between layers of PDMS, but not with hydrogels, makes aerotaxis an ideal model system for the study of microbial navigation in more realistic environments, such as when fluid flow is present.

Temperature gradients have also been created within microfluidic devices and used to study thermotaxis and thermokinesis. One method relied on using a Peltier device together with circulation channels for hot and cold water, and on diffusion of heat through the PDMS, to generate a temperature gradient, which was visualized with a temperature-sensitive fluorescent dye (Demir et al. 2011, Salman et al. 2006). Salman et al. (2006) used this approach to demonstrate a subtle interplay between thermotaxis, metabolism and chemotaxis in *E. coli*: when cells accumulate at their preferred temperature by thermotaxis, locally enhanced nutrient consumption produces a nutrient gradient opposing the temperature gradient. This causes the bacteria to migrate toward the lower temperature. With the same approach, Demir & Salman (2012) demonstrated a further response of *E. coli* to temperature gradients thermokinesis whereby (under low nutrient conditions) higher temperatures cause decreased intracellular pH, which in turns diminishes the swimming speed, resulting in a slow migration toward warmer water.

2.3 In Sea and Soil

One group of organisms for which microfluidics has enabled a range of new insights is marine microbes. Their nutrient sources often come as ephemeral, submillimeter patches, which dwindle by diffusion. The utilization of these patches by microbes has been challenging to study given the scales of traditional oceanographic approaches, but can be conveniently investigated using microfluidics. Seymour et al. (2008) devised a microinjector system consisting of a microchannel used to inject a narrow band of chemoattractants within a wider test channel to model the appearance and diffusion of a nutrient patch. This approach has been successfully employed to quantify the microbial response by video microscopy in a range of marine processes. Stocker et al. (2008) showed that the chemotactic response of the marine bacterium *Pseudoalteromonas haloplanktis* can be an order of magnitude faster than that of *E. coli*, suggesting that marine microbes are well adapted to exploiting small, ephemeral nutrient sources. Strong chemotactic responses are also found among protists and phytoplankton, indicating that microbial trophic interactions in the ocean may occur primarily within microscale hotspots (Seymour et al. 2009b). The ability to replicate microscale nutrient pulses has further enabled the study of marine microbial responses to organic matter exuded by photosynthetic phytoplankton (Seymour et al. 2010a, 2009a), to mucus produced by corals (Garren et al, in review), and to sulfur compounds including the climatically active gas dimethylsulfide (Seymour et al. 2010b) (Fig. 2a,b).

Stocker et al. (2008) used a modified version of the microinjector to simulate the nutrient plume in the wake of a settling marine particle. The particle was represented as a small PDMS cylinder, sitting in front of the microinjector so as to turn the band into a plume. Experiments revealed that *P. haloplanktis* enhanced their exposure to nutrients by 400% over non-motile bacteria through chemotaxis in the nutrient plume of slowly sinking particles. In a different device, the addition of agarose surfaces as models for particles surfaces recently allowed Yawata et al. (in review) to extend quantification beyond chemotaxis and also include observations of surface attachment, revealing a subtle competition-dispersal trade-off as the origin of the ecological differentiation among two closely related bacterial populations within the genus *Vibrio*.

The ability to fabricate complex topographies in microfluidic devices lends itself well to investigations of microbial navigation in highly structured environments, such as soil and porous media. One can produce microchannels that replicate key features of porous media, for example by using a collection of cylindrical pillars to mimic sand grains and the pores between them, with regular (Long & Ford 2009) or random (Durham et al. 2012) arrangements. Using the basic principle of the T-sensor injecting parallel streams of bacteria and chemoattractants Long & Ford (2009) found that flow enhanced chemotactic migration of *E. coli* in porous media. Using a similar device, Singh & Olson (2012) quantified the chemotactic migration of *E. coli* from high- to low-permeability regions, modeled experimentally as areas with and without smaller cylinders amidst a regular array of larger cylinders.

These microfluidic experiments on microbial chemotaxis in sea and soil provide a blueprint for the microfluidic investigation of microbial processes in other natural environments. The flexibility of investigating multiple environments within scenarios that closely mimic natural conditions suggests that a broad range of additional microbial navigation processes in diverse microbial habitats will benefit from microfluidic approaches.

3 EVERYTHING FLOWS

Many microbial habitats are liquid environments frequently experiencing fluid flow, which can be a prevalent influence on the ecology of the microbes (Guasto et al. 2012). Bacteria and phytoplankton consistently experience turbulent flow in oceans and rivers, soil microorganisms are exposed to creeping groundwater flows, and human microbiota are subject to fluid flow in the digestive, respiratory and urinary systems. Flow plays an important role in a broad variety of microbial processes, including nutrient uptake, encounter rates, and fertilization, as well as in many industrial applications, ranging from wastewater treatment to seawater desalination and the production of biofuels. Despite its importance, the effect of flow on microorganisms has been investigated almost exclusively from a theoretical perspective (Locsei & Pedley 2009, Taylor & Stocker 2012), due in large part to the experimental difficulties of visualizing microbial responses in accurately controlled flowing environments.

Microfluidics greatly enhances the ability to study the effect of flow on microbes by allowing one to create controlled flows over a wide range of flow speeds and shear rates. Marcos et al. (2009) used shallow microfluidic channels to show that shear induces a lateral drift across streamlines for non-motile, helically shaped bacteria (*Leptospira biflexa*) owing to their chiral morphology. These authors exploited the precise flow field in a straight microchannel to image bacteria at different depths: the linear variation of the shear rate over the channel depth enabled them to validate the theoretical prediction that the drift velocity is linearly proportional to the shear rate. Marcos et al. (2012) used a similar device to observe the effect of shear on motile bacteria (*Bacillus subtilis*): the ability to precisely quantify the cells cross-streamline migration over a > 1 meter long channel (arranged in a serpentine configuration) allowed them to demonstrate 'rheotaxis', a bias in the swimming direction caused by the coupling between shear and the chirality of the bacterial flagellum. A different effect of flow on motile bacteria was recently discovered through the use of tall (i.e., deeper

than wide) microchannels, where the dominant shear was in the horizontal plane of observation. Using such a setup, Rusconi et al. (in review) observed a fast shear-trapping effect, whereby bacteria strongly accumulate in the high-shear regions. By moving the microscope stage in synchrony with the average flow speed, the authors could track individual bacteria in flow and demonstrate that trapping originated from the cell rotation induced by shear ('Jeffery orbits').

Fluid flows that can be generated in microchannels go beyond unidirectional flows and offer great flexibility in creating spatially-varying flow fields, including extensional flows, hyperbolic flows and vortex flows (Hudson et al. 2004). Marcos & Stocker (2006) created vortices by using a straight microchannel with a cavity on one side: flow in the main channel drives a vortical recirculation in the cavity. This setup allowed them to study the effect on marine microbes of vortices of similar size to those found in the ocean and to observe the preferential alignment of elongated microbes with streamlines, predicted theoretically.

In natural environments, flow often occurs simultaneously with other ambient cues. This is a scenario that is highly conducive to microfluidic analysis. For example, Garcia et al. (2013) investigated the phototactic response (i.e., migration in response to light) of suspensions of the alga *Chlamydomonas reinhardtii* in a Poiseuille flow, showing that self-focusing near the channel center and migration towards the channel walls could be reversibly switched by changing the position of the light source from upstream to downstream and vice-versa. Rusconi et al. (in review) studied the aerotactic response of the bacteria *Bacillus subtilis* in a Poiseuille flow, showing that the trapping caused by shear had a strong quenching effect on aerotaxis. The unexpected interactions among cues revealed by these studied, together with the plethora of cue combinations that microbes experience in their natural environment, highlight the great potential that remains to be exploited in this field.

4 TINY AGITATORS

The swimming motion of microbes themselves can generate flows that enhance mixing or can be harnessed to transport objects. Kim & Breuer (2004) studied the effect of bacterial motility on the diffusion of a tracer molecule using a two-inlet microfluidic channel in which transport between two adjacent streams is due solely to diffusion, when bacteria are absent. They observed that the diffusivity of Dextran was enhanced by up to eight-fold when *E. coli* were added and that the enhancement increased linearly with bacterial concentration. In a modification of this approach, bacteria can be bound to the surfaces of a microchannel via their cell bodies so that the rotation of their flagella mixes the fluid (Kim & Breuer 2007) or pumps it along the device (Kim & Breuer 2008). The performance of these 'bacterial carpets' can be tuned by modulating bacterial motility through chemical stimuli or temperature.

At high cell concentrations, collective motions can not only enhance mixing, but also modify the bulk fluid properties. Gachelin et al. (2013) used a two-inlet microchannel to quantify the rheological properties of concentrated suspensions of *E. coli*, by measuring the position of the interface between the two streams, one with bacteria and one with the suspending fluid. The ratio of the widths of the two streams yielded the ratio of the

viscosities of the two fluids, revealing the presence of a shear-thickening behavior (viscosity increasing with shear) at low shear rates and a shear-thinning behavior (viscosity decreasing with shear) at high shear rates.

Microbes in microchannels have also been put to work as micro-oxen (Martel 2012). Weibel et al. (2005) demonstrated that the alga *C. reinhardtii*, guided by phototaxis, could push microbeads through microchannels. A similar effect was reported for the bacterium *Serratia marcescens* exposed to a chemoattractant gradient (Kim et al. 2012a) and for the gliding bacterium *Mycoplasma mobile* moving along asymmetrically patterned substrates (Hiratsuka et al. 2005). Through the same process, microfabricated gears decorated with asymmetric teeth can be made to rotate (Sokolov et al. 2010). Although these observations remain to be exploited in applications, they show that microfluidics can be used not only to understand, but also to control and exploit microbes.

5 A WORLD WITH BOUNDARIES

Given the pervasive presence of surfaces in microbial habitats, how microbes interact with boundaries is a fundamental question in microbial ecology. Surfaces may act as physical barriers that limit motility and dispersal, as nutrient hotspots in resource-limited environments, or as substrates that cells colonize and infect. Surface interactions play crucial roles in a wide range of ecological, medical and industrial processes including biofouling, fertilization, infection, cell sorting, and bioremediation yet, the biophysical mechanisms governing microbe-surface interactions remain largely unexplored. Once more, microfluidics provides a broad set of experimental platforms to obtain deeper insights on the effects of surfaces on microbes.

5.1 Hitting a Wall

Exquisite control over geometry provides a means to direct and collect microbes in microfluidic devices. For example, bacteria near surfaces swim in circular trajectories and they experience a surface-attracting force, due to the counter-rotating motion and differential drag of the cell body and flagella (Lauga et al. 2006). When *E. coli* cells were confined between an agar surface and a PDMS surface in microchannels only slightly taller than a bacteriums width, they preferentially swam along one sidewall, 'driving on the right' (DiLuzio et al. 2005), as a result of circular swimming and the lower drag near the porous agar surface. Related hydrodynamic considerations explain the pattern of swimming upstream that *E. coli* cells demonstrate near surfaces in the presence of flow (Kaya & Koser 2012).

Appropriately arranged microstructures result in the directional motion of swimming microbes ('rectification') - in a microfluidic analog of Maxwell's demon - and thus in the possibility of concentrating them. The best studied geometry is the 'funnel wall, a line of wedge-shaped barriers with spaces between them through which microbes can swim. Rectification occurs because the probability of crossing the funnel wall is larger in one direction than the other (Fig. 3b). Galajda et al. (2007) used a series of funnel walls to concentrate motile bacteria and separate them from non-motile ones. Concentration also occurs in the presence of flow (Altshuler et al. 2013) and can be destroyed by chemotaxis,

for example when cells create their own gradient through nutrient consumption and, by following it, escape from the trap (Lambert et al. 2010). Funnel walls allowed Kantsler et al. (2013) to show that the surface scattering of *Chlamydomonas* algae is governed by steric interactions between the algas flagella and the surface, rather than by far-field hydrodynamic interactions as previously believed. Taking advantage of the ease of microfabricating multiple geometric configurations, these authors further optimized the funnel geometry for maximal rectification, providing a blueprint for the concentration, separation and eventual collection of different microorganisms (Fig. 3c).

Microstructures have further been used to sort motile bacteria by length. Hulme et al. (2008) took advantage of *E. coli*'s 'swimming on the right' to create a continuous, uni-directional movement of cells in a system of arrowhead-shaped ratchets. By alternating ratcheting sections with sorting junctions, designed to exploit the correlation between the radius of curvature of the circular trajectories and the cell length (DiLuzio et al. 2005), these authors successfully enriched a population in short, and thus young, cells a promising step towards obtaining synchronized cells to study genetic and behavioral changes across generations.

5.2 Flatlanders

Microbes near surfaces can attach to them through a complex process that involves electrostatic, van der Waals, as well as hydrophobic interactions and is mediated by appendages such as pili, fimbriae and flagella. Despite much attention and broad applicability, the mechanisms governing microbial adhesion to surfaces remain poorly understood.

The small dimensions of microfluidic channels allow one to study surface adhesion under a wide range of shear forces and to monitor individual-cell attachment. Accurate control of flow conditions enabled Lecuyer et al. (2011) to show that increased shear stress diminished the number of *P. aeruginosa* cells attached to a surface, but increased their residence time on the surface regardless of the presence of surface appendages. De La Fuente et al. (2007) used microfluidics to study the role pili in anchoring bacteria to the substrate for the plant pathogen *Xylella fastidiosa*. This anchoring can result in cells being flipped by flow and consequently walking upstream (Meng et al. 2005, Shen et al. 2012). Adhesion can be further studied by taking advantage of microcontact printing, a microfluidic technique to spatially control surface chemistry. For example, Holz et al. (2009) used PMDS stamps to print bovine serum albumin (BSA) patches onto glass slides and filled the surrounding surface with fluid-like lipid membranes to study the transition between spreading and clustering in the human pathogens *Neisseria gonorrhoeae* and *N. menigiditis*.

Beyond surface chemistry, microfabrication allows accurate control of surface topography at the scale of individual microbes. Given that the effects of nanometer-sized structures on bacterial surface adhesion have been reviewed elsewhere (Anselme et al. 2010), we only briefly highlight the capabilities offered by surface micro-patterning. Hochbaum & Aizenberg (2010) fabricated arrays of high-aspect-ratio polymer posts to study the growth of *P. aeruginosa* on structured surfaces. Cells spontaneously assembled into patterns dictated by the spacing between neighboring posts. As the spacing approached the cells size, the orientational order and positional order were enhanced, reaching a point where bacteria were

aligned along the length of the posts (Fig. 3d). This assembly phenomenon was likely driven by the cells tendency to maximize their contact area with the surface, a hypothesis supported by experiments showing reduced adhesion upon addition of a micro-texture that minimized cell-surface contact area (Kargar et al. 2012). Further work in this area could result in materials with controlled surface topography with broad implications for anti-biofouling and other biomedical and industrial applications.

5.3 Hanging on by a Thread

A characteristic consequence of microbial attachment to surfaces is the formation of biofilms, surface-attached communities encased in a self-secreted matrix of extracellular polymeric substances (EPS). Bacteria within biofilms have higher chances than their planktonic counterparts of resisting chemical insults and phagocytosis by immune system cells, and the resulting difficulty in combating them makes biofilms a major problem in medicine and industry, where they reduce the lifetime of prosthetic devices, cause chronic infections, and enhance drag and corrosion in pipelines.

Microfluidics has enabled biofilm studies to go beyond flow chambers and to address, in particular, the effect of topographical features (e.g., porous media). Much attention has been devoted to biofilm 'streamers', features frequently found in natural and artificial systems that result from the coupling of complex topography and fluid flow, and are responsible for increased uptake of organic molecules (Battin et al. 2003) and membrane biofouling (Vrouwenvelder et al. 2010). Fabrication of curved channels, coupled with time-lapse confocal microscopy, allowed Rusconi et al. (2010) to demonstrate that *P. aeruginosa* streamers developed where the surface curvature induces secondary, vortical flows that drive the accumulation and extrusion of the polymeric substances that form the streamers backbone. The sharper the surfaces curvature, the more quickly the streamers develop (Guglielmini et al. 2010, Rusconi et al. 2011). By capturing floating biomass, the sieve-like streamers caused rapid clogging of microchannels (Drescher et al. 2013). Similar streamers were observed when vortical flows were produced by the oscillation of a bubble trapped in a horseshoe microstructure and driven by a radio frequency signal (Yazdi & Ardekani 2012). These dynamics have also been investigated in more complex geometries, including ones modeling porous media environments (Kumar et al. 2012, Marty et al. 2012, Valiei et al. 2012), underscoring the potential of microfluidics to mimic multiple components of natural environments with topographic complexity and flow.

6 GROWING UP

Microfluidics provides unique opportunities to study and control the growth of microbes with unprecedented resolution by tuning environmental conditions and spatial confinement. This has already enabled new insights into important biological and ecological processes, ranging from cell morphology during replication, to the controls of long-term population dynamics, to the culturing of previously unculturable or rare populations. By trapping non-septating *E. coli* in individual microchambers, which were made of agarose to keep cells hydrated and nutrient-supplied, Takeuchi et al. (2005) demonstrated that cells could bend during growth to adopt the shape of the chamber. When the confinement was released, cells

retained both the shape of the chamber and their motility (although shape markedly affected swimming).

Under more extreme confinement conditions, bacteria can lose motility yet still traverse constrictions by growth and division. This was demonstrated by Männik et al. (2009), who fabricated microfluidic chambers connected by channels with one dimension as narrow as a few hundred nanometers. Even when considerably flattened, *E. coli* cells were able to grow and divide within these channels such that the lineage of a single ancestor penetrated through constrictions as narrow as half the cell diameter (Fig. 4c). Once bacteria exited the constriction, they exhibited shapes and sizes substantially different from their regular shape, which was recovered after a few days along with motility. This remarkable plasticity of *E. coli* suggested that cell morphology is mostly inconsequential for the accurate partitioning of cell volume during division (Männik et al. 2012). These studies highlight the power of microfabrication for understanding the role of mechanical stress on bacterial growth and morphogenesis, and suggest that sub-micrometer pores may be more prolific bacterial habitats than previously thought.

Similar channel design principles have helped the study of long-term growth and division patterns in bacteria. Wang et al. (2010) used a high-throughput microfluidic device - the 'mother machine' - to follow the growth of single *E. coli* cells for hundreds of generations. The device consisted of a series of growth channels, sufficiently narrow to prevent movement of the 'mother cell' in each of them (Fig. 4a), and connected at the distal end to a trench channel used to replenish nutrients and remove daughter cells as they emerged from each growth channel. They observed that the growth rate of mother cells remained remarkably constant with increasing replicative age, in contrast to previous evidence that it decreased (Stewart et al. 2005), although the apparent conflict may vanish upon accounting for the partitioning of non-genetic damage across the two cell poles (Rang et al. 2011). Long et al. (2013) devised a modified mother machine, with narrower and open-ended growth channels to enable faster loading of cells and replenishment of media, as well as greater immobilization for sub-diffraction, intracellular measurements, which allowed them to probe the viscoelastic properties of the nucleoid by tracking the movement of fluorescently-tagged chromosomal loci. A different approach to multi-generational studies was used for *Caulobacter crescentus* by Siegal-Gaskins & Crosson (2008), who exploited the robust adhesion of this bacterium to surfaces for long-term imaging. These authors showed that growth rates were significantly correlated between mother and daughter cells, providing evidence of epigenetic inheritance of cell division behavior.

Integration of additional microfluidic elements, including peristaltic pumps and micromechanical valves to supply media, remove waste, and recover cells, has enabled the long-term culturing of extremely small populations of bacteria in microfluidic chemostats. Balagaddé et al. (2005) used micro-chemostats to study the planktonic growth of *E. coli*, preventing biofilm formation by alternating circulation and cleaning cycles to eliminate adherent cells. By monitoring individual cells carrying a synthetic circuit, these authors observed greater stability in the programmed behavior compared to macroscale batch cultures and a correlation between oscillations in cell density and specific cell morphologies. Groisman et al. (2005) devised a microfluidic chemostat (and thermostat) made of shallow

chambers to monitor bacterial growth and used auxiliary channels, connected to the chambers via sub-micrometer conduits, to continuously supply fresh media and remove metabolic waste by diffusion, while preventing cell escape due to the conduits' small dimensions. The compliance of PDMS was ingeniously exploited to load cells into the chambers by temporarily inflating the conduits through an increase in pressure inside the device.

The possibility of using extremely reduced volumes or to recreate specific microenvironments, in addition to the ability of direct visualization and parallelization, have been exploited to isolate unculturable microbes and to grow cells that could not be cultured with traditional methods. Marcy et al. (2007) identified a rare subset of microbes from the human mouth by isolating cells with a rod-like morphology, amplifying their genome and performing high-throughput sequencing on a microfluidic chip. By combining microfluidics with cell confinement in aqueous plugs surrounded by an immiscible fluid, Liu et al. (2009) succeeded in isolating and cultivating slow-growing cells from mixed bacterial populations. Further, Kim et al. (2012b) showed how a biomimetic microdevice (Fig. 6a) composed of two channels separated by a porous flexible membrane and lined by human intestinal epithelial cells could mimic the complex structure and physiology of live intestines, allowing the study of the dynamic symbiotic interactions between human and microbial cells.

7 LIVING TOGETHER

In the natural world, a microbial cell will commonly find itself in the vicinity of other cells. Elucidating the mechanisms underlying the interactions among microbes is fundamental to our understanding of how small-scale encounters influence larger-scale processes such as ecosystem function, organismal health, or industrial remediation. Microfluidic devices provide an excellent platform for probing these interactions because they can confine cells in controlled and appropriately designed geometries, allowing one to dissect the specific physical, chemical and biological ingredients of cell-cell interactions.

7.1 Bumping into Each Other

In several environments, including the light organs of some squid and bacterial swarms on surfaces, bacteria can grow to extremely high densities, and the direct, mechanical interactions can generate population-scale patterns. Self-organization can be advantageous for bacterial populations, yet the role played in this process by direct cellular contact has received little attention due to the challenges in visualizing it. Microfluidics has enabled real-time studies of physical interactions in dense bacterial suspensions by providing control over flow and nutrient delivery and allowing single-cell resolution imaging. Using a microfluidic device containing shallow chambers of varying shapes and sizes, Cho et al. (2007) observed that *E. coli* cells self-organized over multiple generations to enhance nutrient transport favoring growth in dense colonies. Similarly shallow microchannels enabled non-motile cells to be grown and imaged as a monolayer to probe the biomechanical interactions arising due to cell growth and division (Volfson et al. 2008). Starting from a randomly oriented initial cell distribution, the rapid increase in cell density observed after a few generations created an expansion flow that triggered a transition to an orientationally

ordered phase (Fig. 5a). The expansion flow was highly inhomogeneous, and characterized by narrow, fast streams amidst regions of stagnant cells (Mather et al. 2010). This phenomenon was attributed to the strong dependence of bacterial mobility on cell size and nutrient availability: larger cells grown in favorable conditions experienced greater friction from the channel walls and were mostly immobile, whereas smaller cells, farther from the nutrient sources, could move around the clusters of stagnant cells, producing the streams.

7.2 Social Sense

Cells interact through a number of chemical signaling pathways, the most heavily studied of which is quorum sensing (QS). Microfluidics has enabled a number of advances in our understanding of cellular signaling, primarily owing to the ability to work in confined environments and with small numbers of cells. For instance, using microfabricated maze topologies, Park et al. (2003b) found that chemotactic self-attraction in *E. coli* due to secreted amino acids could drive rapid accumulation of cells in small confined spaces, triggering quorum-dependent behaviors. With nutrient deprivation (Park et al. 2003a), such chemotactic, self-induced behavior was found to trigger the formation of solitary waves of bacteria that collapsed into the smallest confining structures in their path, emphasizing the ability of a population to induce cell-cell communication under stressful nutrient conditions as a critical component for the population's survival (Fig. 5c).

Until recently, QS was believed to occur only at high cell numbers. In contrast, Boedicker et al. (2009) used microfluidics to produce arrays of droplets, each containing only a few *P. aeruginosa* cells, and visualized QS by GFP fluorescence. Confinement resulted in the onset of QS despite the low cell numbers, due to the rapid accumulation of the cell-secreted auto-inducer molecules, which could not become diluted by diffusion into surrounding liquid. The insight that a few cells can initiate QS - because the concentration of inducer molecules and not cell abundance per se is the primary driver of this behavior - further highlights the role of confinement and chemical transport at the microscale.

By producing reliable chemical signals, microfluidic confinements have been instrumental in demonstrating new synchronization properties of bacterial communities. Danino et al. (2010) achieved a prototypical form of coordination in *E. coli* communities by engineering a synchronizable network of oscillating genes, where neighboring cells exchanged information while keeping the same phase in the oscillation. Trapping the cells in a network of microchannels that supplied them with nutrients while avoiding interference by external flow, led to the observation of intense synchronization waves above a threshold colony size. This work expands on previously engineered synthetic 'time keepers' (oscillators) (Elowitz & Leibler 2000) that did not display synchronization.

Microspatial patterning of bacteria led to the discovery that spatial clustering of bacteria favors the secretion of signaling molecules that induce coagulation in human and mouse blood (Kastrup et al. 2008). These authors patterned prescribed numbers of *Bacillus cereus* and *Bacillus anthracis* cells in different spatial configurations, including a spatially homogeneous arrangement as well as small and large clusters. Only large clusters initiated the coagulation cascade, whereas for other configurations the threshold of signaling molecules required to induce coagulation was not reached. This elegant result illustrates the

power of microfluidics in modeling the microscale spatial structure of bacterial communities and in revealing its macroscopic consequences, a theme we believe to be ubiquitous and poorly understood across microbial ecology.

8 A DYNAMIC NEIGHBORHOOD

There are many questions that have remained unanswered with regard to microbial population dynamics, in large part due to the difficulty of observing these dynamics under realistic microenvironmental conditions. Microfluidics is opening new windows that allow experimentation to overcome this historically recalcitrant barrier and begin building a mechanistic understanding of critical questions such as how a population adapts to a changing environment and what processes determine the stability of multi-species consortia.

8.1 Metapopulations

The dynamics of metapopulations - i.e., populations living in distinct habitat patches connected by dispersal - represents one of the most intriguing topics in ecology, and one that lends itself ideally to microfluidic exploration in the case of microbial metapopulations, because of the ability to create landscapes of patches with great control and flexibility. An early example is the nanofabricated landscape of Keymer et al. (2006), who constructed an array of microscale habitats connected by small corridors, and monitored the cell concentration in every habitat. A series of nanoslits ensured nutrient supply to and waste removal from each habitat, and controllable heterogeneity in habitat quality was introduced by varying the number of nanoslits in a given habitat. The authors found that an *E. coli* population could adapt rapidly to regions of intense nutrient limitation stress in a landscape with high niche diversity. Using the same device, Keymer et al. (2008) discovered that two strains of bacteria, which in a homogeneous culture competed for the same resources to the point of extinction, actually cooperated in the nanofabricated heterogeneous landscape (Fig. 5d). These results suggest that in natural environments such as soil, dominant strains may benefit from the continued existence of less competitive strains, as opposed to their extinction.

The same fundamental principle - allowing the diffusion of physiologically important molecules while preventing cell migration out of habitats - was used by Kim & Breuer (2008) to study the interactions in a synthetic community of three strains of soil bacteria. The three strains were separated physically, but could exchange solutes through a porous membrane that allowed diffusive transport among the habitats of each strain (Fig. 6b). In bulk liquid, the community was highly unstable. In the microfluidic device, "fences made good neighbors": when the wells were placed at intermediate distances from each other, the three populations stably coexisted. These studies illustrate how the integration of membranes into microfluidic devices opened an important window for the study of microbial chemical communication in the absence of physical interaction.

8.2 Predation

An application of microfluidics to microbial ecology that is still in its infancy, yet has great future potential, is the ability to readily visualize individual inter-species cell-to-cell

interactions, in particular predator-prey and virus-host interactions. For example, Park et al. (2011) exploited the facile generation of gradients in microfluidic devices to study predator-prey interactions among bacteria. They used a microfabricated array of picoliter chambers to study the interactions of the bacterial predator *Bdellovibrio bacteriovorus* with its prey, *E. coli*. Integrating two existing microfluidic approaches in novel ways - a Christmas tree gradient generator (Jeon et al. 2000) to create a gradient of prey concentration and an array of arrow-shaped ratchets (Hulme et al. 2008) to sequentially concentrate the predator and the prey cells - resulted in a systematic study of predation rates as a function of the initial concentrations of predator and prey cells.

Microfluidics has also begun to benefit the study of virus-host interactions, providing highly controlled environments that help overcome the challenge of studying the extraordinarily small-scale processes surrounding viral interactions with their hosts (at 100 nm in size, viruses are smaller than the wavelength of visible light) (e.g., Walker (2004)). Advances have been made through the use of devices that allow for trapping and imaging of viruses as well as real-time monitoring of host infection. For example, incorporating dielectrophoretic filters within microfluidic chips has enabled the capture of viral particles from a flowing system and their real-time imaging (Akin et al. 2004), a useful technique for separating and concentrating viruses from mixed communities. Multi-layer devices have been employed to regulate the delivery of viruses to a host population and to image the infection dynamics in real time (Cimetta et al. 2012, Xu et al. 2012).

A major open challenge in microbial ecology is how do we gain a better understanding of the > 99% of environment bacteria that are not culturable and the viruses that regulate these populations? An important advance came when Tadmor et al. (2011) adapted a droplet-based microfluidic PCR method to probe viral infections of uncultured bacteria. By amplifying viral markers (using degenerate primers) and bacterial genetic markers (SSU rRNA gene) from single cells, the authors were able to sequence amplicons and identify host-viral pairs directly from environmental samples. They discovered remarkably structured and specific host-virus relationships, despite the fact that these organisms came from a mixed assemblage in the termite hindgut, where ample opportunity for lateral gene transfer seemingly exists. This application of high-throughput microfluidic PCR opens a new research avenue into the viral-host relationships of unculturable microbes.

9 ANTIBIOTIC RESISTANCE

The effectiveness of antibiotics is one of the major societal challenges of the 21st century, affecting both human health and industries such as agriculture and aquaculture, as greater numbers of microbes develop resistance to antimicrobial compounds. Furthermore, resistance can be transmitted among microbial populations and through different ecosystems, potentially heightening the exposure of human and wildlife populations to such organisms. A range of microfluidic devices has opened a new door for the high-throughput evaluation of the effects of antibiotics on bacteria. Here we focus on selected advances and refer the reader to a recent review for more information (Guo et al. 2012).

Microfluidic-based methods for determining minimum inhibitory concentrations (MICs) have included a wide range of designs, from microwells containing pre-loaded antibiotic

compounds into which cells are added (Cira et al. 2012) to droplet-based systems (Churski et al. 2012) and agarose microparticles (Eun et al. 2011). The effect of antibiotics on cellular repair mechanisms was studied by immobilizing cells on surfaces (Kalashnikov et al. 2012) or in agarose (Choi et al. 2013) within microfluidic chambers. The consequences of low doses of antibiotics on cell morphology during growth have been studied in high-throughput devices made of multiple channels, each equipped with dozens of growth microchambers (Sun et al. 2011).

Within any isogenic population of bacteria there can be dormant cells that tolerate antibiotics, called persisters, which are likely an important reservoir for chronic and recurring infections. A handful of microfluidic studies have recently tackled the question of how these persisters arise within a population. A microfluidic chemostat allowed Vega et al. (2012) to identify the critical role that bacterial chemical communication plays in the formation of persisters. These authors observed that E. coli cells exposed to physiological levels of a chemical signal that affects cellular stress responses, indole, exhibited one order of magnitude higher levels of persistence. By engineering a mutant strain with a fluorescent reporter for this pathway of chemical sensing, the authors could track the levels sensed by individual cells in the microfluidic chemostat and correlate them with that cells subsequent ability to survive antibiotic treatment. This revealed that indole sensing is heterogeneous within a population and plays a significant role in the formation of persister cells.

The growth dynamics of *E. coli* persisters were analyzed at the single-cell level by Gefen et al. (2008) using a microfluidic device that allowed them to modulate the supply of growth media and antibiotics over time. Cells were held in a microfluidic chamber that allowed for continuous supply of growth media and real-time imaging. Using fluorescent reporters the authors observed that persisters continued to synthesize proteins for 90 min at the beginning of what was previously believed to be a dormant stage, and that this phase represented a time window when persisters were vulnerable to antibiotics. This window of vulnerability may prove important for clinical treatment of persistent infections. The observation of single cells under controlled environmental conditions has also led to a degree of predictability on which cells become persisters. Balaban et al. (2004) used a multi-layer microfluidic device to supply nutrients and antibiotics from one layer through a transparent membrane into a second layer, containing an E. coli population arranged in grooves akin to what done in the 'mother machine' (Fig. 4b). The authors were able to predict which cells would become persisters before antibiotics were introduced based on a phenotypic switch in growth rate: cells that eventually became persisters grew more slowly and passed on the slow growth phenotype to daughter cells for several generations. This work has shown that persistence can be a phenotypic switch - gained and then lost again within a single cell lineage - rather than a result of genetic heterogeneity as in the case of antibiotic resistance.

The creative use of microfluidics is broadening our understanding of how environmental conditions and habitat niches influence the development of resistance, a major open question pertaining to the effectiveness of antimicrobials. This is exemplified by the work of Zhang et al. (2011), who used a hexagonal device comprised of 1200 hexagonal wells (Fig. 5e), connected to each other through small corridors and ventilated along the periphery by nanoslits for nutrient and waste exchange and antibiotic dosing. This landscape of

microhabitats, containing both nutrient and antibiotic gradients, resulted in 'Goldilocks points', i.e., wells where conditions were just right for selecting for resistant bacteria. Without the antibiotic gradient, no resistance emerged. At Goldilocks points, resistance developed in as little as ten hours and four single-nucleotide polymorphisms attained fixation responsible for the resistance.

Acknowledgments

ABBREVIATIONS

PDMS	Polydimethylsiloxane
DMSP	Dimethylsulfoniopropionate
EPS	Extracellular polymeric substances
QS	Quorum sensing
AI	Autoinducer molecule
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
GFP	Green fluorescent protein
MIC	Minimum inhibitory concentration

DEFINITIONS

Shear rate	Spatial gradient in fluid velocity. The shear stress is the resulting shear force per unit area on a surface
Chemotaxis	Ability of an organism to sense a chemical gradient and bias its motility accordingly
Ratchet	Device or mechanism that favors motion in one direction of an otherwise isotropically moving organism
Biofilm streamers	Tethered and suspended filamentous aggregations composed of bacteria and the extracellular matrix they secrete
Microhabitat	Confined, microscale volume of fluid within which microbes typically experience uniform environmental conditions
Quorum sensing	A form of chemical communication among microbes occurring when secreted signaling molecules exceed a threshold concentration

| **Persisters** | Cells within a population that survive antibiotic treatment without genetically acquiring resistance |

10 LITERATURE CITED

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SUMMARY POINTS

1. The study of microbial navigation and in particular chemotaxis is an area of microbial ecology that has substantially capitalized on microfluidic technologies, in large part through the tracking of single cells within controlled, spatio-temporally varying gradients created by incorporating porous materials in the microfabrication. This approach has enabled studies that closely mimicked natural environmental conditions and has shed light on a plethora of bacterial behavioral responses, from logarithmic sensing to adaptation.

2. A distinguishing feature of microfluidics is the flexibility to fabricate unconventional geometries and topographical features such as corners, funnels, corridors, nanoslits and posts. This capability has allowed researchers to examine and control the emergence of patterns and self-organization, in particular in the context of interactions with boundaries, adhesion to surfaces and the rectification of bacterial motion.

3. The ability to confine single cells or small populations in highly controlled microenvironments has opened new avenues into biological and ecological questions on growth, morphogenesis, inheritance, cell signaling, and antibiotic resistance, and has provided novel tools to culture rare or mixed populations of cells.

4. The ability to directly visualize the dynamics of single or multiple populations of microbes and to segregate populations physically while allowing them to communicate chemically (achieved through membranes, hydrogels or nanoslits) has provided a new window into the role of spatial heterogeneity on coexistence and cooperation.

5. The ability to impose and manipulate multiple gradients at once using microfluidic technologies has resulted in studies of microbial ecology within environments that reproduce with increasing accuracy the essential physical, chemical and biological features of natural microbial habitats, from the ocean to soils to the human body.

6. These studies exemplify how microfluidics applied to microbial ecology provides a level of environmental control and a richness of data that is nearly impossible or exceedingly time-consuming to obtain in ecological studies at larger scales, and indicates that - when paired with microfluidic arenas - microbial ecology can serve a considerably more powerful function in developing and testing general ecological theories than has occurred to date.

FUTURE ISSUES

1. The navigation, response to chemical cues, and adaptation processes are unknown for most bacteria other than selected model systems, such as E. coli. Can microfluidic platforms facilitate the acquisition of quantitative information about a broad range of microbes and environments to understand the diversity and impacts of microbial navigation and sensing behaviors?

2. Microfluidic studies of microbial interactions with fluid flow and with confining surfaces, two features that are ubiquitous in natural microbial habitats and are easily mimicked through microfabrication, are still in their infancy and promise many new insights into microbial ecology and potentially practical solutions to challenging problems such as biofouling.

3. Microfluidic devices have been underutilized for the study of biofilm formation and development, a complex topic that can greatly benefit from the ability to control the local environment and obtain real-time data at the single-cell level.

4. Exploiting microbes to perform microscale tasks has been elegantly demonstrated in proof-of-concept experiments: can these dynamics be harnessed and implemented in engineering systems?

5. Microbial ecology will greatly benefit from the continued integration of microfluidics with advanced optical methods and spectroscopy techniques to further probe behavioral and metabolic responses of cells under known environmental conditions.

6. A bottleneck for the widespread diffusion of microfluidic technologies to biological and ecological studies is the engineering element in their fabrication and the physics element in the control of flow and transport. In addition to interdisciplinary collaborations, simplified and easily integrated devices with standardized analysis tools will enable a broader cross-section of researchers to take advantage of this powerful approach to microbial ecology.
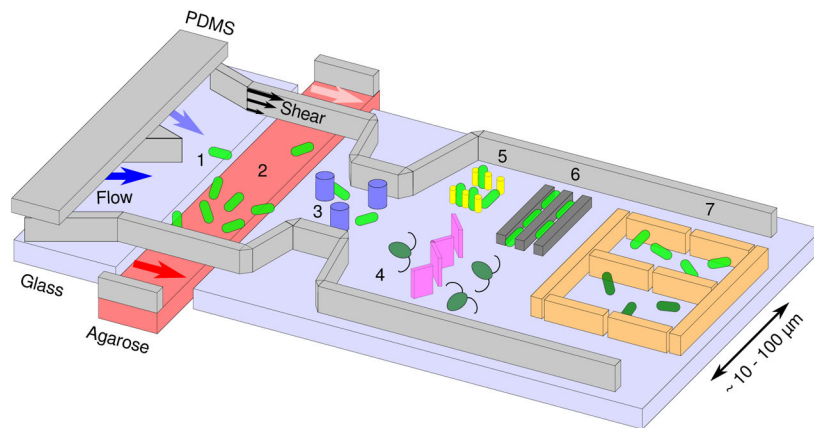
Figure 1. Microfluidics provides a powerful platform for microbial ecology studies
Multiple features of natural microbial habitats can be included in microfluidic studies of the life of microbes: Multiple-inlet (1) and the incorporation of hydrogels (e.g., agarose; 2) to impose spatial gradients; constrictions and topological features to study the effect of flow in porous-like environments (3); funnel-shaped barriers to separate and concentrate swimming microbes (4); microfabricated topography to study bacterial adhesion (5); single-cell confinements to study growth and replication (6); population confinements to investigate competition and population dynamics (7).
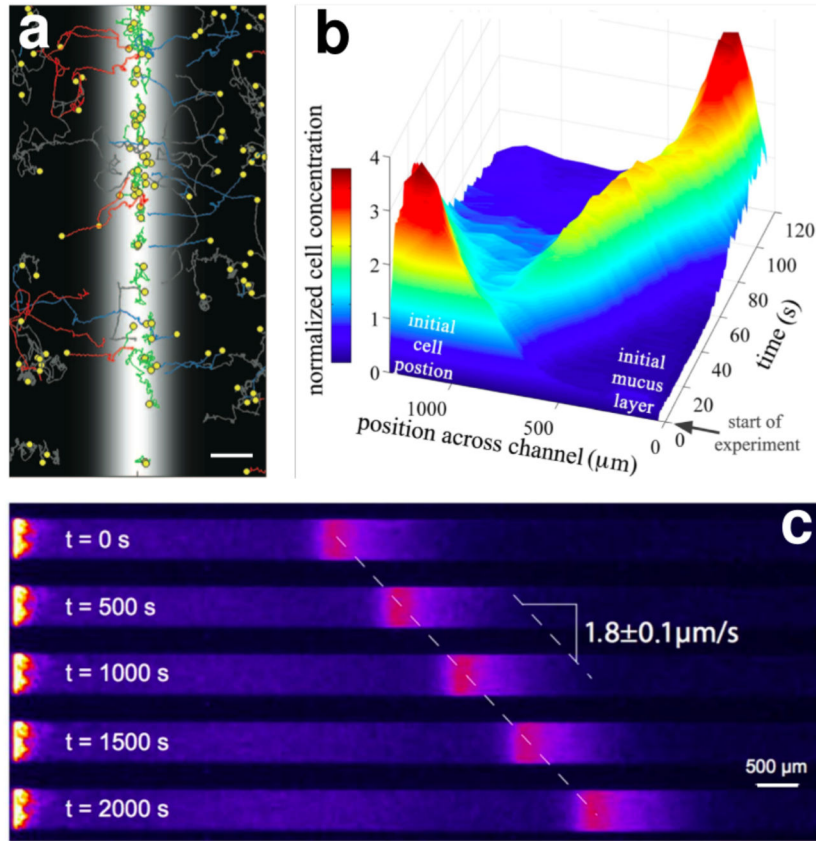
Figure 2. Chemical gradients reveal microbial navigation strategies

a) Swimming trajectories of the dinoflagellate *Oxyrrhis marina* in response to a pulse of chemoattractant (grayscale background). Figure modified from Seymour et al. (2010b) with permission. b) Time evolution of the spatial distribution of a pathogen population (*Vibrio coralliilyticus*) exposed to a diffusing coral mucus (*Pocillopora damicornis*) gradient in a microfluidic channel. Figure modified from Garren et al. (2013) with permission. c) Experimental pulses of *Escherichia coli* traveling across a microfluidic channel at a constant propagation speed. Figure modified from Saragosti et al. (2010) with permission. Scale bars, 500 *μ*m.
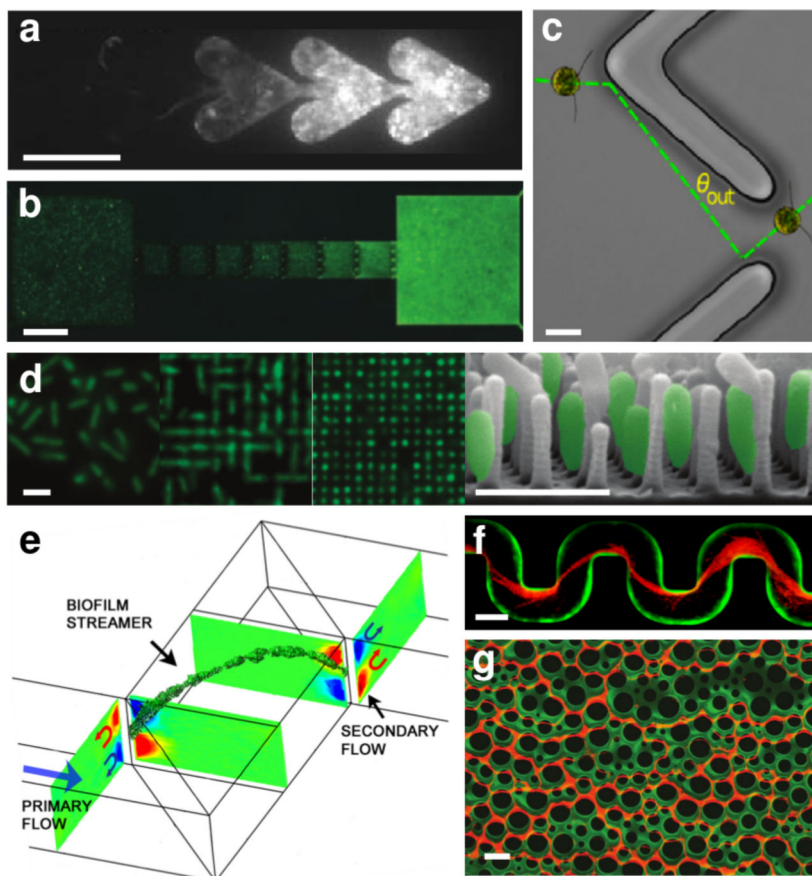
Figure 3. Fine control over microscale topography sheds light on microbe-surface interactions
a) Fluorescent *Escherichia coli* cells trapped and concentrated in arrow-shaped microfluidic ratchets. Figure modified from Hulme et al. (2008) with permission. b) Series of funnel arrays used to separate and concentrate motile from non-motile *Escherichia coli* cells. Figure modified from Galajda et al. (2007) with permission. c) Rectification of algal cells (*Chlamydomonas reinhardtii*) locomotion in microfluidic ratchets via secondary scattering. Figure modified from Kantsler et al. (2013) with permission. d) Fluorescent images (left-hand side) of bacterial cells, *Pseudomonas aeruginosa*, adhering to structured surfaces at decreasing (from left to right) spacing between posts, and a cross-sectional SEM image of the same process (right-hand side; cells are false-colored to highlight their orientation). Figure modified from Hochbaum & Aizenberg (2010) with permission. e) Pictorial visualization of the mechanism underlying the formation of *Pseudomonas aeruginosa* biofilm streamers (rendering from confocal images) in curved microchannels. Figure modified from Rusconi et al. (2011) with permission. f) *Pseudomonas aeruginosa* biofilm streamers highlighted by the subsequent injection of red-fluorescent cells. Figure modified from Drescher et al. (2013) with permission. g) Patterns of biofilm growth (*Escherichia coli* auto-fluorescence in green) and fluid flow (in red, slightly shifted to the right) in a microfluidic device modeling a porous soil environment. Figure modified from Durham et al. (2012) with permission. Scale bars, 2 μm (d), 20 μm (a,c), 200 μm (b,f,g).
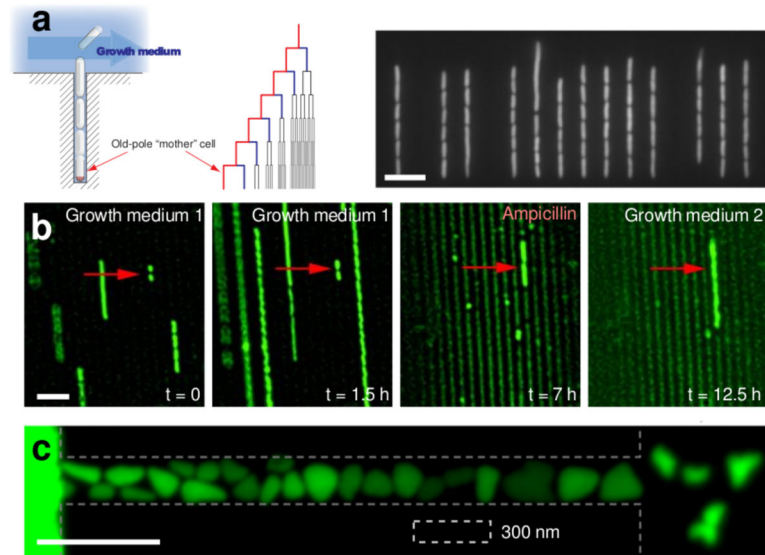
Figure 4. Single-cell microconfinementa open new doors for understanding growth and persistence

a) Schematic representation, lineage tree, and snapshot of the microfluidic "mother machine". Figure modified from Wang et al. (2010) with permission. b) Time-lapse images of a high-persistence mutant of *Escherichia coli* growing in a microfluidic chamber and exposed to ampicillin, showing the location of slowly-growing persisters (red arrows). Figure modified from Balaban et al. (2004) with permission. c) Morphogenesis of *E. coli* in ultra-thin microfluidic channels (dashed lines show approximate boundaries). Figure modified from Männik et al. (2009) with permission. Scale bars, 10 μm.
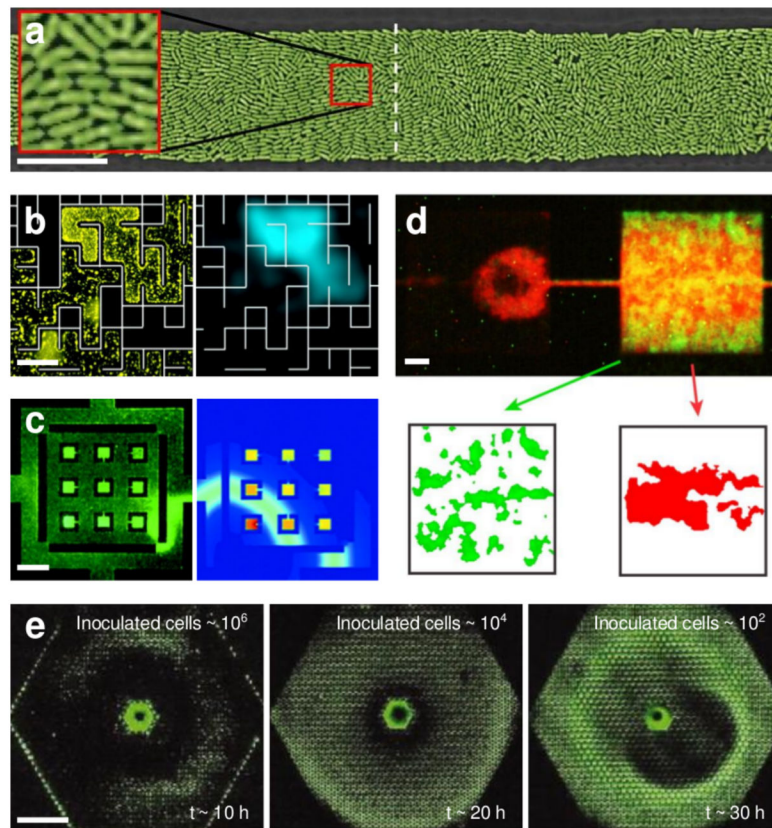
Figure 5. Multiple-cell microconfinements reveal new population-scale microbial processes
a) Bacterial growth and ordering in a quasi-2D microfluidic open channel. Figure modified from Volfson et al. (2008) with permission. b) *Vibrio harveyi* accumulation in a microfabricated maze inducing quorum sensing (dark-field image on the left; photon-counting image of the intrinsic quorum sensing luminescence on the right). Figure modified from Park et al. (2003b) with permission. c) Wild-type *Escherichia coli* collapsing into confining microfluidic chambers (experiments on the left; simulations on the right). Figure modified from Park et al. (2003a) with permission. d) Fluorescence images of competing *Escherichia coli* populations (GASP mutant in red; wild-type cells in green) in microhabitat patches. Figure modified from Keymer et al. (2008) with permission. e) Large array of connected microchambers showing the rapid emergence of antibiotic resistance within an antibiotic gradient for different inoculation densities and different times. Figure modified from Zhang et al. (2011) with permission. Scale bars, 20 μm (a,d), 200 μm (b), 500 μm (c), 5 mm (e).
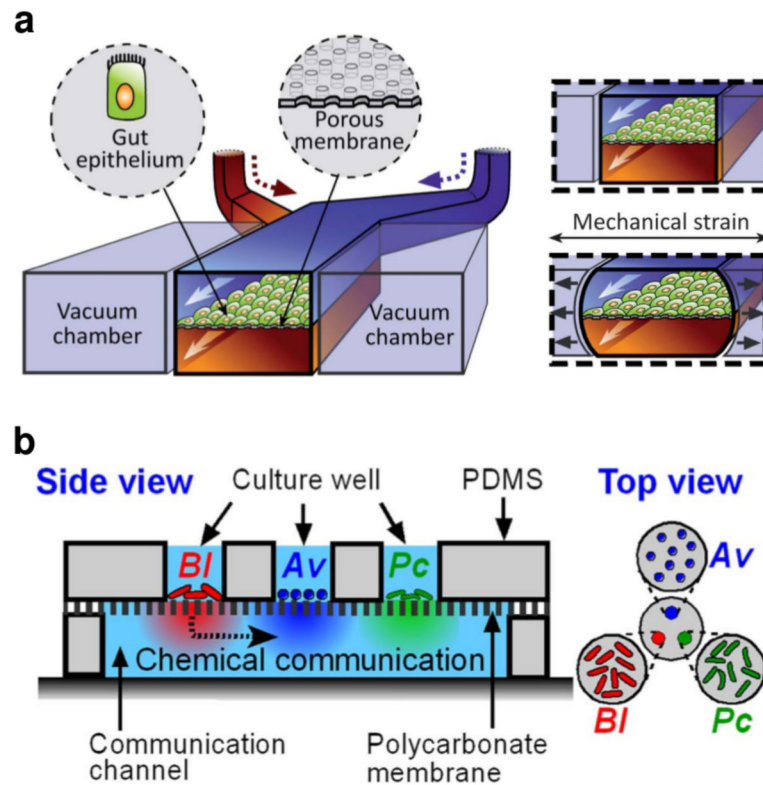
Figure 6. Multi-layer microfluidic devices allow co-culturing
a) Schematic drawing of the gut-on-a-chip device showing the porous membrane lined by gut epithelial cells with or without mechanical strain exerted by suction. Figure modified from Kim et al. (2012b) with permission. b) Schematic drawing of the microfluidic device used to co-culture three species of soil bacteria by imposing spatial structure on three culture wells and providing a chemical communication channel. Figure modified from Kim et al. (2008) with permission.