

Microfabrication meets microbiology

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Abstract | This Review summarizes methods for constructing systems and structures at micron or submicron scales that have applications in microbiology. These tools make it possible to manipulate individual cells and their immediate extracellular environments and have the capability to transform the study of microbial physiology and behaviour. Because of their simplicity, low cost and use in microfabrication, we focus on the application of soft lithographic techniques to the study of microorganisms, and describe several key areas in microbiology in which the development of new microfabricated materials and tools can have a crucial role.

Microtechnology

The fabrication and application of materials, structures and systems with micron or submicron-scale features.

Microenvironment

The region sensed by a cell. The dimensions of this region are usually set by molecular contact, mass transport and diffusion, and range from a few nanometers to perhaps a millimeter.

Soft lithography

A set of techniques that makes microstructures by printing, moulding and embossing using a patterned, elastomeric stamp or mould, and/or a polymeric substrate.

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The field of microtechnology is beginning to impact on microbiology. Its scale of size is well matched to the physical dimensions of most microorganisms, and micron-scale tools make it possible to manipulate individual cells, their immediate extracellular environments (referred to as the microenvironment) and ultimately, their shape and internal organization. This Review describes the intersection of microtechnology and microbiology, with a focus on soft lithography — a set of techniques that are particularly well suited for generating microscale or nanoscale structures in soft materials¹. Soft lithography is a simple technique, and is available to most microbiologists either for their own use, or through shared facilities (public foundries)². This Review is designed to bridge the divide between biologists who study microbial life, and physical scientists and engineers who make new materials and structures.

New tools bring new capabilities to research. In microbiology, genetic techniques revolutionized our understanding of microorganisms by making it possible to manipulate and study their genomes. We believe that the development of physical techniques, including those based on microstructures, will lead to tools that are complementary to genetics and genomics, including new techniques to isolate, manipulate, grow and study single cells and multicellular structures. A number of global problems in microbiology will benefit from the development of these tools, including: the evolutionary origin of cells; the cell as a dynamic, out-of-equilibrium system; intracellular organization and cellular replication; mechanisms of communication between cells; the organization and characteristics of multicellular structures; mechanisms of differentiation and behaviour; and new techniques for the isolation and culturing of microbial strains.

Opportunities in microbiology

To begin a discussion on the representative questions in microbiology that can be addressed using microfabricated tools, we have selected several broad areas of microbiology. In each section, we suggest areas in which new tools and capability are needed and suggest opportunities for the application of soft lithographic techniques.

Structure and intracellular organization of bacteria.

Included among the physical and chemical properties that characterize bacteria are: a specific shape and morphology, organized subcellular components, tightly regulated mechanisms of growth and division, machinery for transporting intracellular cargo (proteins, plasmids and DNA), and mechanisms of polarization^{3–8}. The most fundamental questions regarding the structure of bacteria — how they produce, maintain and replicate their spatial organization — remain unresolved at the molecular level.

The development of new techniques for manipulating the shape of bacteria can complement experimental approaches using genetics and biochemistry. These techniques will make it possible to study the control of cell shape, the relationship between the shape of cells and the organization and function of intracellular machinery, the mechanical properties of cells and the role of physical forces in cellular differentiation. Soft lithography makes it possible to fabricate structures with length scales that match the intrinsic scale of bacterial cells. These techniques are just beginning to be exploited to control and study cell shape.

Phenotypic behaviour. We now appreciate that soluble small molecules and peptides can cause bacterial cells to differentiate into various phenotypes. Many strains of bacteria use small molecules to coordinate and synchronize their behaviour⁹. Some strains grow

and organize into multicellular structures — colonies, biofilms, spores, mycelium and fruiting bodies — that have the characteristics of multicellular organisms; that is, the cells behave in a collective manner in which a division of cellular labour increases access to resources or provides protection and improves the probability of survival against competitors^{10,11}. Although recent advances have revolutionized our understanding of this area of microbiology, we are still just beginning to understand the chemical and physical basis of social interactions between cells.

New tools for controlling the chemical and physical microenvironment surrounding cells will enhance our understanding of the behaviour of bacteria. Related areas in eukaryotic biology have been studied using the techniques of soft lithography with excellent success^{12,13}.

Cell culture. The global diversity of bacteria has been estimated at 10^7 – 10^9 species¹⁴. Only a tiny fraction of these species have been isolated and cultured using traditional microbiological techniques¹⁵. Remarkably, the core set of techniques used for microbial culture has changed little since their development by Koch, Beijerinck and Winogradsky in the nineteenth century; these techniques are, unfortunately, incompatible with the cultivation of most microorganisms.

A detailed understanding of the chemical and physical environment surrounding cells, including the role of abiotic surfaces, stress, temperature and the diffusion of molecules (gas, nutrients, communication substances and metabolic waste) on their homeostasis and replication, will contribute to the development of new techniques for culturing microorganisms. Microfabricated structures that control the local microenvironment of microbial cells might improve culture technology, and contribute to general and medical microbiology. Networks of microchannels could eventually bypass the need for microbial culture by making it possible to perform genetic and biochemical analyses on single cells that are part of environmental communities.

The biology of single cells. The field of microbiology was built largely on studies of the behaviour and biochemistry of populations of cells. Measurements based on ensemble averaging cannot characterize the differences between individual cells. We now appreciate that individual cells — even within a clonal population — can differ from each other genetically, biochemically and behaviourally; however, in many examples, we do not understand the significance of these differences¹⁶. Studies on the behaviour and genetics of individual microbial cells are clarifying our understanding of horizontal gene transfer, chemical signalling, pathogenesis, motility, chemotaxis, microbial viability and persistence, the interaction of cells with surfaces and, in general, the relationship between genetics, biochemistry and behaviour¹⁷. The development of microstructures for isolating and studying single cells will improve our understanding of the structural, biochemical and genetic variation between cells.

Quantitative microbiology. Much of biology is based on qualitative data. In microbiology, many of the techniques that are used — for example, streaking cells and studying their structure and behaviour using optical microscopy — were not designed with reproducibility or quantification in mind. These methods make it difficult (or impossible) to collect data that is quantitative, time-dependent or spatially resolved in experiments in which individual cells, or collections of cells, are studied repeatedly under identical conditions. The development of techniques that facilitate the collection of statistically significant data will accelerate the transformation of microbiology from a science based on qualitative observation into one based on the quantitative analysis of data. One area in which microfabrication can have an important role is in the design and fabrication of structures on which thousands of parallel experiments with single cells — or small groups of cells — can be carried out under identical conditions. Another area in which small structures can impact microbiology is the use of microchannels to transport, mix and deliver fluids to cells. The physics of fluids at microscopic dimensions is predictable and makes it possible to design and carry out experiments under well defined and reproducible conditions. Bacterial chemotaxis is one of many areas of microbiology that can benefit from techniques that take advantage of the properties of fluids at small dimensions.

Systems biology is another emerging field that builds models of biomolecular interactions and networks in cells and organisms, and uses these models to describe biological processes¹⁸. It requires an understanding of the quantitative expression of genes under specific conditions, and of the intracellular location and concentration of metabolites and proteins, and is one field that has much to gain from advances in quantitative microbial biology. Microfabrication has the opportunity to play an important part in the development of new tools and techniques that allow quantitative measurements at the cellular level¹⁹.

New tools open new doors

Many of the techniques that are now used to isolate, purify and study microorganisms have not changed significantly since their initial development. Although new techniques for studying microorganisms have emerged, including mass spectrometry, capillary (gel) electrophoresis, and optical, electron and scanning probe microscopy, the use of these techniques still lags behind genetic and genomic approaches. We believe that new developments in physically based techniques will make it possible to study microorganisms at an unprecedented — even revolutionary — level of detail. Below we describe a set of tools for microfabrication, a key component in this impending revolution, known collectively as ‘soft lithography’. These tools have characteristics that make them particularly useful in addressing a number of the important research questions in microbiology.

Soft lithography and microfabrication

Soft lithography refers to a collection of techniques for creating microstructures and nanostructures based on printing, moulding and embossing¹. The techniques

Soft material

A material (especially a polymer) that is pliable, compressible or elastic.

Public foundry

A facility for the fabrication of microstructured materials and systems.

Elastomeric polymer

A soft, compliant, rubber-like polymer.

Mask

Typically a transparent substrate with a pattern on its surface defined in an opaque material (chrome metal or ink) used in photolithography.

PDMS

Poly(dimethylsiloxane). An elastomeric silicone polymer that is commercially available and has properties that make it well suited to applications in microbiology.

Embossed structure

A structure that is moulded in a surface in relief.

Bas-relief structure

A structure that projects away from a surface.

Bas-relief master

Refers to the master copy and, in soft lithography, consists of patterns of a photoreactive polymer on the surface of a silicon wafer or glass slide.

Photolithography

A process used to transfer a pattern from a mask onto a thin film of photosensitive polymer (photoresist) and then onto the surface of a substrate. Photolithography is commonly used in semiconductor fabrication to fabricate integrated circuits.

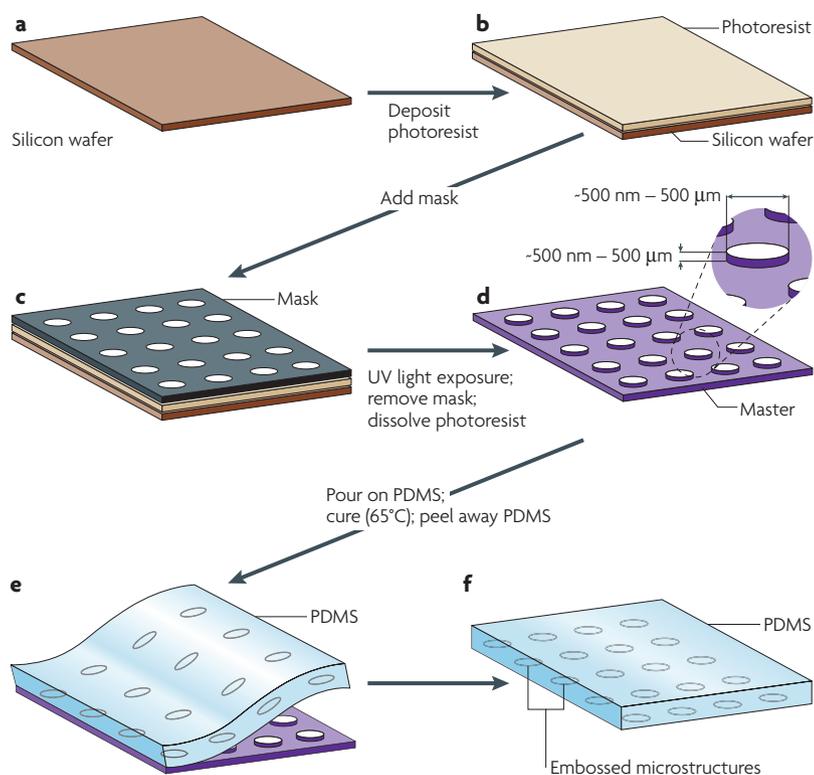


Figure 1 | The fabrication of micropatterned slabs of PDMS. a–b | Photoresist is spin-coated on a silicon wafer. **c** | A mask is placed in contact with the layer of photoresist. **d** | The photoresist is illuminated with ultraviolet (UV) light through the mask. An organic solvent dissolves and removes photoresist that is not crosslinked. The master consists of a silicon wafer with features of photoresist in bas-relief. An expanded view of one of the microfabricated structures with its characteristic critical dimensions is shown. **e** | PDMS is poured on the master, cured thermally and peeled away. **f** | The resulting layer of PDMS has microstructures embossed in its surface. PDMS, poly(dimethylsiloxane).

were developed as an alternative to photolithography and electron-beam lithography, and share the name ‘soft lithography’ because they are all based on using a patterned elastomeric polymer as a mask, stamp or mould, to pattern ‘soft materials’ (for example, polymers, gels and organic monolayers, rather than silicon and glass). The tools of soft lithography are being used with increasing frequency in cell biology because of their simplicity, low cost and compatibility with cells². This section of the Review introduces the core set of soft-lithographic techniques, and describes the characteristics that make them useful in microbiology. This section will not provide an exhaustive review on soft lithography nor will it describe other techniques for microfabrication; other reviews provide more detail on these and related subjects^{1,20,21}.

An introduction to soft lithography

All of the techniques in soft lithography use, as their central component, a layer of poly(dimethylsiloxane) (PDMS), or another polymer with similar characteristics, with embossed structures or bas-relief structures on the surface. The layer of PDMS is fabricated from a bas-relief master by embossing, and typically has features with lateral dimensions of 1–1000 μm and vertical dimensions between 100 nm and hundreds of microns

— the master is produced using photolithography. Y. Xia and G.M.W. have described the procedure for creating microstructures in detail¹; the relevant steps are summarized in FIG. 1.

A pattern is drawn using a CAD tool (computer-aided design). Patterns that have features with lateral dimensions >8 μm are printed on a transparency (a sheet of transparent polymer) using a high-resolution photoplotter²²; the resulting transparency functions as a mask for photolithography. For designs with features that have lateral dimensions <8 μm, the pattern is transferred to a thin layer of metal (usually chrome) on a glass slide using a laser or an electron-beam mask writer. The mask is placed between a source of ultraviolet light and a thin layer of a photoreactive polymer referred to as a photoresist that has been coated on a silicon wafer or glass slide. Exposure to UV light transfers the pattern from the mask to the polymer; the unexposed photoresist is removed using an organic solvent, leaving behind the bas-relief structure in the polymer layer. A silicon wafer with polymeric photoresist structures patterned on its surface is typically referred to as the master copy, or simply the master. The height of the polymer structure is controlled by the thickness of the layer of photoresist that is initially spread on the surface of the wafer; the mask controls the shape and lateral dimensions. New approaches are being developed to reduce both the cost and number of steps required to produce masters; these techniques will make it possible to create microstructures using equipment that is routinely available in many laboratories.

One useful characteristic of soft lithography is that hundreds of replicas of the inverse pattern can be produced in PDMS from one master; this technique (replica moulding) is described in detail in the next section. To make PDMS replicas, the surface of the master is initially treated with a perfluoralkyl trichlorosilane to prevent the PDMS from sticking to the master. The liquid prepolymer of PDMS is mixed, degassed and poured on the surface of the master. After curing thermally (at 65 °C), the layer of PDMS is peeled away and contains the inverse of the original pattern embossed on the surface of the master.

Although each replica takes approximately two hours to prepare, the time can be reduced by curing the layer of PDMS at a temperature >65 °C. A useful characteristic of soft lithography is that it is possible to create prototype structures rapidly²³. In many cases, the entire process — from a CAD design to a completed microfabricated structure in PDMS — requires less than twelve hours. The resulting topographically patterned layers of PDMS can be used as microchambers for isolating and culturing cells, as stamps for patterning cells on surfaces, or as masters for replicating the structure in another material. The structures can also be sealed against flat surfaces and used as microfluidic systems. These applications and others are discussed in more detail in the next section.

PDMS has been the most widely used material for the applications of soft lithography in microbiology, and in biology in general, because of the following characteristics: it is soft, flexible, biocompatible, insulating, unreactive, transparent to ultraviolet and visible light,

CAD tool

Computer aided design. A software program used by engineers and designers for drafting two- and three-dimensional structures.

Photoresist

A photoreactive polymer that undergoes chemical changes that lead to changes in physical properties (such as solubility) after exposure to ultraviolet light.

Microfluidic system

A set of channels that have micron-scale dimensions (typically between 5–500 μm), and are used to manipulate fluids.

permeable to gases and only moderately permeable to water^{2,24}. The prepolymer of PDMS is commercially available, inexpensive, and easy to prepare. It is prepared by mixing two liquid components together, the base and curing agent, followed by degassing to remove air bubbles; the procedure typically takes less than 30 minutes.

Public foundries have been established at several universities (including Harvard University, Stanford University, California Institute of Technology and the University of Washington) to make the techniques of soft lithography available to the biology community. These foundries are sites for training users in the techniques of soft lithography, and they also provide the materials and tools of soft lithography to scientists who want to use the techniques but do not want to be trained in their fabrication; in this capacity, foundries can produce limited numbers of PDMS stamps, moulds or microfluidic systems. Users send their designs and specifications to a foundry and receive masters and replica copies of the masters in PDMS for a reasonable price and without any capital investment in equipment.

In the following sections, we introduce several of the core techniques of soft lithography and describe recent applications of these techniques to the study of microorganisms. Rather than providing a comprehensive review of the literature, we have chosen examples that illustrate the range of applications of these techniques to microbiology.

The tools of soft lithography

Replica moulding. Replica moulding is a technique for duplicating the shape, size and pattern of features on a master and provides a method of patterning a wider range of materials than is possible using photolithography¹. In contrast to photolithography, replica moulding duplicates three-dimensional structures in a single step. A topographically patterned layer of PDMS typically functions as the mould in this technique, and is used to transfer the pattern to the surface of another polymer (FIG. 2a). A prepolymer is deposited on the PDMS mould by casting or spin coating, is cured, and then separated from the master by peeling them apart. This technique has been used to micro-pattern biocompatible polymers, including epoxies, polyurethanes, polyethylene glycol (PEG), agar and agarose. The ability to create micropatterned agar and agarose should be especially useful in microbiology since these materials are so widely used as substrates for isolating and culturing bacterial cells.

Microcontact printing. Microcontact printing transfers patterns from the surface of a topographically patterned PDMS stamp to the surface of a substrate¹ (FIG. 2b). Various 'inks', including small biomolecules, proteins, polyelectrolytes and suspensions of cells, can be patterned directly on surfaces using microcontact printing²⁵⁻²⁷. In this technique, contact between the PDMS stamp and a substrate only transfers the 'ink' from the raised surface of the bas-relief features of the stamp to the substrate, and produces patterns with feature sizes as small as 100 nm over areas as large as ~1 m². The elasticity of the PDMS stamp facilitates

conformal contact between the stamp and the substrate, and makes it possible to pattern non-planar surfaces (including porous, rough or curved surfaces).

Self-assembled monolayers (SAMs) are formed when PDMS stamps 'inked' with alkanethiols (SH-(CH₂)_n-X) are used to microcontact print on surfaces of gold, silver, palladium, platinum or other metals²⁸. SAMs are structures produced by the spontaneous chemisorption and self-organization of alkanethiols. Alkanethiols with long alkyl chains (n = 16 or 18) form hydrophobic monolayers, whereas those with different terminal functional groups (X) can form hydrophilic, hydrophobic or charged SAMs. Many synthetic methods are available for attaching ligands to functional groups (X) on SAMs²⁸.

Another useful characteristic of microcontact printing is that it can be used to pattern surfaces with multiple SAMs. After a SAM of an alkanethiol is patterned on a gold-coated surface by microcontact printing, the substrate is dipped into a solution of a second alkanethiol to form a different SAM on bare regions of the metal surface that were not in contact with the stamp. This technique has been used to make islands of SAMs that adsorb proteins and cells, and which are surrounded by SAMs that resist the adsorption of biomaterial. Patterns of SAMs have been used to study the adhesion of mammalian cells on surfaces². PDMS stamps have also been used to create patterns of proteins, polyelectrolytes and other molecules on glass, polystyrene or silicon^{26,29}. Agarose stamps made by replica moulding from PDMS moulds have been used to microcontact print patterns of proteins on glass substrates³⁰.

Micromoulding in capillaries. Micromoulding in capillaries (MIMIC) is a technique that uses a typographically patterned layer of PDMS in contact with a surface to form a network of microchannels¹. The layer of PDMS is brought into conformal contact with a solid substrate, such as a glass slide, and liquid prepolymer is placed at the edge of the PDMS layer (FIG. 2c). The prepolymer is drawn into the channels by capillary action or by suction, and is subsequently cured to produce solid structures. By peeling the stamp away carefully, a pattern of micropatterned material is left on the substrate surface.

Microtransfer moulding. Microtransfer moulding is a technique for patterning materials in which a thin layer of a liquid prepolymer is applied to the patterned surface of a PDMS stamp¹ (FIG. 2d). The excess prepolymer is removed by scraping the surface with a flat block of PDMS or by blowing N₂ over the surface. The stamp, with its features filled with prepolymer, is brought into conformal contact with a surface, and the prepolymer is cured. When the stamp is peeled away, patterned microstructures remain on the surface of the substrate. The stamp can be refilled and used to pattern more substrates. This technique produces three-dimensional structures with feature sizes as small as ~1 μm in a single step.

Microfluidics. Elastomeric polymers such as PDMS have been widely used for fabricating microfluidic channels and networks^{31,32}. Microfluidic channels

Spin coating

A process for depositing uniform layers of polymer on a substrate. Rotating the substrate at a high speed spreads the material uniformly over the surface. The viscosity of the material and the rotational velocity of the substrate control the thickness of the layer of material; surface tension flattens the surface of the spun film.

SAMs

Self assembled monolayers. Monolayer structures formed by the spontaneous self-assembly of alkanethiols on metal surfaces. In SAMs, the thiol groups are bonded covalently to the metal surface, and the non-covalent, intermolecular packing of the alkane chains causes the molecules to arrange into an ordered, two-dimensional crystal or liquid crystal.

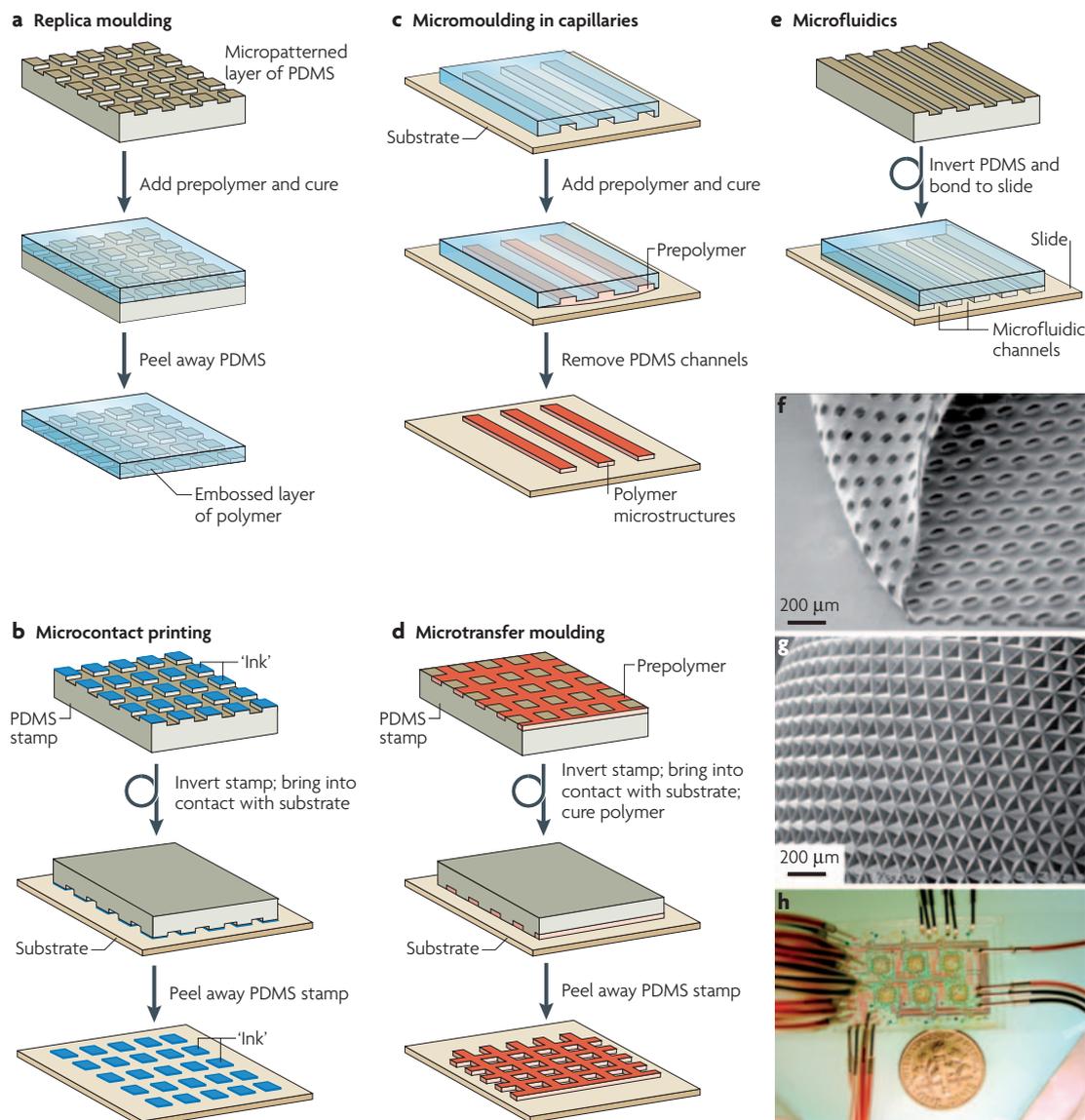


Figure 2 | The core techniques of soft lithography. The key stages of each of the following techniques are shown: **a** | replica moulding; **b** | microcontact printing; **c** | micromoulding in capillaries; **d** | microtransfer moulding; and **e** | microfluidics. **f** | A PDMS membrane with microfabricated holes created by replica moulding from a master with circular posts. **g** | A curved layer of micropatterned polyurethane created by bending a micropatterned layer of PDMS and then replica moulding against it. **h** | A microfluidic chemostat for the growth and culture of microbial cultures. The device incorporates six reactors with an intricate network of plumbing, in a footprint that is approximately 5 cm². PDMS, poly(dimethylsiloxane). Part (**f**) reproduced with permission from REF. 74 © (2000) American Chemical Society. Part (**g**) reproduced with permission from REF. 1 © (1998) Gesellschaft Deutscher Chemiker. Part (**h**) reproduced with permission from REF. 59 © (2005) American Association for the Advancement of Science.

are formed by placing a layer of PDMS with channels embossed on the surface in contact with a glass (or polymer) surface that forms the roof or floor of the channel (FIG. 2e). Channels formed by conformal contact contain a weak physical seal between the two layers of material (the PDMS and a glass slide) and liquid can escape from the channels if the fluid is put under pressure.

PDMS contains surface-exposed methyl groups (-CH₃) and is normally hydrophobic, but its surface can be rendered hydrophilic by exposure to a plasma of air or

oxygen to generate reactive silanol groups (-SiOH) at the surface. Freshly oxidized PDMS that is brought into contact with clean, oxidized glass (or oxidized PDMS) forms an irreversible (covalent) seal through condensation reactions that produce Si-O-Si bonds between the layers. The bonding of PDMS to glass (or PDMS) makes it possible to form sealed microfluidic devices in which fluids can be pumped at pressures as high as 50 psi (~350 kPa) without failure. One interesting characteristic of fluids flowing in microfluidic channels is that the flow is laminar³³. The Reynolds number (Re; dimensionless) describes

the behaviour of fluids. It is defined by $Re = \rho u D_h / \mu$, in which ρ is the density of fluid (g per cm^3), u is the velocity of the fluid (cm s^{-1}), D_h is the hydraulic diameter (cm) of the channel, and μ is the viscosity of the fluid (g per cm per s). When the Reynolds number is less than $\sim 2,000$, fluid flow is laminar; above this value the flow is turbulent. The flow of fluids in microchannels is typically laminar and the velocity of the fluid in a given region of the channel is constant in time. An important aspect of laminar flow in microfluidic channels is that adjacent streams of miscible liquids flow side-by-side, with mixing only by diffusion at the interface between the streams. Networks of branching and recombining microchannels can be used to produce stable gradients of biomolecules^{34–36}. These gradients are perpendicular to the direction of flow, can be generated in solution and on surfaces, and have spatial and temporal stability. As mentioned above, the behaviour of fluids at the micro-scale level is predictable and this characteristic makes it

possible to reproduce experimental conditions that take advantage of liquids moving at a low Reynolds number.

Microfluidic valves. Several groups have used soft-lithographic techniques to develop valves for controlling the flow of fluids and the movement of cells and particles in microfluidic channels. The most frequently used example of this class of structures is the monolithic, pneumatic valves developed by Quake *et al.*^{37,38} Monolithic valves are based on two sets of PDMS microfluidic channels that are perpendicular to each other, and bonded together, one on top of the other. A thin layer of PDMS separates the two layers of channels. One set of channels forms the valves and the other set forms the microfluidic channels in which experiments are carried out. A pressure applied to the valve channels collapses the thin layer of PDMS into the microfluidic channels and controls the flow of fluids. These structures are being used in microbiological studies with increasing frequency.

Applications of soft lithography in microbiology

Immobilizing bacteria on surfaces. The immobilization and patterning of bacteria on surfaces provides new opportunities for sensing and detecting biomolecules using whole cells and for studying cell–cell interactions and interactions between cells and their surroundings.

SAMs have been used to immobilize cells on surfaces in the study of host–pathogen interactions. The covalent attachment of biological ligands to the terminal regions of SAMs provides a way of controlling the density of a ligand on a surface and, importantly, the attached ligands retain their activity. One recent example described the application of SAMs and optical tweezers to measure the force of adhesion between uropathogenic *Escherichia coli* cells expressing type I pili and a mannose-presenting SAM³⁹.

The combination of microcontact printing and SAMs has been used to selectively immobilize bacterial cells on surfaces. For example, Rowan *et al.* used a PDMS stamp to microcontact print patterns of hydrophobic and reactive SAMs on gold to produce ‘enclosures’ that trapped cells of *E. coli*⁴⁰. Several groups have used microcontact printing to capture and detect pathogenic organisms. One study used PDMS stamps to pattern antibodies on silicon substrates and then measured the binding of *E. coli* O157:H7 to the antibodies using optical diffraction⁴¹. Morhard and co-workers detected the binding of *E. coli* O157:H7 to antibodies that were covalently attached to patterned SAMs using optical methods⁴². Another group used microcontact printing to pattern antibodies that were subsequently used to capture cells of *E. coli* O157:H7 and *Renibacterium salmoninarum*⁴³. More recently, Rozhok and co-workers fabricated patterns of surface-bound cells of a motile strain of *E. coli* by microcontact printing SAMs derivatized with anti-lipopolysaccharide antibodies or poly-L-lysine⁴⁴.

Micromoulding in capillaries has also been used to pattern microbial cells onto surfaces. Suh *et al.* used the MIMIC process to selectively confine bacterial cells within PEG microstructures using virus–antibody interactions⁴⁵. The authors fabricated PEG microstructures on the surface of silicon by micromoulding in PDMS

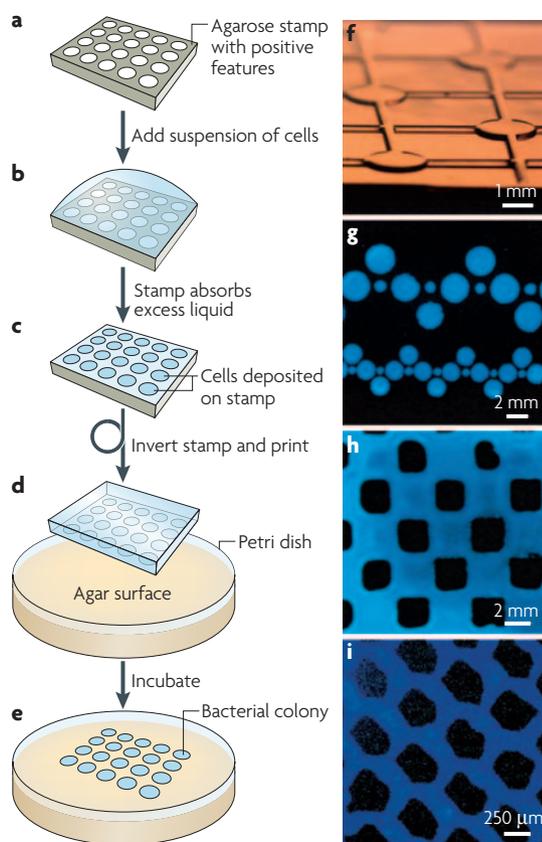


Figure 3 | A method for microcontact printing patterns of bacteria using agarose stamps. **a** | A cartoon of a stamp. **b** | The stamp is ‘inked’ with a suspension of bacterial cells. **c** | The stamp absorbs the excess liquid leaving bacteria on the surface. **d** | The stamp is inverted and brought into contact with agar in a Petri dish (or other surface). Cells are only transferred from the raised features of the stamp. **e** | Incubating the plate produces a pattern of bacteria. Hundreds of replica patterns can be produced from a single stamp. **f** | An image of a stamp. **g–i** | Patterns of *Vibrio fischeri* imaged by recording luminescence. Reproduced with permission from REF. 27 © (2005) American Chemical Society.

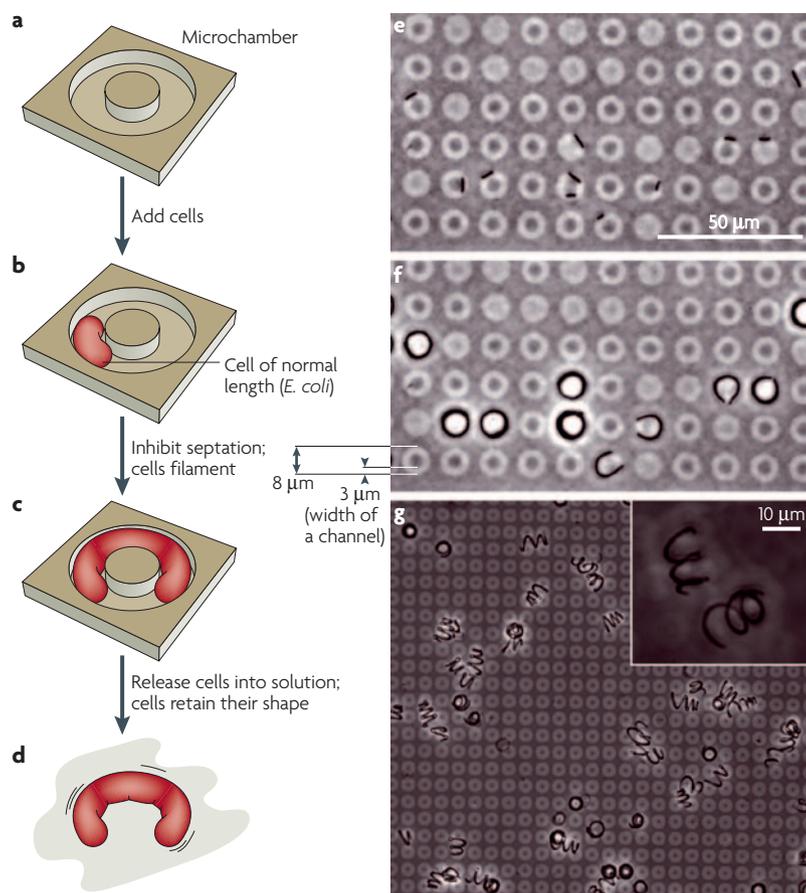


Figure 4 | Engineering the shape of bacteria by physical confinement. **a** | Agarose microchambers fabricated by replica moulding. **b** | Cells are added to the microchambers and a 'ceiling' is placed on top of the agarose to keep the cells confined. **c** | Cells of *Escherichia coli* grown in the presence of cephalixin adopt the shape of the microchambers they are grown in. **d** | The cells retain their shape after they are released into solution. **e** | Cells of *E. coli* in torus-shaped microchambers, imaged using phase-contrast microscopy. **f** | The cells after growth for 90 minutes. **g** | Cells grown in chambers for >2 hours and released form spirals. Spiral cells of *E. coli* superimposed on the chambers they were grown in. Inset: spiral cells at higher magnification. Reproduced with permission from REF. 55 © (2005) American Chemical Society.

capillaries and modified the surrounding (bare) surface of silicon with P3 or P9 antibodies that bind to the surface of the M13 virus. Only *E. coli* cells that were infected with the M13 virus bound to regions of the substrate patterned with antibodies.

Patterning bacterial cells using hydrogels. Agar and agarose form hydrogels. This category of polymer has two characteristics that are particularly useful in microbiology: first, cells grown on their surface remain hydrated; and second, nutrients, gas and byproducts of metabolism diffuse through the polymer network of the gel. There are many other types of hydrogel that also have a use in microbiology. For example, Heo *et al.* filled PDMS channels with a solution of a photoreactive PEG prepolymer that contained *E. coli* cells, and exposed regions of the channel to UV light⁴⁶. Exposure to the UV light resulted in the photopolymerization of the polymer, thereby encapsulating the *E. coli* cells within

Hydrogel
A low-density, crosslinked polymer network containing a high-volume fraction of water.

PEG microstructures inside the channels. Recently, we developed a method for microcontact printing bacteria on agar plates, glass slides and nylon membranes using micropatterned agarose stamps that are formed using replica moulding²⁷. The technique makes it possible to create patterns of bacteria on agar plates with control over the pattern, shape and spacing of features as small as 200 μm over areas as large as 50 cm² (FIG. 3).

Microfluidics: motility, chemotaxis, quorum sensing and population dynamics. Microfluidics has many characteristics that make it useful for the study of cells^{31,47}. We investigated the behaviour of swarmer cells of *E. coli* — a multiflagellated phenotype that is approximately 2–3 times the length of a swimming cell — confined in narrow microchannels in which the floor was agar, and the walls and ceiling were hydrophilic PDMS⁴⁸. Hydrodynamic interactions between the cells and the agar floor caused the cells to swim along the bottom of the channel; the rotation of the cell body made the cells swim preferentially along the right-hand side of the microchannel. A similar principle is being used to sort motile bacteria based on their length as they swim along curved microchannels.

In another study, Mao *et al.* used laminar flow to create gradients of attractants and repellents in microfluidic channels as a system for studying bacterial chemotaxis⁴⁹. The authors found that the microfluidic system was three orders of magnitude more sensitive than traditional capillary-based chemotaxis assays and, using the system, they discovered that L-leucine is a bacterial chemoattractant at low concentrations and a repellent at high concentrations.

Bacteria can alter their expression of genes collectively when the density of cells reaches a certain level. This behaviour is referred to as quorum sensing, and it has a role in many forms of microbial behaviour, including bioluminescence, swarming motility, pathogenicity and biofilm formation⁵⁰. As part of the quorum sensing process, bacterial cells communicate by secreting small, soluble molecules into the surrounding fluid. Park *et al.* studied the growth of *E. coli* and *Vibrio harveyi* in PDMS microfluidic devices and found that cells of *E. coli* accumulated in enclosed areas of a microfluidic maze (for example in dead-ends)^{51,52}. The authors concluded that chemotaxis between cells is capable of producing the density of cells required for quorum sensing.

Microfluidic systems also have applications in studying bacterial population dynamics. In one recent study, a microstructured environment for creating heterogeneity in bacterial habitats was constructed that allowed the authors to study how bacteria adapted to regions of the landscape⁵³. Although these structures were created using traditional methods of fabrication, the application of soft lithography to the fabrication of related structures is clear.

Microfluidics: growth of cells. Recently, a technique was developed for producing motile, filamentous cells of *E. coli* with different shapes by confining and growing single cells in microchambers that were embossed in agarose⁵⁴ (FIG. 4). Replica moulding from a PDMS stamp was used to prepare agarose microchambers contain-

ing nutrients. The microchambers were seeded with cells by adding a drop of a suspension of *E. coli* to the surface of the micropatterned agarose mould and a thin piece of PDMS or agarose was placed on top. In the presence of an antibiotic (cephalexin) that inhibits septation, the *E. coli* cells filamented and adopted the shape of the microchambers in which they grew. Indeed, the cells maintained their shape when they were released from the microchambers.

Bacterial persistence refers to the small percentage of a homogenous population of bacteria that survives after treatment with an antibiotic. Balaban and colleagues studied the persistence of single cells of *E. coli* confined in a flow cell consisting of microfluidic channels fabricated in PDMS⁵⁵. From this analysis, the authors built a model of bacterial persistence based on their observations that persisters cells were not affected by the antibiotic, were not genetically different from other bacterial cells and grew at much slower rates than normal cells.

When microorganisms grow in static cultures, the final density of cells is limited by their depletion of nutrients from their surrounding environment. In chemostatic growth, a small number of cells are constantly removed from the culture and fresh nutrients are added continuously⁵⁶. Cultures can achieve steady-state growth under these conditions. Several groups have designed and used 'microbioreactors' based on microfluidics for growing bacterial and yeast cells^{57–59}. For example, Balagadde *et al.* developed a microfluidic chemostat for the long-term culture of small numbers of bacterial cells⁵⁸. Each microchamber had a volume of 16 nL and was filled with liquid that was constantly recirculated; the chambers were periodically washed with solutions to prevent the formation of biofilms. Groisman and co-workers developed a microfluidic chemostat in which bacteria and yeast cells were confined in microchambers that were connected by channels that allowed the diffusion of nutrients but were impassable to cells⁵⁹.

Microfluidics: harnessing the mechanical work produced by microbial cells. Several groups have demonstrated approaches for capturing the mechanical work produced by motile strains of microbial cells. Although these examples are exploratory in nature, the techniques could also find application in studying fundamental questions related to the structure and behaviour of microorganisms. In one example, researchers created a monolayer of bacterial cells on a surface (referred to as bacterial carpets) by adsorbing cells of *Serratia marcescens* onto a PDMS surface. They found that the motion of the bacterial flagella within the monolayer was coordinated and produced a flow of fluid above the surface of the polymer⁶⁰. Kim and Breuer studied the mixing of two laminar streams of fluids in PDMS microfluidic channels using motile *E. coli*⁶¹. Hiratsuka *et al.* used microchannels to harness the motility of *Mycoplasma* cells to transport polystyrene beads — 500 nm in diameter — that were attached to the cells using streptavidin-biotin chemistry⁶². Recently the same group demonstrated the remarkable rotation of a freestanding object by biasing the coordinated movement of motile *Mycoplasma* cells

using a microchannel⁶³. We have developed a method of moving microscale loads (1–6 µm diameter polystyrene beads) in PDMS microchannels using the unicellular photosynthetic algae *Chlamydomonas reinhardtii* as 'micro-oxen'⁶⁴. Using a combination of photochemistry and surface chemistry, we have demonstrated that motile microorganisms can be guided to pick up, transport and drop off polystyrene beads.

Microfluidics: molecular biology. Several groups have used microfluidic systems for genetic studies and for the specific detection of bacterial cells. Microfluidic platforms have several attractive characteristics for genetic investigations, including the consumption of small volumes of reagents and samples, the generation of small volumes of waste, low unit cost, short reaction times and the capability of analysing single cells.

An important step in many molecular biology techniques and in the detection of pathogens is the lysis of microorganisms to release DNA and other intracellular contents into solution. In one study, *E. coli* and *Saccharomyces cerevisiae* cells were lysed within PDMS microfluidic chambers that were fabricated on compact discs (CDs)⁶⁵. The microfluidic chambers contained spherical beads that collided with cells when the CD was rotated around its horizontal axis (similar to a bead mill). In another study, researchers used optical tweezers in a PDMS microfluidic system to manipulate and sort cells of *E. coli* and a pulsed laser to punch holes in the cell wall⁶⁶. This system has applications in the transformation of single microbial cells with DNA constructs.

The transformation of bacterial cells with foreign DNA inserted into plasmids is one of the most fundamental techniques used in bacterial genetics. Using an array of microchambers in a microfluidic device fabricated in PDMS, Nagamine and co-workers carried out multiple, simultaneous transformations of *E. coli* cells with plasmid DNA⁶⁷. Each chamber contained a different plasmid that had been immobilized on the floor of the chamber. The authors demonstrated that *E. coli* cells in the different chambers were successfully transformed with plasmid DNA by confirming the presence of new phenotypes.

Recent progress toward the miniaturization of PCR-based assays has been reviewed elsewhere^{68,69}. Several groups are working on PCR in microfluidic devices fabricated using soft lithography, and although these systems are still too complex for routine use, this situation should improve as microfluidic technology matures. A fully automated, microfluidic, PCR-based detection system for the detection of bacterial pathogens was recently described⁷⁰. The device contains an integrated microprocessor, pumps, valves, thermocycler and a fluorescent detection system, and was capable of detecting approximately 10^4 – 10^7 cells of *Listeria monocytogenes* per hour. In addition, the automation of gene ligation, transformation and PCR in microfluidic systems fabricated in PDMS has also been described^{71,72}. A recent paper by Ottesen and co-workers elegantly demonstrated the use of microfluidic PCR to amplify and analyse genes isolated from single cells of bacteria harvested from the midgut of termites⁷³.

Outlook

Microfabrication has much to offer microbiology, although the full potential of close and constructive collaboration between the fields is yet to be achieved. It is still unclear to physical scientists and engineers what the needs of microbiologists are in terms of materials and structures, and microbiologists are unsure as to the availability of structures and what types are possible. Soft lithography provides one bridge between these fields. The techniques of soft lithography are sufficiently easy for microbiologists to learn so that it is possible for them to experiment without a major investment in time and resources. Soft lithography makes it possible to fabricate biocompatible materials that have features with dimensions that match the intrinsic scale of microbial cells. The application of these techniques to specific problems by microbiologists can help guide the development of new techniques and materials by chemists, physicists and engineers.

We believe that microfabricated tools will bring new capability to microbiology, and especially to the growing field of quantitative microbiology, in which they will have an important role in furthering our

understanding of the physiology and behaviour of microorganisms. Materials for isolating and studying large numbers of cells in parallel, under identical conditions, has the potential to transform microbiology from a qualitative, observational field into a science that is based on statistically significant data. Microfabricated constraints offer methods for forcing cells to grow into shapes that are otherwise unachievable. The availability of microchannel systems of almost any arbitrary shape and complexity makes it straightforward to explore how microorganisms sense their world, and one another, at a new level of physical detail. In the broadest terms, microfabrication provides new and experimentally straightforward techniques that make it possible to examine microorganisms — either individually or at the population level — in the context of environments in which many of the parameters that are relevant to their behaviour are controlled.

Some tools are already available. The next step is the development of collaborations between fabricators and microbiologists that will lead to the development, refinement and application of new tools and the development of new science.

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

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