Isolation, incubation, and parallel functional testing and identification by FISH of rare microbial single-copy cells from multi-species mixtures using the combination of chemistrode and stochastic confinement†

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This paper illustrates a plug-based microfluidic approach combining the technique of the chemistrode and the principle of stochastic confinement, which can be used to i) starting from a mixture of cells, stochastically isolate single cells into plugs, ii) incubate the plugs to grow clones of the individual cells without competition among different clones, iii) split the plugs into arrays of identical daughter plugs, where each plug contained clones of the original cell, and iv) analyze each array by an independent technique, including cellulase assays, cultivation, cryo-preservation, Gram staining, and Fluorescence *In Situ* Hybridization (FISH). Functionally, this approach is equivalent to simultaneously assaying the clonal daughter cells by multiple killing and non-killing methods. A new protocol for single-cell FISH, a killing method, was developed to identify isolated cells of *Paenibacillus curdlanolyticus* in one array of daughter plugs using a 16S rRNA probe, Pc196. At the same time, live copies of *P. curdlanolyticus* in another array were obtained for cultivation. Among technical advances, this paper reports a chemistrode that enables sampling of nanoliter volumes directly from environmental specimens, such as soil slurries. In addition, a method for analyzing plugs is described: an array of droplets is deposited on the surface, and individual plugs are injected into the droplets of the surface array to induce a reaction and enable microscopy without distortions associated with curvature of plugs. The overall approach is attractive for identifying rare, slow growing microorganisms and would complement current methods to cultivate unculturable microbes from environmental samples.

Introduction

This paper describes using a combination of chemistrode and stochastic confinement to isolate individual microbial cells from diverse mixtures into plugs, then incubate these isolated cells to provide growth without competition, and finally split the resulting plugs containing cells into multiple daughter plugs, each containing clones of the original cell. These daughter plugs can then be used for multiple analyses, such as identification and functional testing, in parallel.

Isolation and functional characterization of microbes from diverse multi-species mixtures is of wide interest because these mixtures perform critical functions in environments ranging from soils, to oceans, to niches inside a eukaryotic host. Metagenomic efforts are documenting the genetic diversity of these mixtures,[1–6] but these methods do not provide access to live cells. Furthermore, short reads do not provide information on which microorganism has which genes.[7] Methods for identifying cells of a specific species within a mixture, for example Fluorescence *In Situ* Hybridization (FISH), may be used to identify bacteria having particular genes,[8,9] but FISH is a killing method and does not provide live, isolated microorganisms.

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Isolation of microorganisms is difficult because many of them appear to be unculturable in traditional experiments, although new techniques are rapidly improving cultivability.[10–12] One problem with culturing rare cells is that they may grow slowly, and, when grown in a mixture, they get out-competed by other species that grow more rapidly or are present at a higher density initially.[13] Limiting dilution can be used to address this problem: by diluting the original mixture to such an extent that individual aliquots (*e.g.* 50 μL aliquots placed into wells of a well plate) contain individual cells, competition is eliminated.[14,15] However, dilution to such a low density makes it more difficult to detect organisms that grow very slowly, undergo just a few divisions, or grow to a low final density.[15,16] In addition, analysis of secreted molecules, such as cellulase enzymes needed for biomass conversion in production of biofuels, becomes more difficult when starting with a low density of cells, because secreted molecules are initially present at low concentrations. Finally, such dilution greatly changes the original microenvironment of the sample and may negatively affect the growth of the organism. Gel microdroplets (GMDs) have been used successfully to overcome some of these problems and confine individual cells of microorganisms in small volumes and enhance growth.[10,17]

The microfluidic technique described here builds on these previous advances. Here we use the chemistrode[18,19] and stochastic confinement in arrays of nanoliter plugs[12,20–24] to construct a system suitable for isolating rare cells. Plugs are aqueous droplets surrounded by an immiscible fluorocarbon carrier fluid,[25,26] and they are useful for monitoring growth of

cells and for analyzing their secretions.[22,23,27,28] The chemistrode relies on multiphase aqueous/fluorous flow to deliver stimuli or to sample responses by trapping a solution into plugs.[18] Separation of competing species into plugs eliminates competition for nutrients and allows all species to grow. Isolation may also prevent toxic compounds released by one species from contacting the other species. Here, isolating cells in plugs provides several other attractive features: i) it eliminates scattering from gels, simplifying analysis by microscopy; ii) it provides an opportunity to confine and analyze secreted molecules,[20,22,27,28] from the level of a few cells all the way down to the single-cell level;[29] and iii) after a few divisions of the original cell within a plug, it enables splitting[18,30] of this plug into daughter plugs, each containing clones of the original cell. This latter feature allows each of the daughter plugs to be manipulated or analyzed by an independent technique. For example, small clonal populations can be analyzed by mutually incompatible techniques, such as those that kill cells *vs.* those requiring live cells.

In this paper, we present a version of the chemistrode that enables sampling directly from the environment, even from heterogeneous samples, such as a soil slurry. We then illustrate that the chemistrode can be combined with stochastic confinement to i) stochastically isolate rare cells in a mixture with more abundant species into plugs, ii) incubate the plugs to grow colonies of the isolated cells, iii) split the plugs into arrays of identical daughter plugs, and iv) perform multiple tests in parallel. We demonstrate this approach by using a mixture of *Paenibacillus curdlanolyticus*, a cellulase-producing species, as the rare and slow-growing species, and *E. coli* as the abundant and rapid-growing species. We also develop a protocol to identify *P. curdlanolyticus* in plugs by using FISH.

Experimental

Design and manipulation of the chemistrode for environmental sampling

A tip of a piece of glass septum theta tubing (1.5 mm, World Precision Instruments) was made sharp by fusing, and then inserted into a piece of Tygon tubing (250 μm I. D., 2000 μm O. D.) (Fig. S1†). Then a piece of a standard, polyimide coated, flexible, fused silica capillary (318 μm I. D., 435 μm O. D., Polymicro) was inserted into the other end of the piece of Tygon tubing. A piece of Teflon tubing (100 μm I. D., 150 μm O. D., Zeus) was inserted into the open end of the glass septum theta tubing, through the Tygon tubing, all the way down to the distal tip of the silica capillary. A second piece of Teflon tubing (200 μm I. D., 250 μm O. D., Zeus) was also inserted into the open end of the glass tubing, and this tubing extended only down to the proximal tip of the glass tubing. The gap between the two pieces of Teflon tubing and the glass tubing was filled with half-cured polydimethylsiloxane (PDMS) glue (Dow-Corning Sylgard 184 A and B at a ratio of 10 : 1, cured at 110 °C for 110 s), and then the device was baked at 65 °C for the PDMS glue to fully cure.

For sampling, the chemistrode was held by the Tygon tubing, and the tip was dipped into the aqueous sample. The carrier fluid was delivered from the 200 μm I. D. Teflon tubing at 0.5 μL min^{-1}, and it flowed to the tip of the chemistrode through the space between the silica capillary and the Teflon tubing. A negative pressure was applied on the 100 μm I. D. Teflon tubing by aspirating at 0.9 μL min^{-1}. Both the carrier fluid and the aqueous sample were aspirated into this Teflon tubing, and the aqueous sample was segmented into plugs by the carrier fluid at the entrance.

Cultivation of microorganisms and culture media

Bacterial species of *Paenibacillus curdlanolyticus* (ATCC 51899) and *Escherichia coli* (*E. coli*, ATCC 25922) were obtained from the American Type Culture Collection. *P. curdlanolyticus* cells were enriched in 30 g L^{-1} of sterilized trypticase soy broth (TSB) media (BD Company) at 30 °C for 12 h, and *E. coli* cells were enriched in Difco Luria-Bertani (LB) broth media (BD Company) at 37 °C for 3 h. Seed inoculum of each species was cultured in a rotary shaking incubator (SI-600 Lab Companion, Jeio Tech) at 180 rpm. During the seed culture of *P. curdlanolyticus*, cellulases were induced by adding filter-sterilized (0.45 μm, Whatman) carboxymethyl-cellulose (CM-cellulose; sodium salt, 0.7 D.S., Sigma-Aldrich) at 1 g L^{-1} final concentration.

The green fluorescence protein (GFP)-labeled *E. coli* (a mutant *E. coli* strain containing PUCP24/EGFP plasmids in *E. coli* K12 YMel-1 host) was constructed in the laboratory. The red fluorescence protein (RFP)-labeled *E. coli* (a mutant *E. coli* strain containing DsRed encoding plasmids in *E. coli* DH10B host) was provided by Professor Benjamin Glick of the University of Chicago. GFP-labeled *E. coli* was cultured in Difco tryptic soy agar (TSA) media (BD company) that included 100 mg L^{-1} kanamycin and 20 mg L^{-1} gentamicin. RFP-labeled *E. coli* was cultured in TSA media that included 100 mg L^{-1} ampicillin.

Preparation of live cells

An inoculum of either *P. curdlanolyticus* or *E. coli* was cultured in either TSB (for *P. curdlanolyticus*) or LB (for *E. coli*) media respectively. Seed inoculum of either GFP-labeled *E. coli* or RFP-labeled *E. coli* was cultured in either LB media including 100 mg L^{-1} kanamycin (for GFP-labeled *E. coli*) or LB media including 100 mg L^{-1} ampicillin (for RFP-labeled *E. coli*) respectively. Cells in a seed culture were harvested at the exponential phase, and then the cells were washed twice with autoclaved 0.9% (w/v) NaCl solution. For immediate use, the number of live cells for each species was approximately estimated by either counting cells under an epi-fluorescence microscope (DMI 6000 B, Leica) (for GFP-labeled *E. coli* and RFP-labeled *E. coli*), or by staining with live/dead fluorescent dye (Live/Dead *Bac*Light Bacterial viability kit, Molecular Probes) then counting cells (for *P. curdlanolyticus* and *E. coli*), or by measuring optical density through a UV/Vis spectrophotometer (Agilent) for *P. curdlanolyticus* and *E. coli*. The number of live cells of each species was determined by the colony counting method in agar plates.

Mixed culture of *P. curdlanolyticus* and *E. coli* in an agar plate

After each cell suspension was serially diluted with sterilized NaCl solution (0.9%, w/v) down to a cell density of 10^1 CFU mL^{-1}, cell suspensions of *P. curdlanolyticus* and *E. coli* were mixed to form suspensions with different density ratios. The cell density of *E. coli* was kept at approximately 10^4 CFU mL^{-1}, and

the cell density of *P. curdlanolyticus* was either 10^4, 10^3, or 10^2 CFU mL^{-1}. Then the mixtures with different ratios were respectively spread on TSA plates, and incubated at 30 °C for 36 h. Based on the different morphology, colonies of either *P. curdlanolyticus* or *E. coli* were distinguished visually.

Isolation of bacterial cells in plugs by stochastic confinement and incubation of the plugs

Plugs, approximately 10 nL in volume, were formed by using the method described previously.[20] Briefly, we formed the plugs in a three-inlet PDMS device with 100 μm wide channels by flowing the cell suspension diluted with TSB at approximately 5×10^4 CFU mL^{-1} at 0.4 μL min^{-1} and the fluorinated carrier fluid (FC40 containing 0.5 mg mL^{-1} RfOEG) at 0.5 μL min^{-1}. A long spacer of carrier fluid was introduced *via* the third inlet after every 30 plugs were formed. Plugs were collected into 200 μm I. D. Teflon tubing, which was inserted into the device up to the inlet junction and sealed in place with wax (Hampton Research). After formation of plugs, the tubing was disconnected from the PDMS device, and the ends of the tubing were sealed with wax. Then, the tubing was incubated in a Petri dish containing 0.5 mL of TSB media at 30 °C. To maintain sterility, all tubing, devices, and syringes used were sterilized by using 70% (v/v) ethanol, and all solutions and media used were either autoclaved or filtered through a PES or PTFE filter with 0.45 μm pore size.

Splitting plugs

We split each individual plug into four daughter plugs by two steps of two-way splitting *via* the method described previously.[18,30] As the plugs moved through the 200 μm I. D. Teflon tubing towards the junction, each plug was split into two daughter plugs, and each of these daughter plugs was also split (Fig. 2c). In other words, four identical daughter plugs were split off from each incoming plug. The daughter plugs resulting from a particular splitting event were collected into four separate segments of 100 μm I. D. Teflon tubing, for a total of four arrays of daughter plugs. After splitting, the plugs in each daughter array were recognized as copies of the initial incoming plugs, and the cells in the initial plugs had been distributed among the daughter plugs. The order of the daughter plugs in each array corresponded to the original order of the incoming plugs from which they were generated.

Cellulase assay on bacteria in an array of plugs

The synthetic substrate resorufin cellobioside (MarkerGene fluorescent cellulase assay kit, MGT Inc.) was used to measure the cellulolytic activity of either *P. curdlanolyticus* or *E. coli* in culture broth. The stock solution of resorufin cellobioside (5 mM) in DMSO was diluted by the reaction buffer (100 mM sodium acetate, pH 6) into 0.5 mM resorufin cellobioside solution. This resorufin cellobioside solution was then injected into each plug in an array by using a PDMS T-junction with a volumetric ratio of 1 : 1. After disconnecting the tubing containing the plugs from the PDMS device and then incubating the tubing at room temperature for 8 h, each plug was imaged (see ESI for details†). High intensity of red fluorescence indicated that the fluorogenic substrate of resorufin cellobioside was cleaved by the cellulolytic enzymes produced by *P. curdlanolyticus*, whereas lower intensity levels of fluorescence indicated either empty plugs or plugs containing non-cellulolytic species, *i.e. E. coli*.

Plate culture of bacteria in an array of plugs

To culture each plug containing either *P. curdlanolyticus* or *E. coli*, plugs were deposited[31] 0.5 cm apart onto a TSA plate using a micro-aspirator (Stoelting) under a stereomicroscope (SMZ-2E, Nikon). One spot on a TSA plate corresponded to a single plug. An Easigrid colony template (Jencons) was attached on the bottom of each plate to guide the deposition of plugs and to track the locations of the deposited plugs. After deposition of plugs, the plate was then incubated at 30 °C overnight. Growth of colonies of either *P. curdlanolyticus* or *E. coli* indicated the presence of that species in the corresponding plug.

Cryo-preservation of bacteria in an array of plugs

For the long-term preservation of plugs, an autoclaved 40% (v/v) glycerol stock solution was injected into each plug in an array by using a T-junction with a volumetric ratio of 1 : 1, so that the final concentration of glycerol in each plug was 20% (v/v). The tubing containing plugs was frozen rapidly by being dipped in liquid nitrogen, and then the tubing was stored in a deep freezer at −80 °C. After the frozen tubing was aseptically thawed on a clean bench at room temperature, each plug was transferred onto the surface of a TSA plate as described above, and then the plate was incubated at 30 °C overnight to revive cells.

Gram staining of bacteria in an array of plugs

The LIVE *Bac*Light bacteria Gram stain kit (Molecular Probe) was used to stain the bacteria. The staining solution was prepared by adding 1.5 μL of 3.34 mM SYTO9 (a green fluorescent dye that stains both live, Gram-positive and live, Gram-negative bacteria) in DMSO and 1.5 μL of 4.67 mM hexidium iodide (a red fluorescent dye that preferentially stains live, Gram-positive bacteria) in DMSO to 1 mL of filter-sterilized water. An array of droplets of the staining solution, each droplet approximately 60 nL in volume, was prepared by spotting the staining solution on a piece of cover slide immersed in FC40 by using 300 μm I. D. Teflon tubing (Weico Wire & Cable). Plugs, contained in 100 μm I. D. Teflon tubing, were then deposited onto the cover slide; each plug was deposited into one droplet of staining solution. Bacteria in each droplet were then imaged. Green fluorescence indicated Gram-negative, live *E. coli* cells, whereas red fluorescence indicated Gram-positive, live *P. curdlanolyticus* cells.

Fluorescence *In Situ* hybridization (FISH) of bacteria in an array of plugs

A 16S ribosomal-RNA targeted probe specific to *P. curdlanolyticus* (Pc196) was designed by Ribocon GmbH and constructed by and purchased from biomers.net – the biopolymer factory (Ulm, Germany) (sequence: 5′-gaa aga ttg ctc ctt ctt-3′ conjugated to the fluorophore Atto550 at the 5′ end). We developed a new protocol for the detection of *P. curdlanolyticus*, and optimal hybridization conditions for the probe were determined

using *P. curdlanolyticus* that had been cultured in bulk in TSB media and then fixed while the *P. curdlanolyticus* cells were in the early stages of exponential growth. The solution for fixing the cells consisted of 50% cold ethanol (4 °C) and 50% RNase-free 1 × PBS (145 mM NaCl, 1.4 mM NaH_2PO_4, 8 mM Na_2HPO_4, in RNase-free water (Thermo Scientific), pH 7.4). The *P. curdlanolyticus* cells in fixative were then stored at −20 °C for 20 h. The fixation procedure for detection of *P. curdlanolyticus* in bulk was modified as follows to detect *P. curdlanolyticus* isolated in plugs. For fixation after stochastic confinement, plugs containing bacteria grown in TSB media were merged with a stream of 100% ethanol by using a T-junction with a volumetric ratio of 1 : 1 (Fig. 6a). The resulting plugs of 50% ethanol and 50% TSB media were incubated at −20 °C for 20 h. After fixation, plugs containing fixed bacteria were spotted on UltraStick glass slides (Gold Seal). While most bacteria adhere to the UltraStick glass slides, to prevent any potential cross contamination of isolated bacterial species between spotted plugs, a PDMS membrane containing 5% carbon and ten 5 cm wells was sealed to the UltraStick slides, and one plug was spotted in each well (Fig. 6b). Prior to hybridization, the hybridization buffer (900 mM NaCl, 20 mM Tris/HCl, 20% formamide, 0.1% SDS, and 5 ng μL^{-1} Pc196 in RNase-free water) was pre-warmed at 48 °C. For hybridization, 100 μL of the buffer was added to each well containing a plug of isolated bacteria. Bacteria were incubated in the hybridization solution for two and a half hours at 48 °C. The hybridization solution was then removed, and bacteria were washed for 30 minutes with washing solution (56 mM NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% SDS in RNase-free water) at 48 °C, and next washed at room temperature with 1 × PBS buffer (pH 7.4). Following hybridization, each spot corresponding to a cataloged plug was scanned using a VT Infinity 2-D array scanner confocal system with an array of 50 μm pinholes (Visitron Systems, Germany) coupled to a Leica DMI6000 inverted microscope (Leica, Germany) and a 568 nm ± 2 nm diode laser (exposure time 800 ms, gain 180). Images were obtained with a back-thinned electron multiplier CCD camera (16 bit, 512 × 512 pixels) (Hamamatsu Photonics, Japan) and a 20 × 0.7 NA objective using Simple PCI software (Hamamatsu Corportation, Japan). All stained bacteria were kept in 50% DABCO during imaging to prevent photo-bleaching.

While the camera used did not provide truly quantitative measurements, intensities of imaged *P. curdlanolyticus* and *E. coli* were compared to ensure reliable identification of *P. curdlanolyticus*, and the intensities were analyzed using MetaMorph Imaging System (Molecular Devices). A line scan with a scan width of one pixel was taken across the bacteria. Data presented is of the intensity along the line scan minus 200 a.u. of background scattering. Images were processed using Adobe Photoshop 6.0.

Results and discussion

We are interested in applying the combination of chemistrode and stochastic confinement to study environmental microbiology and the human microbiome. Some microbes in these environments are "unculturable" *via* traditional plate-based methods. The media used in these traditional methods may selectively enrich certain species in the environmental sample, allowing them to out-compete other species. The losing species in this competitive interaction are not recovered from plates and therefore deemed "unculturable". However, it may be the case that the growth of these "unculturable" species strongly relies on their original media,[11] which may contain specific amounts of certain materials or molecules which are required for growth. If so, culturing these "unculturable" microbes should require using the original sample from the environment, and then incubating the cells in the original media, rather than on plates. One of the challenges of using the original sample from the environment is that samples from some environments – such as soil, sediments, blood, and the human gut – may adhere to and foul or occlude the sampling devices. Plugs can be used to transport solids reliably, including suspensions of inorganic nanoparticles,[32] protein microcrystals,[33] agglutinated red blood cells and clotted blood.[34] To sample microbes directly from their environment, in their original media, and without fouling of the device, we used a modified chemistrode to form plugs immediately as the sample flows into the tip (Fig. 1a). This modified chemistrode was demonstrated by sampling from soil slurry (Fig. 1b). A similar device has been recently described,[35] and we thank the reviewer for pointing out this reference to us.

Having demonstrated that the modified chemistrode can successfully sample directly from the environment, we then tested the rest of the approach (Fig. 2) by forming plugs using a device similar to the one that was previously used for stochastic confinement.[20] With a mixture of two fluorescence-labeled strains of *E. coli*: GFP-labeled *E. coli* and RFP-labeled *E. coli* in equal proportions, we used the principle of stochastic confinement to isolate single bacterial cells into individual plugs. When the number of plugs generated was much higher than the number of cells present initially, among those plugs containing cells, most contained only cells of GFP-labeled *E. coli* or only cells of RFP-labeled *E. coli*. The statistical property of this process can be described by the Poisson distribution.[27] Previous work has shown that encapsulation of cells in plugs does not introduce artifacts to cell growth,[23] and we confirmed that single cells, isolated in plugs and incubated, formed populations with growth rates similar to those observed for cells in bulk solution (Fig. S2†).

After we incubated the plugs to allow time for a few divisions of the original cell within a plug (Fig. 2b), we split each of the plugs in the original array to create four identical, parallel daughter arrays (Fig. 2c). There was no cross-contamination between plugs during the splitting, because the plugs were well separated from each other, as well as from the surfaces of the tubing and the PDMS devices, by the carrier fluid. We calculated the statistical probability of the resultant distribution of cells in the four daughter plugs, assuming that all cells in each initial plug had an equal probability of being distributed into each of the daughter plugs. After five cycles of divisions from a single cell (*i.e.* when there are 32 cells in a plug), there is greater than 99% probability of having at least one cell in each daughter plug. We were also concerned that cells of some bacterial species would form clusters and not split evenly among the daughter plugs. We experimentally confirmed that gentle shear in the plugs (average shear rate ~60 s^{-1}) was sufficient to break up any clusters of all strains of either *Escherichia coli* (*E. coli*) or *Paenibacillus curdlanolyticus* used for this paper. Thus, we presume that splitting

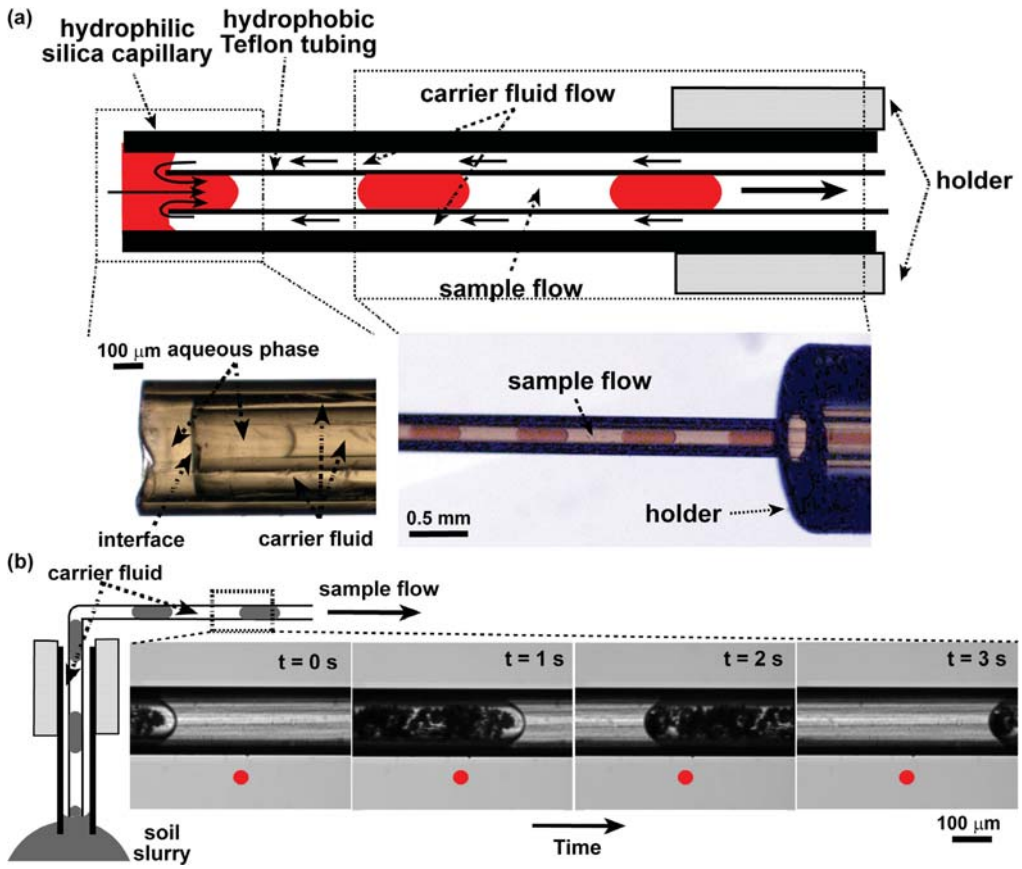

Fig. 1 A modified chemistrode performs sampling of a substrate and enables formation of plugs at the sampling site. **(a)** A schematic drawing of the design of the chemistrode, which consists of a piece of hydrophilic silica capillary with a piece of hydrophobic Teflon tubing inserted inside. The carrier fluid was delivered to the tip of the probe through the space between the silica capillary and the Teflon tubing. Aspiration at flow rate higher than the flow rate at which the carrier is delivered removed both the carrier fluid and the aqueous sample into the Teflon tubing, and the aqueous stream was segmented into plugs by the carrier fluid at the entrance of the Teflon tubing. The carrier fluid was contained inside the chemistrode by capillary forces that keep the carrier fluid adherent to the Teflon walls. A photograph on the lower left shows the tip of the chemistrode sampling from water. A photograph on the lower right shows plugs of a solution of red dye transported by the chemistrode. **(b)** A schematic (left) of the chemistrode sampling from soil slurry; and the sample plugs are shown in the tubing downstream. A series of time-lapse brightfield images (right) show a sample plug containing soil particles passing through the tubing. The red dot is a stationary reference point.

should reliably generate parallel copies of the bacterial populations for multiple tests.

Having demonstrated stochastic isolation and incubation of individual cells from an equal-ratio, multi-species mixture, we then tested whether this approach can be used to do the same for cells of a rare species in a multi-species mixture. In this context, *P. curdlanolyticus* was chosen as the rare, relatively slow-growing species, and wild-type *E. coli* was chosen as the abundant, rapidly-growing species. *P. curdlanolyticus* cells produce and secrete cellulolytic enzymes, a class of enzymes interesting for conversion of biomass into biofuels; whereas, the *E. coli* species that we used does not produce any similar enzymes to cleave cellulose.

We prepared mixtures of *E. coli* and *P. curdlanolyticus* at different ratios of viable cell numbers – 1.5 : 1, 2 : 1, 15 : 1, 20 : 1, and 200 : 1 – and spread suspensions of these mixtures on TSA plates. We were able to detect *P. curdlanolyticus* colonies visually, based on differences in morphology of colonies, in plates spread with suspensions of the mixture with the ratio of either 1.5 : 1 (Fig. 3a) or 2 : 1 (Fig. 3c). However, we could not detect any *P. curdlanolyticus* colonies in plates with the ratio of 15 : 1 (Fig. 3b), 20 : 1 and 200 : 1 (Fig. 3c), indicating that *E. coli* was out-competing *P. curdlanolyticus* on the TSA plates. This result confirms the known difficulty of isolating rare species present at low abundance using conventional plating methods. Here, when the ratio of *E. coli* to *P. curdlanolyticus* was above 15 : 1, no cells of the rare species were recovered.

Then, we isolated the cells in the mixture into individual plugs and incubated the cells of *E. coli* and *P. curdlanolyticus* in plugs. We were able to recover *P. curdlanolyticus* cells that successfully grew up to hundreds of cells in all cases; we tested ratios from 1 : 1 to 40 : 1 (Fig. 3d and 3e). This observation is consistent with what we expected, because the individual species were separated into different plugs so that the slow-growing species (*i.e. P. curdlanolyticus* strain) can have an opportunity to grow without any competition with the fast-growing species (*i.e. E. coli* strain). The fraction of the recovered *P. curdlanolyticus* was always lower than 100%, because there is a minor probability that both a *P. curdlanolyticus* cell and an *E. coli* cell are isolated together in some plugs, according to the Poisson distribution. In such a case, the *E. coli* cells would dominate the growth in those plugs, and the *P. curdlanolyticus* cells in those plugs could not be

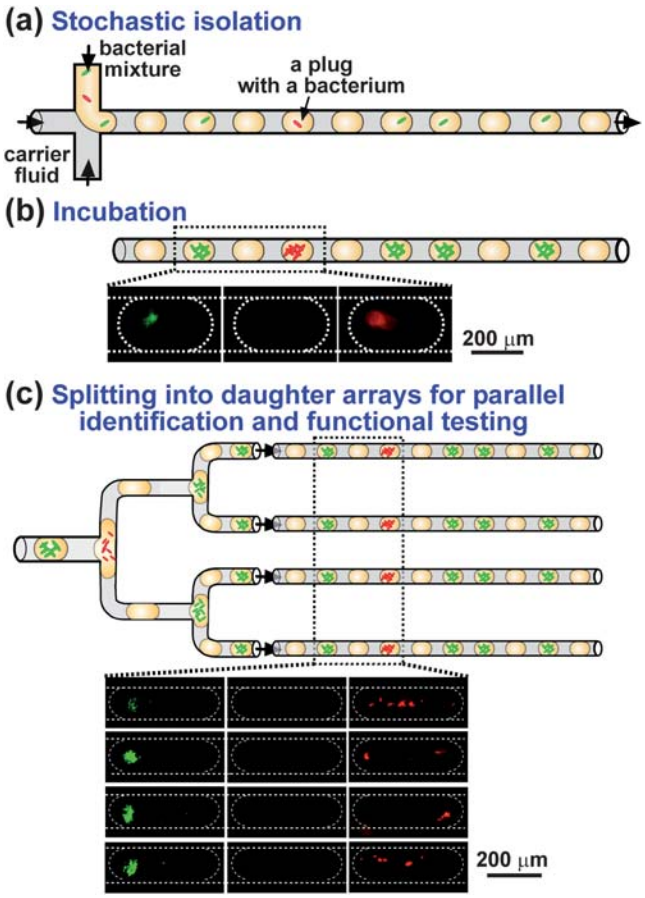

Fig. 2 The chemistrode combined with stochastic confinement can be used to **(a)** isolate individual cells in a multi-species mixture by stochastically encapsulating the cells in an array of individual plugs, **(b)** incubate the plugs to grow colonies of the isolated cells without cross-species interference, **(c)** split the plugs into arrays of identical daughter plugs to perform multiple tests in parallel, such as identification and functional tests. To demonstrate, individual bacterial cells of either GFP-labeled *E. coli* or RFP-labeled *E. coli* were stochastically isolated from a mixture of both *E. coli* strains into plugs. Incubation of the plugs enabled these isolated single bacterial cells to grow. The three images in (b) show three adjacent plugs in an array. The first plug contains GFP-labeled *E. coli*, the second plug is empty, and the third plug contains RFP-labeled *E. coli*. These plugs were incubated for 6 h after formation, and the bacterial cell in each individual plug (if present) divided and grew. After incubation, each plug was split into four identical daughter arrays (c). The four left-most images in (c) show the daughter plugs generated by splitting the left-most plug in (b). The four central images in (c) show the daughter plugs generated by splitting the central plug in (b). The four right-most images in (c) show the daughter plugs generated by splitting the right-most plug in (b).

recovered. The fraction of *P. curdlanolyticus* cells that were recovered was variable among different experiments, presumably because of the stochasticity of the isolation of cells into plugs.

The culturing method of "dilution to extinction" also uses the principle of stochastic isolation.[14,15] However, in these methods there is a very low starting concentration of the inoculum ($\sim 10^0$ CFU mL^{-1}). On the other hand, encapsulating a single bacterium in a plug with a volume of 10 nL provides a cell density of 10^5 mL^{-1} in a single plug. Increasing the inoculum density (*e.g.* 10^5 CFU mL^{-1}) allows a dramatic reduction in the culturing time required to reach detectable cell densities. Creating high inoculum density by isolating individual bacterial cells in plugs is especially important for those bacterial species that have slower division rate at lower concentration,[36] because the rapid accumulation of the molecules or enzymes in the confined environment of the plug may enhance growth of cells.[29] Furthermore, plugs require much lower volumes of reagents for the isolation and the cultivation of bacterial samples. Thus, plug-based methods that rely on stochastic isolation have many advantages over the culturing method of "dilution to extinction".

Methods with gel microdroplets (GMDs) also use stochastic confinement to isolate single cells,[10,17] and isolation by GMDs may be useful for separating and culturing symbiotic species, as compounds can diffuse between GMDs.[10] Some other microfabrication-based methods also have been developed to manipulate and analyze isolated single cells,[37–39] however, using plugs allows splitting of the plugs to form identical copies for analyses with multiple techniques in parallel.

Having demonstrated stochastic isolation and incubation of rare cells in a mixture, we next tested whether these methods could be combined with splitting to create parallel arrays for further testing (Fig. 4). We started with a mixture of *E. coli* and *P. curdlanolyticus* at a concentration ratio of 40 : 1, which does not result in recovery of *P. curdlanolyticus* colonies when the mixture is spread on plates. After isolation and incubation in approximately 1000 plugs, we split 100 plugs into four daughter arrays. Each daughter array also contained 100 plugs, all of which were copies of the original plugs. We then used each array of plugs to perform one of four different tests.

The first daughter array of plugs was used for the cellulase assay. Fluorescence in each plug was detected after each plug was injected with a solution of the fluorogenic substrate resorufin cellobiosides and incubated at room temperature for 8 h. Plugs containing *P. curdlanolyticus* showed more than two times higher fluorescence than plugs containing *E. coli*, and more than four times higher fluorescence than empty plugs (Fig. 4a, Fig. S3†). Injection of reagents into plugs is well established.[18,32,40] Plugs in the second daughter array were deposited onto a fresh TSA plate, and then incubated at 30 °C for 20 h to grow individual colonies. We confirmed that pure colonies of either *P. curdlanolyticus* or *E. coli* grew on the surface of the TSA plate where we deposited the corresponding plugs containing either *P. curdlanolyticus* or *E. coli* (Fig. 4b). Plugs in the third daughter array were used for cryo-preservation. After injection of glycerol, the plugs were frozen by using liquid nitrogen, and then they were stored at −80 °C for one day. To test the recovery of the viability of the cells in the cryo-preserved plugs, those plugs were thawed on the second day and deposited onto a fresh TSA plate for incubation. Colonies grew on all the locations on the surface of the TSA plate where plugs containing cells were deposited (Fig. 4c). The fourth daughter array of plugs was used for Gram staining. We confirmed that the Gram-positive *P. curdlanolyticus* species was stained in red, and the Gram-negative *E. coli* species was stained in green (Fig. 4d) by the fluorescent dye used here.

In this experiment, we developed a new method for analyzing plugs: an array of droplets is deposited onto a surface, and individual plugs are injected into the droplets of the surface array

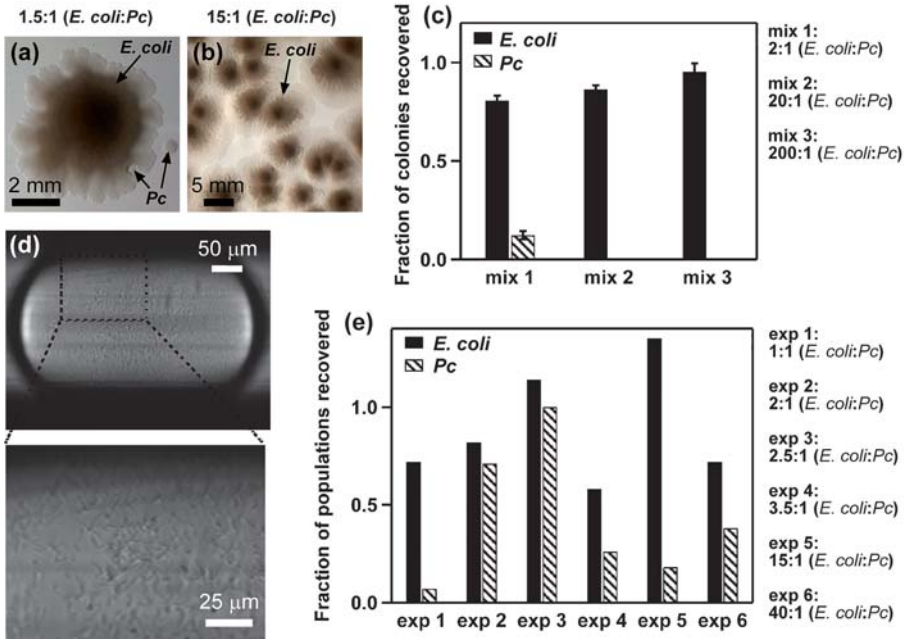

Fig. 3 Rare individual cells in a mixture were isolated by stochastic confinement at ratios much lower than those that were achievable using plate-based methods. **(a)** Colonies of both *E. coli* and *P. curdlanolyticus* were observed after spreading a mixture of *E. coli* and *P. curdlanolyticus* cells, with the ratio of cell density at 1.5 : 1, onto a TSA plate and incubating the plate for 36 h. **(b)** Colonies of only *E. coli* cells were observed after spreading a mixture of *E. coli* and *P. curdlanolyticus* cells, with the ratio of cell density at 15 : 1, onto a TSA plate and incubating the plate 36 h. No *P. curdlanolyticus* colonies were observed after 36 h. **(c)** A plot of the fraction of colonies recovered after spreading mixtures of *E. coli* and *P. curdlanolyticus* with different cell density ratios on TSA plates and incubating the plates for 36 h. The fraction recovered is defined as the ratio of the number of colonies observed on the plates to the number of colonies expected, based on the number of viable cells of each organism, measured by culturing pure cell suspensions prior to mixing them. Error bars denote standard deviations ($n = 2$). **(d)** Bright field images show the population of *P. curdlanolyticus* cells grown from the cell(s) isolated by stochastic confinement in a plug. **(e)** A plot of the fraction of cells recovered after mixtures of *E. coli* and *P. curdlanolyticus* with different concentration ratios were isolated in plugs and incubated. The fraction of cells recovered is defined as the ratio of the number of plugs with each species to the number of plugs expected, based on the number of viable cells of each organism, measured by culturing pure cell suspensions prior to mixing them. See detailed data in Table S1†. *Pc* indicates *P. curdlanolyticus*.

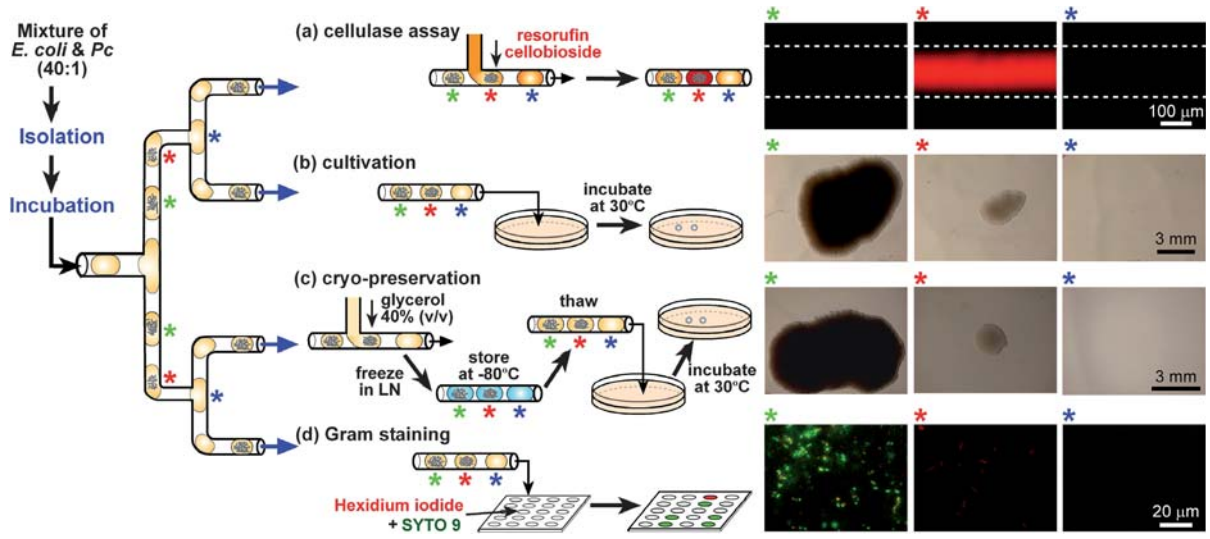

Fig. 4 Populations grown from the isolated single cells were split for parallel tests. Individual cells in a mixture of *E. coli* and *P. curdlanolyticus* (ratio of cell density, 40 : 1) were isolated stochastically in plugs and incubated. Then the plugs were split into four daughter arrays which were used for **(a)** cellulase assay, **(b)** cultivation, **(c)** cryo-preservation, and **(d)** Gram staining, respectively (see the text/ESI†). Each row of microscopic images on the right shows the results of one of the four tests from three sequential plugs in each array. Each column, marked with colored stars, shows the test results for daughters, *i.e.* clones, of a bacterium from a single plug: the green stars trace the four daughter plugs split from a plug containing *E. coli*, the red stars trace the daughter plugs from a plug containing *P. curdlanolyticus*, and the blue stars trace the daughter plugs from an empty plug. The abbreviation LN in (c) means liquid nitrogen. *Pc* indicates *P. curdlanolyticus*.

to induce a reaction. We used this method to perform Gram staining of cells in plugs (Fig. 5). After immersing a piece of glass slide in fluorocarbon, an array of aqueous droplets of the reagent for analysis (here it was Gram staining solution) was first generated on the slide surface (Fig. 5a). Next, each plug in the tubing was deposited into one droplet of reagent, and due to surface tension, the plugs and the droplets merged reliably (Fig. 5b, inset). Readout of reactions in the droplets was imaged optically from the bottom of the slide. Because there was no curved surface in the light pathway (Fig. 5c), aberrations common in imaging of plugs in tubing were eliminated. Two common problems of injecting reagents into plugs by using a PDMS T-junction are that volumetric injection ratio is limited,[40] and that many compounds in the reagents tend to be adsorbed on the PDMS surface. This method circumvents these problems while still enabling analysis with throughput up to $\sim 10^3$ plugs per trial; even higher throughput can potentially be achieved by integration with automatic control. Moreover, immersion in excess fluorocarbon slows down the evaporation of the nanoliter droplets and enables long reaction times – potentially up to a week if at room temperature. This method has the potential to be generally adopted for analyzing the contents of plugs.

An important feature of this approach of combining the chemistrode with stochastic confinement is that splitting of the plugs not only enables performing multiple parallel tests immediately after incubation of the cells in the plugs, but also allows tracing each plug in a daughter array back to the initial plug from which it was split. Because the order of the original plugs is preserved in the order of the daughter plugs, plugs in parallel daughter arrays can be traced by encoding patterns in the sequence of plugs when they are formed. In the experiments for this paper, a long carrier fluid spacing was introduced between every 30 plugs for the identification of sequences of plugs. Thus, the four arrays were reliably aligned, and any potential errors in the alignment, such as might occur if a plug were to split or two plugs were to coalesce, did not propagate. For tests that required transferring plugs from the tubing onto different plates, the locations of the deposited plugs were tracked by using grids. By tracing the sequences of plugs, we matched the order of the four different plugs in the four different arrays that were used for the four different tests. On one hand, rapid classification or identification tests can locate particular plugs containing cells of interest in the array, so that these plugs can be collected for culture. On the other hand, the ability to preserve viable cells in plugs allows for culture of cells long after the splitting event and after knowing the results of a parallel identification test on another daughter array. The splitting also enables the use of incompatible techniques on identical copies of cell populations from the same original single cell. For example, identification techniques such as gene sequencing and FISH require killing of the cells, while live cells are required for cultivation and functional tests as the cellulase assay.

Finally, we demonstrated that FISH can be used to identify *P. curdlanolyticus* cells in one of the identical daughter arrays while using the cells in another daughter array for cultivation (Fig. 6). We developed a new protocol to detect *P. curdlanolyticus* in bulk and modified this protocol (see Experimental) to detect *P. curdlanolyticus* isolated in plugs. After isolating and incubating a mixture of *P. curdlanolyticus* and *E. coli* with a concentration ratio of 1 : 1 in approximately 200 plugs, all plugs were split into four identical daughter arrays. Cells in plugs from the first daughter array were fixed by injecting 100% ethanol into each plug using a T-junction, resulting in 50% ethanol and 50% TSB media (v/v) in each of the plugs. Then the plugs were stored at −20 °C for 20 h and fixation took place (Fig. 6a). After fixation, ten plugs were spotted into wells of a PDMS membrane sealed to an UltraStick glass slide (Fig. 6b). Following staining with a 16S ribosomal-RNA probe specific to *P. curdlanolyticus* (Pc196) (see Experimental), plug 6 (Fig. 6c) was identified as containing *P. curdlanolyticus*. Plug 4 was identified as containing a different species (*E. coli*), judging by weak non-specific fluorescence (Fig. 6c). Plug 3 is an example of a blank plug (Fig. 6c). Using this protocol, the fluorescence intensity of *P. curdlanolyticus* stained with the Pc196 probe was ~9000 a.u. above background, and fluorescence of *E. coli* stained with the same probe was ~100 a.u. above background. This 90 : 1 ratio of intensities facilitated the identification of *P. curdlanolyticus* (Fig. 6d). Fluorescence intensity was similar to that of controls where the procedure was performed in bulk (*i.e.* outside of plugs), indicating that FISH staining from plugs can be performed without loss of staining quality (Fig. S4†). Plugs in the second daughter array were spotted and cultured on a culture plate. Colonies of *P. curdlanolyticus* also grew at the location of plug 6 (Fig. 6e).

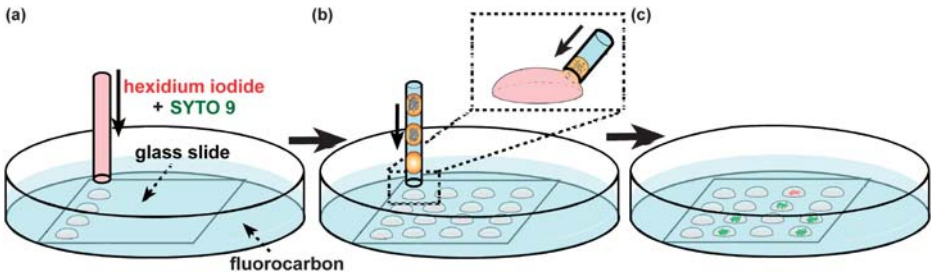

Fig. 5 A schematic drawing of Gram staining, which was performed on a glass slide immersed in fluorocarbon. **(a)** A two-dimensional array of droplets of the staining solution for the Gram test, a mixture of SYTO9 and hexidium iodide, was first spotted onto a piece of glass slide immersed in fluorocarbon (FC40). **(b)** The plugs for the test were then deposited into the droplets of the staining solution. One plug was deposited into each droplet of the staining solution. **(c)** The fluorescence of the stained cells in each droplet was observed from the bottom of the slide by using an epi-fluorescence microscope. The slide was immersed in FC40 during the whole process so that there was no significant evaporation of aqueous droplets.

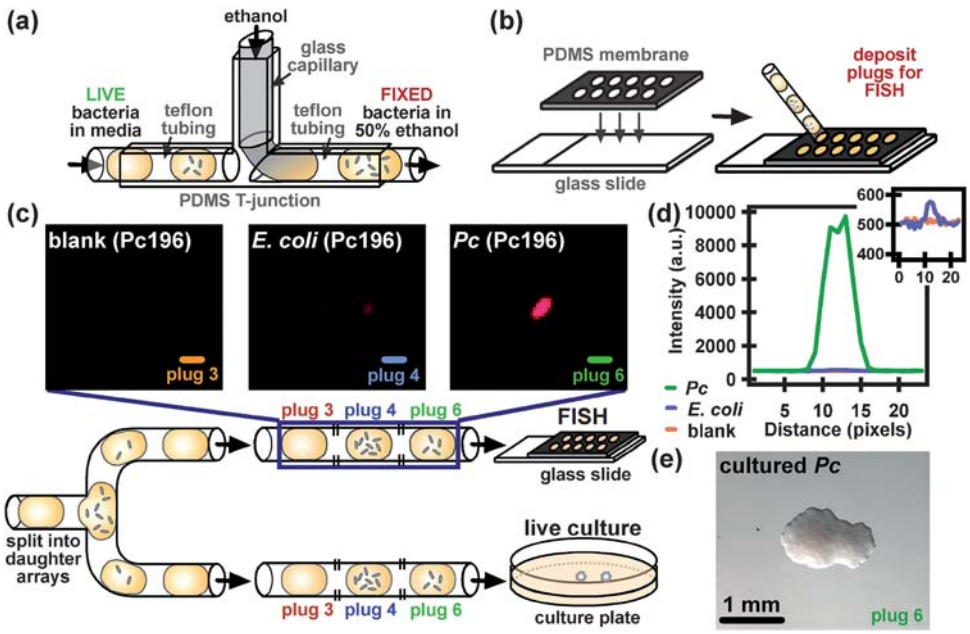

Fig. 6 Parallel live cultivation and killing identification by Fluorescence *In Situ* Hybridization (FISH) on clones of the same cell. FISH was used to identify *P. curdlanolyticus* cells in one of the identical daughter arrays while using the cells in another daughter array for cultivation. **(a)** A schematic drawing of fixation of the cells in an array of plugs for FISH by injection of ethanol into each plug using a T-junction. **(b)** A schematic drawing of attaching a PDMS membrane containing 5% carbon with holes onto an UltraStick glass slide to form staining wells for FISH, into which the plugs containing fixed cells were then deposited. **(c)** Ten plugs in a daughter array were deposited and stained by FISH using the probe Pc196 (see Experimental). *P. curdlanolyticus* cells contained in plug 6 (shown) and plug 5 (not shown) were identified. Plug 3 and plug 4 are examples of blank and *E. coli* containing plugs, respectively. A parallel array of daughter plugs was used for cultivation. **(d)** Fluorescence intensity (a.u.) of the stained cells in plugs shown in (c), as measured by an array scanner confocal microscopy. **(e)** A colony of *P. curdlanolyticus* that grew after culturing plug 6 from the second array. *Pc* indicates *P. curdlanolyticus*.

Conclusions

In this paper, we demonstrate how plug-based microfluidic approaches can be used to accomplish a set of single-cell microbiologic processing: from sampling directly from an environment, to isolating single cells from a multi-species mixture, to identifying cells of interest by killing methods while at the same time providing their clonal copies for functional testing, culturing, or preservation. Sampling directly from the environment with high spatial resolution, as provided by the chemistrode, is attractive for understanding spatial structures present in natural and synthetic microbial communities.[41,42]

This approach requires that isolated cells are culturable individually in plugs. As a result, it is suitable for working with any cells that do not require symbiotic microorganisms for growth, and it is especially useful for attaining rare species in mixtures that are dominated by competitive interactions, or for cells that show enhanced growth in confined volumes.[29]

The modified chemistrode presented in this paper would facilitate culturing isolated cells in their original media, which may contain compounds required for growth. Even if the initial growth in plugs is minimal (just a few divisions), species of interest can be identified by FISH or by other genetic methods, such as 16S rRNA sequencing, which has been applied to identify single cells isolated on a microchip.[39] After identification, alternative techniques and conditions can be used to successfully culture the sample of the species preserved in the parallel array. For example, the hybrid method[43] could be used to generate large numbers of different conditions in plugs to screen optimal growth condition.

The microfluidic approach described in this paper would be potentially useful for studies in environmental microbiology and of the human microbiome or for diagnostics. We demonstrated the approach here for microbes, but it may be applicable for other cells in addition to bacteria. For example, it may be used to propagate and analyze immune cells after isolation from patients. Similarly, we demonstrated the integration of FISH for identification of rare bacterial species with stochastic confinement using *P. curdlanolyticus* as the rare species, but this approach may be applicable for detection of other microbes or cells in single or low numbers, including Gram-positive bacteria, Gram-negative bacteria, or mammalian cells as is performed in prenatal and cancer diagnostics. This approach would also complement current methods for FISH detection.[8,9,44–47]

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References

1 S. Giovannoni and U. Stingl, *Nat. Rev. Microbiol.*, 2007, **5**, 820–826.
2 J. A. Huber, D. Mark Welch, H. G. Morrison, S. M. Huse, P. R. Neal, D. A. Butterfield and M. L. Sogin, *Science*, 2007, **318**, 97–100.
3 D. B. Rusch, A. L. Halpern, G. Sutton, K. B. Heidelberg, S. Williamson, S. Yooseph, D. Y. Wu, J. A. Eisen, J. M. Hoffman, K. Remington, K. Beeson, B. Tran, H. Smith, H. Baden-Tillson, C. Stewart, J. Thorpe, J. Freeman, C. Andrews-Pfannkoch, J. E. Venter, K. Li, S. Kravitz, J. F. Heidelberg, T. Utterback, Y. H. Rogers, L. I. Falcon, V. Souza, G. Bonilla-Rosso, L. E. Eguiarte, D. M. Karl, S. Sathyendranath, T. Platt, E. Bermingham, V. Gallardo, G. Tamayo-Castillo, M. R. Ferrari, R. L. Strausberg, K. Nealson, R. Friedman, M. Frazier and J. C. Venter, *PLoS. Biol.*, 2007, **5**, 398–431.
4 R. Daniel, *Nat. Rev. Microbiol.*, 2005, **3**, 470–478.
5 D. N. Frank and N. R. Pace, *Curr. Opin. Gastroenterol.*, 2008, **24**, 4–10.
6 D. A. Peterson, D. N. Frank, N. R. Pace and J. I. Gordon, *Cell Host Microbe*, 2008, **3**, 417–427.
7 P. Hugenholtz and G. W. Tyson, *Nature*, 2008, **455**, 481–483.
8 R. Amann and B. M. Fuchs, *Nat. Rev. Microbiol.*, 2008, **6**, 339–348.
9 A. Moter and U. B. Gobel, *J. Microbiol. Methods*, 2000, **41**, 85–112.
10 K. Zengler, G. Toledo, M. Rappe, J. Elkins, E. J. Mathur, J. M. Short and M. Keller, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 15681–15686.
11 T. Kaeberlein, K. Lewis and S. S. Epstein, *Science*, 2002, **296**, 1127–1129.
12 A. Grodrian, J. Metze, T. Henkel, K. Martin, M. Roth and J. M. Kohler, *Biosens. Bioelectron.*, 2004, **19**, 1421–1428.
13 N. Kataoka, Y. Tokiwa, Y. Tanaka, K. Takeda and T. Suzuki, *Appl. Microbiol. Biotechnol.*, 1996, **45**, 771–777.
14 F. Schut, E. J. Devries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins and D. K. Button, *Appl. Environ. Microbiol.*, 1993, **59**, 2150–2160.
15 S. A. Connon and S. J. Giovannoni, *Appl. Environ. Microbiol.*, 2002, **68**, 3878–3885.
16 B. C. Ferrari, S. J. Binnerup and M. Gillings, *Appl. Environ. Microbiol.*, 2005, **71**, 8714–8720.
17 A. Manome, H. Zhang, Y. Tani, T. Katsuragi, R. Kurane and T. Tsuchida, *FEMS Microbiol. Lett.*, 2001, **197**, 29–33.
18 D. Chen, W. B. Du, Y. Liu, W. S. Liu, A. Kuznetsov, F. E. Mendez, L. H. Philipson and R. F. Ismagilov, *Proc. Natl. Acad. Sci. USA*, 2008, **105**, 16843–16848.
19 Y. Liu and R. F. Ismagilov, *Langmuir*, 2009, **25**, 2854–2859.
20 J. Q. Boedicker, L. Li, T. R. Kline and R. F. Ismagilov, *Lab Chip*, 2008, **8**, 1265–1272.
21 A. Huebner, M. Srisa-Art, D. Holt, C. Abell, F. Hollfelder, A. J. Demello and J. B. Edel, *Chem. Commun.*, 2007, 1218–1220.
22 A. Huebner, L. F. Olguin, D. Bratton, G. Whyte, W. T. S. Huck, A. J. de Mello, J. B. Edel, C. Abell and F. Hollfelder, *Anal. Chem.*, 2008, **80**, 3890–3896.
23 K. Martin, T. Henkel, V. Baier, A. Grodrian, T. Schon, M. Roth, J. M. Kohler and J. Metze, *Lab Chip*, 2003, **3**, 202–207.
24 K. Martin, K. Lemke, T. Henkel, A. Grodrian, J. M. Kohler, J. Metze and M. Roth, *Biospektrum*, 2006, **12**, 743.
25 H. Song, J. D. Tice and R. F. Ismagilov, *Angew. Chem.-Int. Edit.*, 2003, **42**, 768–772.
26 H. Song, D. L. Chen and R. F. Ismagilov, *Angew. Chem.-Int. Edit.*, 2006, **45**, 7336–7356.
27 J. Clausell-Tormos, D. Lieber, J. C. Baret, A. El-Harrak, O. J. Miller, L. Frenz, J. Blouwolff, K. J. Humphry, S. Koster, H. Duan, C. Holtze, D. A. Weitz, A. D. Griffiths and C. A. Merten, *Chem. Biol.*, 2008, **15**, 427–437.
28 C. H. J. Schmitz, A. C. Rowat, S. Koster and D. A. Weitz, *Lab Chip*, 2009, **9**, 44–49.
29 J. Q. Boedicker, M. E. Vincent and R. F. Ismagilov, *Angew. Chemie Int. Ed.*, 2009, in press.
30 D. N. Adamson, D. Mustafi, J. X. J. Zhang, B. Zheng and R. F. Ismagilov, *Lab Chip*, 2006, **6**, 1178–1186.
31 T. Hatakeyama, D. L. L. Chen and R. F. Ismagilov, *J. Am. Chem. Soc.*, 2006, **128**, 2518–2519.
32 I. Shestopalov, J. D. Tice and R. F. Ismagilov, *Lab Chip*, 2004, **4**, 316–321.
33 C. J. Gerdts, V. Tereshko, M. K. Yadav, I. Dementieva, F. Collart, A. Joachimiak, R. C. Stevens, P. Kuhn, A. Kossiakoff and R. F. Ismagilov, *Angew. Chem.-Int. Edit.*, 2006, **45**, 8156–8160.
34 T. R. Kline, M. K. Runyon, M. Pothiawala and R. F. Ismagilov, *Anal. Chem.*, 2008, **80**, 6190–6197; H. Song, H. W. Li, M. S. Munson, T. G. Van Ha and R. F. Ismagilov, *Anal. Chem.*, 2006, **78**, 4839–4849.
35 J. Schemberg, A. Grodrian, R. Romer, G. Gastrock and K. Lemke, *14. Heiligenstadter kolloquium, Proceedings*, 2008, **27**, 169–176.
36 C. E. Lankford, J. R. Walker, J. B. Reeves, N. H. Nabbut, B. R. Byers and R. J. Jones, *J. Bacteriol.*, 1966, **91**, 1070.
37 W. T. Liu and L. Zhu, *Trends Biotechnol.*, 2005, **23**, 174–179.
38 D. B. Weibel, W. R. DiLuzio and G. M. Whitesides, *Nat. Rev. Microbiol.*, 2007, **5**, 209–218.
39 Y. Marcy, C. Ouverney, E. M. Bik, T. Losekann, N. Ivanova, H. G. Martin, E. Szeto, D. Platt, P. Hugenholtz, D. A. Relman and S. R. Quake, *Proc. Natl. Acad. Sci. USA*, 2007, **104**, 11889–11894.
40 L. Li, J. Q. Boedicker and R. F. Ismagilov, *Anal. Chem.*, 2007, **79**, 2756–2761.
41 H. J. Kim, J. Q. Boedicker, J. W. Choi and R. F. Ismagilov, *Proc. Natl. Acad. Sci. USA*, 2008, **105**, 18188–18193.
42 R. Stocker, J. R. Seymour, A. Samadani, D. E. Hunt and M. F. Polz, *Proc. Natl. Acad. Sci. USA*, 2008, **105**, 4209–4214.
43 L. Li, D. Mustafi, Q. Fu, V. Tereshko, D. L. L. Chen, J. D. Tice and R. F. Ismagilov, *Proc. Natl. Acad. Sci. USA*, 2006, **103**, 19243–19248.
44 M. W. Mittelman, M. Habash, J. M. Lacroix, A. E. Khoury and M. Krajden, *J. Microbiol. Methods*, 1997, **30**, 153–160.
45 V. J. Sieben, C. S. Debes-Marun, L. M. Pilarski and C. J. Backhouse, *Lab Chip*, 2008, **8**, 2151–2156.
46 V. J. Sieben, C. S. D. Marun, P. M. Pilarski, G. V. Kaigala, L. M. Pilarski and C. J. Backhouse, *IET Nanobiotechnol.*, 2007, **1**, 27–35.
47 M. M. M. Kuypers and B. B. Jorgensen, *Environ. Microbiol.*, 2007, **9**, 6–7.

