

Fig. 1 Schematic illustration of the use and applications of different technologies for culturing microorganisms. The horizontal axis spans the extent of control over the liquid handling processes and the chemical environment for growth. The vertical axis expresses the level of parallelization and miniaturization of the bioreactors. Classic methods span a wide range of techniques, from the oldest and simplest such as culture flasks and Petri dishes (a), and extend both towards miniaturization and parallelization, as in well plate cultures (b), and to complex growth protocols, as in automated bioreactors (c). The advent of microfluidics made it possible to control the chemical environments at the scale of individual cells and to observe their growth under chemical and mechanical stresses. This opened new possibilities in microbiology, such as studying the aging of bacterial cells in the 'mother machine' (d).⁸ Single phase microfluidics spans a broad range of applications, both for research and for diagnostics, and includes also highly automated systems (e) for culturing,⁹ yet it is not straightforward with these systems to incubate massively large numbers of single cells or small colonies in isolation. Already in the 1950s droplets have been proposed as isolated, ultra-small compartments for culturing.¹⁰ The bulk emulsion techniques (f), however, have no standardized protocols and use highly polydisperse droplets, which significantly limits their use. The advent of droplet microfluidics led to the development of technologies for controlling practically all the aspects of generation and handling of droplets. The ability to generate thousands or millions of almost ideally identical droplets allows one to controllably encapsulate individual cells (g), culture them in known and controlled environments, take full advantage of the confined volumes and concentration of products of metabolism, detect subtle differences between cells and their phenotypes and sort them. The droplet microfluidic systems span a wide range of sizes of droplet bioreactors. The techniques are also gradually developed towards automation of even highly complex protocols, such as exchange of media in droplets and long-term culturing (h).⁷ Further developments may expand these possibilities, for example, towards a fully automated system for directed evolution (i) supporting thousands of iterations of selection conducted on pools of billions of cultures each originating from a single encapsulated mutant.



microfluidics to the techniques of droplet microfluidics that are pivotal to the use of this technology in microbiology (Fig. 1).

Methods for studying microbes in confinement

In this section, we briefly describe the three main approaches that enable studying microbial cells in parallel in micro-volume bioreactors. The oldest method of growing microbes in bulk emulsions ensures a large number of tiny compartments that can be used for single cell studies. However, this approach does not allow for individual and continuous control of the conditions for each culture. The development of single-phase microfluidic systems and integrated valves opened the possibility of studying cells in a highly controlled manner, yet with limited throughput. Droplet microfluidics synergistically combines the advantages of the massively parallel execution of assay in emulsions with the robust control provided by microfluidic technologies.

Bulk emulsions

Microdroplets were used as micro-bioreactors long before the development of droplet microfluidics. In 1954, Joshua Lederberg presented a method for the isolation and observation of single microbes in droplets deposited on a glass slide covered with a thin layer of mineral oil.¹⁰ Since then, the idea of encapsulation of single cells into droplets was adapted to multiple microbiological studies. Microorganisms were encapsulated in liquid droplets,¹¹ agarose,^{12–16} polyacrylamide¹⁷ and alginate microgel droplets¹⁸ or in double emulsions.¹⁹ In order to increase the throughput of these experiments, droplets and hydrogel particles usually were not deposited on solid substrates but rather suspended in a continuous liquid medium. Agarose microgel particles brought the widest interest due to the possibility of interrogating and sorting them with flow cytometry,^{20,21} even if it required prior filtration of the suspension to select the appropriate size of the beads.¹⁷

Before the advent of droplet microfluidics, several approaches were used to generate droplets and microgels: i) bulk homogenization^{12,13} ii) extrusion¹⁸ or iii) passing the liquid through a vibrating orifice.^{14,15} These straightforward methods were usually used in relatively simple applications, such as i) cell enumeration and metabolic activity monitoring,¹² ii) antibiotic susceptibility testing,^{20,22} and iii) enrichment of slow-growing bacteria.^{23,24} Although simple, these methods continue to be interesting research tools. Bachmann *et al.* have recently demonstrated the use of encapsulation of *Lactococcus lactis* single cells in water-in-oil droplets for passive selection of the slower growing mutants with higher biomass yield – simply by isolating their nutrition from the faster-growing competitors.¹¹ This work shows an interesting use of microdroplets and stochastic confinement in research on microbial ecology, including potential applications to

studies of competition and co-operation in evolving populations or on the relationship between the environment and various metabolic strategies. In another interesting recent demonstration, Fitzsimons *et al.* used droplets to significantly improve a method of sequencing single bacteria.¹⁶ Prior to DNA isolation, they split a diverse microbial community into droplet-microgels, each containing a single cell. Then the cells were allowed to grow inside the microgels to obtain thousands of genetically identical cells as an input for more efficient whole genome amplification with a reduced bias.¹⁶

Despite the numerous applications of single cell encapsulation using bulk emulsification, these approaches suffer from important limitations and until recently could not be translated to other fields of microbiology. First of all, liquid and microgel droplets generated by bulk emulsification are highly polydisperse – and differences in the volume of micro-bioreactors may produce biases in the results of the assays. Moreover, the most commonly used analysis with FACS requires particles of a specific size, and polydisperse populations of droplets must be filtered^{20,21,23} prior to the introduction to a flow cytometer. Usually, the encapsulation of cells with bulk techniques requires separate instruments for each operation: formulation, incubation, detection and sorting. Manipulation over chemical composition in a single experiment is difficult. Also, some operations cannot be conducted, *e.g.* merging and splitting of droplets.

Single phase microfluidic systems

Single phase systems, *i.e.* ones that guide the aqueous solutions and suspensions of microorganisms directly in the channels without the addition of carrier oil, allow for a high degree of integration and automation of operations on the chip. Flow is usually controlled with syringe pumps and pneumatic microvalves²⁵ or with the use of electrokinetic effects.^{26,27} The breakthrough momentum for continuous-flow microfluidics was the development of large-scale integration with elastomer microvalves that enabled manufacturing and handling simultaneously hundreds of individual micro-chambers on a single chip.²⁸ Single phase systems have been successfully used for microbial analysis, including single cell assays. The most important examples include studies of bacterial antibiotic resistance²⁹ and persistence,^{30,31} long-term microbial cultivation in a miniaturized chemostat,⁹ isolation and genetic analysis of single cells of uncultivated microbes,^{32,33} and other examples of single-microbe genomic studies.³⁴ Single phase microfluidics cannot handle the massively large numbers of reactions available to droplet microfluidics; it is more difficult to work with truly tiny volumes in the pico- to nano-litre range and to provide for long-term experiments on small volumes due to the contact of the microbial colonies with solid surfaces. Still, continuous-flow microfluidics offers numerous unique advantages over droplet systems, including *e.g.* possibilities to execute assays involving complex microscopic imaging (*e.g.* on single cells) or



experiments requiring the formation of specified and structured flows and gradients. The applications of continuous-flow microfluidics to the study of microbial physiology and ecology were presented in recent reviews^{35–37} and will not be discussed here further.

Droplet microfluidics

Droplet microfluidics brings together the sample isolation and confinement offered by emulsion systems and the precise liquid handling delivered by single-phase microfluidics. Droplet microfluidic systems use two immiscible phases. One is typically organic liquid (oil) that preferentially wets the walls of the channels. The second, typically aqueous, immiscible phase is introduced into the system and is broken up into droplets. The droplets do not wet the wall of the channels and are carried along them pushed by the oil.

The beginning of droplet microfluidic technology is usually associated with the first demonstrations of generation of aqueous droplets in microchannels using T-junction³⁸ or flow-focusing geometry.³⁹ These chip geometries bring together two immiscible liquids (e.g. oil and water) so that the aqueous phase is broken up by the biocompatible oil phase into uniformly sized droplets. In the following years the physics of droplet formation was described^{40–43} and modules for droplet handling were developed. The first systems demonstrated that droplets can be incubated,^{44–46} passively split⁴⁷ and merged.^{48,49} Detailed reviews of the techniques for handling droplets have been extensively presented in multiple publications recently.^{50–53} From the point of view of usage in microbiology it is important to know that it is possible to manipulate both small (from a few microliters) and large (mL and bigger) volumes of aqueous suspensions of microorganisms. These can be split into droplets ranging in volume from pico- to microliters. The droplets can subsequently be manipulated, *i.e.* split, titrated with additional reagents, incubated, and analysed.

Current state of the art allows generation of droplets with frequencies of more than $\sim 10\,000$ Hz and a coefficient of variation of droplet diameter of less than 2%.⁵⁴ The interrogation and analysis of droplet fluorescence can be handled with speeds of up to $\sim 250\,000$ droplets per second.⁵⁵ One of the most important operations is the active sorting of droplets at high rates using electromagnetic,⁵⁶ acoustic⁵⁷ and mechanical forces.⁵⁸ Monodisperse droplets generated in microfluidics devices can be also sorted with FACS provided that droplets are dispersed in an aqueous solution (they are in the form of double emulsions)^{59,60} or as microgels.^{61,62}

The possibility of single cell encapsulation and the following incubation and analysis is one of the main advantages of droplet microfluidics. In most of the systems the number of cells encapsulated in each droplet is dictated by Poisson statistics. This limits the ability to encapsulate a single cell in each droplet: typically, in order to obtain single-cell occupancy per droplet, the concentration needs to be low enough that only a small fraction of all the droplets is occupied.

Interestingly, it is possible to enhance the efficiency of single-cell encapsulation in droplets.⁶³

Using droplet microfluidics it is also possible to generate droplets with an arbitrarily predefined chemical composition. There are two main approaches for the generation of concentration gradients of chemical agents in the droplets: i) *via* active manipulation of flow velocities using pumps, valves and other external forces⁶⁴ and ii) using passive techniques that are based on physical phenomena such as interfacial tension or gravity. A chemical gradient in droplets can be generated in active ways by varying the input flow rates of the constituent streams,⁶⁵ by drawing liquid from a chromatography column⁶⁶ or by formation of gradients in single-phase streams prior to droplet generation.^{67–69} Other methods are based on merging droplets of different volumes³ or using direct injection of portions of samples to already preformed droplets.⁷⁰

In recent years, various techniques for passive operations on microdroplets have been developed, *i.e.* methods that do not require programmable syringe pumps or other automated instrumentation for controlling the flow of liquids on chips. The latest advances within this area include passive formation of monodisperse droplets^{71–73} and techniques for subsequent operations on droplets. For example, droplets can be passively merged in dedicated geometries such as pillar traps,^{48,74} static arrays^{75,76} or rails and anchors.^{77,78} Passive microfluidics can also be used for more complex operations such as formation of simple gradients in hydrodynamic traps.^{79,80} However, passive methods are of low throughput and typically produce individually addressed compositions of droplets at single Hz rates that are far too low for efficient screening, thus prompting for their implementation in autonomous devices dedicated to point-of-care testing (POCT).⁸¹

Droplet microfluidic techniques offer a very high degree of control over the generation and manipulation of emulsions and droplets.^{82,83} Easy access to a high number of experiments, in turn, provides for supreme statistics and better analytical results of the assays. Importantly, these features depend critically not only on the fluidic operations but also on chemistry. The droplet phase may not even partially wet the surfaces of the microchannels. Surfactants and the carrier liquid should be inert with respect to the microorganisms, often should allow for mass transport of gasses into and out of the droplets, and also should prevent transport of media, active substances and dyes between the droplets.^{84,85} These challenges prompted several breakthroughs in the chemical aspects of microdroplet technology – the most notable examples comprise the introduction of biologically inert fluorocarbons as a continuous phase⁸⁶ and synthesis of biocompatible surfactants that provided long-term stability of emulsions.⁸⁷

Importantly, if properly designed, surfactant-stabilized droplets may reduce the risk of cross-contamination between cultures – a common problem in classical methods (flasks, Petri dishes, multiwell plates) in which crucial operations are performed on cultures that are in contact with the external environment. In droplet microfluidic systems, an organic



continuous phase prevents the transfer of cells between droplets and their adsorption on the walls of microfluidic channels. Lack of contact of the cultures with solid walls prevents formation of biofilms and eliminates additional steps of washing and sterilization of the devices.

In summary, droplet microfluidic systems present the following major unique properties and advantages in comparison to the use of bulk emulsification or continuous flow microfluidics: (i) ability to generate massively large numbers of highly monodisperse liquid compartments for culturing of microbes, from single cells to small populations, (ii) possibility to control and alter the chemical composition and to monitor and sort the droplet bioreactors, and (iii) capability of execution of multistep, iterative, automated long-term reaction protocols.

Below we review the applications of droplet microfluidics solely in microbiology, starting with various approaches to detection of cells and their metabolic activity in droplets, through studies on antibiotics and antibiotic resistance, on bacterial physiology and interactions and, finally, applications in biotechnology.

Applications of droplet microfluidics in microbiology

Detection and identification of microorganisms in droplets

Detection of bacterial cells and their metabolic activity is critical to most of the experimental protocols in microbiology. Different methods of detection contribute to the toolbox used in more complex studies and applications. Some of these

methods have also been developed directly to serve an application in rapid identification of microbial pathogens.

In 2003, Martin *et al.* published their pioneering work that demonstrated the detection of fluorescent proteins synthesized by bacteria that were encapsulated in 60 nL of aqueous droplets.⁸⁸ They used a T-junction chip to encapsulate different bacterial strains and one yeast strain, with the droplets separated by an immiscible oil phase. Microbial growth was detected directly in a droplet by measuring the fluorescence of proteins expressed by *Escherichia coli* and, as a reference, off-chip by agar plate counting.⁸⁸ Since then, detection of the fluorescence of expressed proteins has been widely popular and it has been used, for example, for the analysis of *E. coli* (Fig. 2A),^{89–93} *Salmonella typhimurium*⁹⁴ and *Bacillus subtilis*.⁹⁵

Droplets have also been demonstrated as a suitable platform for assessing the enzymatic activity of microbes. The first paper to demonstrate the monitoring of microbial enzymatic activity in droplets was presented by Huebner *et al.*, who measured the activity of alkaline phosphatase expressed by bacteria.⁹⁶ Other enzymes that have been also used as markers of microbial activity in droplet assays include β -galactosidase,^{46,56,97,98} β -glucuronidase,^{97,99} cellulase,¹⁰⁰ glycosidase,^{101,102} SFP synthase.¹⁰³ Droplet microfluidic assays have also been developed for estimating the alcohol production rate of microbes using enzymatic assays.^{104,105}

Multiple fluorescent markers can be detected/monitored simultaneously in droplet cultures. Shim *et al.* presented a microfluidic device that was capable of trapping and storing picoliter droplets.¹⁰⁶ The authors monitored simultaneously both the gene expression referenced by the red fluorescent

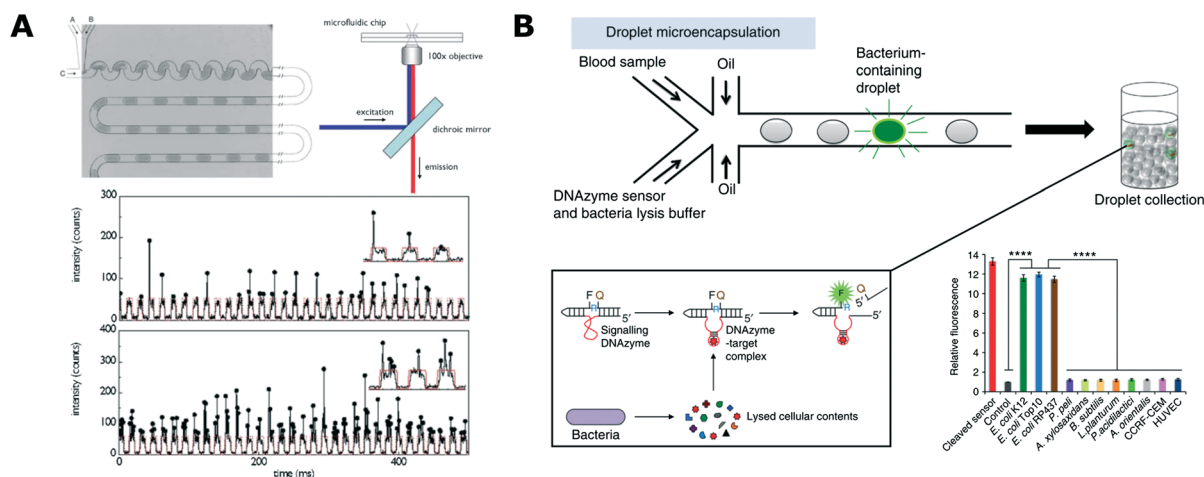


Fig. 2 Detection and analysis of bacteria in droplets. A) Detection of bacteria expressing fluorescent protein. Microfluidic chip for generation of uniformly sized and spaced droplets (top left) together with a schematic of the fluorescence readout setup (top right). At the bottom, exemplary traces of the optical readout of the fluorescence signal. Each arch-shaped signal corresponds to a weakly fluorescent medium that forms the bulk volume of the droplet. Vertical spikes correspond to expressed fluorescence signal of bacteria. Different cell loading conditions are shown with low density on top and high density at the bottom (reproduced from ref. 89 with permission from The Royal Society of Chemistry).⁸⁹ B) Specific detection of bacteria from blood using DNAzyme-based sensor. Blood samples and DNAzyme sensors are mixed prior to encapsulation in droplets (top). Bacteria produce target molecules that bind to DNAzyme, which then undergoes activation *via* a conformational change. Activated DNAzyme autocleaves the domain with a quencher molecule, thus resulting in the increase of fluorescence signal (box, bottom left). Specificity of the DNAzyme designed for *E. coli* detection against the panel of non-target organisms (bottom right) (figures adapted from ref. 108. Copyright 2014 Macmillan Publishers Limited).¹⁰⁸



protein and the enzymatic activity of alkaline phosphatase in bacteria over 20 h cultures starting from single cells.¹⁰⁶ In other works, a similar dual parameter monitoring was carried out in droplet emulsion in order to estimate the level of dye leakage from the droplets.^{107,114}

The presence and the activity of bacteria can also be measured indirectly by monitoring the changes in pH and oxygen level inside the droplets. Polymer micro-particles containing an immobilized pH-sensitive dye were used for determination of pH inside microdroplets. This technology allowed monitoring the metabolic activity of *E. coli* in 300 nL droplets over four days.¹⁰⁹ A similar microfluidic setup was used to measure microbial growth using oxygen-sensitive fluorescent nanoparticles.¹¹⁰ Mahler *et al.* used oxygen-sensitive nanoparticles to analyse the oxygen availability and consumption in emulsions consisting of hundreds of thousands of droplets.¹¹¹

Viability markers commonly used in traditional microbiology have found their way to droplet platforms as well. Boedicker *et al.* used resazurin to test the viability of *Staphylococcus aureus* in droplets.¹¹² Resazurin is converted to fluorescent resorufin by metabolism of viable aerobic organisms. Their work demonstrated how confinement in small droplets shortens the detection time of bacteria comparing to macroscopic cultures.¹¹² Since then, resazurin has been often used for the detection of viable bacteria.^{4,90,113,114} Kang *et al.* demonstrated the use of SYTO® 9 and propidium iodide (PI) dyes as live/dead markers in droplet microfluidics format.¹¹⁵ The membrane permeable SYTO® 9 stains both live and dead cells, while PI stains only dead cells with disrupted cell walls and membranes. This technology was demonstrated in droplets for measuring the fraction of dead and live *E. coli* cells.¹¹⁵

An interesting and important challenge in droplet microfluidics is to develop new schemes of detection with high specificity towards selected strains. Kang *et al.* proposed the use of DNazymes for rapid detection of bacteria in clinical samples (Fig. 2B).¹⁰⁸ DNazymes are synthetic single-stranded DNA oligonucleotides with catalytic activities. These catalytic DNA molecules are activated when they recognize their targets (in a similar way to aptamer binding). DNazymes are generated from a random library using *in vitro* evolution and their catalytic activity can be tied to the generation of a fluorescence signal when the DNzyme meets its target. *E. coli* specific DNzyme was used in a droplet assay combined with a custom developed 'Integrated Comprehensive Droplet Digital Detection' (IC 3D) device for specific detection and quantification of bacteria. This technology enabled absolute quantification of both stock and clinical isolates of *E. coli* in spiked blood within a broad range of concentrations, starting from a single cell per mL, in only 4 h.¹⁰⁸

Lyu *et al.* combined droplet encapsulation with a BlaC-specific fluorogenic probe for quantitative detection of bacteria.¹¹⁶ BlaC is a beta-lactamase enzyme that is naturally expressed by *Mycobacterium tuberculosis* (*Mtb*) and can therefore be considered a suitable marker for specific detection of *Mtb*. The lowest detection limit of 10 colony forming units

per milliliter was demonstrated with BlaC expressing *E. coli*. Quantification was shown with a dynamic range of up to 1×10^7 CFU mL⁻¹ in million-fold higher background concentrations of other bacteria.¹¹⁶ Bacteria can also be detected using immunochemistry, as shown in the work of Sinn *et al.* who used magnetic beads coated with specific antibodies for detecting and monitoring the growth of *E. coli*.¹¹⁷ The detection was performed *via* measurements of the changes in magnetic bead rotation upon binding to a single bacterial cell.¹¹⁷

The droplet platform is also suitable for the direct analysis and detection of bacterial genes and genomes. For example, microbial cells can be sorted according to their gene sequences. Lim *et al.* used real-time PCR in droplets to amplify specific gene sequences from encapsulated bacteria.¹¹⁸ The fluorescence signal generated during PCR allowed sorting and further analysis of the droplets comprising bacteria with a desired DNA sequence.¹¹⁸ In another work, encapsulation in droplets enabled uniform amplification of genomes as demonstrated with single *E. coli* cells. This technique is highly useful as a preliminary step for the next-generation sequencing platforms.¹¹⁹

Stochastic confinement of microbes into small droplets allows for digital quantification (similarly to droplet digital PCR that has recently become available commercially).^{120–122} Assuming Poissonian distribution of bacteria,⁶³ the fraction of positive droplets containing at least one bacteria can be used to calculate the initial microbial concentration in the sample. The positive droplets are usually detected by measuring the increase in fluorescence associated with the metabolic activity of viable bacteria.^{97,99} More specific detection and identification technologies (*e.g.* previously mentioned DNzyme¹⁰⁸- and BlaC¹¹⁶-assisted assays) can also be used for enumeration of their target cells. With these technologies the high detection sensitivities can be complemented by the ability to quantify targets over a wide dynamic range.

While the detection of fluorescence is the most common approach to monitoring and analysing the droplet content, many applications may greatly benefit from label-free schemes. The most straightforward option is to monitor the optical changes in the solution caused by the bacterial growth *via* bright-field microscopy. *Actinobacteria* detection and sorting was demonstrated using such an approach. *Actinobacteria* is a biotechnologically important phylum of microorganisms because many of the modern antimicrobial compounds are derived from them.¹²³ The optical density or turbidity has also been used to monitor bacterial growth and adaptation to antibiotic stress in a micro-droplet continuous culture device.⁷ Recently, Liu *et al.* presented a system for high-throughput label-free detection of proliferating bacterial colonies in droplets using a system based on light scattering.¹²⁴ This method also enabled simultaneous sorting of positive hits, *e.g.* mutants resistant to antibiotics. Another interesting method for detection that is unique to droplet microfluidics is the analysis of evolution of the droplet volume over time. The growth of microorganisms is usually associated with consumption of the nutrients and synthesizing



new metabolites, thus changing the chemical composition of the droplet. As a result, the concentration of osmotically active solutes changes and induces mass transfer of water between droplets, resulting in respective shrinkage or swelling. The decrease in volume of a droplet can be correlated with the number of initially encapsulated cells and their ability to metabolize osmotically active components. This technology is suitable for label-free monitoring of cell metabolism at the single cell level for both yeast and bacteria^{125,126} and also can be used for droplet sorting based on their size.¹²⁷

Antimicrobial susceptibility analysis in droplets

Boedicker *et al.* used droplet cultures to measure the antibiotic susceptibility of methicillin sensitive and resistant strains of *S. aureus*.¹¹² In the study, different antibiotics with fixed concentration were encapsulated with both strains separately in order to test their susceptibility. Later, the minimum inhibitory concentrations (MICs) towards cefoxitin were also determined.¹¹²

Antibiotic susceptibility tests are often conducted in microfluidic droplet systems that control droplets of volume ranging from ~ 100 nL to a few microliters. These systems allow incubation of long sequences of droplets to monitor the growth of bacteria. Very dense gradients of antibiotic concentration in sequences of more than 1000 droplets can be generated for precise MIC determination of encapsulated bacteria (Fig. 3C).^{4,6,128} A similar microfluidic setup was used in another work presented by Cottinet *et al.*, who investigated the phenotypic and genotypic changes in bacterial monocultures in starving conditions.¹²⁹ After various time intervals of starvation in bulk the bacteria were encapsulated so that each droplet contained approximately a single cell. The growth of populations derived from these individual bacteria was then followed over time. Starvation resulted in different growth phenotypes and emergence of corresponding heritable genotypes that were subsequently characterized *via* sequencing.¹²⁹

Churski *et al.* used an automated microfluidic platform to screen the effect of both individual antibiotics and

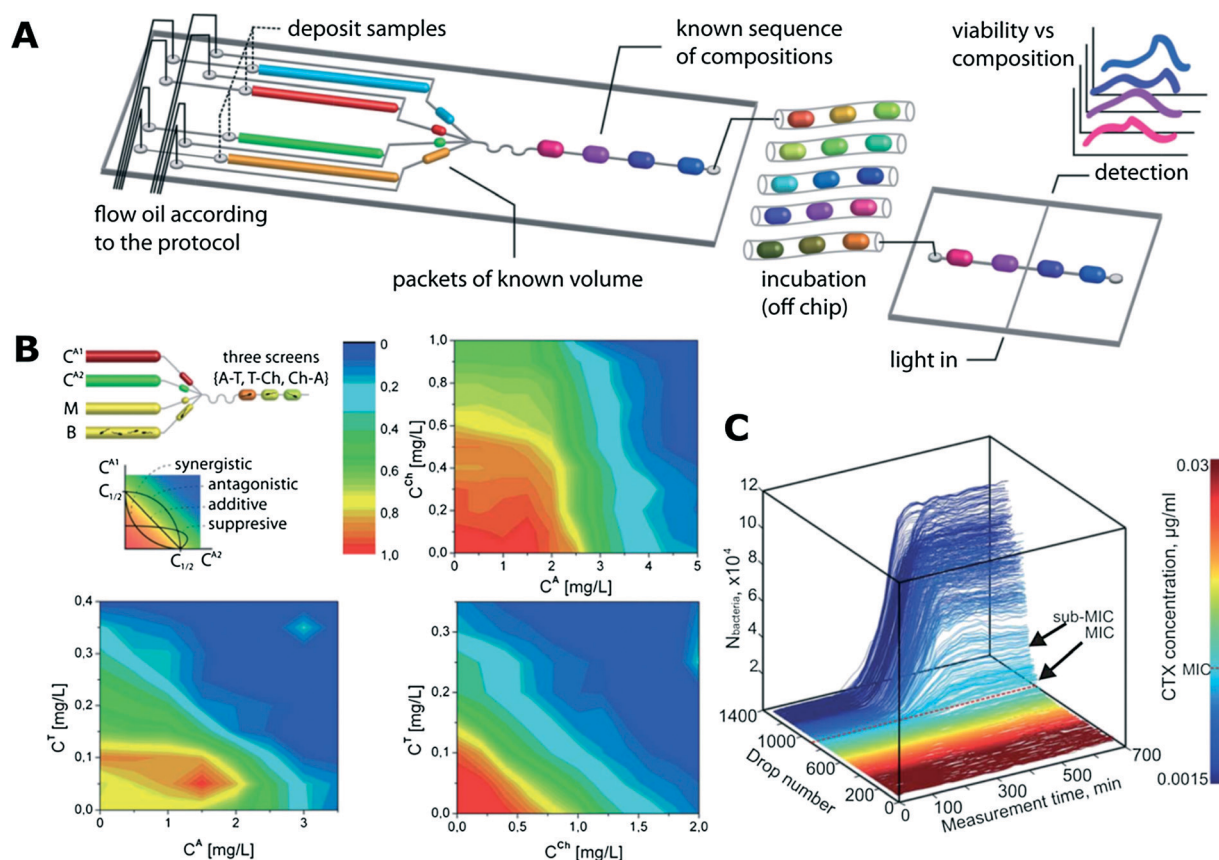


Fig. 3 Antibiotic susceptibility studies using trains of droplets in microfluidic channels. A) A schematic of an exemplary microfluidic system for screening various antibiotic combinations. Microdroplets of controlled and predefined volume and composition (media, bacteria, reagents, fluorescence substrate resazurin) are generated and incubated in long microtubing as droplet trains. After the incubation, the fluorescence of droplets is measured (reproduced from ref. 113 with permission from The Royal Society of Chemistry).¹¹³ B) Interactions between antibiotic pairs measured in the droplet platform shown in (A). Antagonistic (top right), additive (bottom right) and suppressive (bottom left) interactions are demonstrated (reproduced from ref. 113 with permission from The Royal Society of Chemistry).¹¹³ C) Growth curves of bacterial cultures in the gradient of antibiotic concentration. The colour mapping on the right corresponds to droplet number that is linked to antibiotic concentration (reproduced from ref. 128 with permission from The Royal Society of Chemistry).¹²⁸



combinations of drugs (Fig. 3A and B).¹¹³ Automation allowed for rapid determination of MIC levels and pairwise antibiotic interaction mechanisms (synergy, antagonism and suppression).¹¹³

In a series of projects, the group of M. Köhler used automated droplet systems¹⁰⁹ for studies on i) the toxicity of an industrial pollutant 2,4-dinitrophenol (DEP) as a single agent¹³⁰ and in combination with silver and gold nanoparticles;¹³¹ ii) the antimicrobial function of a candidate peptide,¹³⁰ also in combinations with nanoparticles;¹³² iii) the impact of caffeine and a hypertension drug to antibiotic co-administration¹³³ and iv) the combinatorial effect of metal ions and media composition on heavy metal-tolerant bacteria.^{110,134,135} Other similar examples of studies that used droplet microfluidic platforms to generate antibiotic gradients to assess the antimicrobial susceptibility of bacteria can be found in ref. 115, 117 and 136.

Cho *et al.* demonstrated a droplet-based platform for high-throughput screening of photosensitizer activity, that is, a chemical compound that undergoes a controlled light-induced change from a non-toxic to a toxic state for bacterial cells.¹³⁷

Droplet microfluidics enables investigation of antibiotic susceptibility also at the single cell level. This technique is useful for analysis of heterogeneous bacterial populations. In a study presented by Eun *et al.*, *E. coli* cells were encapsulated in agarose microparticles with various concentrations of rifampicin and analyzed using flow cytometry.⁶¹ The MIC of rifampicin was determined, and spontaneous mutants that had developed resistance to the antibiotic were isolated *via* FACS and subsequently characterized by DNA sequencing. The subunit or RNA polymerase was confirmed to be the antibiotic target as several mutations were found there, resulting in resistance.⁶¹ In another work, Liu *et al.* used the droplet platform to screen for antibiotic-resistant bacteria.¹²⁴ They isolated fusidic acid-resistant mutants and estimated the frequency of resistance among a population of *E. coli*.

Droplets can be used as micro-reactors to study the effect of antibiotics over time in quasi-stable nutrient conditions. Jakiela *et al.* designed a system supporting micro-droplet chemostat reactors.⁷ The system allowed for incubation of bacteria in droplets in chemical environments that could be changed and controlled over time in a pre-programmed fashion. Droplets with saturated culture could be split and fused with fresh medium in order to provide stable conditions for bacterial growth. They demonstrated the response and adaptation of bacterial growth to changing antibiotic concentrations over time.⁷

Microbial physiology and interactions between bacteria

One of the great potential uses of droplet microfluidics in microbiology is the isolation of rare or slow-growing species from complex environments. The first to show a proof of concept for this approach were Grodrian *et al.*, who used droplet plugs to isolate different bacterial monocultures from an environmental soil sample.¹³⁸ Liu *et al.* used a similar approach

to demonstrate that slow-growing *Paenibacillus curdlanolyticus* can easily be separated from fast-growing *E. coli* that otherwise would dominate and outnumber the slow-growing species in mixed bulk culture.¹⁰⁰ Isolated single cells were further analysed after the incubation with cellulase assay in droplets as well as using Gram staining and FISH analysis.¹⁰⁰

One of the main challenges in current microbiology is the identification of conditions that support the growth of so far “unculturable” bacteria.¹³⁹ Droplet microfluidics can provide advantageous features in determining such conditions by single cell isolation and low consumption of cultivation medium that is usually supplemented with the liquid substrate obtained from the environment of the investigated organism. Ma *et al.* presented a modified SlipChip device for targeted cultivation of bacteria.¹⁴⁰ In the first step, single cells from complex environmental samples were stochastically confined in an array of nanoliter droplets in order to eliminate competition during growth of various species. After the incubation, each droplet was split into two microcompartments – each on a separate slide of a SlipChip. Subsequently, one ‘half’ of the droplet can be analysed *via* a destructive assay (*e.g.* PCR), while the positive hits (viable colonies) on the corresponding slide can be inoculated for macroscale culture.¹⁴⁰ In the accompanying study, researchers used this device for the first successful cultivation of one of the species from the Human Microbiome Project’s “Most Wanted” list.¹⁴¹

Droplets can also provide a suitable environment to demonstrate and investigate interactions between bacteria. Park *et al.* used droplet microfluidics to encapsulate symbiotic pairs of bacterial strains that both lacked the ability to synthesise one specific amino acid and therefore could not grow alone (Fig. 4A).¹⁴² When encapsulated together with a complementary strain, they could compensate for each other, live long and prosper.¹⁴² Cross-kingdom communication was demonstrated by Jarosz *et al.* who co-encapsulated yeast and bacteria, showing that it takes just a few bacterial cells to switch yeast metabolism from using glucose to other carbon sources *via* prion-based transformation.¹⁴³ This phenomenon is mutually beneficial as yeast cells make less alcohol that is harmful for bacteria and is useful for yeast because their growth and long-term viability is improved with more complex carbon sources.¹⁴³

Huang *et al.* used trapped droplets for long-term (10 day) monitoring of population dynamics in individual droplets⁹³ and demonstrated the oscillating growth pattern of bacteria. After reaching a certain population density, a part of the bacterial population died that led to a sharp decrease in droplet fluorescence intensity. They also investigated and demonstrated the inoculum effect (IE) in their system. IE is a population-dependent phenomenon exhibiting resistance to intermediate antibiotic stress by colonies of high initial densities and susceptibility of populations at low densities.⁹³ Droplets were also used as 3D microenvironments to investigate the biofilm formation of *B. subtilis*. A dual-labelling system was implemented so that the bacteria expressed one





Fig. 4 Microbial interaction studies in droplets. A) Co-encapsulation of symbiotic bacteria in droplets. Both strains are unable to synthesize one amino acid (W, tryptophan; Y, tyrosine) and they are unable to proliferate when encapsulated alone in separate droplets (droplets 2 and 3; 0 h left, 18 h right). Co-encapsulation allows bacteria to grow symbiotically (droplet 1) (figures adapted from ref. 142). B) Communication of bacteria between droplets. The system consisted of inducer sending (S) and receiving strains (R) that were encapsulated separately (top left). Inducer (*N*-acyl-L-homoserine lactone, AHL) diffuses from one droplet to another where it initiates expression of fluorescent reporter proteins. Fluorescence microscopy time-series showing propagation of the signal from the droplet with sender bacteria (middle). Evolution of the average fluorescence intensity for various distances from sender droplet (bottom left) and intensity profiles at different time-points (bottom right) (adapted with permission from ref. 147. Copyright 2013 American Chemical Society).¹⁴⁷

fluorescent protein while swimming and another protein when forming a matrix. Biofilm formation was explored upon specific interfaces in double and triple emulsions and upon negative and positive radii of droplet curvature.¹⁴⁴

Droplet systems can also be engineered to allow for chemical signals to pass into the droplet bioreactors. Quorum sensing (QS) initiation was demonstrated with bacteria encapsulated in droplets held in traps for time-lapse analysis. The porous structure of the PDMS microfluidic chip allowed for diffusion of the QS trigger molecule into droplets from a nearby reservoir.¹⁴⁵ Bai *et al.* trapped two droplets containing different bacterial strains and proved that the diffusion of a QS inducer from one droplet to another can initiate QS in a neighbouring droplet with other complementary bacterial strains.¹⁴⁶ In a similar work, Weitz *et al.* used droplets with different bacterial strains and inducer molecules to show various chemical communication patterns between droplets (Fig. 4B).¹⁴⁷ This approach enabled the demonstration of different interaction patterns: inducer reservoir(s)-to-bacteria, and bacteria-to-bacteria.^{147,148}

Droplet microfluidic techniques for microbial biotechnology

Perhaps one of the most obvious and powerful applications of the features offered by droplet microfluidics is in high-throughput experiments for directed evolution. In their seminal work, Agresti *et al.* transformed members of a library of randomly mutated genes (coding horseradish peroxidase) into yeast cells and then enzymes were expressed and presented on the cell surface.¹⁴⁹ The yeast cells were then co-

encapsulated with non-fluorescent substrate into droplets. After a short incubation the droplets were sorted on a chip and as a result, selected droplets with higher fluorescence contained the most efficient variants of the enzyme. A tenfold increase in enzymatic activity was achieved after four rounds of selection. As the authors stated, droplet technology provided a 1000-fold increase in speed and a 1-million-fold reduction in cost of the assay compared to modern robotic screening platforms.¹⁴⁹

Kintses *et al.* used droplet microfluidics to perform directed evolution for enhanced hydrolytic activity of arylsulfatase from *Pseudomonas aeruginosa* (PAS).¹⁵² Three rounds of evolution led to enrichment of bacterial clones with a 6-fold increase in both enzyme activity and expression.¹⁵² In the follow-up experiment, the authors combined double emulsion droplets with FACS to demonstrate a 100 000-fold enrichment of droplets containing active arylsulfatases.⁶⁰ UV-mutagenesis was used to create spontaneous mutations in a library comprising 10^5 yeast cells in order to screen for improved secretion of industrially relevant enzymes. As a result, several mutant strains were isolated with increased α -amylase production rates (Fig. 5A).¹⁵³ Whole genome sequencing revealed mutations among genes related to protein secretion as well as in genes that have not been linked to secretion before.¹⁵⁰ In another study, Larsen *et al.* used a droplet platform to evolve polymerase capable of synthesizing nucleic acid polymers with unnatural backbone structures.¹⁵⁴ Droplet microfluidic technology has also been used to perform directed evolution of other enzymes such as CotA laccase¹⁵⁵ and phosphotriesterase.⁶²



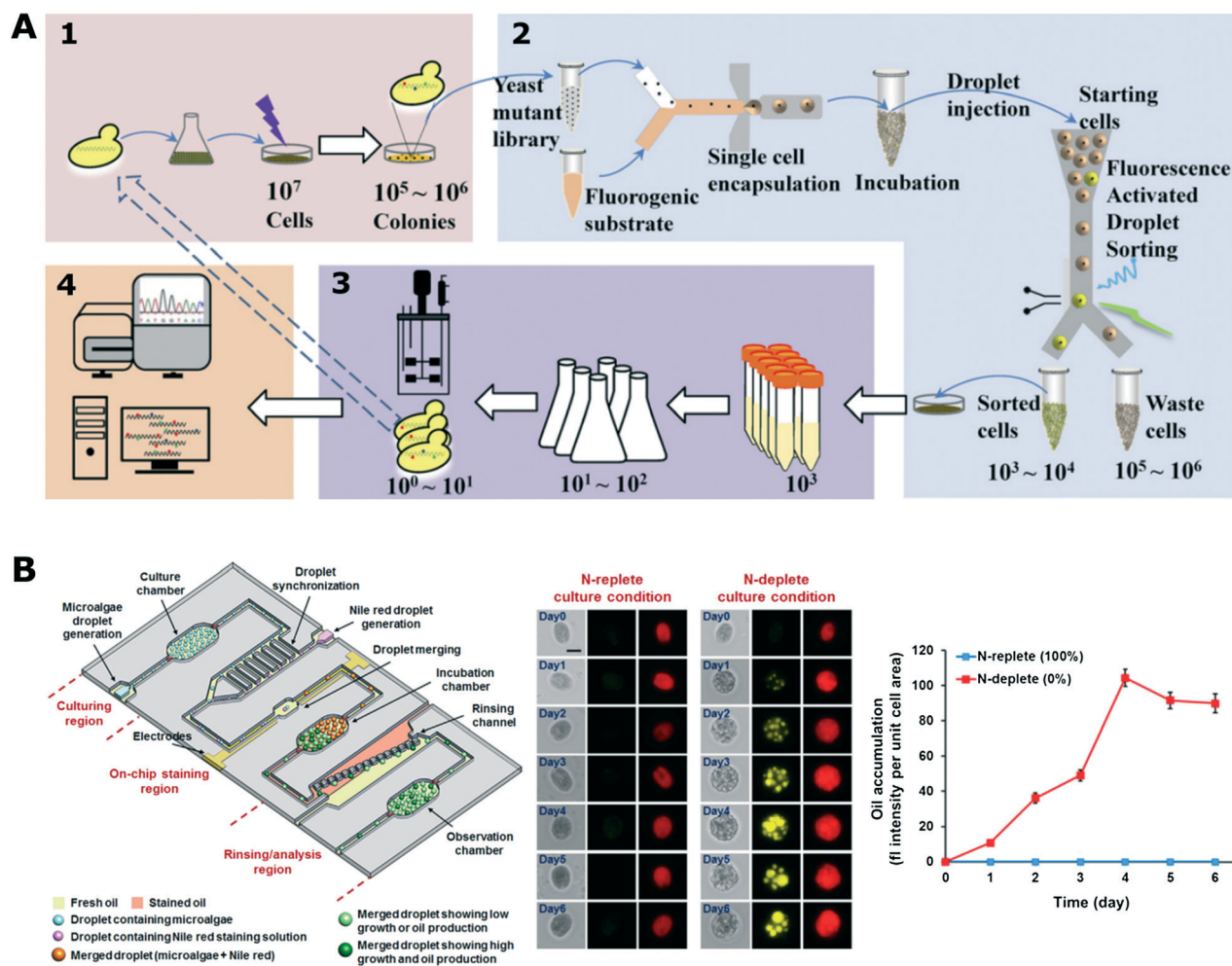


Fig. 5 Biotechnological applications using droplet microfluidic platform. A) High-throughput screening for enhanced secretion of enzyme (α -amylase). The mutant library was generated by UV irradiation and then randomly encapsulated with fluorogenic substrate chemical so that each droplet contained a maximum of a single cell. Droplets with enhanced fluorescent signals were sorted and recovered for further analysis (adapted with permission from ref. 150. Copyright 2015 National Academy of Sciences).¹⁵⁰ B) Droplet platform for microalgae screening for their growth and oil production. The platform comprised three functional parts (left): 1) droplet generation and incubation, 2) module for addition of fluorescent dye and 3) section for analysis and quantification of oil production rate. (Middle) Comparison between stressed (N-deplete) and non-stressed (N-replete) growth conditions for oil production (red – chlorophyll auto-fluorescence, yellow – labelled oil). (Right) Average fluorescence intensity of stained algae cells (adapted from ref. 151. Copyright 2016 John Wiley & Sons, Inc.).¹⁵¹

The ability to consume xylose by microorganisms is important in research on biofuels. Microdroplet technology has been used in a proof-of-principle study that demonstrated isolation of xylose-overconsuming yeast strains initially outnumbered 10 thousand times in the background of native cells.¹⁵⁶ A similar technology was applied for the enrichment of cellulose-producing yeast cells¹⁵⁷ and lactate-producing *E. coli* clones.¹⁵⁶ In a different study, uncultured bacteria from a wheat stubble field were screened directly in droplets containing a fluorogenic cellobiohydrolase substrate for their ability to hydrolyse cellulosic biomass. Remarkably, sorting of droplets depending on cellobiohydrolase activity resulted in the selection of a bacterial population with respectively 17- and 7-fold higher cellobiohydrolase and endoglucanase activity.¹⁵⁸ Jiang *et al.* developed a microfluidic device for the formation and streaking of over a thousand droplets on a Petri

dish.¹⁵⁹ The microfluidic streak plate (MSP) approach enabled the analysis of microbial consortia from soil samples and led to the isolation and discovery of species with a high efficiency of degradation of polycyclic aromatic hydrocarbons (PAHs) or fluoranthene.¹⁵⁹ Dong *et al.* presented a system for enrichment of chemotactic bacteria from a mixture of microbes.¹⁶⁰ First, chemotaxis assay was performed in single-phase parallel-flow wide channel; next, separated single bacterial cells were encapsulated in droplets for further cultivation.¹⁶⁰ Jang *et al.* presented a trapped droplet array to enrich the sample for bacteria that produce higher levels of l-tryptophan .¹⁶¹

In another work, a metagenomic DNA library was cloned into a vector that was expressed in *E. coli* hosts while encapsulated in droplets together with the respective enzyme substrates. The droplet platform enabled the discovery of new

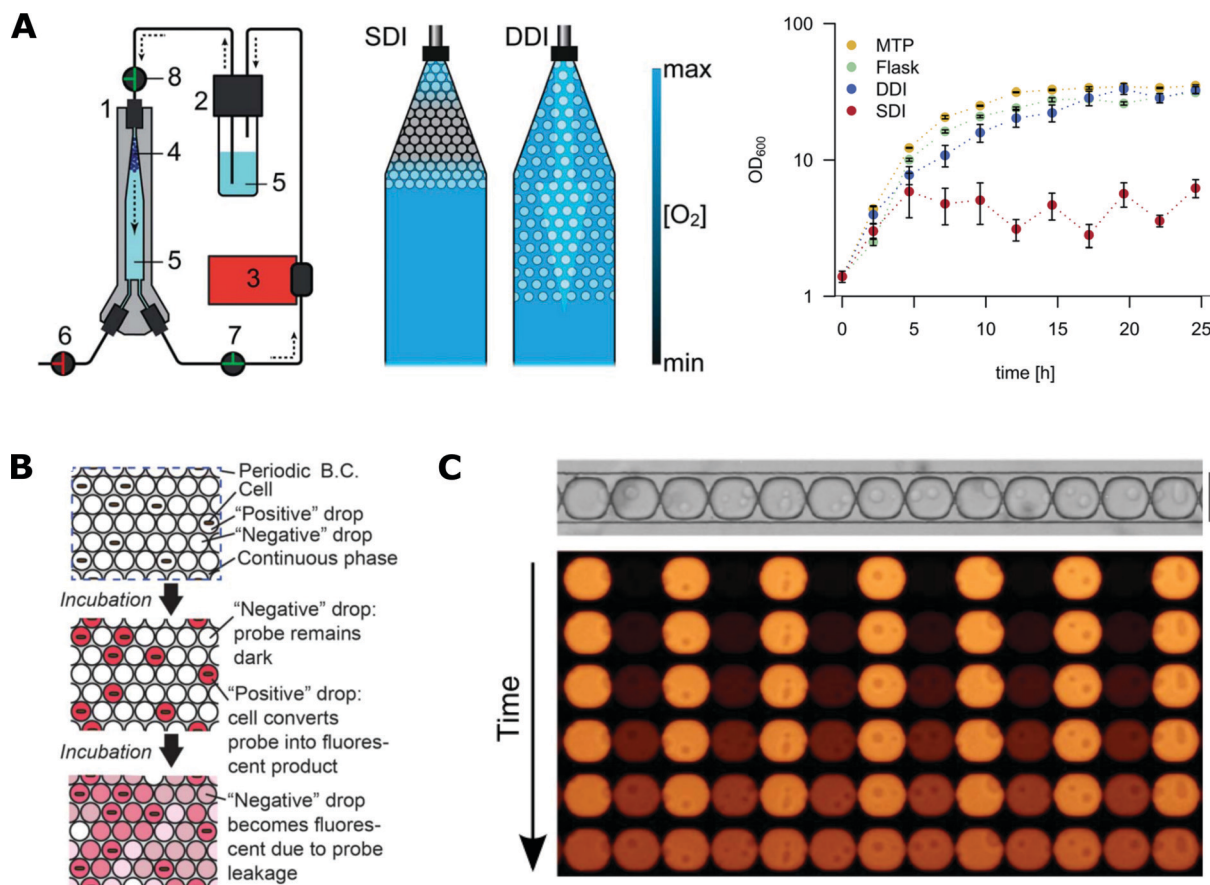


Fig. 6 Challenges with microfluidics for microbiological applications. A) Oxygen availability during incubation in microfluidic droplets. On the left, droplet incubation setup with different oxygen availability rates during static (SDI) and dynamic (DDI) incubation. The numbers stand for 1) droplet incubator, 2) oil reservoir, 3) peristaltic pump, 4) droplet population, 5) perfluorinated oil, 6–8) valves. On the right, comparison of *E. coli* growth kinetics in cultures grown in 96-well plates (MTP), shaking flask, SDI and DDI (adapted from ref. 111 with permission from The Royal Society of Chemistry).¹¹¹ B) The scheme for the dye leakage model in a 2D array of drops (B.C. stands for boundary condition). A positive drop contains one cell and a negative drop contains no cells. The drops are immersed in and separated by a continuous phase. Cells in positive drops convert the probes from the dark state into a fluorescent state (denoted by the pink colour), increasing the fluorescence intensity of positive drops. The fluorescent probe can also leak into the neighbouring negative drops and increase their intensity (adapted from ref. 177 with permission from The Royal Society of Chemistry).¹⁷⁷ C) Experimental studies of the leakage process of fluorescent dye into neighbouring droplets that are assembled in traps in a row. (Figures adapted from ref. 178. Copyright 2016 Macmillan Publishers Limited).¹⁷⁸

specifically engineered strains and cannot be easily translated to other microorganisms.

Marker dyes for microbial metabolism or enzymatic activity can be used for a wider range of species and strains. A common problem associated with their use is the possible “leakage” of fluorescent signal molecules from droplet to carrier oil and to neighbouring droplets (Fig. 6B and C).^{99,107,177,190,191} The main mechanisms of leakage can be diffusion into oil or formation of micellar or vesicular structures that act as carriers between droplets.^{107,178,192} Decrease and averaging of the measured signal may compromise the sensitivity and resolution of analysis of the reactions occurring inside the droplets. Suggested solutions include replacement of the most common HFE-7500 liquid with FC-40 fluorocarbon oil,¹⁹⁰ decreasing the surfactant concentration,^{107,192} and addition of bovine serum albumin (BSA)¹⁰⁷ or sugar additives to the water phase.¹⁹³ Also, less leaky dyes like coumarin-dyes¹⁰¹ or dodecylresorufin^{114,191} can

be used as reporter dyes. The most interesting and so far the most promising approach is using nanoparticles for the stabilization of droplets and prevention of leakage of hydrophobic molecules.⁹⁰ Recently this method has been supplemented with coating of the nanoparticles with bovine serum albumin to increase the compatibility of the interface with enzymes.¹⁹⁴

An interesting area for further developments is finding new fluorescent dyes and metabolic markers that are stably maintained in droplets. Alternatively, there could be advancements in complementary technologies that prevent such leakage of small molecules from droplets. We also foresee a surge in labelling technologies that are designed with a specific target in mind like DNazymes¹⁰⁸ or species/strain specific enzymes similar to BlaC.¹¹⁶ Definitely, the development of a sensitive label-free detection technology for small (nano-liter and smaller) droplet reactors, similar to the large-scale conventional OD readouts, would provide a great new thrust for the droplet culturing systems.



Engineering challenges in automation and simplification of droplet microfluidic systems

There are two major directions for further development of microfluidic systems for handling droplet bioreactors for experimentation in microbiology – further automation of complex liquid handling protocols and increasing the accessibility of the basic technologies for users without expertise in microfluidics.

Control of the concentration of reagents during the assay is a very important aspect in microbial cultures. Microfluidic systems provide many approaches for the generation of sets of droplets with arbitrary combinations of reagents.^{80,113,195} Still, manipulating the concentration of reagents during the assay is very challenging. Usually this task requires multiple valves and optical feedback loops.⁷ The introduction of hydrodynamic and passive geometries can reduce the number of valves needed.^{79,94,161} Long-term cultures with on-line monitoring of growth typically use microliter scale droplets containing up to tens of thousands of microbes.^{7,128} Although a technique for splitting microliter plugs into libraries of nanoliter droplets has been demonstrated,¹⁹⁶ there is still a great outstanding challenge of integrating long-term culturing in controlled chemical environments and monitoring of the resulting single-cell distributions of growth and other phenotypic factors.

The second important challenge is to make the droplet microfluidic technology more widely available for the microbiologists and research communities in general. Currently the technology is mostly used by engineering laboratories that design the microfluidic systems and “early adopters” in life sciences who collaborate with these engineering groups.¹⁹⁷ In order to be truly successful and popular any novel and useful technology has to be widely available and cost-effective.

The situation is promising for droplet technologies as the first commercial devices are already available for nucleic acid research (droplet digital PCR). There is also a number of companies that already provide basic droplet microfluidic tools and custom chip development options that are suitable for research on microbiology as well as in life sciences in general. We believe that the most pivotal technologies could be adopted to simplistic and inexpensive formats. For example, the recent revival of interest in passive techniques for generation of droplets^{71,73,198} should lead to the development of chips that allow the transformation of a suspension of bacteria into a monodisperse emulsion simply by injecting it with an automatic pipette. Supplementing this with inexpensive optical detectors and sorters could boost the use of emulsion technologies in microbiology.

Opportunities

The ability to encapsulate single cells or small colonies in massively large numbers of bioreactors for growth, monitoring and selection provides numerous opportunities in tackling the most important problems in fundamental and applied microbiology.

Currently, the most obvious and important problem is the massive build-up of resistance to antibiotics that already presents a global threat to public health. The imminent threat of transiting into the “post-antibiotic era” – in which common infections and minor injuries can kill – is calling for immediate and drastic measures.¹⁹⁹ In 2015 the World Health Organization endorsed a global action plan on antimicrobial resistance to combat this grim apocalyptic vision.²⁰⁰ Among other suggestions, the plan calls for the development of new technologies for tackling the full extent of the problem.

Improved and widely accessible methods for screening of resistance to antibiotics are needed. Droplet technologies can certainly be used both for simple manual antibiotic susceptibility tests⁸¹ and for automated analyses. While the use of cocktails of antibiotics (or antibiotics and adjuvants) is one of the primary strategies for tackling bacteria,^{201–203} in the current state of the art there are practically no standardized methods for rapid evaluation of susceptibility to combinations. We believe that both passive and automated droplet techniques could offer solutions allowing the establishment of sensitivity to multiple combinations (*e.g.* 10 × 10) of drugs, each combination tested across a range of concentrations. Providing access to such technologies would surely have a major impact on tracking the epidemiology of resistance and understanding the evolution and patterns in spreading the mechanisms of resistance. It could also aid directly the clinical practices in choosing effective combinations in critical infections.

Recent studies have demonstrated that use of a specific combination of drugs can invert or modulate the selective advantage of resistant bacteria competing with sensitive strains and reverse the evolution of antibiotic resistance.^{201,204–206} The automated droplet microfluidic systems could also be engineered for massive *in vitro* screening of combinations of drugs for use in designing new combination treatments. The technological need in this field is truly outstanding and the ability to culture bacteria constitutes a technical bottleneck in research. Providing a technology that would allow screening, *e.g.* double or triple combinations of a library of a hundred drugs (*i.e.* 100² or 100³ times a matrix of dilutions for each combination) or *e.g.* a pool of 30 drugs in combination, each mixed with one of a 10 thousand member library of candidate adjuvants per day would pose a high chance of finding new, unexpected, combinations of drugs and probably also elucidate the biomolecular mechanisms behind resistance.^{207–209} Also, the long-term culturing systems⁷ may be engineered to manipulate i) the chemical environment in time in each of hundreds of colonies, ii) change this environment in response to the observed growth patterns, and iii) transfer bacteria between the droplets. All these features provide unique opportunities to i) uncover the dynamics of development of resistance in the space of chemical composition of media and ii) program and test various ‘algorithms’ of administration of drugs aimed at promoting the desired evolutionary traits.

Classical diagnostics realized by *in vitro* culturing can be assisted or sometimes replaced by modern methods of



