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Droplet Microfluidics for Microbial Biotechnology

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Abstract Droplet microfluidics has recently evolved as a prominent platform for high-throughput experimentation for various research fields including microbiology. Key features of droplet microfluidics, like compartmentalization, miniaturization, and parallelization, have enabled many possibilities for microbiology including cultivation of microorganisms at a single-cell level, study of microbial interactions in a community, detection and analysis of microbial products, and screening of extensive microbial libraries with ultrahigh-throughput and minimal reagent consumptions. In this book chapter, we present several aspects and applications of droplet microfluidics for its implementation in various fields of microbial biotechnology. Recent advances in the cultivation of microorganisms in droplets including methods for isolation and domestication of rare microbes are reviewed. Similarly, a

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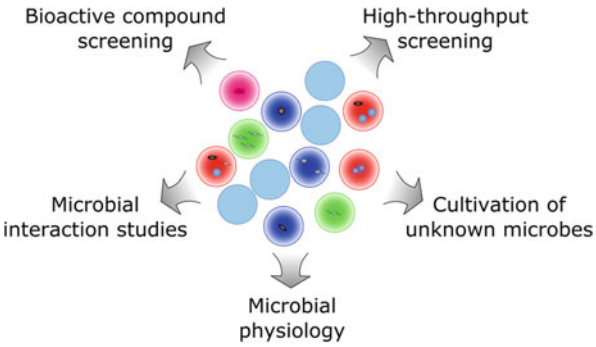
comparison of different detection and analysis techniques for microbial activities is summarized. Finally, several microbial applications are discussed with a focus on exploring new antimicrobials and high-throughput enzyme activity screening. We aim to highlight the advantages, limitations, and current developments in droplet microfluidics for microbial biotechnology while envisioning its enormous potential applications in the future.

Graphical Abstract



Keywords Antibiotic screening, Cultivation of rare microbes, Droplet microfluidics, Enzyme screening, Fluorescence-activated cell sorting, Ultrahigh-throughput microbial cultivation

1 Introduction

Within the rapidly growing field of microfluidics, droplet-based microfluidics refers to systems based on the combination of immiscible phases, which results in the formation of drops of one phase embedded in the other. This simple approach has revolutionized various experimentation platforms as it combines microfluidic miniaturization and ultrahigh-throughput with compartmentalization, one of nature's (life's) oldest key strategies. When generating aqueous droplets surrounded by an inert carrier phase, it is possible to reduce the working volume by more than six orders of magnitude, specifically from μL to pL or even fL. Furthermore, the stringent and controllable conditions during droplet formation allow the production of thousands of compartments per second with a minimal volume variance. Thereby, not only costs but also time can be spared in comparison with traditional liquid-handling methods while maintaining excellent experimental quality. These conditions have led to thousands of technological developments and applications based on droplets in the fields of chemistry and biology, some representing particularly high-impact breakthroughs enabling omics techniques at single-cell resolution with very

high throughput [1–8], or harnessed to screen for improved antibodies [9–11], or enzymes [12–15].

From the microbiologist perspective, droplets provide a paradigm-changing experimental approach. Ironically, except from microscopy, the science of studying microorganisms has been the science of growing them to scales fitting our hands and volumes, given that further experimentation at the micrometer scale was impossible until recently. This inadvertently biased microbiological research towards the development of techniques, strategies, and equipment that restrict the window of diversity that can be analyzed. Microfluidic approaches enable experimentation controlling and monitoring physical scales much closer to those of the microbial world, albeit with some challenges that must be addressed for broad-range applicability.

In droplet microfluidics, even single cells will be compartmentalized in a volume between 1 and 100 pL, i.e., droplets of approx. 10 to 100 μm in diameter. In terms of concentrations, this is similar to 10^7–10^9 cells/mL, which is the standard working range at which large-scaled methods work. Therefore, droplets provide a platform in which the biochemical and physiological parameters of a single cell can be studied in a similar fashion as normally done for millions of cells. This, in combination with the extremely fast production of droplets, results in an experimental platform with the capability to explore the enormous diversity of microbiological samples (Fig. 1).

The specific features of microfluidic droplets should render these a promising starting point to isolate, culture, and identify a significant fraction of the until now unculturable and undiscovered microbial biodiversity along with its vast metabolic potential (Fig. 2a). Due to the ultrahigh-throughput, complex heuristic experimental design can be performed to identify ideal nutrient conditions (Fig. 1). Additionally, the minimal volume requirements inherent to microfluidic techniques enable the preparation and analysis of rare and limited – therefore mostly unexplored – samples in their natural conditions, such as microbiota from small animals and plants. Moreover, discretization of microbial cells in compartments with a similar order of magnitude is accompanied by crucial physiological advantages. First, isolation eliminates competition for nutrients, providing the possibility to culture strains commonly hidden under faster-growing communities [16, 17] (Fig. 2b). Alternatively, the droplets can also be exploited to foster microbial interactions, which in many cases have been shown to be critical for growth and metabolite production [18, 19]. Second, higher cell and metabolite effective concentrations are easily detectable and activate concentration-based processes such as quorum sensing [20]. Finally, micro-compartmentalization enables the separation and distinction of otherwise identical cells that present different expression profiles, e.g., scout cells [21] or silent vs. activated gene clusters.

Similarly, droplets can also be exploited to screen human-made microbiological diversity, such as mutant [22] and metagenomic [23] libraries (Fig. 2). For the goal to detect and isolate microbial variants that expand the boundaries of industrial microbiology, droplets provide a platform for the implementation of ultrahigh-throughput assays for improved enzymes and producer strains. The diversity in mutant or metagenomic libraries can easily reach more than 10 million different variants that are impossible to analyze in detail with traditional methods. Yet, in a field where

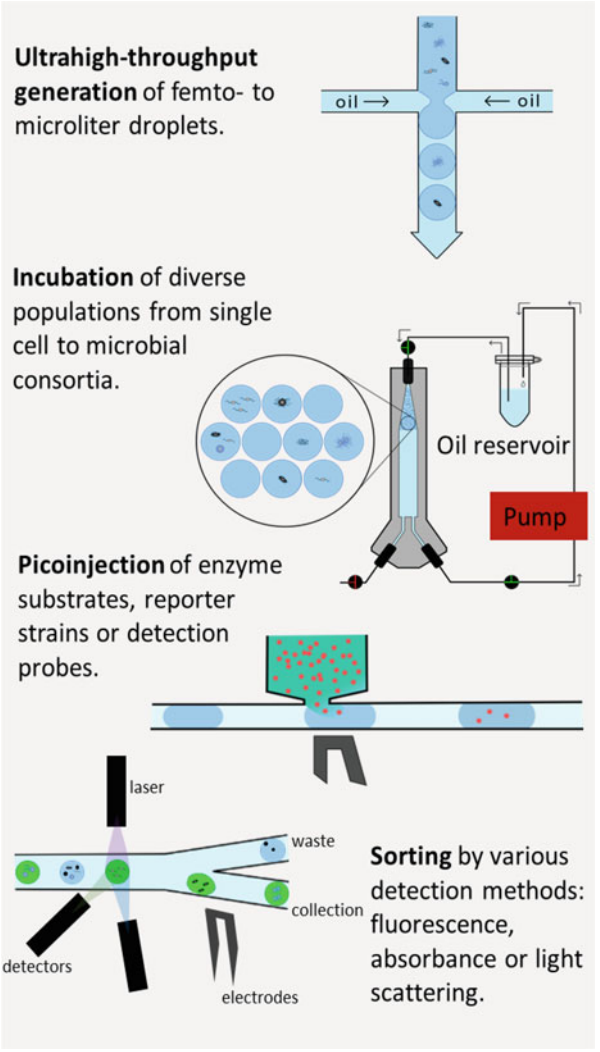


Fig. 1 Droplet microfluidics workflow overview. A microfluidic device encapsulates single cell or microbial consortia into monodispersed droplets containing growth medium. Microbes are grown in microdroplets inside the incubator which maintains the continuous flow of oxic oil. Electric field-activated picoinjection modifies droplet contents by allowing the delivery of an external solution in a regulated fashion. At designated time points, droplets of interest are recovered by on-chip active sorting. Integration of different detection methods generates trigger, leading to deflection of desired droplets into the collection channel through dielectrophoresis

improvements of a fraction of a percentage may define the viability of an application [24], it is essential to explore the full extent of the created diversity to find the most promising variants and understand the corresponding performance improvements.

2 Droplet Microfluidics for Microbial Cultivation

During the development of droplet microfluidic techniques, microorganisms have been used as effective models for proof-of-concept studies [25]. This is especially the case for the isolation of individual cells in ultra-small volumes. Confinement in

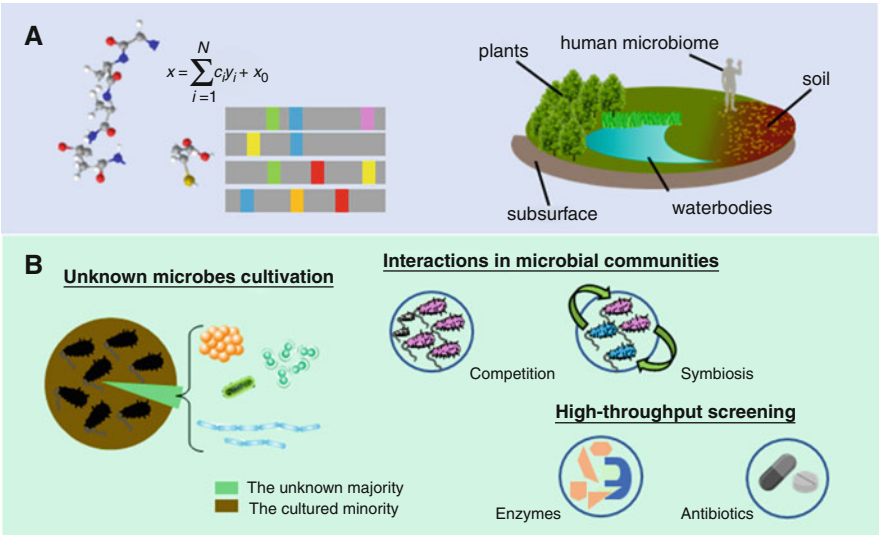


Fig. 2 From biological diversity to applications. (**a**) Microbial resources for droplet cultivations: droplet microfluidics enables ultrahigh-throughput screening of mutant libraries generated from random mutagenesis, statistical modelling and computational design (left), as well as tremendous reservoir of microbial communities in diverse environments (right). (**b**) Applications for droplet microfluidics: droplet microfluidics have been demonstrated as a capable platform for detection and identification of microbes. Compartmentalization allows isolation of unknown and slow-growing species and the study of interactions between microbes from complex environments. Finally, droplets can be employed as microreactors for high-throughput enzyme and antibiotics screening within a controlled chemical environment

such reduced volumes decreases detectable growth time and eventually increases the effective concentration of secreted molecules [26]. Moreover, the ability to create and analyze millions of droplets per day enables the examination of large and diverse samples, find rare cells, and analyze whole populations in terms of genetic and phenotypic varieties.

Most of the initial applications have focused on single-cell campaigns, mostly because droplets are the first high-throughput experimental platform that enables this high impact approach [1, 3, 8, 10, 34–36]. Yet, a number of studies have also used the singularization of cells in droplets with subsequent incubation that results in growth as growth is a powerful yet easy strategy that can be used as a response variable or signal amplifier [22, 37–44]. This is particularly relevant when microorganisms for biotechnological applications are being evaluated or screened. Often initial incubations were performed in tubing loops or arrays on chip [28, 29, 45]. Simple off-chip cultivation in contrast was performed in syringes or reaction tubes [30]. Measuring growth is essential when searching for microorganisms or variants under different nutrient sources or stress conditions or simply when the desired product is biomass or strongly correlated to biomass production. In addition, a number of studies aiming to explore global microbial phenotypes [46–48],

antibiotic resistance [49–51], or community culture and composition [52–60] have relied on growth inside of droplets.

However, as more complex and comprehensive protocols are envisioned, a higher degree of microbiological craftsmanship [61] should be implemented for droplet-based experimentation. Such is the case for studies involving more complex microorganisms with distinct metabolic profiles. Therefore, incubation conditions must be appropriately controlled in order to provide ideal conditions for the microorganisms and the different experiments being performed. Maximizing homogeneity for the millions of droplets per experiment can result in either maximized growth or production of the molecules of interest. In this context, oxygen and pH control in millions of droplets [32, 33] provide the tools to effectively link droplet microfluidics and classic microbiological standards (Fig. 3). The possibility to measure and control oxygen availability and pH provides natural or artificial incubation conditions in droplets that could be adjusted to imitate the original bacterial habitat (soil pores, static or agitated water, animal intestines, etc.) or bioreactor conditions. In fact, it is of great advantage for biotechnological screening applications to provide bioreactor-like conditions and control, as the selected variants must be scaled up for their implementation in industrial production processes. Further applications of optimized incubation setups include the exploration of hypoxic conditions, the usage of gases as growth or enzymatic substrates, and the screening for molecules and microbes active under adverse pH conditions.

3 Detecting Microbial Activity in Droplet Microfluidics

The development and integration of effective detection techniques to a microfluidic system is a critical step for any biotechnological and microbiological analysis. Implementation of traditional laboratory techniques, which provide high sensitivity and accuracy, requires proper integration strategies, since they typically require expensive and bulky instruments, skilled personnel, and extensive analysis time [62, 63]. In addition, miniaturization of sample volume and fast-flowing droplets in a microfluidic system poses significant challenges for rapid and sensitive detection. Therefore, an ideal detection technique for droplet microfluidics includes features like simple, rapid response, high sensitivity, compact, flexible, and low cost.

For analysis and quantification of microbiological samples in droplet microfluidics, different detection methods have been developed and implemented including optical-, electrical-, and mass spectrometry-based detection (for an overview see Table 1) [64]. Among others, optical methods have become very popular with the advancement of detection instruments, miniaturization of optical components, and development of dyes and biomarkers. Optical methods have been demonstrated for diverse chemical and biological applications along with research focusing on improving detection sensitivity and dynamic range [65, 66]. Various spectroscopic methods used for analysis include fluorescence, absorbance, light scattering, and Raman signals.

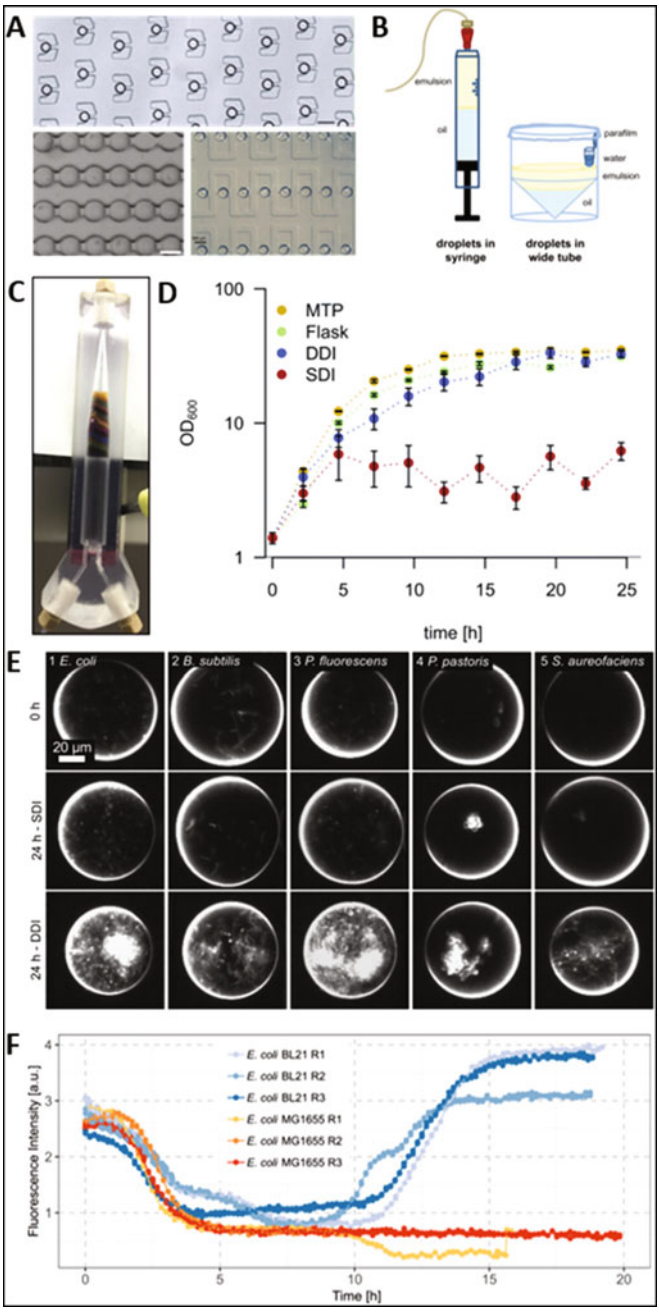


Fig. 3 Examples for droplet incubation strategies and microbial growth characterization. (**a**) Droplets incubated inside microfluidic structures in traps or delay lines (with reprints from [27–29]. (**b**) Off-chip droplet incubation is usually done in reaction tubes or inside syringes [30]. (**c**) Dynamic droplet incubator to enhance oxygenation and homogeneity of droplet populations [31]. (**d**) Picoliter cultures of microorganisms grow similar to larger-scaled methods when dynamically incubated [32]. (**e**) Colonies formed inside of dynamically incubated droplets reach much

Fluorescence is the most widely used detection technique for chemical and biological analysis considering various factors like high-signal intensity, highly sensitive dyes, selective fluorescent labelling biomarkers, well-established protocols, high-end instruments, etc. Using fluorescence detection method, microbial activity can be detected by various approaches like direct measurement of fluorescence from cellular metabolites, labelling cellular metabolites with fluorescent dyes, using fluorogenic substrates for enzyme assays, using reporter strains expressing fluorescent proteins, or using fluorescent-based probes. Availability of several fluorogenic substrates with higher quantum yield has enabled development of sensitive fluorescence detection and efficient sorting mechanism for high-throughput enzyme screening. Details regarding these applications are discussed in the respective subchapter below.

On the other hand, microbial viability markers based on fluorescent dyes have been used in droplets for growth and survival analysis. Assays based on highly fluorescent resorufin, which is formed through metabolic activity from resazurin [67] and dodecylresorufin [68], have been implemented for bacterial and antibiotic inhibition analysis. Resorufin-based substrates have also been used for detection of ethanol-producing cyanobacteria [69] and screening for high xylose-consuming yeast strains [39]. However, leakage of resorufin from droplets [68] has restricted its application to microbial growth assays requiring long time incubation. Recently, a FRET-based RNA probe has been demonstrated for the detection of growth, sorting, and analysis of a microbial community from environmental samples [57]. Similarly bacterial cells stained with SYTO 9, propidium iodide [70], and SyTox Orange [71] dyes have been used for drug susceptibility testing and screening metagenomics library for antibiotic producers.

Bacterial strains expressing fluorescent proteins have been popular as a sensor or reporter for various microbial assays including antibiotic screening and microbial interaction studies. Fluorescent proteins are expressed continuously with fluorescence intensity depicting the concentration of cells. Such reporter strains were used to demonstrate growth and long-term cultivation of microorganisms in droplets [30, 72, 73], analyzing bacterial dynamics [74], and performing MIC (minimum inhibitory concentration) assays [75]. *Escherichia coli* and *Bacillus subtilis* strains expressing fluorescent proteins like GFP, mKate, and mCherry have been extensively utilized. For high-throughput screening of antibiotic producers from complex environmental samples, reporter strain expressing mCherry proteins was picoinjected to pre-cultivated droplets containing environmental microorganisms, and fluorescence signals were measured to determine the inhibition activity [38, 76]. Similarly, multiple fluorogenic strains with auxotrophic variants have been used to investigate microbial interactions between *E. coli* and *Pseudomonas putida* in a microfluidic system [77].

Fig. 3 (continued) higher biomass levels [32]. (**f**) Using dynamic incubation, it is also possible to monitor and control the pH of the entire droplet population [33]. *Images are reprints of the indicated publications with permission of the original publisher*

Table 1 Analytical methods for detecting microbial activity in droplets

Detection method	Screening technique	Application	Microorganism	Remarks	Refs.
Fluorescence	Direct detection of cellular metabolites	Monitoring microbial production of riboflavin	*E. coli*	Continuous monitoring for long-term incubation	[73]
		Screening for enhanced riboflavin producers	*Y. lipolytica*	FACS analysis using double emulsion	[41]
		Detection of chlorophyll production	*E. gracilis* and *C. reinhardtii*	Gel droplets with FACS analysis	[37]
	Labelling with fluorescent dyes	Live/dead assay	*E. coli*	Cell viability assay and drug susceptibility testing by staining cells with SYTO 9 and propidium iodide	[70]
		Screening for bacteria inhibiting *S. aureus*	*S. aureus*	Double emulsion droplets with FACS analysis	[38]
		Screening for high lipid-producing microalgae	*E. gracilis and C. reinhardtii*	Gel droplets with FACS analysis	[37]
		Screening for antibiotic producers	*S. aureus*	Screening metagenomic library by co-encapsulating *E. coli* and *S. aureus* and staining dead cells with SyTox Orange	[71]
	Fluorescent-based substrates for enzyme screening	Alkaline phosphatase	*Tetraselmis* sp.	Measurement of enzymatic activity at single-cell level	[102]
			E. coli	Enzyme kinetics study	[103]
		Cellulase	Bacterial community from soil sample	High-throughput screening	[42]
			T. reesei	Screening of filamentous fungi in droplets	[104]
		Lipase	Environmental water and soil sample	Compact optical system for fluorescence measurement	[66]

(continued)

Table 1 (continued)

Detection method	Screening technique	Application	Microorganism	Remarks	Refs.
		Amylase	*A. niger*	Growth and analysis of fungi in droplets	[105]
		Xylanase	*Y. lipolytica*	High-throughput screening technique for improved enzyme activity	[106]
		Esterases	*E. coli*	Screening of metagenomic library from environmental sample	[23]
		Cot A laccase	*E. coli*	Flexible screening platform and picoinjection of substrate	[107]
	Reporter strains expressing fluorescent proteins	Growth and cultivation of microorganisms	*E. coli*	Easy detection of microbial growth in droplets	[72]
		Growth analysis	*B. subtilis*		[30]
		Analysis of bacterial dynamics	*E. coli*		[74]
		Screening antibiotic producers from environmental sample	Environmental soil sample		[76]
		Demonstration of microbial co-cultivation	*E. coli* and *B. subtilis*	Simultaneous detection of multiple colors using a single sensor	[78]
		Bacterial growth monitoring	*E. coli*	Continuous monitoring for long-term incubation	[73]
		Cultivation of microorganisms	*E. coli* and *P. aeruginosa*	Cultivation in plates by streaking droplets	[53]
		Growth kinetics and MIC assays	*E. coli*	Automated fluid handling	[75]
		Microbial interaction study	*E. coli* and *P. putida*	Cultivation of microbes in adjacent chambers separated by nanochannels, demonstration for	[77]

(continued)

Table 1 (continued)

Detection method	Screening technique	Application	Microorganism	Remarks	Refs.
				metabolic cross-feeding and microbial gene transfer	
	Reporter strains based on product specific expression of fluorescent proteins	Screening for vitamin B2 producers	*B. subtilis*	*E. coli* sensor cells with a riboswitch specific for vitamin B2	[108]
	Fluorescence-based dyes/ probes	Growth analysis	Environmental sample	FRET-based RNA probes cleaved by RNase produced by growing microorganisms, sorting droplets with high growth	[57]
		Microbial viability assay	*E. coli* and *E. aerogenes*	Use of dodecylresorufin-based dyes	[68]
		Antimicrobial susceptibility assessment	*E. coli*	Use of resorufin for detection of cell growth	[67]
Absorbance	Absorbance-based substrate	Directed enzyme evolution	*E. coli*	Absorbance-based screening for high producers of phenylalanine dehydrogenase	[79]
		Study fermentation process	*Z. mobilis*	Determination of ethanol concentration using colorimetric assay	[80]
	Direct monitoring of droplet content	Measurement of viscosity and optical density	*E. coli*	Lower throughput	[109]
		Sorting colonies of similar cell density	*S. cerevisiae*	Multi-parametric analysis and sorting	[81]
Light scattering	Detection of scattered light from droplet content	High-throughput screening of antibiotic-resistant bacteria	*E. coli*	Label-free screening	[51]
	Imaging of scattered light pattern	Detection of *E. coli*	*E. coli*	Label-free detection with high sensitivity	[86]

(continued)

Table 1 (continued)

Detection method	Screening technique	Application	Microorganism	Remarks	Refs.
Raman signal	Surface-enhanced Raman spectroscopy	Bacterial strain identification	*E. coli*	Fast recording of SERS spectra from droplets	[110]
Image-based	Image analysis of droplet content	Enrichment of droplets with growth	*S. puniceus*	Triggered imaging and sorting of droplets, label-free method	[87]
		Identification and recognition of cells in droplets	*D. tertiolecta* and *P. tricornutum*	Discrimination of cells based on morphologies and sorting	[88]
	Colored beads for encoding droplets	MIC assay	*E. coli*	Encoding various experimental conditions using colored beads	[31]
	Immunoassay with fluorescent-labelled antibodies	Detection and identification of bacteria	*E. coli*	Use of magnetic beads to capture *E. coli* and labelling with fluorescently labelled anti-*E. coli* antibodies	[89]
Electrical conductance	Measurement of conductivity signals with integrated electrodes	Quantification of *E. coli*	*E. coli*	3D-printed chip with integrated electrodes allowing contactless measurement	[97]
	Impedance measurement	Monitoring cell differentiation		Label-free and noninvasive detection of cells	[96]
Mass spectrometry	Electrospray ionization	Mass-activated droplet sorting		Splitting droplets and sorting based on mass	[101]
		Detection of microbial secondary metabolites	*S. griseus*	Intensity analysis at known mass-charge ratio	[76]
			S. griseus	Combined fluorescence and MS detection	[100]

Recent developments also demonstrated the simultaneous detection of multiple fluorescence signals for antibiotic screening and microbial co-cultivation assays [78]. With such setups, multiple parameters can be analyzed from individual droplets, which open the door to multiplexing biochemical and microbiological assays.

Though fluorescence-based detection setups are popular in droplet microfluidics, one should take into account the degradation of fluorogenic substrates over time, stability, and inter-droplet transfer of fluorophore molecules. A general prerequisite for all droplet-specific fluorescence assays is a containment of the fluorophore to the respective droplet. If fluorophores can move out of the droplet into the oil phase, the specificity of the signal to a droplet gets lost and the signal intensity decreases. However, the mobility of specific fluorophores can also be exploited for monitoring bulk droplet properties. Measurements of several parameters critical for microbial growth and metabolite productions, like change in pH and oxygen level, have been assessed by using fluorescence detection methods [32, 33].

Absorbance is a label-free technique and can be measured in droplets by monitoring change in optical properties of droplet content. Absorbance-based techniques have been demonstrated for monitoring cell density and screening of microbial libraries. A chromogenic substrate, WST-1 resulting in the absorbing dye WST-1 formazan, was used for screening of an *E. coli* mutant library producing phenylalanine dehydrogenase [79]. A similar colorimetric assay was implemented for monitoring ethanol production by *Zymomonas mobilis* during fermentation [80]. Furthermore, absorbance signals were utilized for monitoring droplet content and sorting colonies of similar cell densities to minimize assay variability arising from growth phenotypes [81]. However due to miniaturization in microfluidic system, the active optical path length for absorbance measurement is highly decreased in comparison with traditional optical readers, thus resulting in lower detection sensitivity. Recently, several optimizations and modifications, including elongated channel designs [82, 83], lock-in-based detection [84], and optofluidic approaches [85], have been demonstrated in realizing absorbance measurement in microfluidic platform.

Light-scattering-based analysis of microorganisms has also been demonstrated in droplets. A high-throughput label-free detection setup was developed to analyze bacterial growth in droplets and screen antibiotic-resistant mutants [51]. In the presence of the antibiotic fusidic acid, growth of a normal *E. coli* strain is inhibited, while antibiotic-resistant mutant bacteria could grow resulting in higher scattered light signals. Furthermore, a recent microfluidic droplets study monitored microbial growth and quantified microorganisms by imaging 2D light-scattering patterns [86].

Image-based analyses of droplets have also been utilized for microbial analysis. Bright-field images were analyzed for sorting and enriching droplets with grown microorganisms from empty droplets [87]. Similarly, different morphologies of cells [88] or fluorescent microscopy images based on immunoassays [89] have also been utilized for the identification and detection of microbial samples in droplets. With advanced image analysis algorithms based on machine learning and deep neural networks, microbial samples have been analyzed in 3D culture system [90] and in multiplexed assays [31]. Within the latter work, different experimental conditions were coded using colored beads, which were decoded by droplet image analysis.

Another approach is to modify droplets either to gel droplets [37, 71] or to double emulsions [38] for analysis with conventional FACS (fluorescence-based cell sorting) instruments. This allows simultaneous analysis of multiple fluorescent

signals and scattered light parameters, enabling multi-parametric analysis and sorting of droplets. This approach significantly increased the throughput of the screening process. However, generating double emulsions and gel emulsions is not straightforward and limits the fluid handling operations like merging and splitting.

Most of the current optical detection setups are based on bulky microscopes along with complex and often expensive optical configurations. Recent developments in the combination of optical and fluidic systems have resulted in the emergence of optofluidic devices, synergistic integration of optical components with a microfluidic device [91, 92]. The integration of optics into microfluidic chips allows alignment-free setups with higher sensitivity and multiplexing capability of chemical and biological assays [62], which also greatly benefit microbial experimentations [93].

In addition to optical methods, other detection methods including electrical signals and mass spectrometry (MS) have also been utilized for the detection of microbial activity. Electrical conductance and impedance measurement have been demonstrated for measuring droplet dimensions including velocity [94, 95] and characterizing cell growth in droplets [96]. Label-free detection and counting of *E. coli* cells were demonstrated in droplets by contactless conductivity measurements [97]. Mass spectrometry (MS) is a label-free method and provides information about analytes depending on mass-to-charge ratio. Several studies have combined a droplet microfluidic system with MS [98, 99]. Microfluidic droplets are sprayed into a MS head either by using a capillary connector or by modifying chip designs with cone-shaped outlets. A droplet MS platform was developed for detecting secondary metabolites produced by *Actinobacteria* [76, 100]. However, MS is a destructive method, resulting in a loss of possible hits. Recently, new microfluidic handling techniques have been introduced with the capability of splitting droplets, analyzing one daughter droplet by MS, and sorting other daughter droplets based on MS results [101]. Such setup possesses broad prospects for microbial screening using droplet microfluidics.

In addition to the above mentioned online analysis tools, several offline detection techniques have been demonstrated for droplet microfluidics. In most cases, droplets are broken and analyzed for droplet content. These include DNA sequencing, liquid and gas chromatography, and mass spectrometry among others.

4 Droplet Cultivations of Rare Microbes and to Search for New Antimicrobials

Cultivation of microorganisms in very small droplets has several critical advantages for some of the most pressing challenges in environmental microbiology: the cultivation of microorganisms considered unculturable and the search for new anti-infective natural products. This advantage particularly is generated by (1) the ultrahigh-throughput of droplets experiments, which allows very deep sampling of the environmental microbial consortia; (2) the very small size of the cultivation

vessel between 50 and 200 pL, which allows to conduct high numbers of experiments with a minimum input of resources and preparative work; and (3) an increasing portfolio of high-speed functional assays to evaluate microbial growth and activity.

It has long been acknowledged that the majority of today's anti-infective compounds have first been isolated from microbial producers or are derivatives of microbial natural products. However, after the golden age of antibiotics discovery in the 1940–1960s, no novel antibiotic structures and functionalities have been discovered. Intensive efforts to rationally and statistically generate new lead compounds through chemical synthesis proved not very fruitful. Today, with the urgent need for discovery of functionally new lead structures because of emerging antibiotic resistances, scientists increasingly go back to the initial source of anti-infective compounds: the unlimited pool of microbial natural products. The central challenge for this approach is to reduce the level of rediscovery of already known molecules. Thereby, three strategies are followed: (1) genome-based mining for new biosynthetic gene clusters possibly with different structural properties, which might point to new chemical functionalities [111–113]; (2) the search for natural products in so far under explored environmental habitats like marine resources or host-associated microbial communities [114, 115]; and (3) a deeper and more strategic microbial cultivation effort to access natural products from slow-growing, previously uncultured, or synergistically growing microorganisms. Often even a combination of these three strategies is applied.

Especially for the last two strategies, droplet microfluidics has shown great potential. A statistical distribution for inoculating either a single microbial cell or a small consortium into one droplet as an individual growth vessel enables the parallel cultivation of millions of microbial cultures in a bulk volume of one milliliter or less (Fig. 3). Controlled cultivation conditions at defined temperature, oxygenation level, pH, and time provide a large operational window for successful cultivations. Such strategy has been used, for example, to characterize the diversity and ability to grow on different carbon sources for the microbiota from human fecal samples [116]. Functional screening of these individual droplets can be achieved at around 100–1,000 droplets per second, resulting in a capacity to evaluate almost 10 million cultivations per day. Since the individual droplet size is very small, specific chemical analysis for natural products is challenging, but not impossible. For example, it has been successfully shown that individual droplets can directly be injected into a mass spectrometer to quantify the production of a microbial natural product in a droplet [76, 100]. Droplets of *Streptomyces griseus* producing streptomycin were analyzed and validated for efficient detection from single droplets. However, so far the valuable microbial droplet content is sacrificed during such an invasive chemical analysis. Alternatively, functional reporter assays can predict the presence of a bioactive natural product and can be applied to preselect high potential microbial droplets from either empty droplets without growth or droplets without bioactivity. In this case, a reporter agent is added to the pre-cultivated microbial droplet. In the search for antimicrobials, this is typically a microbial strain or defined strain mixture sensitive against inhibition by antimicrobials in the droplet (Fig. 4) [38, 76, 117]. A

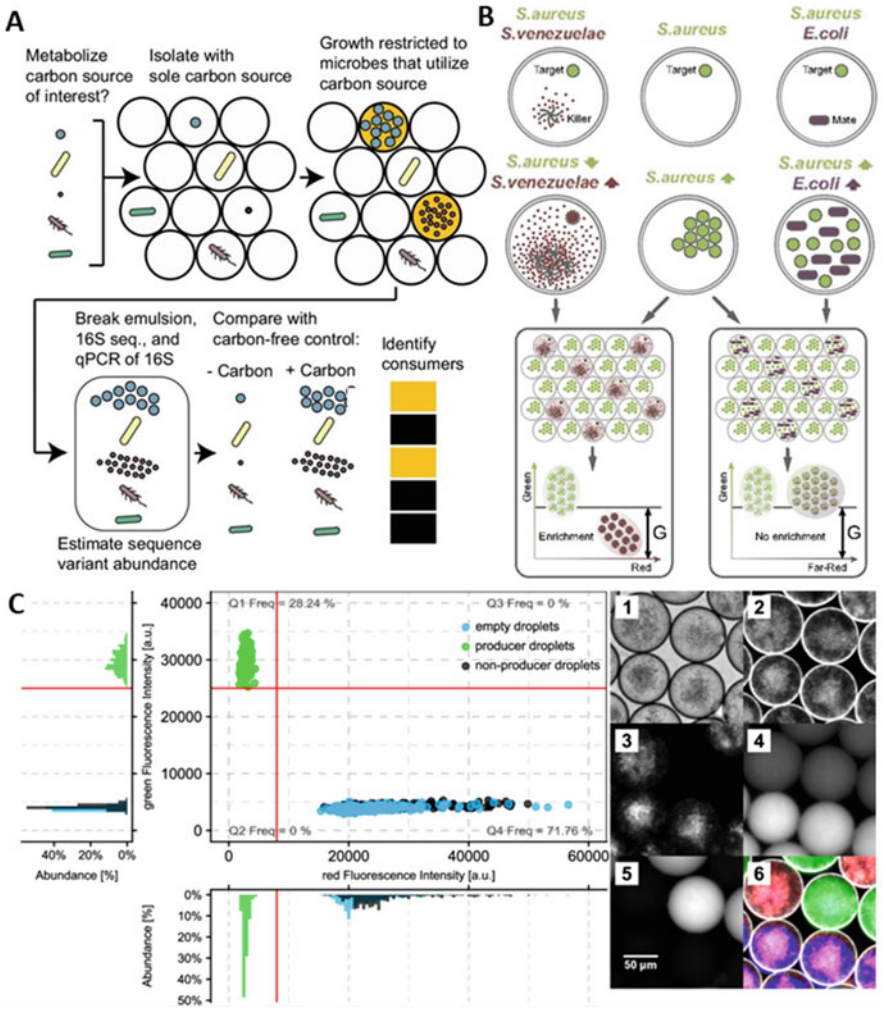


Fig. 4 Applications of ultrahigh-throughput microbial cultivations in picoliter droplets. (**a**) Characterization of carbon source (prebiotic) consumption within human gut bacteria [116]. (**b**, **c**) Negative interaction assays (i.e., antibiotic production) using co-encapsulation of possible producers and fluorescently labelled reporter strains [38, 76]. *Images are reprints of the indicated publications with permission of the original publisher*

growth-based fluorescence signal of the reporter strain indicates uninhibited growth (clear increase in fluorescence signal) or inhibition (no signal). With this approach a much more specific and targeted initial isolation of microorganisms with antimicrobial potential is possible. Even more, through the combination of reporter strains of different target groups (e.g., Gram positive and Gram negative), a differential selection of antimicrobial strains against a specific target group (e.g., active against only gram negatives) would become possible. Beyond this, for different screening

assays, reporter strains, which can report even on the mode of action of antimicrobial compounds, are already available [118].

An even simpler application of droplet microfluidic cultivation is the high-throughput determination of microbial antibiotic resistances by detecting growth or growth inhibition of a target strain in presence of antibiotics [31, 51, 67, 119]. In case the target compound does not have antimicrobial activity, biosensor strains can be employed, which induce antimicrobial activity or sensitivity to the reporter strain in presence of the target compound. This has, for example, been realized for the detection of muconic acid producing strains of *P. putida*, where muconic acid induces sensitivity of a normally resistant *E. coli* reporter strain against streptomycin [120]. If the target natural product is an enzyme, a selection assay based on an enzyme activity screen with a fluorogenic substrate can be employed to select the most active microbial droplet subpopulation [42].

Increasing resolution of culture-independent sequencing-based techniques has enriched us with better understanding of the identity and role of rare microbes. Nevertheless, culture-dependent methods are still essential for the surveying of microbial functional biodiversity. With the vast majority of microbial community members in diverse environments ranging from human guts to plant rhizosphere yet unknown or considered unculturable, new strategies are needed for the isolation of uncultured species and the study of the interaction within natural microbial consortia. In addition to the high-throughput and minimal input of resources, droplet microfluidics offers exceptional advantages over conventional methods in the cultivation and analysis of unknown microbes through single-cell technology, compartmentalization, and parallelization.

Rare microbes in large sample volume are difficult to detect and isolate since they are usually present in low numbers in their natural environment and coexist with other microorganisms, which are often much faster-growing species [26]. To overcome the outcompetition by fast-growing populations, microfluidics enables the stochastic confinement of single cells in discrete droplets. Stochastic confinement refers to the separation of a sample into small volume such that the number of small volumes is greater than the total number of cells in the sample [121]. This blocks the effect of outgrowth and influence of inhibitory signals by competitors and predators therefore allowing a more accurate representation of rare taxa. Accumulation of products of metabolism and quorum sensing molecules by bacterial cells confined in small droplet volumes attain the critical threshold faster than in bulk culture so that their growth can be promoted [20]. Enhanced detection of cellular activities can also be achieved as the dilution of secreted molecules would be limited [26]. Isolation of single cells in droplets has been demonstrated to improve recovery of slow-growing environmental species [57, 122, 123]. Liu et al. employed microfluidic single-cell isolation to separate slow-growing *Paenibacillus curdlanolyticus* from the competition of fast growing *E. coli*, which would otherwise dominate in mixed bulk culture [124]. Isolation and characterization of rare populations from soil, mouse, and human gut microbiomes have been achieved by single-cell encapsulation using a microfluidic platform [56, 117]. Compared to conventional culture methods, a larger representation of rare taxa was achieved, attaining up to four-fold increase in

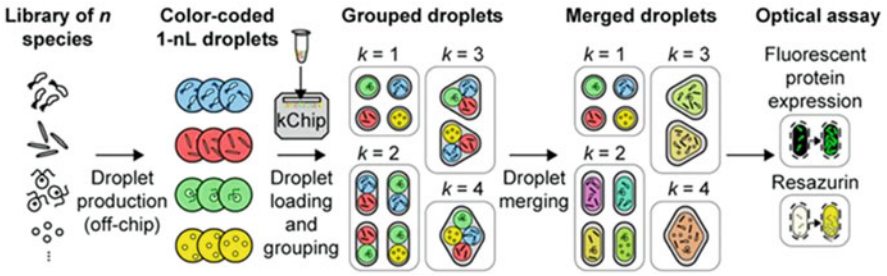


Fig. 5 Testing synthetic microbial communities in randomized combinations of different microorganisms [59]. *Images are reprints of the indicated publications with permission of the original publisher*

richness of microbial growth. Automated sorting based on colony density further enhanced the relative abundance of slow-growing species. A previously unknown *Blastococcus* species with high polycyclic aromatic hydrocarbons degradation efficiency was discovered in a soil community using a microfluidic streak plate method of single-cell droplets [53].

Natural microbiota or microbiomes are governed by the interactions between microbes and those with the environment. Deciphering these interactive networks can help to unravel the composition, functions, and dynamics of these complex microbial ecosystems. While most cell-cell communication and interactions are mediated by the secretion or consumption of small diffusible molecules [125], conventional laboratory bulk cultivation techniques largely overlook and severely limit the study of these interactions. Alternatively, microfluidic approaches allow miniaturized compartmentalization, which creates well-controlled environments in massively parallelized fashion to investigate interaction between microbes (Fig. 5) [55, 59, 126]. Dilution of microbial communities to multiple cells per droplet permits the co-cultivation of symbiotic microbial communities and therefore the study of partner-dependent relationships. For instance, Park et al. demonstrated a proof of concept study using a synthetic model system constructed with cross-feeding *E. coli* mutants to mimic various compositions of natural consortia [126]. Microbial Interaction Network Inference in microdroplets (MINI-Drop) was developed by Hsu et al. [55] to analyze the microbial interactions mediated by distinct molecular mechanisms in droplets containing one- to three-member consortia. Complex interplay between the presence of antibiotics and change in temperature on species interactions was also revealed in a three-member consortium. Cross-kingdom communication was shown by Jarosz et al. through co-encapsulating yeast and bacteria [127]. Carbon metabolism of yeast was transformed in the presence of bacteria which produce [GAR(+)], a protein-based epigenetic element, resulting in the mutual benefit of both organisms. KChip, a droplet-based platform that permits rapid and highly parallel screening of microbial communities, was recently introduced by Kehe et al. [59]. This platform enabled the identification of soil isolates that promoted the growth of a model plant symbiont *Herbaspirillum frisingense* and

therefore can potentially be adopted for the characterization of microbial consortia possessing functions in environmental remediation.

In a slightly different approach, micro- and nano-fabricated growth chambers provide a spatial separation of microbial cells in droplets while allowing the diffusion of growth compounds and secreted metabolites through porous chamber walls [128]. In situ and in vitro culture of unknown microbes from river water, soil, and human gut has been demonstrated with micro-compartmentalization devices, but technologies that allow the elucidation of microbial interactive networks are still lacking. The recently reported nanoporous microscale microbial incubator platform enables size-dependent control and transport of chemical factors and signaling molecules, facilitating the monitoring of growth dynamics of microbes by various stimuli [129]. Thus, the incubator is foreseeable to be useful to investigate the community interactions of uncultivated biosphere members. Challenges remain in the development of high-throughput microchamber devices for spatial isolation and cultivation of uncultured microbial species. However, overall the advancement of droplet microfluidic technologies provides a completely new and highly promising toolbox to access the metabolic potential of so far uncultured microorganisms.

5 Ultrahigh-Throughput Enzyme Activity Screening and Selection

Microbes provide a rich source for many novel enzymes, which are inherently eco-friendly, nontoxic, and adaptable for large-scale production through fermentation [130]. Hence, such enzymes are sought for various biotechnological applications. For instance, they can replace harsh chemicals in cleaning products mitigating their negative effects on the environment and increase sustainability [131]. They might even help in pressing problems, such as degradation or recycling of plastics [132, 133]. Therefore, the discovery and improvement of biological catalysts are of paramount importance. The environmental enzyme pool is fairly unlimited, and the synthetic creation of enzyme variants, e.g., through mutagenesis approaches, results in enzyme libraries of indefinite numbers of variants. However, finding the desired variants is comparable with searching for the needle in the haystack due to the huge diversity of microbes or enzyme versions in either natural or experimental samples.

State-of-the-art high-throughput screening techniques for biocatalysts are based on conventional microtiter plates (MTP) with automated liquid handling robotic platforms and approaches based on fluorescence-activated cell sorting (FACS) [40, 134]. In general, a MTP screening approach covers maximal 10^5 individual samples, with a maximum theoretical throughput of one assay event per second. Such campaigns require extensive equipment investments and exaggerated consumable consumption (mostly plastic tips and plates). On the other hand, FACS enables ultrahigh-throughput, allowing the analysis of more than 10^3 events per second, easily reaching millions per experiment or day. MTPs are not feasible to be operated

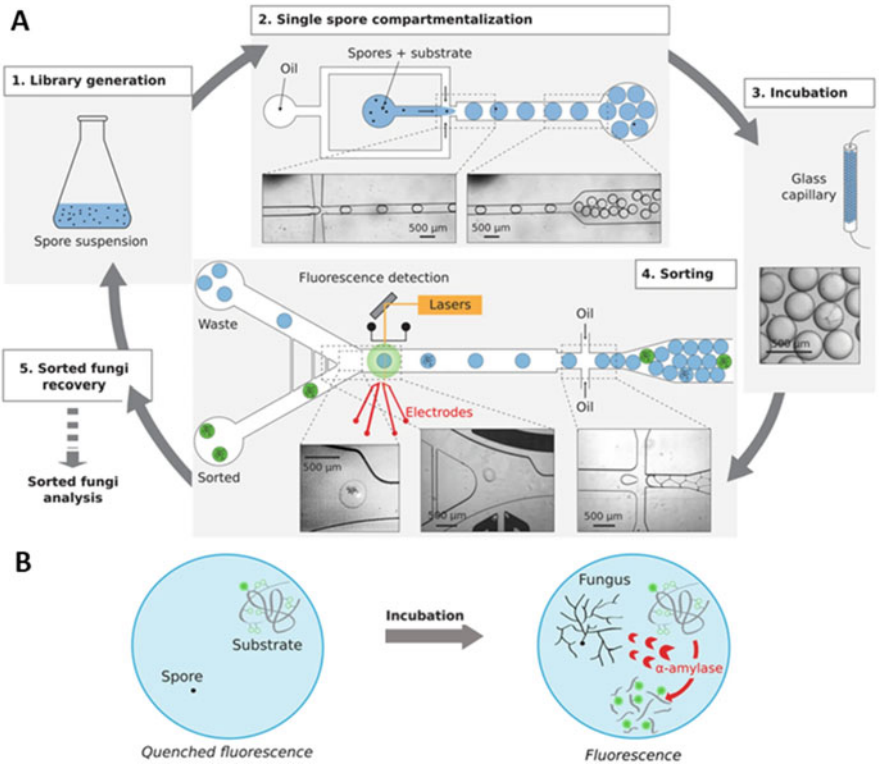


Fig. 6 Droplet-based microfluidic platform for enzyme screening. (**a**) Schematic showing different steps of enzyme screening. (**b**) Example concept for indication of enzyme activity through fluorogenic assay [105]

for single-cell analysis preventing the ability to unveil cell-to-cell heterogeneity. In contrast, with FACS, single cells can be measured and sorted, but this approach lacks compatibility with screening of secreted compounds and is therefore mostly limited to intracellular or cellular membrane-associated enzyme activity [135], which are not so relevant for industrial biotechnology. Droplet microfluidic approaches take advantage of the best properties of both technologies (Fig. 6a). They can be operated at a throughout similar to FACS, with fluorescence-activated droplet sorting (FADS) working in the range of 100–30,000 Hz [136]. Yet, encapsulation of single cells in droplets provides the genotype-phenotype linkage even for secreted molecules. The study by Obexer et al. clearly demonstrated the throughput benefit of droplets isolating a high potential variant of a combinatorial designed retroaldolase in only one round of directed evolution and FADS [137]. The catalytic efficiency of the isolate is comparable to a variant obtained from five rounds of conventional directed evolution using MTP screening. Wagner et al. compared FACS to droplet sorting by screening for better producers of riboflavin, concluding that the droplet approach is able to isolate higher titer producers [41].

Droplets do not have to contain liquid content. They can also be gelled by either agarose or alginate [37, 138]. For these gel microdroplets or for double emulsions (drop within a drop), even established FACS devices can be used. Multiple studies have used this approach [37, 138, 139]. It was successfully shown that gel microdroplets, which were embedded in a biomimetic polyelectrolyte shell, yielded an eightfold improved *Pseudomonas diminuta* producer of a phosphotriesterase after FACS screening [13]. In a similar fashion, a *Pichia pastoris* mutant library was encapsulated in gel microdroplets and screened via FACS [140]. The best performing clone showed 1.3-fold higher xylanase expression compared to the parent strain. In any case, more optimization of microfluidic processes for generating gel microdroplets is required, since currently the stability of the operation is generally more error prone. Furthermore various manipulation techniques, like picoinjection, splitting, and coalescence of droplets, which have been extensively developed for water-oil droplets, are not compatible with gel microdroplets [25, 141, 142].

Commonly, fluorescence assays are implemented for the indication of enzyme activity in droplets. This is mainly due to the available approaches for detecting and quantifying droplet contents (see previous sections). Fluorogenic substrates, with quenched fluorophore molecules, are either co-encapsulated or picoinjected into droplets. The fluorophore molecule is released in the presence of an enzyme, resulting in higher fluorescence intensity and demonstrating higher enzyme activity (Fig. 6b). For every screening task, reliable assay development is of critical importance, and fundamental differences must be taken into account. Unlike in MTP-based assays, the usage of expensive reagents does not hinder the screening campaigns because the total required amounts are in the microliter scale. It is important to understand the mechanisms of the microbial host for enzyme expression, in particular if it is either intracellularly located or secreted out of the cells. The former often requires the addition of cell lysis agents upon droplet generation or after a first round of incubation. Such additives can be either co-encapsulated or picoinjected into the droplet. Once the enzyme is released from the cell, its activity results in the release of the fluorophore molecule. Typically, the subsequent increase of fluorescence intensity is correlated to a higher enzyme activity. For example, miniaturized cell lysate assays have led to sixfold higher sulfatase activity and expression after three rounds of screening [143]. However, the addition of lysis agents holds several risks, such as affecting the enzymatic reaction, releasing interfering cell components, preventing subsequent cell growth after selection, reducing droplet stability, and increasing deviation due to inhomogeneous lysis across the droplets [144]. In contrast, the screening of secreted compounds can be performed under milder conditions. Since the cells are not getting damaged, sorted isolates can be recovered easily on agar plates or in liquid media. However, cell cultures in droplets can affect the assay both positively and negatively. On one hand, incubated cells have probably increased in numbers, and thus the effective concentration of enzyme is higher, increasing the signal intensity. However, microorganisms also produce by-products that can interfere with the assay signal or stability. Therefore, it is a common practice in MTP-based assays to include centrifugation or

washing steps. This is not possible in the microdroplet format. Nevertheless, mutant and metagenomics libraries of microorganisms have been successfully screened to find strains with higher enzyme activity. Several microbial producers including *E. coli*, *Trichoderma reesei*, and *Yarrowia lipolytica* have been screened for several enzymes like cellulases, amylases, xylanases, alkaline phosphatases, esterases, etc. [23, 42, 66, 102–106]. However, it should be noted that due to the usage of substrate analogues with fluorescence properties, the selected variants are often less active with the native substrate. To circumvent the necessity for fluorogenic model substrates, coupled enzyme assays are also applicable in droplets. Here the product of a realistic enzyme reaction functions as the substrate for another easily detectable reaction. With this concept, an oxidase mutant library has been screened in droplets by activating a horseradish peroxidase cascade resulting in a red fluorescence signal directly proportional to the target enzyme activity [145].

Overall, the strategies for the discovery and improvement of enzymatic activity and production can be divided into three fundamental steps. The first is bioprospecting for novel activities. Here, the potential of droplets is unprecedented. Ultrahigh-throughput is essential to consequently screen natural samples of extracted microbes or metagenomes, which contain millions of variants [23, 38, 42, 60]. Subsequently, enzymes are subject to a directed evolution approach in order to understand key residues in their structures [146] but also to increase the activity under general and particular operational conditions [12, 79, 143, 147, 148]. Finally, production hosts can be analyzed and optimized to achieve the highest possible production activities and yields [22, 105, 106, 108, 149, 150]. This relatively simple pipeline, when combined with a targeted industrial interest, has the potential to dramatically reduce costs and development time while achieving superior results compared to traditional screening campaigns.

6 Conclusions

Droplet microfluidic has emerged as a high potential tool for ultrahigh-throughput microbial cultivation with applications in single-cell analysis, cultivation of rare microbes, discovery of new natural products, and biocatalyst evolution. Despite the major advantages such as speed of throughput, reduction of costs, and resolution to single cells, the penetration rate of the technique into microbiology labs is still slow. This is probably based on two reasons. First, adopting microfluidic techniques is not yet straightforward. As a very multidisciplinary approach, a variety of skills and equipments is required to establish the necessary competences to handle these platforms. As for now, there is no commercially available device that offers a plug and play solution despite various ongoing efforts. Second, microfluidics provides an experimental perspective, which in many cases is counterintuitive to the well-established and validated methods in microbiology. This implies that some parameters monitored and controlled for quality under classic approaches might be sometimes irrelevant or not yet possible to monitor or control. For example, optical

density in a picoliter droplet cannot be measured, and therefore cell proliferation must be controlled with other methods. Another major difference/challenge is associated to the nature of droplet production and handling. While thousands or even millions of droplets will be produced per experiment, it is extremely cumbersome to distinguish one from the other, in contrast to what is done with labelled reaction tubes or wells. That means, predefined conditions and variables might be difficult to track on a droplet-by-droplet basis. A similar difficulty arises with single-droplet handling. While sorting operations have been developed, isolating a particular single droplet for further processing remains elusive.

Yet, most of the works reviewed here highlight both the rapid technical advancements taking place in the field and applications which clearly showcase the potential for groundbreaking research. Importantly, as more research groups and companies adopt microfluidic approaches, more creative solutions and applications arise.

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