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Review

Growing microbes in millifluidic droplets

Microbiology has continuously pushed efforts towards understanding microbial diversity. Technologies and methods have also evolved, from plating, and use of microscopes and cytometers, towards micro-well handling robots and, finally, fluidic-based devices. The aim of this review was to bring microbiologists attention to the outstanding analytical and handling power of millifluidic droplet technologies for analysing and sorting phenotypic diversity in the microbial world. This new format overcomes many limitations of previous approaches. It provides outstanding reproducible growth conditions over droplet reservoirs allowing unprecedented sensitive read-out over thousands of colonies over time. The confinement of the millifluidic train within tubes and the implementation of a three phases format excludes any contamination issues. The automation and handling of reservoir droplets is inherently facilitated. We show as a proof of principle the efficiency of capturing phenotypic diversity within a bacterial sample submitted to a sub-minimum inhibitory concentration of antibiotic. The precision offered by the millifluidic format allows the detection of a variety of resistance strategies that compete and coexist. The review finally explores the potential of this approach to address new challenges such as community-based growth of multiple-strain systems.

Keywords: Diversity / Droplet / Microbiology / Millifluidics / Screening

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1 Introduction

Microbes exist in every conceivable habitat: from oceans to soils, from sedimentary rocks to deep-sea thermal vents [1], on the surfaces and insides of animals, plants and insects [2]. Human has a long-term relationship with microbes as they are at the same time implicated in health and in pathogenesis of multiple diseases. Also, they have been exploited for the past thousands of years for food transformation and production of biochemicals ranging from industrial enzymes to pharmaceuticals.

To study microbes, microbiologists have developed methods and technologies based on growth, both on solid and liquid media, which allow further phenotypic and genotypic analysis. Due to their small sizes – the typical microbe's scale is the micrometre – and their diversity, microbiology has seen constant developments in miniaturising and parallelising microbial cultures and analysis. Indeed, the idea of in parallel cultivation of microorganisms is not new. Gel plating can be considered as the first method (early 19th century) able to parallelize the growth of clonal colonies. This approach is particularly useful in isolating species and phenotypes by the growth amplification of single inoculated cell into colonies. Current diagnostics methods for microbial infection still rely on this basic clonal isolation step. Later, micro-well platform technologies (late 70s) have opened the possibility to implement homogeneous culture and read-out in high-throughput formats, driven by pharmaceutical industry, which developed those tools for compounds screenings. A further step towards miniaturisation is reached with the development of microfluidics for biology (2000s), which allows single-cell cultivation and analysis [3], opening new perspectives for microbiology [4].

Digital microfluidics, defined as two phases fluidics, use aqueous micron-sized droplets as bioreactors circulated by an inert oil continuous phase. It has been extensively used for genomic analysis, including sequencing [5] and digital PCR [6]. Indeed the very large throughput (more than 10^6/h) offered by droplet microfluidics technologies is particularly adapted to the size of genomic DNA libraries. More recently, digital microfluidics has shown considerable promises in screening single cells libraries in order to identify the most interesting phenotypes (enzymes [7,8], clonal antibodies [9] production and xylose consumption [10]). This review will focus on growth and phenotyping of microorganisms using micro-droplets.

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Abbreviations: AST, antibiotic susceptibility testing; **MBRs**, microbioreactors; **MIC**, minimum inhibitory concentration

In the first part we will highlight the advantages and limitations of using droplets for phenotyping compared to traditional methods. We will dwell on the use of droplets having a size ranging from 1 to 100 nL (half a millimetre in diameter), which is defined in the literature as 'segmented flow', 'pipe-based bioreactors' or 'millifluidics'. In a second part we will present some applications of micro-droplets for microbiology ranging from industrial biotechnology to clinical diagnostics.

2 Bacterial growth

To better understand the use of droplets as micro-bioreactors (MBRs) for growing and studying microorganisms, we will look firstly at traditional methods, their automation and miniaturisation. We will try to outline the specific possibilities and limitations offered by each method.

2.1 State of the art in microbiology

Microbiology is the study of microorganisms which implies growth and isolation to better understand their functions and interactions with their environment (host, community, etc.).

Solid media have been used since early 19th century and remain the golden standard for microbiologists. It allows isolation of cells according to their growth pattern and ability to form a colony. Those media can be selective if they contain biocide agents or have a composition on which only a fraction of the microbes can grow. This method is very advantageous. It is simple and inexpensive. It allows isolating phenotypes and can provide an absolute numeration in colonies forming units of the growing bacterial inoculum (load) within a sample. One major drawback concerns its intrinsic heterogeneity. Bacteria are inherently subjected to gradients inside the colony as it grows. This implies that all the cells are not subjected to the same culture conditions. For example, this can lead to diversification inside a colony [11]. Recent efforts have been made to automate their use so as to improve throughput for diagnostics (PrevIsola, Biomérieux) or screening (Colony Piking robot, Tecan). Those solutions rely on robotic arms. They remain complicated and expensive.

Broth cultures were developed even earlier using meat extracts to amplify populations from a few individuals to larger populations. The size of the containers can vary from millilitres, in the case of test tubes or flasks in the laboratory, to thousands of litres in the case of bioreactors for industrial production. Culture conditions are considered as homogeneous under good agitation conditions, although it is more and more difficult as the cultures become larger. However, in this format all types of read-out will inform on mean parameters that integrate all types of cell behaviours. Therefore, such approach does not identify individual differences [12] and does not discriminate clones. Indeed, the 'fittest' clones, the ones that grow the most rapidly in this defined environment will take over the rest of the population.

2.2 Towards miniaturisation

Some of the drawbacks of traditional methods can be solved by miniaturising down to scales closer to the ones of microorganisms. In some case this allows to look at individual comportments [13] and to manipulate larger numbers of samples.

Micro-wells remain the standard solution for parallelisation, high-throughput screening, chemicals or mutants libraries handling as well as micro-dilution antibiotic susceptibility testing (AST) [14]. Micro-wells-based automation primarily developed for the pharmaceutical industry can be extended to small volume and higher throughput analytical rate by implementing more and more complex and refined automation: each well can be processed independently and various protocols can thus be directly adapted.

However, micro-wells suffer from inherent limitations: poor mixing, multiple interfaces (with air and cuvette) and evaporation for small volumes, which create a source of gradients and heterogeneities that obscure the possibility of homogeneous readings. Furthermore, it is expensive, technologically heavy and not affordable for most microbiology labs.

Flow cytometry was developed in the 70s and was one of the first instruments allowing the measurement of diversity at the single-cell level. With throughputs as high as 10 000 single cells analysed per second, it remains the most powerful tool for statistical analysis of populations. While it is well adapted to eukaryotic cells, its use for bacterial analysis is limited because of their smaller size. Furthermore, only internal or surface markers can be traced because excreted products are lost in solution. Flow cytometry is well adapted to describe parameters such as size distribution or instantaneous expression of internal protein, but does not inform on strain productivity, which involve excreted products. Because the individuals are measured once, individual kinetic monitoring is also impossible. However, fluorescence-activated cell sorting makes sorting and isolation of single cells possible.

Microbiology is born with the invention of microscopy by Leeuwenhoek. It remains the most reliable method to count cells one by one. Also, many methods rely on Time Lapse Microscopy to monitor cells parameters over time. The implementation of microfluidics has allowed the miniaturisation of agar plates and flow chambers because these formats allow for fast enough diffusion of nutrients across a permeable membrane [15]; such cell confinement permits the monitoring of inheritance along the first microbes divisions starting from single ones [16]. Tracking viability and growth at single-cell level is particularly relevant to address questions related to dormancy and viable but non-growing cells in natural isolates [17]. In most methods viability is reduced to the ability to grow. Homogeneity of the growth conditions is ensured by the continuous perfusion of growth medium using microfluidic channels. However, the enclosed nature of these systems makes the collection of the cells from the device and their subsequent use difficult.

For microbial cultivation, efforts have been undertaken to miniaturise MBRs [18]. Microfluidic MBRs have clear advantages, such as small volume, little or no need for cleaning (one time use), and high throughput (multiple MBRs in parallel), better mass transfer and heat exchange. Such μMBRs are implemented with sets of valves for multiplexed batch [19] and

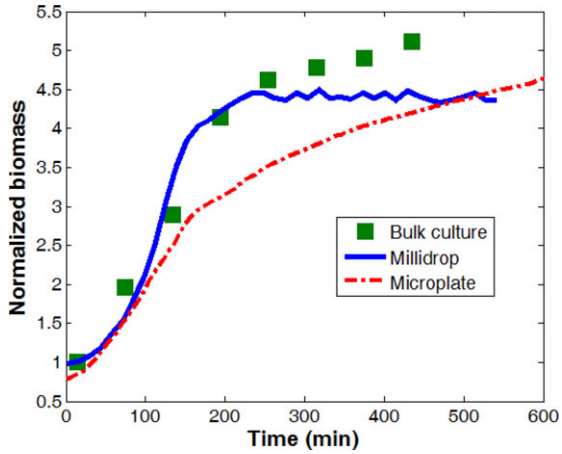

Figure 1. Comparison of bacterial growth as a function of miniaturisation.Bacterial growth monitored, in microtiter plate (red dotted line), in flask culture (green squares) and in a 200 nL droplet (blue line). While the initial slopes are quite similar (growth rate are the same at the beginning) it slows down in the flask and microtiter plate cultures because of bad oxygenation. Continuous and homogeneous oxygenation is ensured by millimetre scale droplet interfaces.

continuous (chemostat) cultivation [20]. Those devices remain quite complex to be fabricated, since they require multi-step micro-fabrication printing.

2.3 Droplet-based microfluidics

The idea of using droplets as MBRs for the isolation of microorganisms is not totally new. André Lowff proposed more than 60 years ago to encapsulate single *Bacillus megatherium* to study lysogenesis [21]. The use of droplet as bioreactors has grown in the past 10 years with the development of droplet-based microfluidics also called digital microfluidics [22]. Droplets have volumes ranging from a few picolitres to hundreds of nanolitres. Each droplet can be individually manipulated as the content of micro-wells using a pipette. The large range of applications of this technology is out of the scope of this review and is well described by Teh et al. [23], Guo et al. [24], Joensson et al. [25] and Theberge et al. [26].

It has been reported that the perfectly controlled microenvironment within droplets provides robust and reproducible growth of microorganisms, when single cells from the same clone are inoculated into droplets of a few picolitres volume [27, 28] to nanolitre volumes [29–31]. As illustrated in Fig. 1, growth conditions are different in micro-droplets compared to flask and microtiter plate culture. Oxygenation is more efficient in the case of micro-droplet growth [32] because diffusion is faster at this scale while heterogeneity exists in the other methods even under stirring conditions.

When droplets are smaller than a few nanolitres, one can still detect and measure individual cell output within each drop, including non-dividing cells [33]. Droplets can then be collected by methods similar to fluorescence-activated cell sorting [27].

2.4 Millifluidics

To allow for amplification, i.e. sufficient growth of colonies starting from single cells, the scale of usual microfluidics droplets (~10–50 μm in diameter), as broadly used today, is not large enough. Millifluidics, also referred to as 'micro-segmented flow' [34] or 'pipe-based bioreactors' [35] in the literature, has been developed to provide a simple and versatile technology. At the scale of millimetre, the droplet train stability, and its manipulation, is facilitated by a connected tubing strategy (Fig. 2), instead of chip printing, as for microfluidics. All the manipulations are performed within tubes, which prevent evaporation and contamination; this allows experiments to be run for more than 3 days. Millifluidics is at the cross-section between micro-wells and flow cytometry technologies. It offers to the microbiologist a sufficient growth dynamic, reproducible growth conditions, the possibility to tune the inoculum size down to one, vary the composition (gradients) over the train, and a great variety of read-outs.

The digital millifluidic format provides extremely reproducible surface and bulk properties of each individual reservoir with a perfect control of mixing via flow recirculation, and gas permeation without any cross-contamination [36]. The microorganisms are never in contact with solid surfaces. In such volumes, microorganisms can expand from one up to 10^5 individuals and therefore a sufficient amplification of inherited characters is possible. As a trade-off, it does not allow to measure single cells properties before amplification as can be done with Time Lapse Microscopy or with microfluidics.

The size of the inoculum can be adjusted from 1 to 1000 initial cells per droplet depending on the application and still offer a sufficient growth dynamic, up to 20 generations when starting with a single cell. These millimetric bioreactors enable both investigation of single ancestor associated phenotypes (such as Time Lapse Microscopy, gel plating and microfluidics) and population testing (such as micro-wells and bulk).

Because the droplets are spatially separated, it is possible to generate gradients of composition [37] and multiplex experiments in the same droplet train. Because of the millimetric size of the droplets, diffusion between droplets (which would tend to average the composition) is absent as compared to microfluidics [33].

Finally, millifluidics has the same read-out versatility as flow cytometry or micro-wells offer. Regular optical measurement of biomass using fluorescence [30, 38] (Fig. 2C) or camera imaging [39] has been demonstrated including Raman Spectroscopy [40] and Capacitance [41] measurement of biomass inside droplets.

Figure 3 provides a synthesis of the possibilities and limitations of the phenotyping methods we described in this part. We used eight important characteristics to compare them: volume of the sample, final number of cells reached at stationary phase, size of the library that could be interrogated and whether single-cell analysis, compartmentalisation, non-growing cell and growth monitoring and sorting were possible.

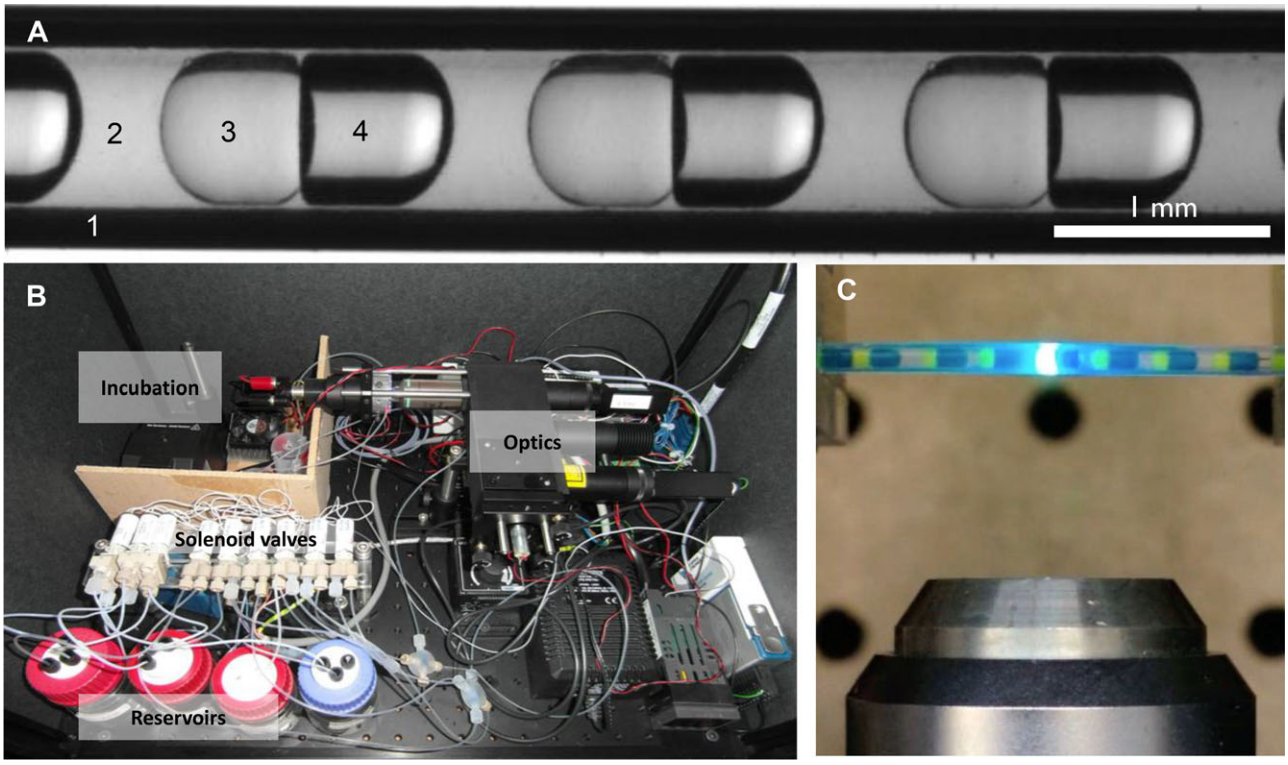

Figure 2. Millifluidics. (A) Aqueous bioreactors (3) are stored in FEP tubing (1), separated by a spacing fluid (4) and carried by perfluorinated oil (2); (B) MilliDrop prototype in Bibette's lab. (C) Epifluorescence scheme for measurement inside bioreactors.

3 Applications of micro-droplets in microbiology

As explained above, micro-droplets are particularly suited to probing microorganism community output, as well as offering all necessary droplet manipulation requirements, which opens a vast range of applications.

3.1 High content screening

Microorganisms are responsible for many biotransformation processes. It is therefore of great importance to select for individuals which have the best activities. Depending on the size of the diversity interrogated, the throughput rate of traditional methods is not sufficient. Droplet-based microfluidics is thought to be a game changer by providing screening capabilities of above 10^7 clones per day [27,7,8]. To achieve these throughput rates, small droplets must be used. Most of the assays rely on fluorescence, which is not systematically an appropriate reporter. Furthermore, for some microorganisms such as filamentous fungi, small droplets can be destroyed by the growth of hyphae through interfaces.

Millifluidics is a good alternative to circumvent this issue still providing a medium throughput capability but with higher content information. In this respect it is quite similar to microtiter plate robots, but the ease of use and lower costs because of reaction volumes, about three orders of magnitudes lower, are improved.

3.2 Susceptibility/toxicity testing

The standard methods for toxicity and AST are based on micro-wells and logarithmic scale concentration. As mentioned before, micro-wells filling can be adapted to various growth medium compositions, but the limitation of this technology beside its inherent limited sensitivity comes also from the operating time and investment and operating costs of robots. For instance, the preparation of a linear composition gradient within a micro-well plate array is a laborious operation. One commercial alternative can be found in the mass preparation of pre-filled plates (Biolog).

In contrast, segmented flow allows rapid preparation of various composition gradient profiles by simply tuning flow rates. Figure 4 illustrates how high-resolution minimum inhibitory concentration (MIC) can be obtained in less than 4 h. The antibiotic (here chloramphenicol) is co-encapsulated with a red fluorophore. Measurement of red fluorescence allows monitoring precisely the antibiotic concentration (colour map) across the droplet train. At the same time, growth is monitored in each droplet using resazurin as reporter, which becomes fluorescent when bacteria are metabolically active. With this tool, both refined MIC investigation (unpublished data) and rapid combinatorial toxicity screens are enabled [42].

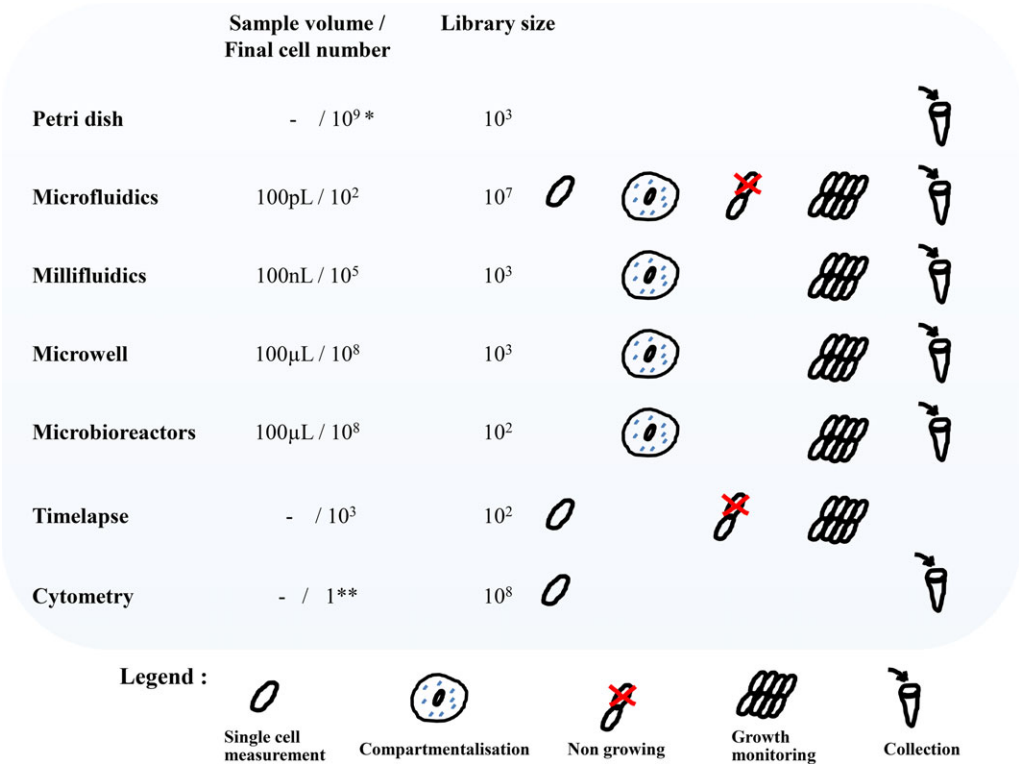

Figure 3. Synthesis of the possibilities and limitations of phenotyping methods. Final cell number is given for bacterial population such as *E. coli*. Library size refers to the total number of clones you may reasonably test with the method. Single-cell measurement requires direct monitoring of each cell, and therefore excludes traits measured on the colony derived from single cell. 'Growth monitoring' refers to any monitoring on a group of related cells but also to acquisition over time. Compartmentalisation covers secretion concentration in a closed volume and co-culture sequestration. Collection indicates the possibility to dispense the biological material out of the phenotyping system. *For comparison Petri dish volume is given as 100 μL estimated from a total Petri dish volume of 20 mL that supports the growth of 100 colonies. **For the comparison, the cytometry sample size is 1 for each single cell, but one cytometry measurement would count $\sim 10^5$ cells.

This approach has been exemplified in different academic labs on toxicology [43] and AST measurements [30,31,44]. The first example revealed subtle cross-toxicity between particles and drugs with a complete mapping of toxicity interplay in a 2D composition space. In 2013, the same group performed toxicity assay in multi-dimensional composition spaces thanks to segmented flow parallelisation, which would have been hardly possible with other methods. For a review, readers should refer to Cao et al. [37].

3.3 Single-cell phenotyping and population adaptation to antibiotics

The scale of segmented flow bioreactors enables the isolation and measurement of single cell initiated process. Such measurement at the level of individuals may reveal diversity within clonal populations or within ecological communities. This diversity measurement is relevant for clinical challenges such as drug resistances [45] or virulence factors [46–48], and for a better understanding of microorganism ecology [49]. Interest in measurement of single cell is rising thanks to various technological approaches. There have been several reviews on microfluidics approaches for single-cell analysis in the past years [25,50] and recently [51]. Outside the scope of this review, millifluidic approaches have already been validated for mammalian cells [52], algal cells [28], whole organisms [53] and tissues [54]. To highlight the potential of millifluidic single-cell analysis, we will focus here on the adaptation process of a bacterial population.

Microorganism adaptation to new environments or to new stress may involve diversification. Diversity fuels adaptation because under new conditions some phenotypes within this diversity will grow better or survive better, and then outcompete the rest of the population. Genetic mutations, some stochasticity in gene expression or epigenetic regulation, generate phenotypic mutants constantly. Those mutants constitute a minority of cells that are different from the dominating phenotype. They are rare and therefore difficult to detect before they expand within the population thanks to new conditions advantageous to them. When a specific challenge occurs, the population fraction of the adapted phenotypes would increase and become simultaneously observable. Such population dynamics measurement requires to first sample and then genotype or phenotypes as many single cells as possible. As stated before millifluidics provides a solution to parallelize the phenotypic analysis of individuals and isolate them. It combines measurement flexibility of micro-plates reader and simple isolation principle of gelose plating.

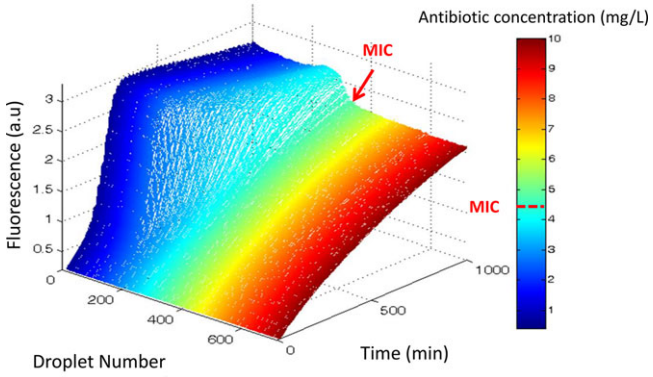

Figure 4. High-resolution minimum inhibitory concentration.The curves correspond to the fluorescence of the resorufin formed over time by metabolic reduction of resazurin (80 μM initial concentration) in droplets prepared with an inoculum of 100 *Escherichia coli* ATCC 25922 and chloramphenicol (bacteriostatic). The concentration of chloramphenicol is linearly increasing from 0 to 10 mg/L according to the colour map (monitored by red fluorescent sulforhodamine 101). The linear gradient of antibiotic reveals the continuous dose response of the antibiotic on bacteria viability (assessed by the metabolic activity). Free growth of bacteria leads to blue curves and resazurin reduction without growth produce red curves. The MIC as marked by a red arrow and a red bar can be determined, and the measurement provides a high content dose–response relationship.

In the context of antibiotic resistance, the process of resistance acquisition within a population is most often considered as a single-step jackpot. Indeed, such selection of a happy-few is observed when large populations face an unprecedented high antibiotic concentration. Then, extremely rare mutants carrying the modification, which increase efficiently their resistance by several decades are the sole survivors or the largely dominating survivors. Such high-resistance mutants correspond to highly specific mutations on the protein targeted by the antibiotic or on the enzyme degrading the antibiotic [55]. For smaller populations or less challenging antibiotic concentrations, the population dynamics may be less dramatic. In a small population, the chance to observe a given rare mutation is reduced and if it does not appear in the population then it will not outcompete all others phenotypes. For low antibiotic concentration challenge, the sufficiently adapting modifications are numerous, they may occur at higher frequency and the expected selective advantage given by those modifications is smaller. Co-existence or transient co-existence is therefore more likely.

Here, we present results from a study performed on an *Escherichia coli* clonal population adapting to a low concentration of ampicillin, an antibiotic of the beta-lactam family. The initial MIC of the strain was measured at 0.6 mg/mL of ampicillin. The MIC is high due to the presence of an *ampR* gene on the chromosome which codes for a beta-lactamase enzyme degrading ampicillin. The population is grown in batch with 0.1 mg/mL of ampicillin, which can be considered as a low concentration with respect to the initial MIC. Figure 5A and B show the growth curves in Luria Broth medium (no antibiotic) acquired in parallel for hundreds of single cells from the adapting population using the millifluidic droplet analyser MilliDrop [30]. Phenotyping is based on the acquisition of the fluorescence curve (see Fig. 5) resulting from growth of single bacteria isolated in the droplets. In Fig. 5A, phenotypic analysis of the initial clonal population (derived from recent single-cell bottleneck) shows extremely reproducible growth curves. With our phenotyping approach, the initial population reveals a unique phenotype. After 70 generations of cultivation with ampicillin, the growth curves collection (Fig. 5B) reveals at least three phenotypic classes. These coexisting phenotypes underline a soft process of adaptations where several strategies were selected and maintained within the population.

As far as we know, no other technics would provide such phenotypic diversity picture. Plating on gelose may sometime reveal colony morphology diversity but such phenotypic readout is less consistent and less flexible. The cytometry may be appropriate especially in this particular case where modifications are observed on fluorescence signal but as illustrated by Fig. 5C one limitation of cytometry is the high level of non-inherited cell-to-cell variability [56]. The analysis of the mixed population in cytometry would show enlarged distribution or may be bimodal distributions as can be observed in Fig. 5C. Cytometry would hardly discriminate the three phenotypes as well as they are with the millifluidic machine. Such difference is simply due to the measurement of single-cell trait on its whole progeny. The measurement of an average trait on the small colonies resulting from a single cell is analogue to filtering fluctuations.

Interestingly, the situation observed with ampicillin exposure leading to coexisting subpopulations is related to co-culture which will be discussed in the next section. The MIC of two phenotypes isolated after 70 generations was measured. Both resisted to higher concentration of ampicillin than the ancestral population. But one exhibits a significantly higher resistance than the other (data not shown). This suggests some commensalism within the population. The less resistant would benefit from the resistance mechanism of the more resistant phenotype. Such scenario is perfectly in line with the degradation of ampicillin by the beta-lactamase activity conferred by the resistance gene. Such interaction between co-cultured types is indeed difficult to track, but segmented flow technologies provide new opportunities.

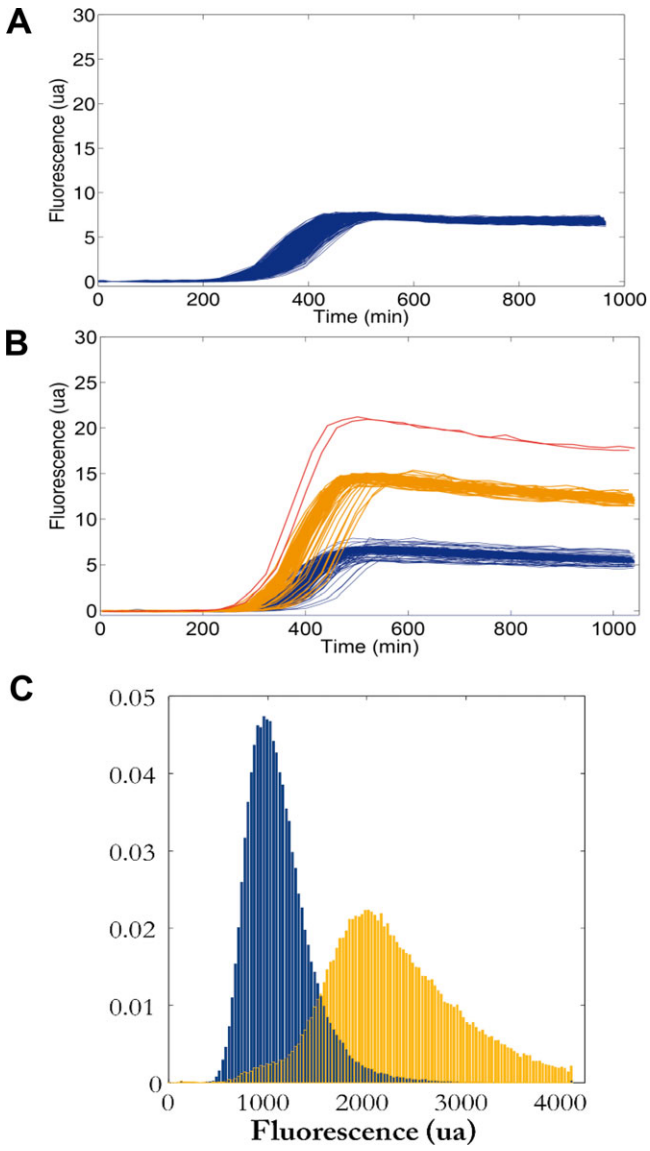

Figure 5. Phenotypic diversification under low antibiotic stress. (A) The fluorescence growth curves measured on 200 droplets inoculated with single fluorescent bacteria before exposure to ampicillin. (B) The fluorescence growth curves measured for single fluorescent bacteria after 70 generations of growth with 0.1 mg/mL of ampicillin. Three phenotypes are identified in blue, yellow and red. (C) Fluorescence distribution measured in cytometry for two phenotypes isolated after the 70 generations of exposure to ampicillin and identified with corresponding colours used in B. In all experiments, *E. coli* MC4100 YFP strain was used.

3.3 Co-cultivation and domestication

Less than 1% of the total bacterial species can be recovered by traditional microbiological methods, and the easy cultured portion is not representative of the total diversity [57]. Indeed, classical cultivation strategies supply an excess of nutrients so that only fast-growing bacteria that are capable of colony or biofilm formation are selected. Microbial interactions in natural microbiota are, in many cases, crucial for the sustenance of the communities, but the precise nature of these interactions remains largely unknown because of the inherent complexity and difficulties in laboratory cultivation. Conventional pure-culture-oriented cultivation does not account for these interactions mediated by small molecules, which severely limits its utility in cultivating and studying 'unculturable' microorganisms from synergistic communities.

Recent works have demonstrated droplet-enabled co-cultivation [48]. Its extension can effectively decompose complicated microbiota [58] and thus facilitate the elucidation of underlying interactions.

4 Concluding remarks

Liquid droplet reservoirs, from 10 to 100 nL in volume (a droplet of less than a millimetre in diameter, the so-called

millifluidic scale), have been revealed over these last years to afford outstanding sensitivity in characterising the diversity of the microbial word. This volume offers a sufficient growth dynamics: microorganisms can expand from one up to 10^5 individuals and therefore it allows a sufficient amplification of inherited characters. Moreover, the digital millifluidic format provides extremely reproducible surface and bulk properties of each individual reservoir with a perfect control of mixing and gas permeation. Our initial studies allowed us to conclude that 100 nL droplets are particularly suited to probing microorganisms colonies output, as well as offering all necessary droplet manipulation requirements. There is also good reason to expect development of intellectual property surrounding the use of such technology. Indeed, this millifluidic tool presents new possibilities for applications in green chemistry (including new routes for bio-based resources, bioremediation and soil agricultural efficacy) and for human health (probiotics, or assemblages tailor-made for faecal transplants and new antibiotics). Our laboratory is currently pushing substantial efforts towards these directions, including the possibility for external labs to acquire such instruments.

Practical application

Millifluidics has a broad spectrum of practical applications: in genomics with PCR amplification, in chemistry with polymer or nanoparticles synthesis and in diagnostics or in microbiology with the high-throughput screening of industrial strains and antimicrobial compounds. In this review, we focus on the microbiology applications. We highlight the strength of compartmentalisation in the diversity of assessment of microbial populations.

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LB, NB, JB and JB are founders of MilliDrop Instruments SAS.

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