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REVIEW PAPER

# Recent Progress in the Development of Droplet-based Microfluidic Technologies for Phenotypic Screening using Cell-cell Interactions

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**Abstract** Specific cell-cell interactions enable the complex metabolic tasks associated with natural microbial communities and facilitates their improved adaptation to environmental changes when compared to monocultures. Understanding these interactions can help resolve many of the underlying mechanisms regulating these complex microbial ecosystems and supply novel insights for various applications. However, the complexity of microbial interactions makes it difficult to evaluate them individually. However, droplet-based microfluidic methods can be used to compartmentalize individual responses to specific conditions in a massively parallel manner allowing for evaluations at a single-cell resolution. Moreover, individual droplets can be withdrawn from these systems without washing or dilution and can thus be used to determine the impact of specific substances used in intercellular interactions via further analysis such as next-generation sequencing or mass spectrometry. In this review, we summarized the recent progress around droplet-based microfluidic technologies for phenotypic characterization and screening using cell-cell interaction, which continues to diversify over time expanding its application to a variety of topics.

**Keywords:** droplet, microfluidic technologies, phenotypic screening, cell-cell interaction

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## 1. Introduction

Native microbial communities perform complex metabolic tasks and adapt better to environmental changes compared to monocultures [1]. Most of the essential functions in the Earth's ecosystems are regulated by these kinds of natural consortiums [2] and many of these natural communities are already being applied in bioproduction [3], bioremediation [4], and probiotic-mediated therapies [5].

These natural microbiomes are governed by cell-cell and cell-environment interactions [6,7] which means that understanding these interactions can help resolve the underlying regulatory mechanisms controlling these complex microbial ecosystems and aid in their application in a variety of fields [8]. However, due to the complexity of these microbial interactions and their dependence on the environment, it can be difficult to predict behavior even in seemingly simple communities, making it difficult to efficiently design and apply these microbial communities [9,10]. However, if we can develop systems that allow us to more accurately observe these cell-cell interactions we may be able to apply these natural consortia in a more predictable manner and design more sophisticated synthetic consortia enabling the production of more efficient functional communities when compared to the conventional monoculture process. In addition, any platform that enables the individual evaluation of specific microbial strains may facilitate the identification of more novel strains from natural sources or help identify strains with improved performance among existing microbial libraries. Cell-cell interaction-based screening can be used for discovering novel strains producing natural products such as antibiotics and secondary metabolites for organ transplantation, cancer treatment, and cholesterol control [11]. Moreover, these platforms can be used for screening the strains with enhanced phenotypes that may be difficult

to distinguish in monoculture.

However, the evaluation of cell-cell interactions remains challenging as the microscopic behavior of individual contributors in these interactions may be hindered or obscured by the macroscopic behaviors in conventional laboratory scale cultivation [8,12]. Thus, it is necessary to apply microfluidic methods to evaluate these interactions as these offer insights into systems at the single-cell level. Flow cytometry is a common and versatile tool for examining single cells for various purposes [13]. However, flow cytometry is not an ideal tool for gathering information about cell-cell interactions or cell behaviors over time [7,14,15]. Microfluidic perfusion culture is another commonly used microfluidic technology and enables culture in small-scale microchambers equipped with constant perfusion. These systems make it easy to modify the environment often facilitating the addition of medium or stimulants to cultures at predefined concentrations for defined time intervals. This means that the reactions of single cells can be monitored using live-cell microscopy [16-19] facilitating the evaluation of complex environmental conditions such as concentration gradients, with several studies using this approach to evaluate chemotaxis [20-22]. However, this technique is generally limited to microscopic readouts and is difficult to apply to large-scale phenotypic analysis and screening [23].

Droplet-based microfluidic methods were introduced to overcome the limitations associated with the aforementioned techniques, and are designed to facilitate the creation of small, compartmentalized environments that can be manipulated in a massively parallel manner [2,6,24]. These systems allow the user to vary the concentration of the microbial community, allowing for the development of microdroplets with different ratios of cells which can then be co-cultured in a high-throughput environment allowing for nuanced evaluations of these complex communities. Since several studies have shown that the ratios between strains in specific communities have a significant effect on cell signaling, analyzing these large numbers of different ratios within similar communities can be of significant value when evaluating specific cellular interactions [25-27]. The recent automation of these droplet-based microfluidic systems has even made it possible to generate droplets with complex combinations using many different strains simultaneously [28-31]. Moreover, these systems make it easy to evaluate the impact of specific secretory signals in complex intercellular interactions as cell responses in individual droplets can be monitored without washing or diluting [32-37] the individual droplets and these withdrawn droplets can be used for strain population or secretory protein evaluations via next generation sequencing and mass spectrometry [37].

In this review, we summarized the applications of the study of cell-cell interactions via droplet-based microfluidic evaluations (Fig. 1). We hope that this will help those interested in these technologies apply them more broadly and facilitate the early adoption of this rapidly expanding technology in more complex evaluations in the future.

## 2. Observation of Changes in Cellular Behavior in Response to Specific Cell-cell Interactions

Droplets generated by microfluidic devices were often utilized to co-cultivate microbes and observe their cell-cell

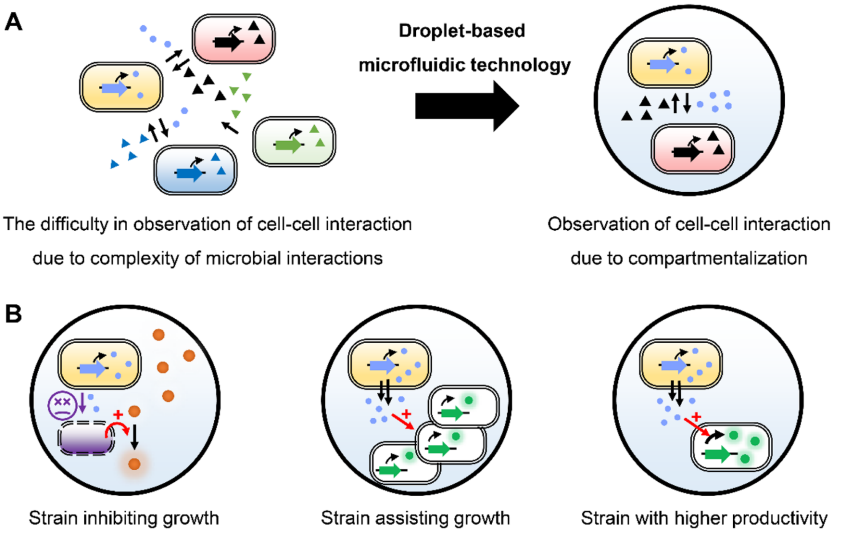


**Fig. 1.** Droplet-based microfluidic technologies facilitate phenotypic screening using cell-cell interactions. (A) Observation of cell-cell interactions via compartmentalization of complex communities facilitates improved understanding of these interactions. (B) Several fluorescent screening methods can be combined to facilitate the high throughput screening of various strains facilitate the identification of inhibitors, growth promoters and more productive strains depending on the screening technology applied.

interactions [24]. This research used the co-encapsulation of low concentrations of two strains auxotrophic for different amino acids using a microfluidic device which produced four types of droplets simultaneously, (1) empty droplets, (2 and 3) droplets containing only one of the two strains, and (4) droplets containing both strains. This allowed for the growth of only the droplets containing both strains and facilitated the in-depth evaluation of the symbiotic interactions between these strains. This study confirmed that highly parallel compartmentalization was possible, and that no molecular exchange takes place between these droplets, making this an ideal system for these types of applications. A different study used droplet-based microfluidics to evaluate the effects of bacterial cells on yeast cell growth [38], facilitating the observation that [$GAR^+$] prions can be induced by a small number of bacterial cells in just a few yeast doubling cycles.

This system also allowed for the evaluation of changes in morphological and doubling-time in response to the presence of peptide mating pheromones in budding yeasts [23]. This study varied the concentration and ratio of MATα and MATa, and added synthetic MATα-type mating factor to these cells and then evaluated the resulting behavioral changes in these microbes during mating. These experiments revealed that these interactions are dependent on various mutations and specific interactions. In addition, several experiments using consortia consisting of 2-3 members were completed in an effort to evaluate a variety of cell-to-cell and environmental interactions and the individual contribution of specific consortium members to these outcomes. These experiments made several important discoveries but also highlight the high-throughput screening capacity of microdroplet based microfluidic systems when combined with fluorescence tagging of target cells [6]. Cell-cell interaction in diverse networks and cell-environment interactions such as their response to various combinations of antibiotics and cultivation temperatures were also observed using this method. These evaluations produced a novel model that can predict the probability of strain growth using the initial proportion of the strain within the community and the total cell density of these communities. Taken together the results of these studies may aid in the design of more effective synthetic consortia by facilitating the precise contribution of each strain to provide the optimal outcome.

## 3. Phenotypic Screening Using Cell-cell Interactions

As the accurate observation of cell-cell interactions has become increasingly feasible, we have seen a concurrent increase in the number of studies using these techniques to screen for specific phenotypes (Fig. 1B, Table 1).

**Table 1.** Summary of several recent phenotypic screening completed using microdroplet mediated evaluation of specific cell-cell interactions

| Screened phenotype | Initial population | Principle underlying the fluorescence-based screening method | Reference |
|---|---|---|---|
| Strains inhibiting growth of *Staphylococcus aureus* | *Escherichia coli* harboring metagenomic libraries | Fluorescent viability dye specific to antibiotic mediated cell death | [39] |
| Strains inhibiting growth of *S. aureus* | Human oral microbiome | Constitutive fluorescent protein-expressing cells (sorting of droplets with reduced fluorescence) | [37] |
| Strains inhibiting growth of *S. aureus* | Siberian bear oral microbiome | Constitutive fluorescent protein-expressing cells (sorting of droplet with reduced fluorescence) | [40] |
| Strains promoting growth of *Chlorella sorokiniana* | Microbes from various natural environments | Increased chlorophyll autofluorescence of *C. sorokiniana* | [41] |
| Strains promoting growth of *Herbaspirillum frisingense* | Soil microbes | Constitutive fluorescent protein-expressing cells | [2] |
| Strains with increased of 2-ketoisovalerate and L-tryptophan production | Chemically mutagenized strain library | Constitutive fluorescent protein-expressing auxotrophs for target chemicals | [42] |
| Strains with increased of p-coumaric acid production | 28 variants known to produce various levels of p-coumaric acid | Cells harboring biosensors that express fluorescent protein in response to p-coumaric acid concentration | [43] |
| Strains with increased vitamin B2 production | Libraries generated via genome shuffling | Cells harboring biosensors that express fluorescent protein in response to changes in FMN concentration | [44] |
| Strains with increased conversion efficiency of benzene into phenol | 5 methanotrophs expressing soluble methane monooxygenase | Cells harboring biosensors that express fluorescent protein in response to changes in phenol concentration | [45] |
| Strains with enhanced 3-hydroxypropionic acid secretion | Library of strains overexpressing random endogenous genes from *E. coli* | Cells harboring biosensors that express fluorescent protein in response to changes in 3-hydroxypropionic acid concentrations | [46] |

FMN: flavin mononucleotide.

## 4. Identifying Growth Inhibiting Strains

Microdroplet-based cell-cell interactions can also be used to screen for antibiotic secreting strains. One example of this is the enrichment for antibiotic-producers via their co-culture with *Staphylococcus aureus*, a bacterial pathogen known to be a major causative factor for respiratory and other pathogenic pneumonias. These systems can also be used to evaluate recombinant antibiotic-producing *Saccharomyces cerevisiae* [39]. The droplets containing strains expressing effective antibiotics targeting *S. aureus* were sorted using a fluorescent viability dye that selectively stains dead cells. This system allowed for a more than 1,000-fold enrichment factors via a single round of sorting. The system was also used to identify the antibiotic genes that effectively inhibit *S. aureus* using recombinant *Escherichia coli* harboring metagenomic libraries generated from environmental competitors of *S. aureus*.

Strains inhibiting *S. aureus* were also identified from the oral microbiome of humans and Siberian bears using a similar approach [37,40]. This compartmentalized culture method allows for the critical evaluation of slower growing strains which might be outcompeted in conventional bulk culture environments by facilitating the elimination of competition [37]. *S. aureus* was also co-cultured with *Streptomyces venezuelae* which acts as a killer and *E. coli* which acts as a mate. Each of these three strains expressed different fluorophores, allowing for the distinct fluorescence distribution of clear evaluation of the target-killer/target-mate relationship. A subsequent paper went on to describe the strains identified in these evaluations and found that most were from the Siberian bear oral microbiome and that most of these produced novel antibiotic amicoumacin A [40]. These researchers where then able to evaluate the effects of varying concentrations of this antibiotic on the oral microbiota of the Siberian bear, human fecal microbiota from a patient with colitis, and a healthy donor using metagenomic sequencing and bioinformatic analysis. This method can also be applied to the high-throughput evaluation of the minimum inhibitory concentration of each strain within each microbiome.

## 5. Identifying Growth Promoting Strains

Several studies have also used this technology to identify microbial strains or communities that promote the growth of a specific target. One example of this includes a study that co-cultivated *Chlorella sorokiniana*, a candidate algal biofuel production strain, and the microbes from the natural environment for isolating bacteria promoting algal growth [41]. This was evaluated via chlorophyll autofluorescence and subsequent sequence analysis of the 16S rRNA indicated that the bacterial populations in these droplets decreased from 4,120 operational taxonomic units to 11 over four rounds of iterative screening. This produced a 30% increase in *C. sorokiniana*, growth in comparison to its pure culture. Similarly, soil microbes were co-cultivated with *Herbaspirillum frisingense*, a model plant symbiont, to identify strains likely to improve the growth rate of *H. frisingense* [2]. These researchers screened droplets with an improved growth rate by co-culturing fluorescent protein expressing *H. frisingense* and various microbial communities allowing them to automatically construct and identify 100,000 synthetic communities with various strain proportions per day.

## 6. Identifying Better Producer Strains

These systems can also be used to identify strains with specific properties. For example the combination of droplet microfluidics and the introduction of auxotrophs or specifically fluorescence-labelled strains might facilitate the identification of better producer strains. One example of this was a study in which cross-feeding auxotrophs were co-cultured in droplets to screen for strains with improved target metabolite production [42]. One of the auxotrophs was unable to produce the target substance, an essential metabolite for survival, and the other strain could not produce lysine. Lysine auxotrophic strains which produce higher amounts of essential metabolite increase growth rate of the target chemical auxotroph producing fluorescent proteins. Through positive feedback that enhances each other's growth, even a small increase in production is amplified and causes a large difference in the fluorescence signal, which can be easily evaluated through fluorescence-based monitoring. This study identified a strain with increased 2-ketoisovalerate, a precursor of isobutanol, and L-tryptophan production and allowed for a 5-fold improvement in isobutanol production from the chemically mutagenized library when compared to the parental strain. In addition, several other recent studies have applied biosensors for similar experiments. In this case the biosensor detects the target molecules and converts them into fluorescent signal allowing for the evaluation of components not associated with cell growth. Strains with high productivity of p-coumaric acid were enriched using this method via the co-culture of the relevant yeast strains and an *E. coli* based p-coumaric acid biosensor [43]. In the initial rounds of evaluation, the yeast strains producing p-coumaric acid made up 80% of the total; however, after the second screening, this was increased to 96%. Strains with higher vitamin B2 production were also screened using this

methodology and 9000 *Bacillus subtilis* libraries generated via genome shuffling [44]. These libraries were co-cultured with an *E. coli* strain which converts B2 into flavin mononucleotide (FMN) and harbors a FMN biosensor. As a result of measuring the productivity of each of the 96 variants in the randomly selected and screened strains through intercellular interaction, the former obtained 10 variants and the latter obtained 40 variants with increased vitamin B2 production when compared to their parental strain. Subsequent genotyping of these strains identified several genes associated with B2 production. This method was also effectively used to identify several soluble methane monooxygenase (sMMO) producing methanotrophs capable of converting more benzene into phenol [45]. Studies on the interactions between methanotrophs and phenol sensing strains can also be used for the high-throughput screening of strains with enhanced sMMO expression from large-scale methanotroph libraries. These microdroplets were also shown to facilitate the identification of chemical secretion-related genes [46]. One example of this was the evaluation of a 3-hydroxypropionic acid (3-HP) producing strain library using randomly overexpressed endogenous genes of *E. coli* and an 3-HP biosensor. The secretion-related genes were effectively identified via the cell-cell interaction mediated sorting of specific droplets.

## 7. Application to Mammalian Cells

This co-culture technology can also be applied to mammalian cells to study the interactions between cytokines and mammalian cells or mammalian antibodies and their targets. One such evaluation notes that the viability of human megakaryoblastic leukemia cell line M07e was modulated according to the total number and ratio of co-encapsulated interleukin-3 secreting cells (MBA2) [47]. *S. cerevisiae* strains secreting functional murine interleukin-3 were enriched from a 1:10,000 dilution when compared to the negative control [48]. These were then co-encapsulated with murine BA/F3 reporter cells, which respond to mIL-3 stimulation by producing fluorescent protein. *Pichia pastoris* producing positive antibodies were also screened using co-cultivation of the antibody-producing library and mammalian target cells via the addition of a fluorescently labeled secondary IgG [49]. These evaluations revealed that the strains producing the positive antibody were efficiently enriched to at least a 10,000-fold excess of negative control within 3 days. These systems have the advantage of being easy to prepare target cells and allowing for the efficient confirmation confirming the effect of antibodies that act directly on the target.

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## Ethical Statements

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

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