Microfluidic cultivation and analysis tools for interaction studies of co-cultures

Alina Burmeister[1,2] and Alexander Grünberger[1,2*]

[1] Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich, 52425 Jülich, Germany

[2] Multiscale Bioengineering, Bielefeld University, Universitätsstr. 25, 33615 Bielefeld, Germany

*Corresponding author:

E-Mail: alexander.gruenberger@uni-bielefeld.de

Phone: +49-521-106-5289

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Highlights:

- A major challenge for the investigation of microbial consortia is a lack of analytical tools
- Microfluidic tools are emerging for the analysis of microbial consortia
- Microfluidic cultivation allows to investigate contact-based and contactless interactions
- Microfluidic cultivation will expand its full potential in combination with traditional lab-routines

Abstract

Microbial consortia are fascinating yet barely understood biological systems with an elusive intrinsic complexity. Studying microbial consortia and the interactions of their members is of major importance for the understanding, engineering and control of synthetic and natural microbial consortia. Microfluidic cultivation and analysis devices are versatile tools for the study of microbial interactions on a single-cell level. While there is a vast amount of literature on microfluidics for the investigation of monocultures only few studies on co-cultures have been developed in this context. Here we give an overview of different microfluidic single-cell cultivation tools for the analysis of microbial consortia with a focus on their physiology, growth dynamics and cellular interactions. Finally, central challenges and perspectives for the future application of microfluidic tools for microbial consortia investigations will be given.

Introduction

Unraveling microbial interactions is of utmost importance for understanding, engineering and controlling natural and synthetic microbial consortia [1]. For instance, microorganisms play key roles in the human gut for food digestion and as a cause of diverse diseases [2,3]. Moreover, they are important for diverse environmental processes such as natural decomposition of organic matter [1] and are essential producer organisms in biotechnology [4].

An improved understanding of consortia can be gained by either the analysis of naturally occurring consortia [5] or the analysis of artificial/selected consortia members or simplified synthetic consortia (Figure 1A)[6]. Different analytical methods are available for their analysis (Figure 1B)[7]. They range from optical methods such as optical density measurements [8], cell plating [9,10], flow cytometry (FC) and fluorescence activated cell sorting (FACS) [11,12] to different "omics" methods such as metabolomics, transcriptomics as well as metagenomics. In recent years, novel microfluidic methods complemented the portfolio for the investigation of microbial consortia (Figure 1B)[7,13].

Classical optical density measurements are easy to use for bulk average measurements of monocultures, but are of limited use in mixed cultures as different strains cannot be distinguished by simple light absorption [8]. Plating is a laborious method with a low time resolution and therefore not useful for growth dynamic analyses [9,10]. FC is frequently used to investigate population dynamics within mixed culture processes [11,12]. Multi-"Omics" technologies are applied to understand metabolic processes within consortia, but still often lack the ability to perform subpopulation analysis. Metagenomics are used for understanding natural consortia by identifying consortia strains in "microbial dark matter", but the study of individual microbiome members is challenging [7,13,14].

Recently novel microfluidic methods were developed and applied for the analysis of microbial consortia. These devices offer the analysis of single-cell dynamics with full spatio-temporal resolution, defined and controllable environmental conditions (physical, biological and chemical stimuli) in a high-throughput manner [15,16]. Additionally, microfluidic fabrication methods enable the fabrication of diverse geometries which can mimic natural habitats [17] or give insights into the formation of biofilms [18,19].

In combination, all these methods allow an improved understanding of growth dynamics, heterogeneity, culture stability, spatial organization etc. in mixed cultures (Figure 1C). Consequently, this knowledge can then be used to control and engineer natural and synthetic consortia [20].

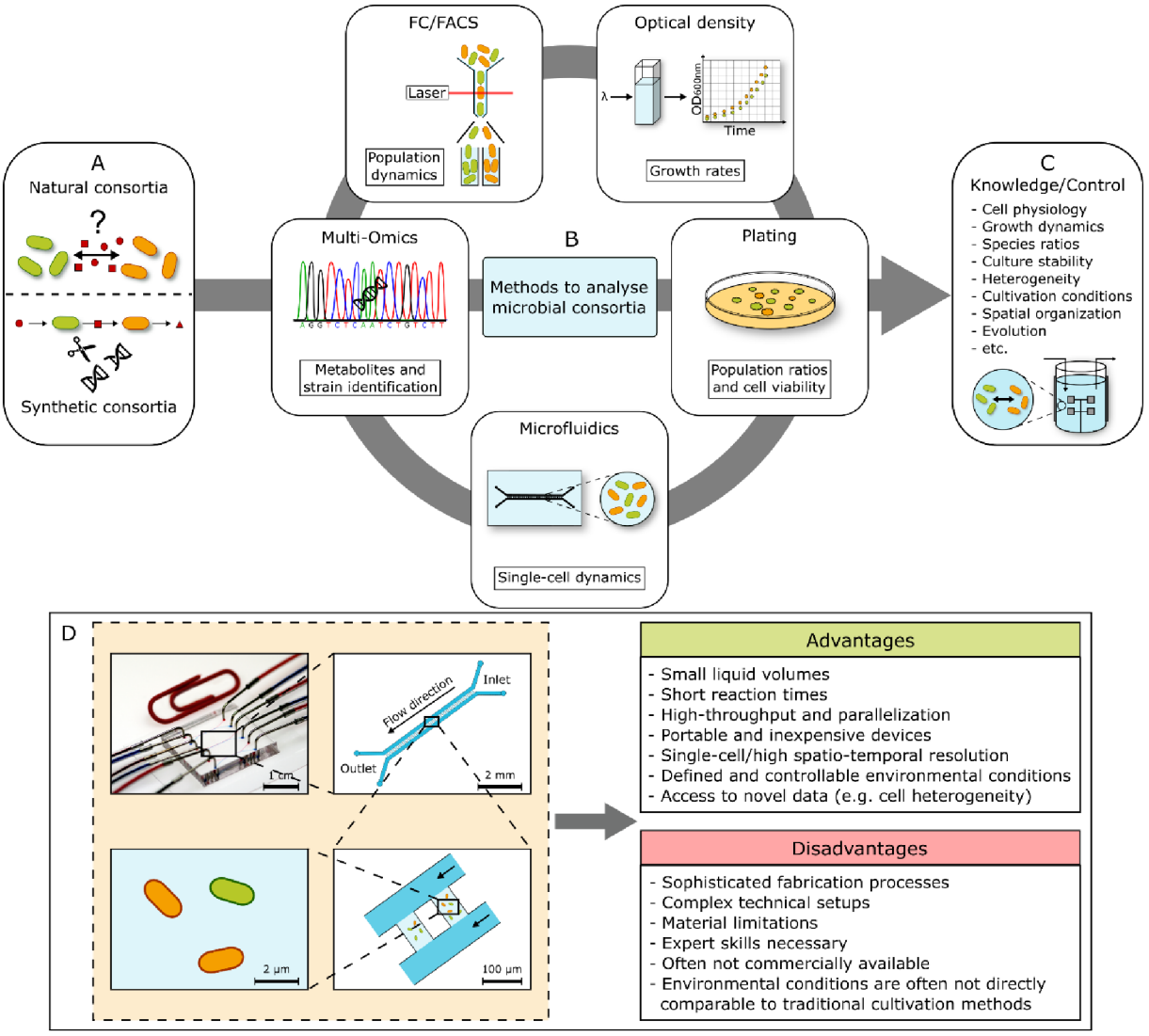

Figure 1: Understanding microbial consortia. (A) Understanding interactions inside complex natural and simplified synthetic consortia requires different analytical methods. (B) These methods include flow cytometry, optical density measurement, "multi-omics" technologies, plating and microfluidics methods. (C) The obtained knowledge (e.g. cell physiology, growth dynamics etc.) improves the understanding of natural consortia and the development and control of novel synthetic consortia. (D) Structure and layout of a microfluidic cultivation device, typically containing several arrays of cultivation habitats for cells in nanoliter to picoliter range (left). Advantages and disadvantages of microfluidics as an analysis tool (right).

Microfluidic systems for studying microbial consortia

Classical cultivation and analysis methods provide only limited information about interactions inside consortia. One milestone in cultivating bacteria from environmental samples was the development of the ichip [21]. This microfluidic tool facilitates the cultivation of previously unculturable bacteria inside microwells allowing the cells to exchange metabolites with their natural consortium *in situ*. After cultivation, offline analysis such as colony count or metagenomics of the grown microcolonies gives an insight into the population composition [21,22]. In an alternative approach, the influence of surface topography in biofilm formation was studied by Bhattacharjee *et al*. [23]. Different micro-patterned surfaces were developed for the creation of biofilms to analyze antibiotic susceptibility in an *Escherichia coli* and *Pseudomonas aeruginosa* consortium. For further information regarding artificial microfluidic habitats for microbial consortia, the reader is referred to a recent review by Wondraczek and co-workers, discussing artificial microbial arenas for microbial consortia [24]. For recent literature focusing on microfluidic systems for the investigation of complex community behavior especially in soil the reader is referred Stanley *et al.* and Aleklett *et al.* [25,26].

Both, for the understanding of natural communities as well as for development of synthetic cultures detailed understanding of a consortium's physiology, especially of interactions between consortia members and their growth dynamics, is of interest. As a result, first microfluidic tools were applied for the study of cell-cell-interactions and growth dynamics of mixed cultures as well as their individual members. In this review we will focus on systems allowing the study of microbial consortia dynamics and interactions with single-cell resolution [27–29]. For microfluidic systems investigating e.g. the physiology within monocultures or interactions within isogenic colonies (e.g. growth, heterogeneity) and behavior (e.g. chemotaxis, quorum sensing) the reader is referred to existing reviews such as Grünberger *et al.* and Fritzsch *et al.* [16,30].

Microfluidic setups for cell-cell interaction studies

An overview on published microfluidic systems applied for cultivations of microbial consortia is given in Figure 2. These systems can be divided into four main categories based on the microfluidic cultivation chamber geometry and spatially arrangement of cells. The geometry of microfluidic cultivation devices directly influences the cell physiology as it restricts the spatial degree of freedom for cell growth. In terms of cellular resolution, microfluidic cultivations can either be performed on the population level in 3D environments (Figure 2 A, B) or on the single-cell level with a 2D, 1D or 0D spatial degree of freedom (Figure 2 C, D). Additionally, cells within a consortium can have direct cell contact (Figure 2 A, C) or indirect contact through porous membranes which are only permeable for metabolites, e.g. signaling molecules (Figure 2 B, D).

Droplet microfluidic systems or 3D habitats in form of connected microwells are the most frequently applied tools to study microbial consortia (Figure 2 A)[31–35]. These systems allow for the cultivation of two or more species in a closed cultivation area and are often applied for screening and subpopulation studies [36]. Especially for the investigation of population dynamics microwells are frequently applied [33]. The throughput and temporal resolution of microwell and droplet systems are relatively high, while the handling is comparatively simple. However, the 3D cultivation chamber are not optimal for microscopic analysis and these systems mostly lack any environmental control. For the use of morphologically similar strains fluorescence labelling is necessary, making it impractical for quantitative interaction studies of mixed cultures.

The exchange of metabolites within mixed cultures is often based on secretion of molecules and diffusive transport between cells. This behavior can be considered in microfluidic designs by the separation of cells through membrane or membrane-like structures (Figure 2B)[37]. Alternatively, cells can be separated in solid, but porous systems such as hydrogels for modelling natural habitats and studying spatial organization of consortia [38]. For both systems analysis and environmental control is difficult and the throughput is limited.

Cultivation with full single-cell resolution may be the most favorable method in terms of analysis (mostly image analysis), environmental control and temporal resolution (Figure 2 C, D)[39–41]. Microfluidic devices are ideal to investigate cell-cell contact based interactions of population growth dynamics on the single-cell level. The implementation of a spatial separation of cells improves the versatility of these systems in terms of cultivation control, especially for the investigation of unknown metabolic interactions (Figure 2D). Membranes [42] or porous agarose [43] allow the exchange of metabolites via diffusion between different cells/cell colonies, while preventing direct cell-cell contact. Combined with live cell imaging, the

microfluidic tools with full single-cell resolution (Figure 2 C, D) allow precise and fast environmental control and the measurement of several cellular parameters, such as morphology, growth rates, heterogeneity and gene activity or intracellular metabolite concentration via fluorescence markers [39,42,43].

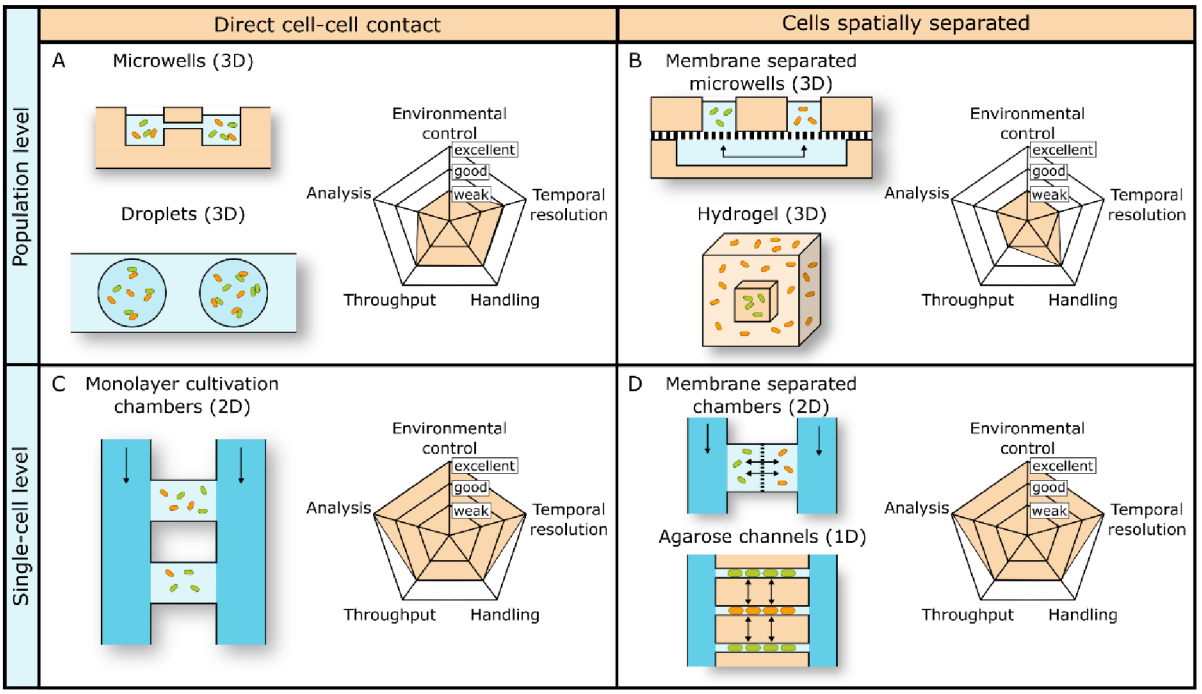

Figure 2: Schematic overview of different microfluidic systems for microbial interaction studies. (A) 3D cultivation systems with direct cell-cell contact in the form of microwells [31–34] or droplets [44]. (B) Spatially separated chambers for cultivation of two different strains in liquid [37] and solid medium [38]. (C) Cultivation of microbial consortia in monolayer growth chambers with direct cell-cell contact [39,40]. (D) Spatially separated cultivation with single-cell resolution in membrane separated chambers [42] and agarose channels [43].

Investigation of microbial cell-cell interactions

Understanding microbial cell-cell interactions is the key for understanding and controlling mixed cultures in nature, medicine and biotechnology [45]. Table 1 gives an overview of microfluidic systems used for the investigation of microbial interactions published in the last years. Applications include the investigation of quorum sensing [39,46–48], cross-feeding dynamics [9,37,38,42–44,49], and predator-prey as well as habitat competition dynamics [31,33].

Vliet *et al.* applied an array of microwells (100x100x5 µm³) which were connected by narrow channels to study the migration behavior of two different *E. coli* strains (Figure 3A)[33]. Different fluorescence labelling allowed to monitor their competing colonization of the array. The cultivation of a three-strain consortium in microwells was described by Kim *et al.* (Figure 3B)[37]. Cells inoculated in microwells were spatially separated but could communicate through a communication channel, that was separated from the microwells with a membrane on the bottom of the wells. The authors could show that the distance between the wells had a vital influence on the stability and survival of the consortium. A similar setup was applied by Nagy *et al*. for the study of quorum sensing between two different *E. coli* strains [47].

Chen *et al*. presented a system for the analysis of direct cell-cell contact with single-cell resolution (Figure 3C)[39]. Cells were continuously supplied with medium via adjacent deeper medium channels. Fluorescence coupled oscillating gene circuits in two *E. coli* strains were used to observe cellular interaction in microfluidic 2D cultivation chambers. Oscillating gene circuits in *E. coli* were recently also studied in a comparable setup by Alnahhas *et al.* [40]. Similar setups were used to investigate gene transfer via conjugation between different strains [42,50].

Burmeister *et al.* developed a 2D cultivation system with spatially separated cultivation chambers (Figure 3 D)[42]. Here, strains were separated by a sieve structure and growth was restricted to a monolayer in several parallel arranged cultivation chambers. The sieve structure allowed exchange of metabolites via diffusion. This was verified by co-cultivation of a lysine producing *Corynebacterium glutamicum* with a lysine auxotrophic *C. glutamicum*. Adjacent fluid channels allowed constant and controllable environmental conditions. A similar setup, but with a nano-cellulose filter between the chamber compartments, was developed by Osmekhina *et al.* to study quorum sensing based interactions in *E. coli* [51].

These examples demonstrate that microfluidic single-cell tools can be used to investigate a wide range of microbial cell-cell interactions of mixed microbial consortia and lay the foundation for systematic studies of interactions such as crossfeeding interactions and quorum sensing.

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183 **Table 1** Studies of different bacterial interactions in microfluidic systems.

 **Population level Direct cell-cell contact**			
System characteristics	**Organisms**	**Interaction mode**	**Reference**
Microfluidic glass device with structures for generation of microdroplets	*E. coli* (tryptophane auxotroph), *E. coli* (tyrosine auxotroph), *E. coli* (serine auxotroph)	Syntrophic interactions with direct cell-cell contact in droplets	Park *et al.* [44]
PDMS device with one-dimensional arrays of linked patches/chambers	*E. coli, E. coli*	Competition for habitat space with direct cell-cell contact	Van Vliet *et al.* [33]
PDMS device with one-dimensional arrays of linked patches/chambers	*E. coli, Bdellovibrio bacteriovorus*	Predator-prey dynamics	Hol *et al.* [31]
Agar plate co-cultivation	*E. coli* (histidine/tryptophane auxotroph), *Acinetobacter baylyi* (histidine/tryptophane auxotroph)	Amino acid cross-feeding via diffusive exchange on agar	Pande *et al.* [9]
Microwells	*E. coli, Enterobacter cloacae*	Syntrophic interactions	Guo *et al.* [49]
 **Population level Cells spatially separated**			
System characteristics	**Organisms**	**Interaction mode**	**Reference**
PDMS embedded microwells separated by polycarbonate membrane	*Acetobacter vinelandii, Bacillus licheniformis, Paenibacillus curdlanolyticus*	Syntrophic interactions via diffusive exchange of metabolites	Kim *et al.* [37]
PMMA device with two cultivation chambers separated by a silicon nitride microsieve	*E. coli* (inducer), *E. coli* (inducible)	Induced quorum sensing with spatially separated and fluorescence labelled strains	Hesselman *et al.* [46]
Gelatin-based 3D printed microscale cavities with embedded cells	*S. aureus, P. aeruginosa*	Protection from antibiotics due to shell of β-lactamase producing strain	Connell *et al.* [38]
Two reservoir chambers and observation channel separated by porous membrane	*E. coli* (chemotactic), *E. coli* (non-chemotactic)	Quorum sensing interaction	Nagy *et al.* [47]
Cells embedded in alginate hydrogels adjacent to chitosan membrane	*E. coli* (transmitter), *E. coli* (enhancer/reducer), *E. coli* (reporter)	Quorum sensing autoinducer-2 system	Luo *et al.* [48]
 **Single-cell level Direct cell-cell contact**			
System characteristics	**Organisms**	**Interaction mode**	**Reference**
Monolayer growth chambers connected to deeper supply channels	*E. coli* (activator), *E. coli* (repressor)	Oscillating up and down regulation of genes via quorum sensing molecules	Chen *et al.* [39]
Monolayer growth chambers connected to deeper supply channels	*E. coli* (sender), *E. coli* (receiver)	Oscillating up and down regulation of genes via quorum sensing molecules	Alnahhas *et al.* [40]
Monolayer growth chambers with adjacent deeper supply channels	*E. coli, Pseudomonas putida*	Gene transfer via conjugation	Burmeister *et al.* [42]
 **Single-cell level Cells spatially separated**			
System characteristics	**Organisms**	**Interaction mode**	**Reference**
Agarose-based microfluidic device with linear tracks for cell growth	*E. coli* (arginine auxotroph), *E.coli* (isoleucine, leucine, valine auxotroph)	Syntrophic interaction by diffusive exchange of amino acids	Moffitt *et al.* [43]
Monolayer growth chambers separated by sieve structure and adjacent deeper supply channels	*C. glutamicum* (lysine auxotroph), *C. glutamicum* (lysine producer)	Syntrophic interactions by diffusive exchange of amino acids	Burmeister *et al.* [42]

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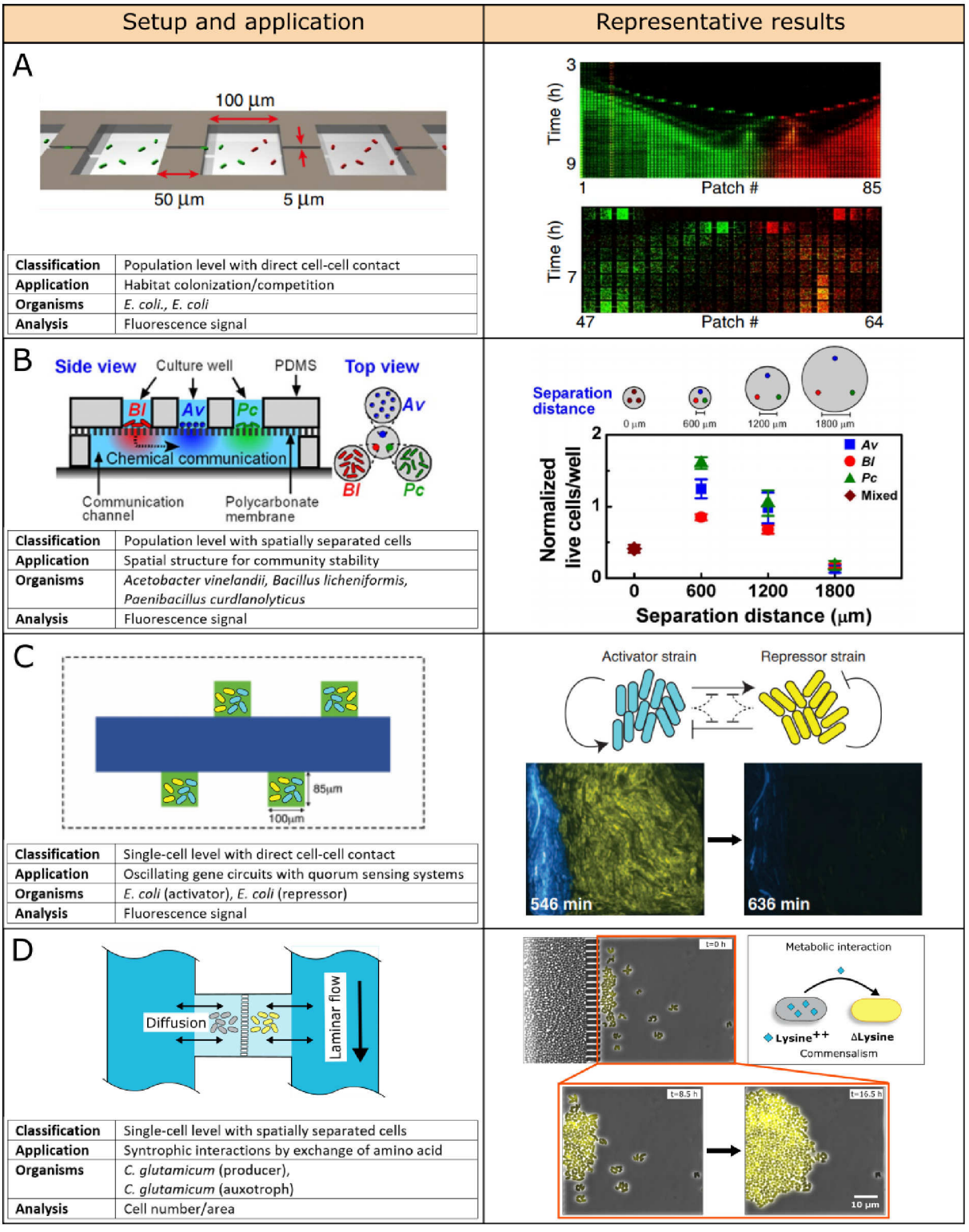

Figure 3: Examples of microfluidic systems for studies of bacterial communities. (A) Microfluidic setup with connected microwells for the study of habitat colonization [33]. (B) System for the investigation of community stability in membrane-separated culture wells [37]. (C) Monolayer growth chambers for the analysis of oscillating gene circuits [39]. (D) Monolayer growth chambers with spatial separation of cells for syntrophic interaction studies via diffusible metabolites [42]. Images adapted, modified and reprinted with permission from [33,37,39,42].

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Investigation of microbial interaction with higher organisms

Interactions in natural communities can also be found between different species [52]. This includes for example bacterial-fungal [53], bacterial-mammalian [54,55] and bacterial-plant interactions [56] (Figure 4). Different microfluidic proof-of-concept systems have successfully been developed and applied.

A PDMS-based device for investigation of bacterial-fungal interactions was developed by Stanley *et al.* (Figure 4A)[53]. For the observation of interactions, hyphal growth was restricted to one plane inside a shallow cultivation channel. With this system an antagonistic behavior of *Bacillus subtilis* towards *Coprinopsis cinerea* was identified. In the presence of a *B. subtilis* wildtype strain growth of the fungus was inhibited.

Hong and colleagues established a device which revealed that bacterial cells are more attracted to cancer cells than to normal cells (Figure 4B)[54]. In their device a central fluid channel with *Salmonella typhimurium* was connected via collagen filled chambers to cultivation chambers for normal mammalian cells and cancer cells. Bacterial cell migration was triggered by chemical stimuli that could diffuse through the collagen and most bacterial cells migrated towards the cancer cell side.

Ellett and co-workers have developed a microfluidic assay for the investigation of antimicrobial activity of neutrophils against pathogens like *Staphylococcus aureus* (Figure 4C)[55]. They implemented an array of several round microchambers (200 µm diameter x 50 µm height) in which growth of both cell types could be observed. Different ratios of *S. aureus* and neutrophils as well as *E. coli* and neutrophils were observed on the single-cell level. The success or failure of the immune cells depending on the cell density and ratio was analyzed.

A detailed investigation of bacterial-root association was realized with a microfluidic tracking root system (TRIS) by Massalha *et al.* (Figure 4D)[56]. TRIS had several parallel arranged fluid channels with a height of 160 µm and three inlet holes each. Here, bacteria cells and plant roots were cultivated within several cultivation channels, allowing direct cell-cell contact between both species. Cultivation experiments revealed that *B. subtilis* always accumulated very fast near the root elongation zone of *Arabidopsis thaliana* forming a dense biofilm around the root tip.

Taken together, these examples demonstrate the wealth of inter-kingdom consortia that can be investigated and the information that can be acquired about interactions by applying different microfluidic single-cell cultivation systems. In many of these cases, the knowledge gathered about the interactions would have been impossible to obtain without microfluidics.

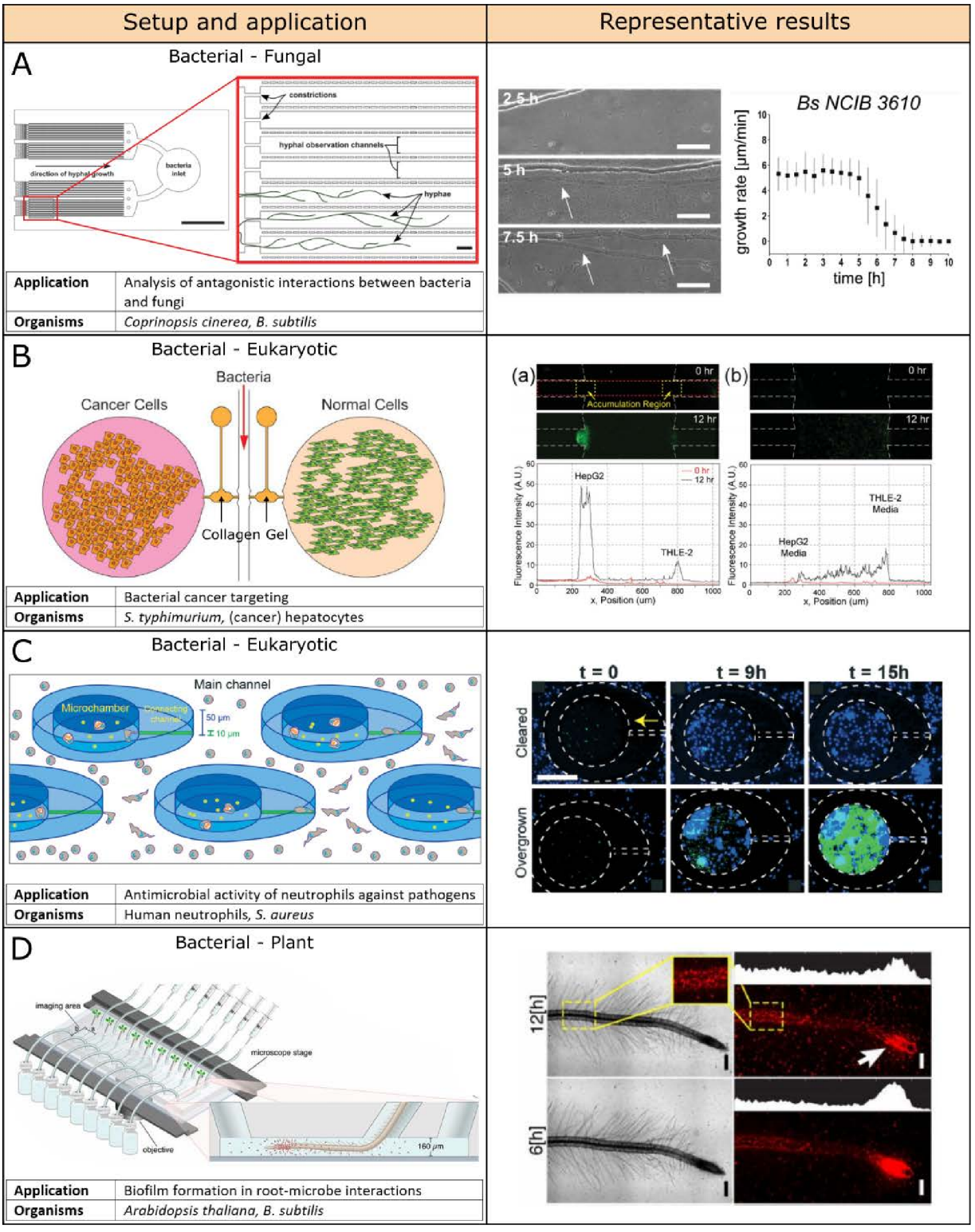

Figure 4: Exemplary microfluidic studies between bacteria and higher organisms. (A) Bacterial-fungal interaction: Wild-type strain *B. subtilis* NCIB 3610 inhibited fungal growth upon

direct cell-cell contact [53]. (B) Bacterial-eukaryotic interaction: Normal hepatocytes, cancer hepatocytes and *S. typhimurium* were spatially separated by collagen gel. Bacteria were attracted to cancer cells side [54]. (C) Bacterial-eukaryotic interaction: Human neutrophils entered microchambers filled with *S. aureus* and attacked the pathogen [55]. (D) Bacterial-plant interaction: *B. subtilis* was preferably attracted to the root tip and built up a biofilm around the whole root [56]. Images adapted, modified and reprinted with permission from [53–56].

Current challenges and future perspectives

Several technical challenges need to be tackled to fully realize the benefits of microfluidic tools for the analysis of microbial consortia: (i) overcoming material limitations to create functional cultivation devices; (ii) controllable environments; (iii) fast and reliable (image) analysis tools; and (iv) the integration of microfluidic cultivation systems into traditional analysis workflows.

Many systems described here have been fabricated by photolithographic methods [57]. This restricts the fabrication to planar surfaces. Progress in technical fabrication techniques e.g. multiphoton lithography [58] and microscopy e.g. 3D confocal laser-scanning microscopy [59] will allow the fabrication and analysis of growth in flexible 3D habitats and chambers that allow the emulation of more natural habitats. Furthermore, versatility for the analysis of interactions between organisms with different morphological structures will be increased. Advancement in fabrication also enables to control and modify environmental factors in a precise and dynamic manner. Here, the methods currently developed for single-cell cultivation of monocultures serve as a blueprint for microbial consortia studies on single-cell level. This will allow to accurately emulate natural and complex environmental conditions [60,61].

Most of the demonstrated microfluidic methods presented in this paper rely on advanced image processing tools for analysis and visualisation of live-cell imaging data [62]. These tools need to be adapted for the analysis of microbial consortia to get deeper insights into microbial interactions. For fast screening of synthetic communities, color-coded droplet microfluidics in combination with optical assays can reveal growth-promoting interactions with a high throughput [63]. Alternatively, novel strategies need to be developed for reliable sampling of cells during or at the end of different microfluidic cultivations. This will shift pure image-based visualization and analysis to quantitative offline analysis with conventional protocols adjusted to a few numbers of cells [64].

In future, full potential of microfluidic methods relies on the successful integration of microfluidic tools into existing working routines and methods (see Figure 1B). Both, traditional methods

and microfluidics have their own advantages and disadvantages and they can complement each other in practical applications. This can be achieved by a wise application of microfluidic technologies for questions and topics, which cannot be analyzed or are difficult to analyze with conventional methods. Examples include the dynamics within heterogeneity of single cells but also consortia behavior at defined environmental conditions. In addition to experimental data, computational modelling can help to understand complex systems. Especially for bottom-up approaches and to predict the behavior of communities with more than three strains, mathematical models may give deeper insights [65,66].

Conclusion

The application of novel microfluidic single-cell cultivation systems opens up novel possibilities for qualitative and quantitative understanding of microbial interactions within synthetic and natural mixed cultures. The combination of traditional methods and microfluidic single-cell tools will improve the understanding of cell-cell interactions within mixed consortia, both on spatial and temporal scale. We are convinced that in future microfluidic tools will undoubtedly become an increasingly used tool for microbial interactions studies especially on cell phenotypes, growth dynamics and interactions occurring within microbial consortia. This will lay the foundation for an improved understanding of natural and synthetic mixed cultures but also the development and engineering of synthetic microbial consortia with application in medicine and biotechnology.

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Outstanding Paper

* of special interest

** of outstanding interest

*Van Vliet, 2014:	One of the first demonstration for colonization of a structured habitat by two different populations with competition for habitat space.
*Hol, 2016:	This study describes the predator-prey dynamics in a microfluidic patchy landscape of *Bdellovibrio bacteriovorus* and *Escherichia coli.*
*Connell, 2013:	A three-dimensional hydrogel printing strategy that allows to arrange different microbial populations in diverse geometries.
*Chen, 2015:	One of the few publications on the analysis of quorum sensing interactions with direct cell-cell contact and single-cell resolution.
**Burmeister, 2019:	Microfluidic platform for cultivation of microbial consortia in direct cell-cell contact or spatially separated with single-cell resolution.
*Kim, 2008:	Demonstration of a three-strain consortium in spatially separated microwells with communication channel for exchange of metabolites and signaling molecules.
**Wondraczek, 2019:	An overview of artificial microbial habitats and novel materials for the fabrication of habitats for the analysis of microbial consortia is given.
*Massalha, 2017:	A microfluidic setup for the observation of bacteria-root interactions.
*Grünberger, 2014:	Review on single-cell microfluidics for bioprocess development.
**Ghosh, 2016:	An insightful review on the significance of microbial interactions and its application in biotechnology.