Resolving spatiotemporal dynamics in bacterial multicellular populations: approaches and challenges

Suyen Solange Espinoza Miranda*[1], Gorkhmaz Abbaszade*[2], Wolfgang R. Hess[3], Knut Drescher[4], Antoine-Emmanuel Saliba[1,5], Vasily Zaburdaev[6,7], Liraz Chai[8,9], Klaus Dreisewerd[10], Alexander Grünberger[11], Christian Westendorf[12], Susann Müller[#2] & Thorsten Mascher[#13]

1. Institute for Molecular Infection Biology (IMIB) - University of Würzburg, Josef-Schneider-Straße 2/D15, 97080 Würzburg, Germany

2. Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, D-04318 Leipzig, Germany

3. University of Freiburg, Faculty of Biology, Genetics and Experimental Bioinformatics, Schänzlestraße 1, D-79104-Freiburg, Germany

4. University of Basel, Biozentrum, Spitalstrasse 41, 4056 Basel, Switzerland

5. Helmholtz Institute for RNA-based Infection Research (HIRI), Helmholtz Center for Infection Research (HZI), Würzburg, Germany

6. Friedrich-Alexander-Universität Erlangen-Nürnberg, Cauerstr. 11, D-91058, Erlangen, Germany

7. Max-Planck-Zentrum für Physik und Medizin, Kusmaulallee 2, D-91054, Erlangen, Germany

8. The Hebrew University of Jerusalem, Institute of Chemistry, Jerusalem 91904, Israel

9. The Harvey M. Krueger Family Center for Nanoscience and Nanotechnology, The Hebrew University of Jerusalem, Jerusalem 9190401, Israel

10. University of Münster, Institute of Hygiene, Robert-Koch-Str. 41, D-48149 Münster, Germany

11. Karlsruhe Institute of Technology (KIT), Institute of Process Engineering in Life Sciences (BLT), Microsystems in Bioprocess Engineering (µBVT), Fritz-Haber-Weg 2, D-76131 Karlsruhe, Germany

12. Peter Debye Institute for Soft Matter Physics, Leipzig University, Linnéstrasse 5, D-04103 Leipzig, Germany

13. Technische Universität Dresden, General Microbiology, Zellescher Weg 20b, D-01217 Dresden, Germany

*joined first authorship

#joined last authorship

Table of contents

Introduction 3
1. Analysis of spatiotemporal distribution of single cells in intact biofilms by microscopy 8
2. Analysis of the spatial distribution of metabolites and structural lipids in bacterial colonies and biofilms using mass spectrometry imaging 10
3. Analysis of single-cell heterogeneity and its local dynamics by microbial flow cytometry 13
4. Analysis of early biofilm development and behavior by microfluidic cultivation systems 16
5. Analysis of cellular function in single cells: Single-cell transcriptomics 19
5.1. Challenges associated with capturing the RNA of a single bacterium 20
5.2. Single-bacteria RNA-seq using combinatorial methods 21
5.3. Single-bacteria RNA-seq on plates 22
5.4. Single-bacteria RNA-seq in droplets 22
5.5. Spatial transcriptomics for bacteria 22
6. Analysis of biophysical properties of intact biofilm populations 25
6.1 Fluorescence microscopy-based microrheology 26
6.2 Optical elastography 26
6.3 X-ray synchrotron diffraction and fluorescence 28
Conclusions 29
Acknowledgements 30

Abstract

The development of multicellularity represents a key evolutionary transition that is crucial for the emergence of complex life forms. Although multicellularity has traditionally been studied in eukaryotes, it originates in prokaryotes. Coordinated aggregation of individual cells within the confines of a colony results in emerging, higher-level functions that benefit the population as a whole. During colony differentiation, an almost infinite number of ecological and physiological population-forming forces are at work, creating complex, intricate colony structures with divergent functions. Understanding the assembly and dynamics of such populations requires resolving individual cells or cell groups within such macroscopic structures. Addressing how each individual cell contributes to the collective action requires pushing the resolution boundaries of key technologies that will be presented in this review. In particular, single-cell techniques provide powerful tools for studying bacterial multicellularity with unprecedented spatial and temporal resolution. These advancements include novel microscopic techniques, mass spectrometry imaging, flow cytometry, spatial transcriptomics, single-bacteria RNA-seq, and the integration of spatiotemporal transcriptomics with microscopy, alongside advanced microfluidic cultivation systems. This review encourages to explore the synergistic potential of the new technologies in the study of bacterial multicellularity, with a particular focus on individuals in differentiated bacterial biofilms (colonies). It highlights how resolving population structures at the single-cell level and understanding their respective functions can elucidate overarching functions of bacterial multicellular populations.

Keywords: microbial multicellularity, population ecology, phenotypic heterogeneity, cell differentiation, single-cell technologies, single-cell transcriptomics, population dynamics

Introduction

The development of multicellularity represents a pivotal evolutionary transition, laying the foundation for the emergence of complex life forms on Earth (1). Unlike many evolutionary events, multicellularity has risen independently and multiple times, particularly in the bacterial kingdom (2–4). While the emergence of complex multicellularity in prokaryotes dates back some three billion years, the current knowledge of this process is still predominantly derived from studies of eukaryotic organisms (5). The general concept of multicellularity encompasses several critical developmental requirements including cell adhesion, cell-cell communication, cell-cell coordination, functional diversification and interactions among cells, defense mechanisms or programmed cell death.

Ever since Robert Koch's postulates, which required isolating pure cultures of microorganisms from individual cells, bacteria were – and still are – perceived as archetypically unicellular. While multicellular traits are well documented for some species, most prominently the filamentous cyanobacteria, streptomycetes, or myxobacteria, these examples were appreciated as fascinating multicellular exceptions from an otherwise unicellular bacterial world (6–9). However, an appreciation of 'common' bacteria as multicellular organisms started only very slowly, with two seminal conceptual articles by James Shapiro (10, 11). Since then, the diversity and inherent phenotypic heterogeneity of bacterial populations has been recognized by an increasing number of studies. What once seemed homogeneous – as the average of a population – suddenly became visible as highly heterogeneous. Isogenic populations diversify into phenotypically different subpopulations and cell types that are endowed with astonishing individuality (12, 13).

Developing a concept for bacterial multicellularity is still at its beginning, but the underlying field of research is currently fast evolving, as also highlighted by this review. Accordingly, no generally accepted definition exists and any attempt remains an approximation towards a universally acceptable concept to come. For many, bacterial multicellularity is only applicable (if at all) to terminally differentiated cell states within larger population aggregates, such as fruiting bodies, (endo)spores or heterocysts. While such a definition is in line with the eukaryotic concept of multicellularity, in which cells terminally differentiate to assume their functions in the context of tissues and organs (14), it neglects the important role of non-terminally differentiated cells in the context of bacterial biofilms. While it can be argued that phenotypically diversified cells in biofilms and structured colonies only follow, and then adapt to, local gradients and accordingly should not be considered as multicellular states, this restricted perception neglects the truly multicellular driving forces behind the evolution of such traits.

For the purpose of this review, we propose a more comprehensive definition of bacterial multicellularity as characterized by spatially structured bacterial populations that exhibit at least transient stability. Multicellularity encompasses several key elements: (i) cellular differentiation (15) and the dynamic distribution of different cell types (**Figure 1A**), (ii) positional determinants that govern cell positioning and influence cell fate (**Figure 1B**), (iii) a distinct architectural organization of the microbial population governed by (iv) extracellular cues and biophysical properties, including chemical gradients (nutrients, oxygen, antibiotics), which determine and are shaped by (v) intercellular interactions and the interplay between cells and their environment (**Figure 1C**). Collectively, these aspects determine the form of bacterial multicellularity (filament, biofilm, differentiated colony) that provides a structural framework, from which emergent multicellular functions can arise.

‘Form’ and ‘Function’ are therefore tightly interconnected aspects of bacterial multicellularity that critically depend on each other. True multicellular functions can only emerge in the context of a differentiated multicellular form. The multicellular form presumably evolved only, because it provided an overall fitness advantage to the entire population that outweighed any costs associated with a multicellular lifestyle. Importantly for this concept of multicellularity, such functions either exclusively occur in a multicellular form or at least only make physiological sense within its context. These functions provide a fitness gain for the population. Accordingly, the differentiated multicellular population rather than the individual cell is the unit of evolution for such traits to manifest, irrespective of whether the underlying physiology leads to a terminally or only transiently differentiated cell within a stable multicellular population.

The ability to process large amounts of data using powerful bioinformatics tools, together with breakthroughs in instrumentation and imaging technology, now allows capturing molecular processes within structured macroscopic bacterial populations (mm scale) at near single-cell resolution (µm scale, (12, 16–18). Multi-scale, high-resolution approaches leading to high-dimensional readouts based on techniques such as 3D imaging, flow cytometry and cell sorting, imaging mass spectrometry and single-cell transcriptomics are now readily available to gain insights into subpopulation structures and the functions of the individual cell within them (**Figure 2**). But a mechanistic understanding of the underlying biological processes from the cellular to the population level and finally to the emerging overarching functions requires tightly linked correlative data analyses by combining different approaches.

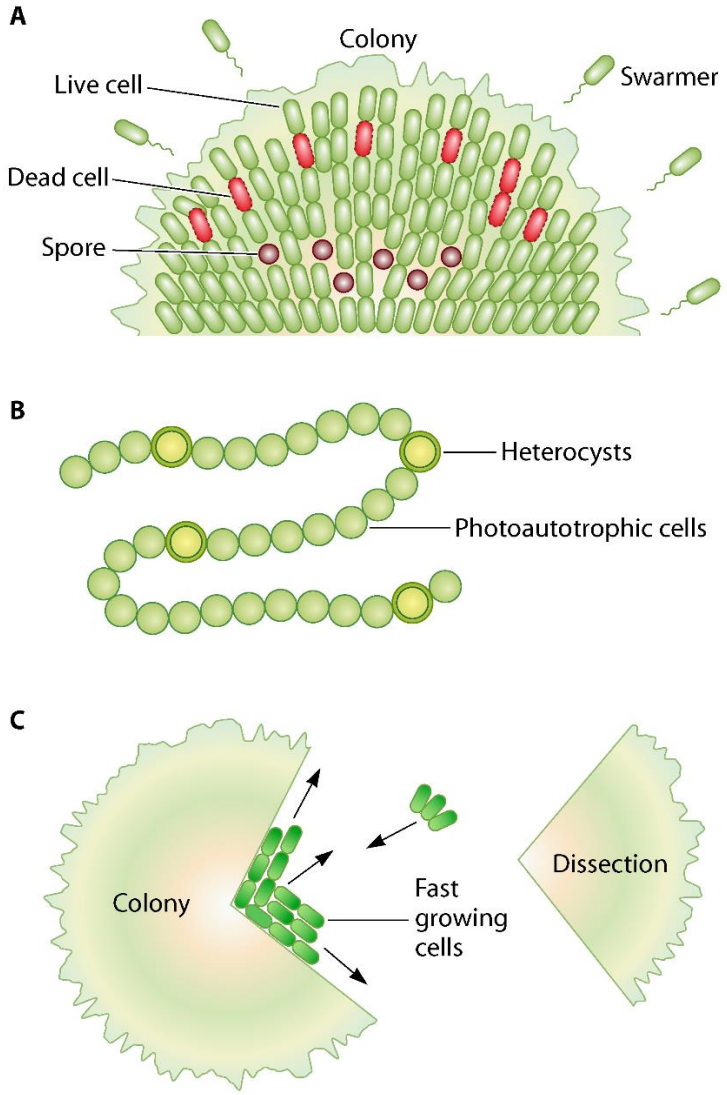

Figure 1: Schemes of pure population multicellularity, where divergent functions of individuals contribute to overarching functions of the whole population. **A**: Interaction of different individual cell types in a colony of *Bacillus subtilis* in response to nutrient scarcity: An increasing number of spores can be found in the center of the colony. Near the outer edge, some of the cells produce toxins to initiate a suicide mechanism in favor of the surviving cells. Swarming cells penetrate to the outer edge of the colony and search for new nutrient resources. **B**: Division-of-labor as possibility to fix dinitrogen gas as nitrogen source. The photoautotrophic cyanobacterium *Anabaena* sp. develops heterocysts within a filamentous chain of cells when facing nitrogen starvation (19). **C**: Resilience to perturbation in a *B. subtilis* biofilm after dissection of a part of the colony. Wound healing occurred by an initially fast outgrowth of residual cells that remained on the substrate after the biofilm piece removal (20).

This review aims to succinctly summarize available advanced technologies for the analysis of emergent functions of structured multicellular bacterial populations down to the single-cell level. It also discusses the challenges and opportunities associated with studying multicellular bacterial populations using these technologies. The review emphasizes the need to apply high-resolution methods, both at different scales and in combination, to penetrate the complex nature of microbial populations, for example, by elucidating defense mechanisms in response to perturbations, or by elucidating how nutrients are utilized within a structured multicellular population. The technologies discussed aim to, and can, provide a gateway to understanding how each individual cell contributes to the collective action of a confined population.

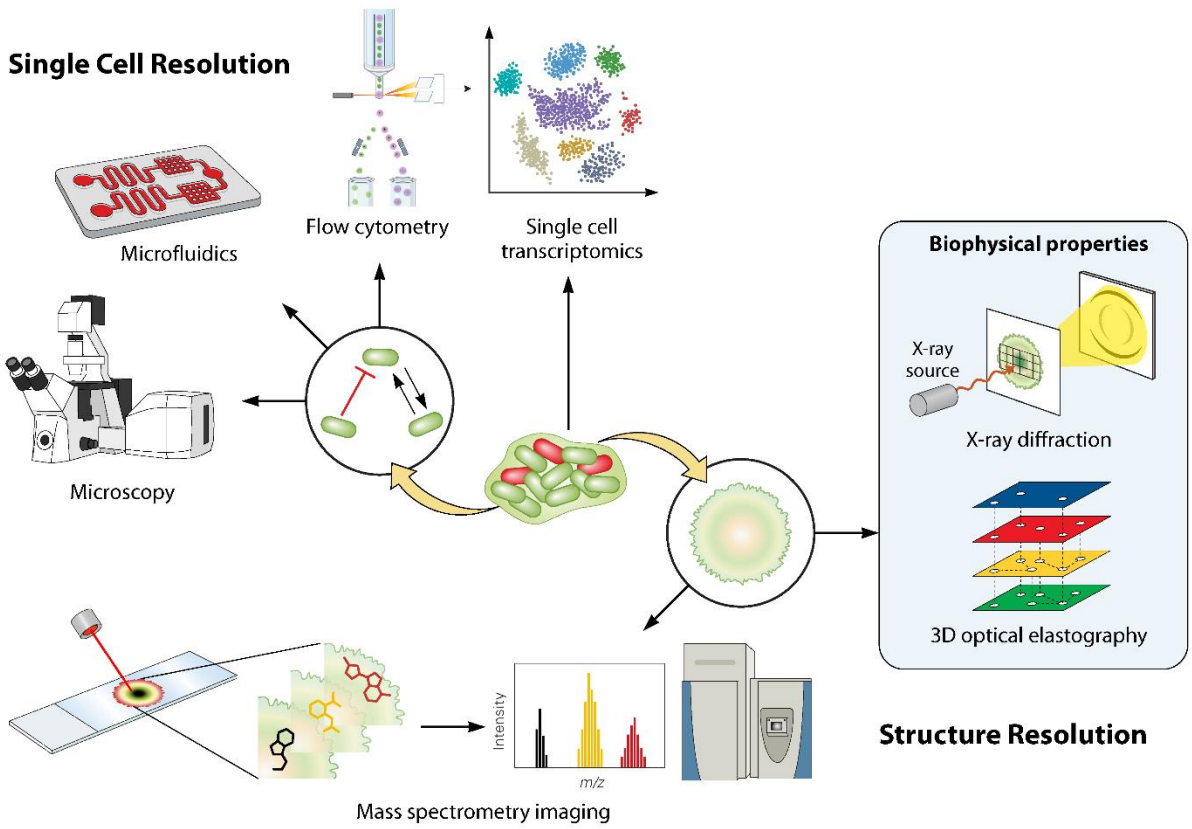

Figure 2. Overview of the recent technologies used for the study of multicellularity in bacterial biofilms (greenish and reddish dots represent cells that form a colony). From analyzing population structure to resolving single cells, these techniques are helping to uncover the overarching functions of the entire population.

1. Analysis of spatiotemporal distribution of single cells in intact biofilms by microscopy

Analyzing the spatiotemporal distribution of single cells within biofilms through microscopy provides critical insights into the dynamic organization and interactions of microbial populations. This section delves into the methodologies employed to achieve high-resolution imaging and the computational tools used to interpret the complex spatial and temporal patterns observed within these biofilms.

Fluorescence microscopy techniques, including single-point-scanning confocal microscopy, multi-point-scanning confocal microscopy, or selective plane illumination microscopy, have emerged as important methods for achieving 3D spatial resolution (21–23). Of particular note is the ability of these microscopy techniques to capture time-resolved, 3D images of living cells within population structures without significantly disrupting their behavior. This is made possible by the use of fluorescent proteins that are fused to proteins of interest, or antibodies conjugated to a fluorescent dye, or fusions of fluorescent proteins to promoters of interest, which report on the levels of a particular protein, or the expression of a particular gene. However, the use of fluorescent proteins and 3D fluorescence microscopy has technical caveats that need to be carefully evaluated when using this technique. The use of fluorescent protein-based reporters can alter the biological processes under investigation. Fusions of a protein of interest coupled to a fluorescent protein may not have the same level of activity as the native protein, which may modify the biological process under investigation. Similarly, using antibodies to label a protein of interest may interfere with the function of this protein. Furthermore, promoter reporters that are supplied on a plasmid may alter the levels of transcriptional regulators that bind to the promoter of interest at the native chromosomal locus. An additional issue is that the currently available bright fluorescent proteins require oxygen for folding into a functional conformation, which severely limits their application in biofilms above a certain size range, as the center of these biofilms are anaerobic (24). Current anaerobic fluorescent proteins unfortunately display a low level of brightness. When performing time-resolved imaging of 3D biofilms using fluorescent proteins, the amount of laser light that is deposited into the sample usually causes photobleaching of the fluorescent proteins over time, and may also lead to phototoxicity that alters the biological processes under investigation (25). However, when the laser exposure is minimized, and care is taken that the reporters are functional and do not significantly interfere with the cellular activities, time-resolved 3D imaging of bacterial biofilms using fluorescence microscopy is a powerful tool for studying the organization and dynamics of these populations.

Upon acquisition of fluorescence microscopy images of biofilms with 3D spatial resolution, potential temporal resolution, and one or more fluorescence channels, the analysis of bacterial properties in such multidimensional images typically presents challenges (21). As manual image analysis is typically biased, or even impossible for very large datasets with millions of images, computational image analysis techniques have been developed for analyzing bacterial populations. The earliest software solutions performed image analysis without (or with very limited) spatial resolution inside the biofilms, such as COMSTAT, COMSTAT2, or PHLIP (26–28). More recent software, such as Daime and BiofilmQ enable analysis of images with spatial resolution (29, 30).

To analyze microscopy images of entire bacterial populations with spatial resolution, presently, there are two broad options: the first option is relevant when image resolution is not sufficient to detect individual cells or single-cell analysis is not required, or the research focus lies on non-cellular entities such as the biofilm matrix. In this case the software BiofilmQ provides an algorithm that can perform a spatial analysis based on the dissection of the image of the biofilm population into pseudo-cells with cubical shape (30). The second option is to detect the individual cells in the population structure, followed by an analysis of the detected cells and their spatial context. Numerous software solutions, including BacStalk, Oufti, the imageJ-based tool MicrobeJ, DeLTA, and SuperSegger, facilitate the detection of individual cells and intracellular analysis in 2D images, such as those implemented in (31–35). These tools often also enable the intracellular analysis of the fluorescent signal distribution within cells. Although detection of bacterial cells in 3D images have received comparatively less attention, traditional image analysis methodologies have been established several years ago for coccoid-shaped and rod-shaped bacterial cells (21, 36, 37). More recently, convolutional neural networks have strongly improved the detection accuracy of single bacterial cells in 3D (22, 38, 39), with the adaptation of the StartDist neural network for bacterial cell shapes, termed StarDist OPP, demonstrating the highest accuracy so far (38). After the detection of the individual cells in 2D or 3D, or the dissection of low-resolution images into pseudo-cells, BiofilmQ offers numerous tools for computing spatial relationships between cells and determining their positioning relative to the biofilm surface, biofilm center, or substrate surface (30).

Despite the advances in fluorescence microscopy techniques and image analysis techniques, fluorescence microscopy-based analysis of bacterial population attributes is limited. The limited imaging depth that confocal microscopy techniques can achieve, restricts the analysis to the outer layers of a colony - studies that image single cells in biofilms loose single-cell resolution at depths beyond approximately 30 µm, due to scattering of photons in densely-packed bacterial biofilms (21). Even if single-cell resolution is not required or desired, the optical scattering of photons reduces the signal-to-background ratio further into the biofilm. Additional imaging depth can be obtained by

using two-photon microscopy, but this technique suffers from lower spatial resolution compared with the widely-used single-photon confocal microscopy techniques. Additionally, phototoxicity from excitation light, which is particularly relevant for 3D imaging with confocal and two-photon microscopy techniques, can potentially alter the natural behavior of the cells. Furthermore, fluorescence microscopy-based analysis is typically constrained to artificial biofilm setups with a limited number of fluorescent proteins or fluorescent probes, which may not fully reflect the complexity of even pure colony biofilms. However, the outlook is promising as ongoing research efforts are focused on improving advanced microscopy techniques, such as light-sheet microscopy and adaptive optics, to overcome the imaging depth limitation.

Another useful technique is microscopic Raman spectroscopy imaging, which can non-destructively characterize physiological states of bacterial cells (40). In most cases, changes in macromolecular composition between cells can be determined, but biomarkers such as autofluorescent molecules or storage molecules (41) can also be detected locally in individual bacterial cells. Functional interconnectivity between cells can be traced using metabolite-linked heavy isotopes by Raman-SIP, although this is currently mainly performed on community samples where metabolic exchange occurs between different species (42–44).

For all technologies, the integration of machine learning algorithms and the development of end-to-end automated pipelines for image analysis hold the potential to address the challenges of accurate segmentation and high-throughput analysis in the future.

2. Analysis of the spatial distribution of metabolites and structural lipids in bacterial colonies and biofilms using mass spectrometry imaging

Microscopy can provide detailed insights into the spatial organization of single cells within biofilms, but is limited by the requirement of using fluorescence reporters. Mass spectrometry imaging (MSI) as a label-free technique offers a complementary approach to analyze the spatial distribution of metabolites and structural lipids within structured microbial populations (45). MSI builds on the annotation of molecules of interest based on their mass to charge (*m/z*) ratio as registered by a mass analyzer. This flexibility, together with a generally high detection sensitivity and the ability to use tandem MS technologies for further structural characterization and identification are reasons why MS-based methods serve as primary analysis techniques in several omics-fields, such as proteomics, metabolomics and lipidomics (46). The most widely used method for imaging of bacterial metabolites is matrix-assisted laser desorption ionization (MALDI-MSI). With this technique, samples are coated with a thin film of a “matrix” – a small organic compound that is absorbing the laser light and assists in transferring thermally labile biomolecules into the gas phase as well as their ionization. To obtain the information about the spatial position, the so-prepared samples are scanned with a laser beam on a

pixel-by-pixel basis. Typically, this occurs in a vacuum ion source. The mass spectra registered at the individual positions are evaluated using dedicated software to generate presentations of the molecular profiles and for further bioinformatic evaluation, such as a segmentation analysis (47). New developments in MSI instrumentation increasingly enable a robust single-cell detection level for eukaryotic systems at about 5 µm pixel size (47, 48). To improve this value to the low micrometer range, MALDI-2 and more recently transmission-mode (t-)MALDI-2 have been introduced (49, 50).

Compared to eukaryotic systems, the analysis of bacterial cultures and biofilms poses several additional challenges: First, the high rigidity of bacterial cell walls along with their small cell size require advanced protocols for an efficient extraction of cytoplasmic compounds (45, 51). Second, the complex composition of bacterial biofilms, consisting of cells with very different functions and eventually bacterial spores (e.g. for *B. subtilis* colonies), together with the chemically extraordinarily diverse nature of the extracellular matrix and excreted signaling compounds, places high demands on any chemical analysis technique. For the MALDI-MSI analysis, both features are exacerbated by the need for preserving the spatial information on a meaningful scale, thus to prevent analyte diffusion across the biofilms.

As illustrated in **Figure 3**, in most microbial MALDI-MSI studies performed to-date, biofilms were analyzed in “top view-geometry”, i.e. whole colonies were coated with the MALDI matrix and the ensemble profiled with the MALDI laser from the front side. A limitation of this acquisition mode is the averaging effect that is caused by the only poorly defined extraction of bacterial metabolites from different depths in the sample. To overcome this restriction, embedding and sectioning protocols similar to those already established for animal and plant tissue are currently being developed (51, 52). The combination of optimal sample preparation, high-resolution t-MALDI-2-MSI and its amendment with further correlative imaging modalities, such as fluorescence microscopy, could form the basis for a close-to-single-cell resolution also for bacterial ensembles in the near future.

Third, unlike different to MALDI-based biotyping systems that are routinely used in clinical settings (53, 54), the technically more complex MALDI imaging instruments are typically not placed in a dedicated microbiology laboratory. To safely inactivate pathogens prior to the MALDI-MSI analysis, protocols have been developed, in which bacterial biofilms are grown on filter media, such as mixed cellulose ester membranes (**Figure 3**, 50, 51). At defined time points, the biofilm-containing membranes can be removed from the agar and then, for example, be applied to a brief steam inactivation step (51). Alternatively, a brief chemical fixation, for example with 10% formaldehyde solution to kill heat-resistant spores, may be applied. While steam inactivation has been found to preserve the general morphology of the biofilms on a macroscopic level (51), shrinkage by dehydration will

need to be taken into account in high-resolution studies of cross-sections. Similarly, the cross-linking of NH_2 groups (e.g. in bacterial peptides or phosphatidylethanolamines of the cell walls) is potentially leading to unwanted modifications.

By applying the filter membrane-based culturing method and the signal enhancement that is provided by MALDI-2 laser postionization, more than 30 different 2-alkyl quinolones and 11 mono- and di-rhamnolipids were visualized from *Pseudomonas aeruginosa* colonies challenged with co-cultured *Staphylococcus aureus* in a spatially-resolved manner (51). This result indicates a previously unknown multicellular complexity of the associated quorum sensing and defense pathways. Other important classes of bacterial metabolites that have been visualized in this way were *B. subtilis* surfactins and plipastatins (51). The spatially resolved detection of spore delaying protein (SDP) and sporulation killing factors (SKF), two endotoxins involved in cannibalism mechanism of *B. subtilis* (55), by MALDI-MSI was previously further correlated with fluorescence microscopy of a GFP-expressing mutant (56) .

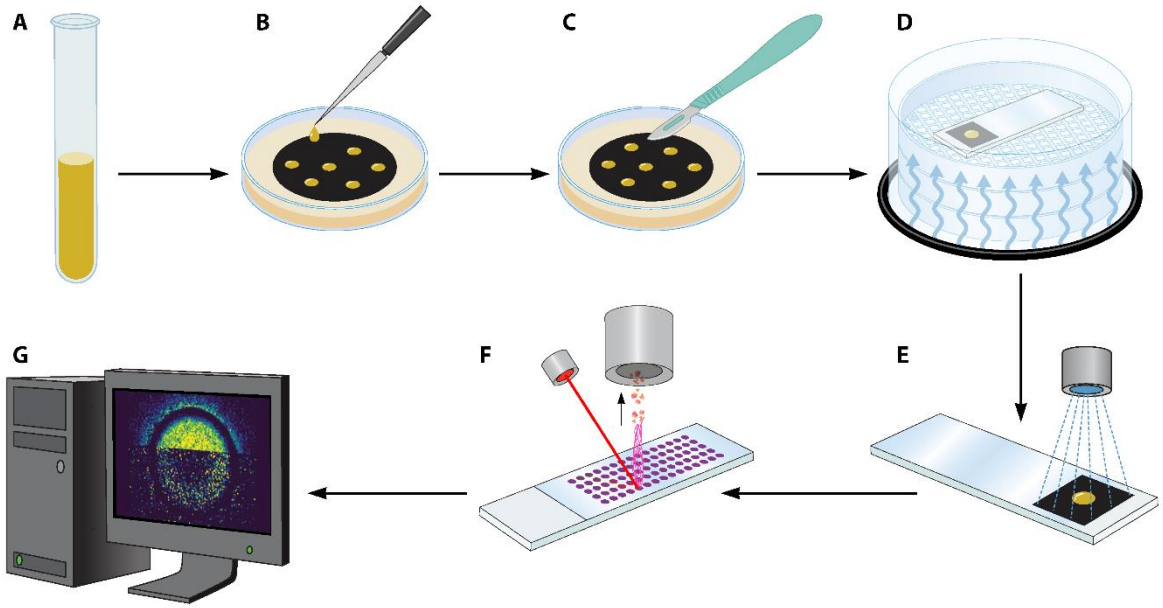

Figure 3: **Scheme for MALDI-MSI compatible culturing and sample preparation of bacterial colonies for top-view analysis. A**: cultivation of cells in LB-broth. **B**: Inoculation of 1 µL bacterial suspension on a mixed cellulose-ester membrane placed on LB-agar. **C**: Dissection of a colony together with the membrane. **D**: Optionally: Steam inactivation. **E**: MALDI matrix coating. **F**: MALDI-MSI experiment. **G**: Data processing.

3. Analysis of single-cell heterogeneity and its local dynamics by microbial flow cytometry

The different molecules detected by MALDI-MSI at specific sites within a colony in situ are produced by cells that have differentiated into functionally diverse cells. To further study spatiotemporal variations in phenotypic functions, either a robotic arm can be used for cell sampling based on phenotypic imaging data (57), or biopsies can be taken from colony biofilms at different locations for subsequent flow cytometry analysis (58). Flow cytometry is a powerful method for analyzing bacterial colonies at the single-cell level to decipher phenotypic heterogeneity. The method is widely used in medical research to characterize individual eukaryotic cells and is also very powerful for the rapid and quantitative analysis of individual microbial cells in a high-throughput process (**Figure 4**). A flow cytometer typically measures between 3,000 to 5,000 cells/sec by aligning cell after cell in a liquid stream through hydrodynamic focusing. Light scattering is detected at small angles for forward scattering (FSC) or at 90° for side scattering (SSC), while fluorescence of the cells is also recorded at 90°. The emitted photons are collected by photomultipliers and are electronically amplified. Cytometers can have several laser lines for excitation and are calibrated with bead or cell standards and the obtained data is commonly displayed in so-called 2D plots, in which two of the measured cell parameters are visualized (**Figure 4**). Bacterial cells pose a challenge in differentiating cell types due to their small size, morphological similarity and smaller number of cellular components. Consequently, flow cytometers used in microbiology require higher resolution due to lower dye binding and the lack of widely available cell-specific markers, necessitating customized protocols for each species (59).

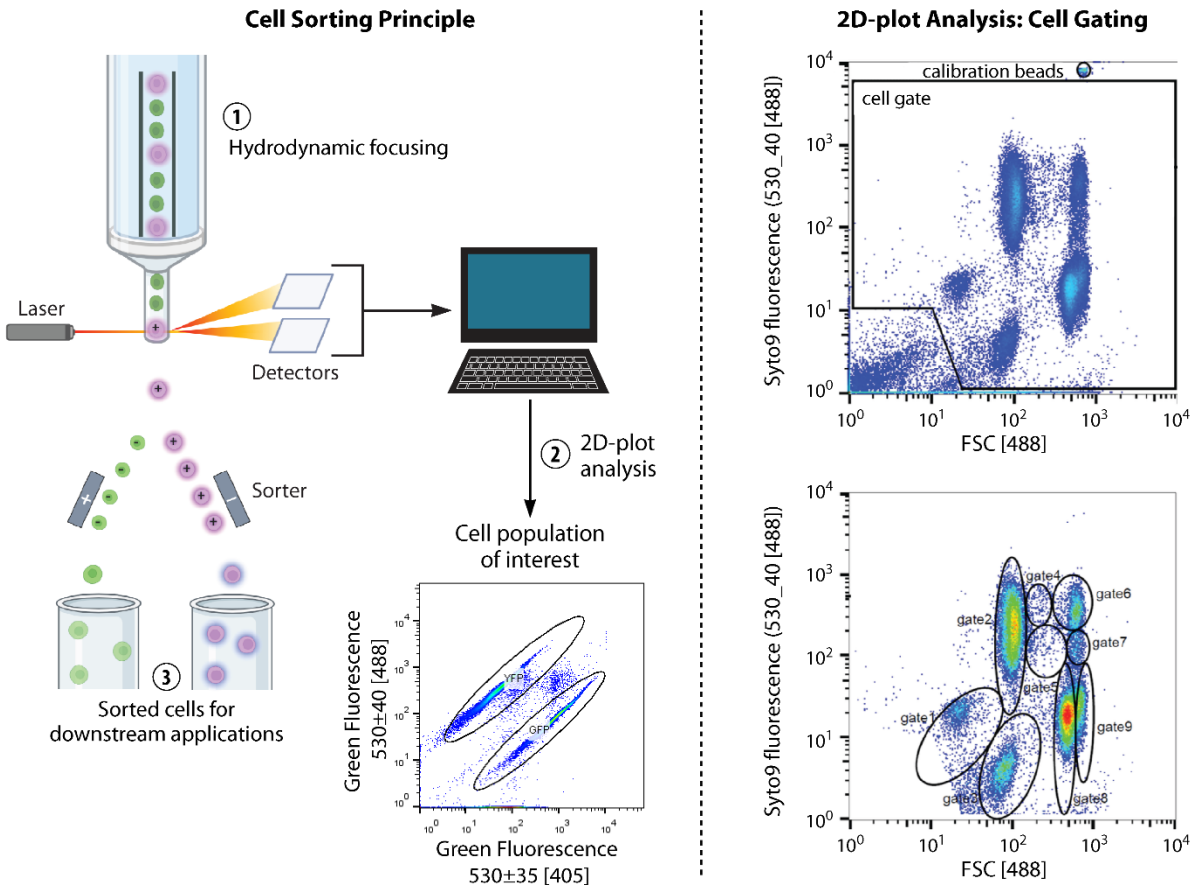

Figure 4. **Flow cytometric analysis of cells obtained from a *B. subtilis* colony**. Left: Intrinsic (e.g., scattering) and extrinsic (e.g., fluorescence) parameters of the cells are measured by flow cytometry, and the resulting cell subsets are displayed as a 2D plot. From here, a sorting decision is made and the cells are sorted by FACS (fluorescence activated cell sorting) accordingly for subsequent analysis. Right: *B. subtilis* cells are characterized with FSC and Syto9 staining (for nucleic acids). The population diversifies into several heterogeneous subpopulations with different vegetative cell types and spore types. Top, a cell gate is set to separate cells from all other events. Bottom, cell distributions are shown without instrumental noise and calibration beads. Each subpopulation is separated from the others by gating for both cell number determination and cell sorting. The colors from blue to red indicate the increasing number of cells per subgroup of cells.

The assessment of cellular characteristics can be achieved through intrinsic or extrinsic parameters. Intrinsic parameters do not require cell treatment and convey light scattering properties, using both forward scattering, which provides information about cell size, and side scattering to measure cell density and surface roughness (60).

Gene expression can be quantitatively visualized using fluorescent reporter gene fusions (16). Additionally, autofluorescent pigments in autotrophic cells (61) or fluorescent marker molecules of metabolic pathways (62) identify cell types and quantify metabolic activities. The advantage of analyzing intrinsic parameters lies in the ability to assess cells without altering their physiological state. On the other hand, extrinsic methods involve labeling cell functions using fluorescent dyes, such as functional FISH probes or antibodies, although these labels may not always be quantitative due to non-penetrable cell walls (63). These methods can also measure viability states (e.g. live/dead ratio, membrane potential, pH (64) or quantify cellular macromolecular components such as protein or DNA levels (65). Despite the large number of fluorescent markers available, only two or three dyes can be simultaneously applied to a single bacterial cell to avoid (or not to disturb) phenomena like fluorescence resonance energy transfer or compensation depletion (66)

Flow cytometry is often used to accurately quantify absolute cell numbers in microbiological populations. A recent study examined wound healing in *B. subtilis* colonies, where flow cytometry was used to measure fractions of live and dead cells, after a quarter of a biofilm was removed and compared to normally growing biofilms (20). A remarkable finding was that cell growth in the biofilm “wound” initially outpaced normal growth but then later lagged behind due nutrient depletion (see scheme **Figure 1C**). This initial rapid growth was attributed to residual cells remaining in or on the agar (up to 10% of the cells) that was overlooked in optical microscopy-based studies. In addition to analyzing live and dead cells, also various cell cycle states can be differentiated. The dynamics of heterogeneous cell types and proportions can be tracked over time, providing a complex view of spatially and temporally coherent physiological processes in bacterial populations. An example for dynamic cytometric fingerprinting of growth-related cell states is shown for *Prestia megaterium* in SI MOVIE 1. Furthermore, fluorescent reporters can be used to study the broad functional behavior of cells in pure populations (67, 68). Interestingly, in the absence of reporter genes or functional fluorescent dyes, functional diversity can also be identified using correlation analysis by combining cell information (coming from flow cytometry) and cultivation related abiotic parameters. The associated different cell types and abiotic factors, including measurable gradients of oxygen or carbon and energy sources, as well as synthesized macromolecules such lipopeptides (e.g. surfactants and plipastatins in the case of *B. subtilis* and 2-alkyl-quinolones and mono- and dirhamnolipids for *P. aeruginosa*, (51)) could serve to estimate the cell-cell and cell-abiotic interactions. In addition, the strength of correlations between cell types as well as cell types and abiotic factors can be used for sorting decisions. The 2D data generated by flow cytometry can be automatically gated using the tools PhenoGMM (69) and flowEMMi (70) or evaluated and visualized by flowCHIC (71), flowCybar (72, 73) or Flowsofine (74).

The potential of flow cytometry is greatly enhanced by its ability to separate cell types locally by cell sorting (FACS - fluorescence activated cell sorting). Functions of sorted subpopulations can then be identified in detail by subsequent omics technologies such as proteomics (75, 76), or transcriptomics (77, 78). A pioneering study that applied proteomics analysis on sorted bacterial cells revealed the differences in protein compositions of *E. coli* and *P. putida* strains (79). Such cell separation techniques can provide insight into the interactions between cell types and, by excluding unsorted cells, facilitate the identification of protein biomarkers and protein-protein interactions associated only within the sorted cell phenotypes (76, 80). Additionally, recent advancements in sequencing technologies, including single-cell RNA-seq, permits targeting single cells for functional assessment and facilitating the identification of various functional subgroups in bacterial colonies (81, 82). Although very promising, these methods are still limited by the structure of prokaryotic cells, which may cause higher sequencing costs and detect low numbers of transcripts with higher uncertainties (78, 83, 84). Alternatively, the prior sorting of bacterial subpopulation by FACS, coupled with traditional bulk mRNA-seq analysis, can generate high precision gene expression profiles of cell subgroups (77). In addition, vital cells with certain physiological properties can be sorted for further cultivation approaches or specific viability tests (85). Nevertheless, there are still limitations when performing cell sorting on bacterial single cells or relatively small numbers of cells, especially if subsequent omics applications are to be conducted. It is also important to note that, unlike live cell imaging, e.g. in microfluidic devices, individual cells cannot be tracked over time.

4. Analysis of early biofilm development and behavior by microfluidic cultivation systems

Microfluidics deals with the handling and control of fluids on the micrometer scale (86, 87) and is therefore an ideal tool for analyzing microbial biofilm development in a well-controlled environment. Established microfabrication techniques employing soft lithography and replica molding (88, 89), as well as emerging 3D-printing (90–92) allow the generation of channels with lower micrometric features. These features may range from simple straight channels to various complex geometries (**Figure 5A, B**). Within the microfluidic channels, well-controlled, stable and reproducible environments can be established and maintained. Controllable environmental parameters are for instance the composition of the medium, its flow speed, chemical gradients and if applicable, the ambient temperature. The use of transparent materials such as polydimethylsiloxane (PDMS) and glass enables the integration of microfluidic devices with optical methods for readout, such as life-cell imaging and high-resolution microscopy. The resulting spatial and temporal resolution of the recordings are precise enough to track individual cells

within a cell cluster or even smaller biofilms and thus monitoring growth, cell division and fluorescence coupled metabolic parameters (21, 36). Both large-scale imaging of developing biofilms and recording of small cell clusters require an integration of microfluidics with automated high-resolution microscopy image acquisition and will benefit from advanced imaging methods such as confocal laser scanning microscopy.

Various microfluidic systems have been developed to investigate and characterize biofilms (93, 94). Naturally, the unbounded growth of a bacterial biofilm is best studied in a straight, large aspect ratio microfluidic channel (36, 95) (**Figure 5 A-B top**). This maximizes the observable area and avoids unnecessary complex flow profiles. While a straight channel is a simple geometry, the flow may still be tightly controlled by the integration of multiple inlets (96, 97). Defined flow conditions allow to determine the impact of the medium and molecules of interest, such as for instance antibiotics, on biofilm formation (98, 99). The flow itself may be characterized experimentally by employing and tracking fluorescent tracers, or numerically through computational fluid dynamics (100–102). In nature, a bacterial biofilm is often opposed to a dynamic environment, defined by changing shear forces (103–105). The impact of flow shear on bacterial adhesion, onset of biofilm formation and subsequent development can be investigated in microfluidic devices in a defined manner (106). Furthermore, the chemical composition of the biofilm surrounding medium may also change dynamically. Over the years, microfluidic assays were developed to overcome the limitations posed by static biofilm methods and are capable of tracing the physiological response of bacterial populations to well defined chemically fluctuating environments (107–109). Finally, the architectures of natural environments are inherently complex and heterogeneous (110, 111). These heterogeneities can be modeled using microfluidic devices and their micrometer resolution. For instance, the cavity-like pores of the human skin and mammalian gut surface (112) were simulated by creating microfluidic cavities (107, 113, 114), **Figure 5 A-B middle**). Similarly but even more complex, soil as a habitat of water, air and particles of varying length scales was recently mimicked in microfluidic channels using small diameter circular PDMS pillars or even fully heterogeneous PDMS structures (115–117) **Figure A-B bottom**).

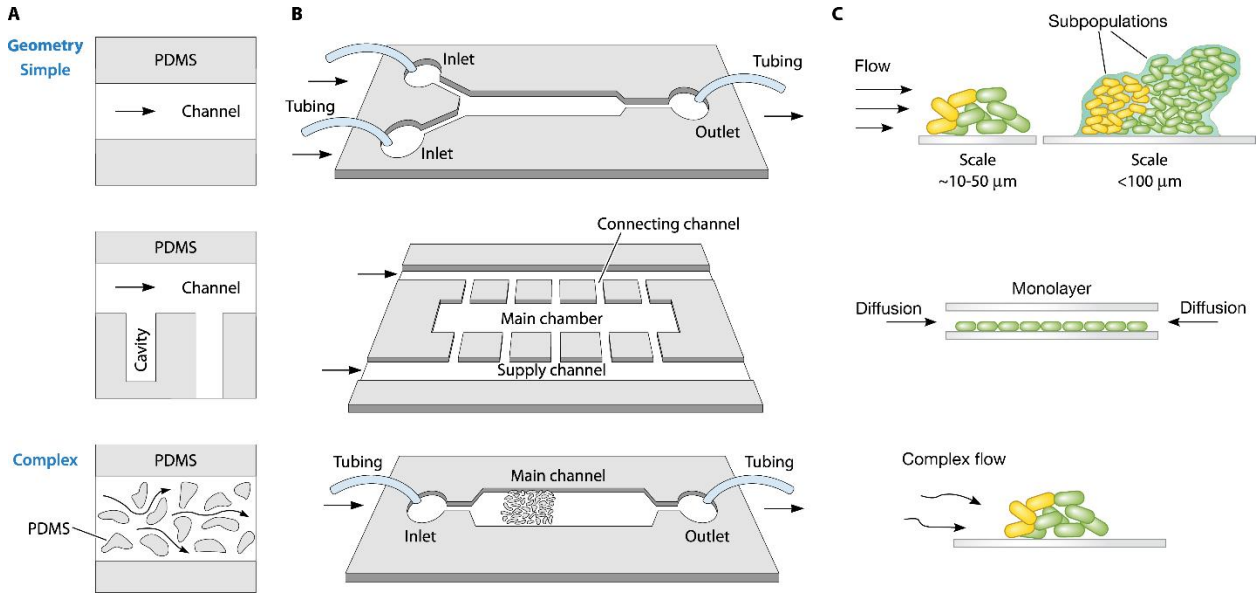

Figure 5. Overview of a microfluidic approach to biofilm analysis. A: The microfabrication process allows for a large variety of channel geometries. Three exemplary geometries representing a straight channel (top), a microfluidic cultivation chamber with dead end or open cavities (middle) and a complex soil like geometry (bottom) are shown. **B**: Respective examples of such geometries. A wide aspect ratio channel with multiple inlets (top), a microfluidic cultivation chamber (middle), a microfluidic channel with irregularly shaped obstacles (bottom) (see (97, 118, 119) for more details). In general, one or more syringe pumps drive a fluid flow (black arrows) through the microfluidic channel(s) and connected tubing. **C**: Sketched examples of different biofilm growth and experimental conditions regarding the respective microfluidic approaches. The growth and development of bacterial biofilms, and potentially also fluorescently labeled subpopulations (yellow, green) therein, can be studied from single or few cells up to small biofilms of several 10,000 cells.

Microfluidic systems have improved the understanding of the mechanisms underlying biofilm formation and early biofilm development. They provide a useful tool for investigating the dynamic interactions within the biofilm structure and function, its composition and the external environment, determined by the applied flow, medium and surrounding geometry. Ultimately, natural microbial biofilms exhibit significant heterogeneities themselves, often comprising diverse species or genotypes in dynamic environments (120). In contrast to well mixed scenarios, the fundamental evolutionary forces of genetic drift and selection may be altered in a bacterial biofilm (121, 122). In conjunction with automated microscopy and lineage tracing, such heterogeneities may be introduced into a microfluidic grown biofilm by pre-mixing fluorescently labeled bacteria with a pre-determined fitness difference. Naturally, a multitude of different microcolonies and biofilms with varying fractions of two genotypes will be present and their growth and

development can be recorded (**Figure 5C**). For instance, (123) described the biphasic competitive dynamics between *E. coli* strains with and without a growth rate difference in microchannels. Furthermore, the potential of microfluidics to create micrometric features was used to characterize the colonization resistance of packed bacterial populations in small cavities (113) and, more recently, to demonstrate the impact of the local cavity geometry on the evolutionary fixation probability (114). Another recent study examined the dynamics of polarization at different stages of colony development by using microfluidic flow chambers in combination with microscopy and fluorescence single-cell analyses. It was found that hyperpolarization emerges at the colony center and progresses outward to the periphery, which marks a transition to collective membrane potential dynamics (124) that is associated with the growth resources and oxygen concentration.

In the last few years, several microfluidic single-cell cultivation devices, based on 'mother machine' like micro-chemostats (95, 125) have been developed. These enable the analysis of single cells and small cell clusters with full spatiotemporal resolution (126). Furthermore, microfluidic monolayer growth chambers allowed investigating various biofilm-related aspects, such as the onset of biofilm formation in *B. subtilis* at single-cell level (118, 127). While a closed system is paramount for a stringent environmental control and long-term operation, it opposes the selective sampling of cells or subpopulations. Recently, this was overcome by integrating a robot-assisted sampling device, which pierces the PDMS above and subsequently samples from the microfluidic channel below in a pre-defined manner (128, 129). This approach would allow integrating e.g. FACS-sorting and single-cell transcriptomics, as described in the next chapter.

A potential limitation during long term operation is the formation of large clumps by non-adhering bacteria and/or flow induced shedding of extracellular matrix (130). This may ultimately clog the inlet, outlet or main channel and thus alter or even fully stall the applied flow. To prevent clogging the experiment may require the application of undesirably high flow rates and thus shear stress. Biofilm experiments employing microfluidic channels usually focus on relatively small but well-resolvable biofilms. Probing the properties of large biofilms (millimeter scale) regarding gene expression profiles, 3D structure, mechanical stiffness, cell density, etc. therefore need to be studied with a different set of methods.

5. Analysis of cellular function in single cells: Single-cell transcriptomics

It has long been recognized that isogenic bacterial populations can exhibit a significant phenotypic diversification, e.g. under specific stimuli (131). Thus, microbial populations exhibit intricate structures and nutrient gradients resulting in the emergence of locally

distinct subpopulations (132). While traditional methods, such as the already mentioned FACS and microscopy help quantifying cell phenotypes, these and other techniques fail to capture the full spectrum of bacterial behavior within biofilms. This limitation can now be overcome with omics technologies. RNA sequencing (RNA-seq) has proven to be a powerful tool to capture the full spectrum of bacterial physiology, but has mainly been applied in approaches averaging the signal over millions of bacteria (133). Yet, a new generation of technologies has emerged in 2020 to capture the transcriptome of single bacteria. Such approaches hold immense promise for revolutionizing our knowledge of biofilm dynamics. The following sections discuss the variety of the different protocols and the challenges associated with implementing such emerging protocols.

5.1. Challenges associated with capturing the RNA of a single bacterium

Single-cell RNA-seq (sc-RNA-seq) approaches have become a standard application in eukaryotic molecular biology (134). Adapting these techniques to bacterial systems must take the molecular, physiological and morphological differences into account. Bacterial cells contain only around 1/100 of the total RNA of an eukaryotic cell (78), the average half-life of bacterial mRNAs is often only in the range of seconds to a few minutes in contrast to the minute-to-hours range of eukaryotic cells (135–138) and mRNA enrichment via poly-A tails is not directly possible in bacteria. Moreover, there is no universal lysis method that works at the single-cell level and usually conditions must be adjusted for each bacterial species (139). Another obstacle is the isolation of single cells from multicellular bacteria, such as *Anabaena* and *Nostoc* species, which grow in one-dimensional filaments covered by a robust cell wall and sheath, have a periplasm continuous along the entire filament length (140) and are connected by a particular type of prokaryotic gap junctions (141). There are multiple protocols for the isolation of mature heterocysts from these filaments (142, 143). These protocols have been successfully used for the separation of heterocysts and vegetative cells with the objective of investigating their respective transcriptomes using microarrays (144).

To summarize, several approaches have been developed for bacterial single-cell transcriptomic analysis to overcome these different roadblocks (see below and **Table 1**). Beyond these general bacterial challenges, studying bacteria within biofilms at a single-cell resolution adds additional complexities: i) Biofilms are densely packed populations encased in an extracellular matrix, which itself is a physical barrier to access all members of a population (145), ii) Variation in oxygen and nutrient concentration can result in a myriad of metabolic changes and capturing the full spectrum of these behaviors can be challenging without the proper techniques to resolve single-cell dynamics (146), iii) The different growth stages of a cell within the biofilm life cycle is dynamic and capturing the transcriptome of attachment, expansion, maturation and dispersion states might be essential for elucidating the temporal gene expression patterns and regulatory mechanisms driving the complex behaviors exhibited by bacteria within biofilms (147).

Considering the challenges of just performing transcriptome studies in bacteria alone, it has been a major achievement that single-cell transcriptomics has been successfully established in the biofilm context. In the next section, the latest advancements of sc-RNA-seq in bacteria and their applications are described.

5.2. Single-bacteria RNA-seq using combinatorial methods

Split Pool Ligation-based Transcriptome sequencing (SPLiT-seq) was originally developed for eukaryotic single cells (148) and was then adapted for bacterial cells (*Escherichia coli* and *B. subtilis*) leading to the microbial split-pool ligation-based transcriptomics (microSPLiT) and prokaryotic expression profiling by tagging RNA in situ and sequencing (PETRI-seq) (149) protocols. These methods do not require the prior isolation of single cells. Instead, cells fixed with formaldehyde are distributed into individual wells, permeabilized and subjected to in situ combinatorial indexing. For this, cells are split three times across 96-well plates for three rounds of barcoding by reverse transcription and two ligations (149). In the first step, reverse transcription is primed with barcoded random hexamer primers specific to each well. For the following two steps, cells are pooled and redistributed across new microplates for two rounds of barcoding by ligating another set of barcoded primers to the cDNA. Alternatively, the RNA was polyadenylated with *E. coli* poly(A) polymerase I and reverse transcribed *in situ* using bar-coded oligo(dT)-primers (150). Finally, the cells are pooled, lysed, cDNA libraries prepared and sequenced. In this way, the transcripts of every single cell finally carry a unique combination of barcodes and therefore can be assigned to individual cells. 5′-phosphate-dependent exonuclease was used to remove rRNA contaminations. Additionally, RNase H was used to digest rRNA after reverse transcription primed by rRNA-specific oligonucleotides (150). In a recent publication, the PETRI-seq protocol was modified through the depletion of rRNA-derived cDNA fragments through hybridization to a set of DNA probes resulting in a significant reduction in the fraction of uninformative rRNA reads from ~96-90% to 46-8% (151).

These methods have been used to identify cell-specific expression patterns that would not have been accessible by bulk sequencing. In the PETRI-seq approach (149), different populations of *E. coli* and *S. aureus* were sequenced and rare subpopulations exhibiting distinct gene expression programs were detected (149). Using a modified version of the PETRI-seq protocol, a small number of cells were identified at the bottom of a static *E. coli* biofilm that expressed the gene encoding PdeI, predicted as a phosphodiesterase (151). However, the authors demonstrated that the expression of PdeI rather correlated with elevated levels of the second messenger c-di-GMP and that this effect was associated with the formation of ampicillin-resistant persister cells (151). Recently the PETRI-seq approach was used to propose a new classification of regulation based on measuring the transcriptional response of each gene in individual cells of *S. aureus* and

E. coli following its replication (152). These combinatorial approaches are limited by the higher number of cells that are needed to achieve an optimal mRNA capture efficiency.

5.3. Single-bacteria RNA-seq on plates

MATQ-seq was also originally developed for single-cell sequencing of total RNA from eukaryotic cells (153). In the implementation for bacterial cells, single bacteria are sorted into the wells of a 96-well plate using FACS followed by cell lysis and cDNA synthesis and amplification(154). The authors used RNAlater to maintain the RNA integrity during the sorting step. To avoid domination of the resulting cDNA libraries by rRNA- and tRNA-reads, a more efficient, Cas9-dependent rRNA depletion step was integrated in an improved version of this protocol, yielding much higher numbers of non-rRNA reads (155). By this approach, all classes of different bacterial transcripts were detected and the sensitivity should allow the identification of low-abundance transcripts by MATQ-seq (154, 155). If the approach is combined with a suitable technique to isolate cells in a reproducible way from a microbial population, it is in principle suitable for analyzing biofilms at high resolution and high sensitivity. Reproducibility and high sequencing depth per cell are achieved in plate-based methods, however the number of cells that can be multiplexed simultaneously is limited to just a few hundreds of cells.

5.4. Single-bacteria RNA-seq in droplets

The M3-seq approach combines plate-based, *in situ* indexing with droplet-based indexing and post hoc rRNA depletion, that is, using RNase H and rRNA-specific DNA probes after cDNA synthesis (156). M3-seq allows transcriptome-scale sc-RNA-seq at higher cell numbers and across multiple conditions (156). Another recently developed droplet-based method has been called BacDrop (84). This workflow involves fixation and permeabilization of cells followed by rRNA and gDNA depletion before reverse transcription and indexing using RNase H and DNase I. The first barcoding is performed during the reverse transcription and the resulting cDNA is polyadenylated at the 3’ end using terminal transferase. The second barcoding then is part of the 2nd strand cDNA synthesis within droplets. BacDrop was tested on several species, including the gram-positive *Enterococcus faecium* for broader applicability and indicated heterogeneity within the investigated bacterial populations (84). While this droplet-based approach provides a higher throughput power of multiplexing, it requires lab to lab optimization of the available protocol, which challenges reproducibility.

5.5. Spatial transcriptomics for bacteria

An aspect of particular relevance to the study of bacterial biofilms is the assignment of an obtained transcriptome profile not only to a single cell, but to a specific cell, located at a distinct position within the respective microbial population. Par-seqFISH is an approach connecting gene expression and spatial context at single-cell and single-molecule

resolution (157). This method was developed from a seqFISH approach targeting individual cells within the neural crest stem cell niche of chicken embryos (158). The authors addressed about 600,000 *P. aeruginosa* cells across multiple conditions, in planktonic and biofilm cultures (157). The mRNAs were hybridized by two sets of probes. First, a set of 12 to 20 non-fluorescent probes was used. In this approach, all probes targeting the same mRNA carry identical sequence tags that in a second hybridization interact with short, fluorescently labeled oligonucleotides called “readout” probes. Therefore, multiple mRNAs can be detected at once using readout probes labeled with different fluorophores. The pattern is detected using automated microscopy, then these probes are stripped and washed away, so the cycle can be repeated, measuring mRNA levels by a new set of readout probes. The authors used a library of probes targeting 105 marker genes (157). Once the sequential hybridizations were completed, the resulting images could be combined into spatially resolved multigene profiles at the single-cell level. Analyzing planktonic and biofilm populations of *P. aeruginosa* by Par-seqFISH, Dar et al. (157) showed that different transcriptional states emerged dynamically during the development of a biofilm as well as during planktonic growth, an insight that would have been impossible to obtain by more traditional approaches. The authors detected 20 different subpopulations with likely different metabolic capabilities and involving the differential expression of virulence-related genes (157).

This technique is very attractive for the characterization of cells in a microbial biofilm. It can be adapted to different species and conditions, but appears to be technically more demanding. A certain limitation arises from the fact that only mRNAs can be detected, for which probe sets were predesigned.

This overview summarizes the different approaches for single cell isolation, fixation, treatment and sequencing from which the most suitable protocol for a given project can be derived. One should notice, however, that all methods were tested on a very narrow set of model species (**Table 1**). Therefore, it is important to expand the taxonomic breadth of bacteria chosen for single-cell transcriptome analysis and to ultimately also aim at more heterogeneous microbial populations.

Table 1. Comparison of different approaches developed for single-cell RNA sequencing.

Method	**par-seqFISH**	**SPLiT-Seq (microSPLiT)**	**PETRI-Seq**	**MATQ-seq**	**M3-seq**	**BacDrop**
Cell fixation	Paraform-aldehyde	Formaldehyde	Form-aldehyde	RNAlater	Form-aldehyde	Formaldehyde
Bacteria	*P. aeruginosa*	*E. coli, B. subtilis*	*E. coli*, *S. aureus*	*S. enterica, P. aeruginosa*	*B. subtilis, E. coli*	*K. pneumoniae, E. coli, P. aeruginosa, E. faecium*
Ribosomal RNA	16S rRNA signals used as reference. Potential for assigning taxonomic information.	Degradation of rRNA by terminator exonuclease and RNase H.	No depletion for rRNA, leading to 84.9% (*E. coli*) and 75% (*S. aureus*) rRNA reads.	DASH for efficient rRNA depletion.	Depletion of rRNA after library amplification using RNase H.	Depletion from total RNA using RNase H.
Advantages	Transcriptome information is assigned to individual cells at spatial resolution.	Microfluidics-independent.	Microfluidics-independent, rare sub-populations of cells detectable.	Sensitivity, rare transcripts detectable. No limitation in sequencing depth.	High throughput and high cell numbers possible.	10x Genomics platform is used.
Technical hallmarks	Parallel hybridization of probes over sequential cycles *in situ.*	Polyadenylation of mRNAs, combinatorial barcoding over 3 to 4 cycles of adapter ligations.	RT using barcoded random hexamers, combinatorial barcoding over 2 cycles.	FACS-sorting, sensitive RT protocol.	10x Genomics microfluidic droplets.	Droplet- based, enabling use of the 10x Genomics platform.
Suitability for analysis of clonal biofilm populations	Excellent, but limitation in the number of detectable genes because a set of prede- signed probes is needed.	Assignment to a specific cell at spatial resolution is not possible.	No spatial information possible.	Potential for high resolution and high sensitivity analysis if cells are tracked.	Potential if cells are tracked.	Potential if cells are tracked.
Limitations	Limited by number of probes used			Low throughput protocol (100s of cells profile at once)	Not enough sequencing depth achieved. High number of cells required to perform protocol and rare subpopulations might not be captured. .	
Reference	(157)	(150)	(149)	(154, 155)	(156)	(84)

6. Analysis of biophysical properties of intact biofilm populations

As was mentioned above, alternating gene expression patterns lead to heterogeneous distribution of cell phenotypes in biofilms. Locally guiding the formation of biochemical microenvironments, these processes may then be accompanied by gradients of biophysical cues such as accumulation of mechanical and osmotic stresses (159, 160), active cellular processes (161), varying water content and state (162) as well as hierarchical molecular organization across multiple space- and time-scales (163). Indeed, a bilateral feedback of mechanical stresses and biochemical patterning is now recognized to be essential for understanding proper embryonic development of eukaryotes (164). Similarly, following the close analogy of biofilms to multicellular organisms (165, 166), proper characterization of biofilm mechanical properties and the reciprocal effect of molecular gradients and forces currently comes into the spotlight (167).

Bacteria in colony biofilms embed themselves in a polymer meshwork, composed of secreted polysaccharides and fiber-forming proteins, including polysaccharide-binding proteins, proteases, and nucleases (168). The formation of this extracellular matrix is a dynamic process, affecting biofilm mechanical properties on the cellular and bulk scales and as a result its composition varies with spatial position in the biofilm and during biofilm development (169). To understand biofilm development as a whole, it would be necessary to view biofilms as composite materials and solve the central puzzle of material scientists, namely how cells and molecular structures organize to form the complex soft biofilm material.

To determine the density and shape of biofilm colonies optical coherence tomography (170) can be used. However, specific tools are needed to both quantify material properties in biofilms with spatial resolution, while also getting insights on the underlying microscopic structure. The spectrum of biofilm material properties changes from soft colonies grown on catheters to stiff calcified biofilms on the teeth surface. There is a broad range of methods to probe viscoelastic properties of biofilms at different scales (171, 172). These range from standard rotational rheometers, which require large amounts of biomass (often multiple colonies) (173, 174), to atomic force microscopy (AFM), which provides spatial resolution of mechanical properties down to the nm scale, but with low throughput. AFM measurements are also limited by complex interactions of the AFM probe with the biofilm surface and thus often performed under non-physiological dry conditions or via ethanol immersion (or other solutions, e.g. NaCl) (175). Bridging the gap between the macroscopic scale of the entire biofilm and the cellular and molecular scales has been achieved by fluorescence microscopy techniques that image biofilms at subcellular resolution, and by 2D electron or X-ray microscopy, which scans large areas with beam sizes on the order of molecules. Methods, such as transmission electron microscopy and

micro-computed tomography (using X-rays) may both be expanded to 3D mapping via reconstruction of 2D images (tomography). But despite the 3D visualization, the spatial resolution of these methods still falls behind electron or X-ray diffraction techniques.

How to better accomplish high resolution mapping of biofilms in a space- and time-resolved manner will be discussed in this subsection. The focus will be on fluorescence microscopy-base rheological measurements (176), a newly developed optical elastography approach (177, 178) and hierarchical structural analysis with X-ray diffraction and X-ray fluorescence techniques (163, 179, 180) with the latter two allowing for label-free, whole biofilm measurements.

6.1 Fluorescence microscopy-based microrheology

Given the benefits of fluorescence microscopy techniques for 3D live-cell imaging across multiple length scales, several techniques have been developed to determine spatially-resolved mechanical properties of biofilms using fluorescence microscopy. Early implementations have relied on introducing micron-sized beads into the biofilms, which can then be tracked using microscopy and image analysis. By following the passive diffusion of the beads (181–183), or by actively actuating magnetic beads inside biofilms using a magnetic tweezer (184, 185), the local rheology around the beads can be studied. However, bacterial cells are expected to be anchored differently to the extracellular matrix within biofilms than micron-sized beads that were introduced artificially. Therefore, further approaches have been developed to use the bacterial cells themselves as tracers for studying the local mechanical properties of biofilms (176, 186). Individual cells are tracked using microscopy and image analysis, while the biofilm is perturbed by an external stress, such as strong shear flow (**Figure 6A**). By tracking all cells in biofilms before, during, and after the mechanical perturbation, it was possible to obtain a map of the elastic modulus of 3D biofilms with micron-scale spatial resolution (176). Interestingly, the spatial distribution of the elastic modulus was found to match the spatial distribution of the polysaccharide component of the extracellular matrix, whereas the other matrix components did not display a spatial distribution matching that of the elastic modulus.

6.2 Optical elastography

Elastography, a technique used to quantify tissue and organ stiffness variations using ultrasound or magnetic resonance imaging, has emerged as a promising tool for biofilm research. *B. subtilis* colonies grown on agar were transferred with a piece of underlying agar (thus not perturbing the colony) onto a piezo actuator (**Figure 6B**). An actuator was vibrated horizontally with a short burst (~2 s) of frequencies in the kHz range. For thin-layer samples like biofilms growing on agar, shaking gives rise to shear waves propagating in the plane of vibrations in biofilms. For opaque biofilms, optical imaging is able to trace surface displacements due to horizontal shear waves. By using multi-

frequency inversion, a spatially resolved map of the elastic modulus (**Figure 6B**) could be created. These measurements showed that the central part of the colony was softer compared to its periphery, softening of the biofilm with its age, and changes of material properties in the regions of biofilm wrinkles that cover water filled channels (187). Importantly, the obtained absolute values of the storage modulus in the range of 1-4 kPa were consistent with previously reported bulk measurements for biofilms (174). This promising novel technique is the first to deliver non-invasive, highly spatially-resolved mechanical characterization of whole living biofilm colonies with µm resolution. Since the measurement just takes several seconds, it can be repeated almost as a time lapse sequence following the development of the biofilm. The relatively inexpensive and simple setup makes it accessible to any laboratory. It needs to be mentioned, though, that the interpretation of results in complex architectures containing slip interfaces such as water-filled channels under biofilm wrinkles is still open (178).

Extremely short and non-invasive measurements allow this technique to be combined with any other tool in a sequential manner. It will be interesting to study how the biofilm material properties change in response to mechanical perturbations (i.e. stretching, bending, or local damage) and osmotic or biochemical stresses.

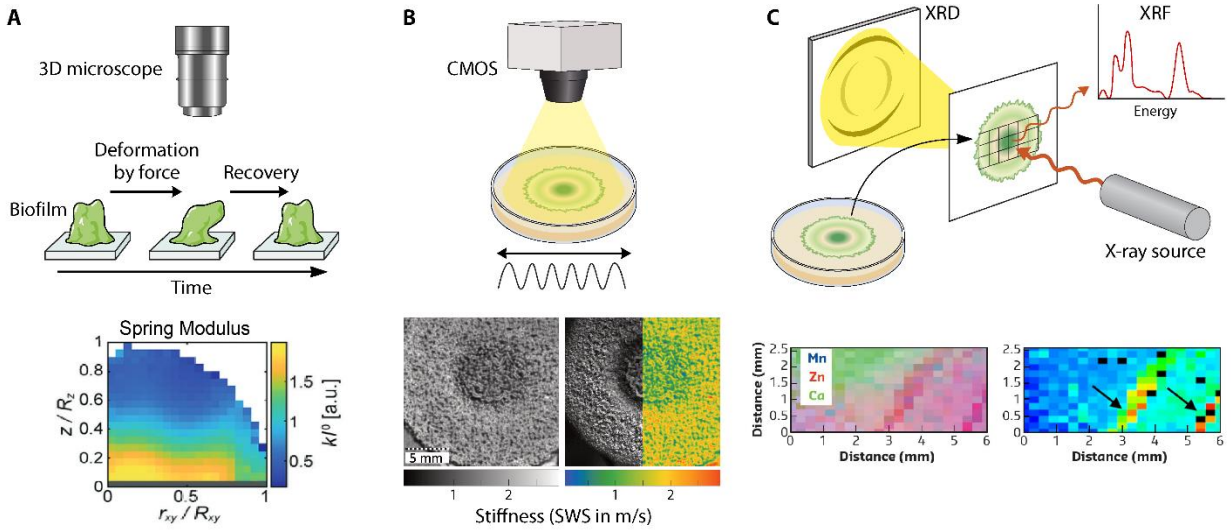

Figure 6. Methods for mechanical and structural characterization of biofilms. **A**: 3D imaging of biofilms and tracking all individual cells during the deformation and recovery of the biofilm upon a mechanical deformation (top) allows to computationally infer the elastic modulus of the biofilm with spatial resolution (bottom) (176). **B**: Schematics of the image-based elastography setup, where a biofilm sample is vibrated by a piezo-actuator in horizontal direction (top). Displacements on the surface of the sample due to thus

generated shear waves are imaged by a fast camera. With the help of reconstruction algorithms shear wave speeds are calculated and linked to the material properties. An example of a biofilm elastography sample (bottom) with a zoom in into the central area of the biofilm. Wrinkles on the surface of the biofilm are also visible in the maps of the shear wave speeds (SWS). Image courtesy J. Jordan and I. Sack, for experimental details see (178). **C**: Schematics of the X-ray diffraction (XRD)/fluorescence (XRF) setup, with a biofilm sample irradiated with a synchrotron X-ray beam and XRD/XRF signals collected simultaneously (top). XRD signal map of a biofilm piece, showing increased spore signal along biofilm wrinkles (marked with arrows) (bottom right). XRF signal map of the same biofilm piece (at the bottom right), revealing metal ion accumulation (e.g. Mn and Zn but not Ca) at the biofilm wrinkles (bottom left). Images reproduced from (163) with permissions of authors. Schematics of setups are not drawn to scale.

6.3 X-ray synchrotron diffraction and fluorescence

X-ray radiation has been used in medicine to discriminate soft from hard tissues (e.g., teeth and bone) and in scientific laboratories mostly to decipher the properties of organic crystals and inorganic minerals. Thanks to the atomic scale wavelength of X-rays, diffraction (X-ray diffraction, XRD) exposes periodic atomic structures, for example of teeth and bone (188, 189), whereas scattering (e.g. small angle X-ray scattering, SAXS) reveals dimension shape of larger scale and soft objects, such as brain tumors (190). Just like eukaryotic tissues, biofilms are soft materials and even structurally ordered molecules therein are often isotropic and therefore they are not expected to diffract. Yet, it was recently shown that mapping the X-ray scattering of whole biofilms may become handy in spatially resolving the distribution of macromolecules and water and even discriminating between the different states of water (163).

Synchrotron radiation facility beamlines provide brilliant X-ray beams allowing fast mapping with remarkable spatial resolution. The experimental conditions for biofilm measurements vary depending on the synchrotron facility and the specific beamline, and the acquisition time may vary between ms/point and tens of seconds/point, depending on the photon flux and the sample thickness. The choice of a synchrotron facility and tuning the measuring conditions are therefore important for the success of an experiment, and they should reflect the balance between scanning area and the measurement resolution (for XRD) and the absorption cross-section of the elements under study (for X-ray fluorescence, XRF). Above all, given that beam-induced radiation damage biofilms may extend beyond the dimensions of the beam size, especially for hydrated samples, correct experimental conditions are crucial to minimizing beam damage (**Figure 6C**).

Combining XRD with XRF, as is now commonly feasible in synchrotron facilities (179, 180), reveals complementary knowledge on the spatial distribution of periodic structures (XRD) and metals ions (XRF) in whole biofilms (see **Figure 6C** top panel for schematics

and bottom panel for results). Such a combined XRD/XRF study across whole biofilms suggested that enhanced water evaporation from the surface of longitudinal biofilm wrinkles (187) assists the accumulation of metal ions in biofilms but more so along the wrinkles. Interestingly, cells displaying characteristic diffraction patterns, in this case bacterial spores, are also mapped on the positions of the wrinkles. This suggests that the evaporation driven accumulation of metal ions in the wrinkles may be a driver of sporulation (see the mapping of metal ions XRF and spore XRD signals in **Figure 6C** bottom left and right panels respectively). Therefore, a combination of XRD/XRF enables the direct observation of sporulation processes without requiring genetic labeling techniques or specific markers (163) along with the distribution of water and essential metal ions.

While XRD/XRF mapping provides structural information on spatial heterogeneities of molecular structure and metal ion composition, the possible coupling between metal ion distribution and genetic expression on a macroscopic length scale calls for a combination of additional methods. To obtain an even more complete picture of the biofilm as the result of coordinated gene expression and secreted small molecules, information from complementary methods such as MALDI-imaging and spatial mRNA sequencing – as described above – should be considered.

Conclusions

The central focus of this review was to provide an overview of emerging cutting-edge technologies that are just beginning to conquer studies of bacterial populations. Although these technologies are still challenging to use, they are very useful for resolving macroscopic structures (mm range) down to micron resolution, i.e. at the level of individual microbial cells. Achieving this resolution within structured populations is only now becoming available or is still in development. But the key technologies are in place to study truly multicellular physiological traits that are the result of phenotypic heterogeneity and spatiotemporally controlled differentiation programs within structured and at least transiently stable microbial populations, giving rise to properties not observed at the single-cell level or in dispersed cultures. The current body of evidence already indicates that such emerging multicellular functions can only be detected and understood by combinations of the methods described in this review. Accordingly, research is only at the very beginning of gaining a mechanistic insight and molecular understanding of the multicellular nature of bacterial life in structured populations.

This review highlights examples of the application of these techniques for resolving emergent functions in multicellular bacterial populations, with a special emphasis on bacterial colonies as a structured form of biofilms. From a physiological point of view, this

review focused exclusively on resolving the multicellular properties of bacterial colonies, i.e. a clonal form of spatiotemporally differentiated but isogenic biofilms, and their emergent functions.

The exciting discoveries that are currently being made on the evolutionary questions surrounding the traits of multicellularity indicate that even for traditionally unicellular organisms, the multicellular population may be the unit for evolution and selection – at least for some multicellular functions. In this context, complexly structured biofilms and colonies can represent a multicellular organism without the individual cells having become terminally differentiated. Addressing this fundamental question promises to yield a wealth of novel insights, as anticipated from the studies outlined above.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) within the SPP 2389 priority program "Emergent Functions of Bacterial Multicellularity" (Grants SSEM, AES: 503267551; GA, SM: 503905203; WRH: 503940974; VZ, LC: 504222949; KD: 504072468; KD, TM: 504017689, AG: 504072468; CW: 503995533).

References

1. Grosberg RK, Strathmann RR. 2007. The evolution of multicellularity: A minor major transition? Annu Rev Ecol Evol Syst 38:621–654.
2. Fisher RM, Cornwallis CK, West SA. 2013. Group formation, relatedness, and the evolution of multicellularity. Curr Biol 23:1120–1125.
3. Bingham EP, Ratcliff WC. 2024. A nonadaptive explanation for macroevolutionary patterns in the evolution of complex multicellularity. Proc Natl Acad Sci 121:e2319840121.
4. Lyons NA, Kolter R. 2015. On the evolution of bacterial multicellularity. Curr Opin Microbiol 24:21–28.
5. Oldewurtel ER, Kouzel N, Dewenter L, Henseler K, Maier B. 2015. Differential interaction forces govern bacterial sorting in early biofilms. Elife 4:1–21.
6. Arnaouteli S, Bamford NC, Stanley-Wall NR, Kovács ÁT. 2021. *Bacillus subtilis* biofilm formation and social interactions. Nat Rev Microbiol 19:600–614.
7. Bretl DJ, Kirby JR. 2016. Molecular mechanisms of signaling in *Myxococcus xanthus* development. J Mol Biol 428:3805–3830.
8. Zambri MP, Williams MA, Elliot MA. 2022. Zambri MP, Williams MA, Elliot MA. How *Streptomyces* thrive: Advancing our understanding of classical development and uncovering new behaviors. Adv Microb Physiol 80:203-236.
9. Zeng X, Zhang CC. 2022. The making of a heterocyst in Cyanobacteria. Annu Rev Microbiol 76:597–618.
10. Shapiro JA. 1998. Thinking about bacterial populations as multicellular organisms. Annu Rev Microbiol 52:81–104.
11. Shapiro JA. 1 June 1988. Bacteria as multicellular organisms. Sci Am 1988 DOI:10.1038/scientificamerican0688-82
12. Veening JW, Smits WK, Kuipers OP. 2008. Bistability, epigenetics, and bet-hedging in bacteria. Annu Rev Microbiol 62:193–210.
13. Steenackers HP, Parijs I, Dubey A, Foster KR, Vanderleyden J. 2016. Experimental evolution in biofilm populations. FEMS Microbiol Rev 40:373–397.

14. Bich L, Pradeu T, Moreau JF. 2019. Understanding multicellularity: The functional organization of the intercellular space. Front Physiol 10:1170
15. Hengge R. 2020. Linking bacterial growth, survival, and multicellularity – small signaling molecules as triggers and drivers. Curr Opin Microbiol 55:57–66.
16. Jahn M, Vorpahl C, Türkowsky D, Lindmeyer M, Bühler B, Harms H, Müller S. 2014. Accurate determination of plasmid copy number of flow-sorted cells using droplet digital PCR. Anal Chem 86:5969–5976.
17. Keegstra JM, Kamino K, Anquez F, Lazova MD, Emonet T, Shimizu TS. 2017. Phenotypic diversity and temporal variability in a bacterial signaling network revealed by single-cell FRET. Elife 6:e27455.
18. Manina G, Griego A, Singh LK, McKinney JD, Dhar N. 2019. Preexisting variation in DNA damage response predicts the fate of single mycobacteria under stress. EMBO J 38:1–19.
19. Flores E, Herrero A. 2010. Compartmentalized function through cell differentiation in filamentous cyanobacteria. Nat Rev Microbiol 8:39–50.
20. Ye Y, Ghrayeb M, Miercke S, Arif S, Müller S, Mascher T, Chai L, Zaburdaev V. 2023. Residual cells and nutrient availability guide wound healing in bacterial biofilms. Soft Matter 20:1047–1060.
21. Hartmann R, Singh PK, Pearce P, Mok R, Song B, Díaz-Pascual F, Dunkel J, Drescher K. 2019. Emergence of three-dimensional order and structure in growing biofilms. Nat Phys 15:251–256.
22. Zhang M, Zhang J, Wang Y, Wang J, Achimovich AM, Acton ST, Gahlmann A. 2020. Non-invasive single-cell morphometry in living bacterial biofilms. Nat Commun 11:1–13.
23. Yordanov S, Neuhaus K, Hartmann R, Díaz-Pascual F, Vidakovic L, Singh PK, Drescher K. 2021. Single-objective high-resolution confocal light sheet fluorescence microscopy for standard biological sample geometries. Biomed Opt Express 12:3372.
24. Díaz-Pascual F, Lempp M, Nosho K, Jeckel H, Jo JK, Neuhaus K, Hartmann R, Jelli E, Hansen MF, Price-Whelan A, Dietrich LE, Link H, Drescher K. 2021. Spatial alanine metabolism determines local growth dynamics of escherichia coli colonies. Elife 10:1–29.
25. Yordanov S, Neuhaus K, Hartmann R, Díaz-Pascual F, Vidakovic L, Singh PK, Drescher K. 2021. Single-objective high-resolution confocal light sheet fluorescence microscopy for standard biological sample geometries. Biomed Opt Express 12:3372–3391.
26. Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersboll BK, Molin S. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 146:2395–2407.
27. Vorregaard M. 2008. PhD thesis. Comstat2 - a modern 3D image analysis environment for biofilms. Technical University of Denmark, Kongens Lyngby, Denmark
28. Mueller LN, de Brouwer JFC, Almeida JS, Stal LJ, Xavier JB. 2006. Analysis of a marine phototrophic biofilm by confocal laser scanning microscopy using the new image quantification software PHLIP. BMC Ecol 6:1.
29. Daims H, Lücker S, Wagner M. 2005. Daime, a novel image analysis program for microbial ecology and biofilm research. Environ Microbiol 8:200–213.
30. Hartmann R, Jeckel H, Jelli E, Singh PK, Vaidya S, Bayer M, Rode DKH, Vidakovic L, Díaz-Pascual F, Fong JCN, Dragoš A, Lamprecht O, Thöming JG, Netter N, Häussler S, Nadell CD, Sourjik V, Kovács ÁT, Yildiz FH, Drescher K. 2021. Quantitative image analysis of microbial communities with BiofilmQ. Nat Microbiol 6:151–156.
31. Hartmann R, van Teeseling MCF, Thanbichler M, Drescher K. 2020. BacStalk: A comprehensive and interactive image analysis software tool for bacterial cell biology. Mol Microbiol 114:140–150.
32. Paintdakhi A, Parry B, Campos M, Irnov I, Elf J, Surovtsev I, Jacobs-Wagner C. 2016. Oufti: An integrated software package for high-accuracy, high-throughput quantitative microscopy analysis. Mol Microbiol 99:767–777.
33. Ducret A, Quardokus EM, Brun Y V. 2016. MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. Nat Microbiol 1:16077.
34. Lugagne JB, Lin H, Dunlop MJ. 2020. Delta: Automated cell segmentation, tracking, and lineage reconstruction using deep learning. PLoS Comput Biol 16:1–18.
35. Stylianidou S, Brennan C, Nissen SB, Kuwada NJ, Wiggins PA. 2016. SuperSegger: robust image segmentation, analysis and lineage tracking of bacterial cells. Mol Microbiol 102:690–700.
36. Drescher K, Dunkel J, Nadell CD, Van Teeffelen S, Grnja I, Wingreen NS, Stone HA, Bassler BL. 2016. Architectural transitions in Vibrio cholerae biofilms at single-cell resolution. Proc Natl Acad Sci U S A 113(14):E2066-72.

37. Stewart EJ, Satorius AE, Younger JG, Solomon MJ. 2013. Role of environmental and antibiotic stress on Staphylococcus epidermidis biofilm microstructure. Langmuir 29(23):7017-24.
38. Jelli E, Ohmura T, Netter N, Abt M, Jiménez-Siebert E, Neuhaus K, Rode DKH, Nadell CD, Drescher K. 2023. Single-cell segmentation in bacterial biofilms with an optimized deep learning method enables tracking of cell lineages and measurements of growth rates. Mol Microbiol 119:659–676.
39. Zhang J, Wang Y, Donarski ED, Toma TT, Miles MT, Acton ST, Gahlmann A. 2022. BCM3D 2.0: accurate segmentation of single bacterial cells in dense biofilms using computationally generated intermediate image representations. NPJ Biofilms Microbiomes 8(1):99
40. Cui D, Kong L, Wang Y, Zhu Y, Zhang C. 2022. In situ identification of environmental microorganisms with Raman spectroscopy. Environ Sci Ecotechnology 11:100187.
41. Ciobotă V, Burkhardt E-M, Schumacher W, Rösch P, Küsel K, Popp J. 2010. The influence of intracellular storage material on bacterial identification by means of Raman spectroscopy. Anal Bioanal Chem 397:2929–2937.
42. Wang Y, Song Y, Tao Y, Muhamadali H, Goodacre R, Zhou N-Y, Preston GM, Xu J, Huang WE. 2016. Reverse and multiple stable isotope probing to study bacterial metabolism and interactions at the single cell level. Anal Chem 88:9443–9450.
43. Wang Y, Xu J, Kong L, Liu T, Yi L, Wang H, Huang WE, Zheng C. 2020. Raman-deuterium isotope probing to study metabolic activities of single bacterial cells in human intestinal microbiota. Microb Biotechnol 13:572–583.
44. Berry D, Mader E, Lee TK, Woebken D, Wang Y, Zhu D, Palatinszky M, Schintlmeister A, Schmid MC, Hanson BT, Shterzer N, Mizrahi I, Rauch I, Decker T, Bocklitz T, Popp J, Gibson CM, Fowler PW, Huang WE, Wagner M. 2015. Tracking heavy water (D2O) incorporation for identifying and sorting active microbial cells. Proc Natl Acad Sci 112:E194–E203.
45. Parmar D, Rosado-Rosa JM, Shrout JD, Sweedler J V. 2024. Metabolic insights from mass spectrometry imaging of biofilms: A perspective from model microorganisms. Methods 224:21–34.
46. Couvillion SP, Zhu Y, Nagy G, Adkins JN, Ansong C, Renslow RS, Piehowski PD, Ibrahim YM, Kelly RT, Metz TO. 2019. New mass spectrometry technologies contributing towards comprehensive and high throughput omics analyses of single cells. Analyst 144:794–807.
47. Hu H, Yin R, Brown HM, Laskin J. 2021. Spatial segmentation of mass spectrometry imaging data by combining multivariate clustering and univariate thresholding. Anal Chem 93:3477–3485.
48. Bien T, Koerfer K, Schwenzfeier J, Dreisewerd K, Soltwisch J. 2022. Mass spectrometry imaging to explore molecular heterogeneity in cell culture. Proc Natl Acad Sci U S A 119:1–12.
49. Niehaus M, Soltwisch J, Belov ME, Dreisewerd K. 2019. Transmission-mode MALDI-2 mass spectrometry imaging of cells and tissues at subcellular resolution. Nat Methods 16:925–931.
50. Soltwisch J, Kettling H, Vens-Cappell S, Wiegelmann M, Müthing J, Dreisewerd K. 2015. Mass spectrometry imaging with laser-induced postionization. Science 348(6231):211-5.
51. Brockmann EU, Steil D, Bauwens A, Soltwisch J, Dreisewerd K. 2019. Advanced methods for MALDI-MS Imaging of the chemical communication in microbial communities. Anal Chem 91:15081–15089.
52. Rivera ES, Weiss A, Migas LG, Freiberg JA, Djambazova K V., Neumann EK, Van de Plas R, Spraggins JM, Skaar EP, Caprioli RM. 2022. Imaging mass spectrometry reveals complex lipid distributions across *Staphylococcus aureus* biofilm layers. J Mass Spectrom Adv Clin Lab 26:36–46.
53. Singhal N, Kumar M, Kanaujia PK, Virdi JS. 2015. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front Microbiol 6:791.
54. Calderaro A, Chezzi C. 2024. MALDI-TOF MS: a reliable tool in the real life of the clinical microbiology laboratory. Microorganisms 12(2):322.
55. Höfler C, Heckmann J, Fritsch A, Popp P, Gebhard S, Fritz G, Mascher T. 2016. Cannibalism stress response in *Bacillus subtilis*. Microbiol (Reading) 162(1):164-176.
56. Si T, Li B, Zhang K, Xu Y, Zhao H, Sweedler J V. 2016. Characterization of *Bacillus subtilis* colony biofilms via mass spectrometry and fluorescence imaging. J Proteome Res 15:1955–1962.
57. Jeckel H, Nosho K, Neuhaus K, Hastewell AD, Skinner DJ, Saha D, Netter N, Paczia N, Dunkel J, Drescher K. 2023. Simultaneous spatiotemporal transcriptomics and microscopy of *Bacillus subtilis* swarm development reveal cooperation across generations. Nat Microbiol 8:2378–2391.
58. Abbaszade G, Stückrath K, Müller S. 2024. Bacterial colony biopsies: spatial discrimination of heterogeneous cell types by cytometric fingerprinting. Methods Ecol Evol (under review).

59. Cichocki N, Hübschmann T, Schattenberg F, Kerckhof FM, Overmann J, Müller S. 2020. Bacterial mock communities as standards for reproducible cytometric microbiome analysis. Nat Protoc 15:2788–2812.
60. Rao NS, Lundberg L, Palmkron S, Håkansson S, Bergenståhl B, Carlquist M. 2021. Flow cytometric analysis reveals culture condition dependent variations in phenotypic heterogeneity of *Limosilactobacillus reuteri.* Sci Rep 11:1–12.
61. Schulze K, López DA, Tillich UM, Frohme M. 2011. A simple viability analysis for unicellular cyanobacteria using a new autofluorescence assay, automated microscopy, and ImageJ. BMC Biotechnol 11:118.
62. Lambrecht J, Cichocki N, Hübschmann T, Koch C, Harms H, Müller S. 2017. Flow cytometric quantification, sorting and sequencing of methanogenic archaea based on F420 autofluorescence. Microb Cell Fact 16:1–15.
63. Harris RL, Vetter MCYL, van Heerden E, Cason E, Vermeulen JG, Taneja A, Kieft TL, DeCoste CJ, Laevsky GS, Onstott TC. 2022. FISH-TAMB, a fixation-free mRNA fluorescent labeling technique to target transcriptionally active members in microbial communities. Microb Ecol 84:182–197.
64. Müller S, Nebe-Von-Caron G. 2010. Functional single-cell analyses: Flow cytometry and cell sorting of microbial populations and communities. FEMS Microbiol Rev 34:554–587.
65. Müller S. 2007. Modes of cytometric bacterial DNA pattern: a tool for pursuing growth. Cell Prolif 40:621–639.
66. Keegstra JM, Kamino K, Anquez F, Lazova MD, Emonet T, Shimizu TS. 2017. Phenotypic diversity and temporal variability in a bacterial signaling network revealed by single-cell FRET. Elife 6:e27455.
67. Armbruster CR, Lee CK, Parker-Gilham J, De Anda J, Xia A, Zhao K, Murakami K, Tseng BS, Hoffman LR, Jin F, Harwood CS, Wong GCL, Parsek MR. 2019. Heterogeneity in surface sensing suggests a division of labor in pseudomonas aeruginosa populations. Elife 8:1–29.
68. Nguyen TM, Telek S, Zicler A, Martinez JA, Zacchetti B, Kopp J, Slouka C, Herwig C, Grünberger A, Delvigne F. 2021. Reducing phenotypic instabilities of a microbial population during continuous cultivation based on cell switching dynamics. Biotechnol Bioeng 118:3847–3859.
69. Rubbens P, Props R, Kerckhof F-M, Boon N, Waegeman W. 2021. PhenoGMM: Gaussian mixture modeling of cytometry data quantifies changes in microbial community structure. mSphere 6(1):e00530-20.
70. Bruckmann C, Müller S, Höner zu Siederdissen C. 2022. Automatic, fast, hierarchical, and non-overlapping gating of flow cytometric data with flowEMMi v2. Comput Struct Biotechnol J 20:6473–6489.
71. Koch C, Fetzer I, Harms H, Müller S. 2013. CHIC-an automated approach for the detection of dynamic variations in complex microbial communities. Cytom Part A 83 A:561–567.
72. Koch C, Günther S, Desta AF, Hübschmann T, Müller S. 2013. Cytometric fingerprinting for analyzing microbial intracommunity structure variation and identifying subcommunity function. Nat Protoc 8:190–202.
73. Günther S, Koch C, Hübschmann T, Röske I, Müller RA, Bley T, Harms H, Müller S. 2012. Correlation of community dynamics and process parameters as a tool for the prediction of the stability of wastewater treatment. Environ Sci Technol 46:84–92.
74. Kupschus J, Janssen S, Hoek A, Kuska J, Rathjens J, Sonntag C, Ickstadt K, Budzinski L, Chang HD, Rossi A, Esser C, Hochrath K. 2022. Rapid detection and online analysis of microbial changes through flow cytometry. Cytom Part A 103:419–428.
75. Jahn M, Seifert J, von Bergen M, Schmid A, Bühler B, Müller S. 2013. Subpopulation-proteomics in prokaryotic populations. Curr Opin Biotechnol 24:79–87.
76. Végvári Á, Zhang X, Zubarev RA. 2023. Toward single bacterium proteomics. J Am Soc Mass Spectrom 34:2098–2106.
77. Freiherr von Boeselager R, Pfeifer E, Frunzke J. 2018. Cytometry meets next-generation sequencing – RNA-Seq of sorted subpopulations reveals regional replication and iron-triggered prophage induction in *Corynebacterium glutamicum*. Sci Rep 8:1–13.
78. Imdahl F, Saliba AE. 2020. Advances and challenges in single-cell RNA-seq of microbial communities. Curr Opin Microbiol 57:102–110.
79. Jehmlich N, Hübschmann T, Gesell Salazar M, Völker U, Benndorf D, Müller S, Von Bergen M, Schmidt F. 2010. Advanced tool for characterization of microbial cultures by combining cytomics and proteomics. Appl Microbiol Biotechnol 88:575–584.

80. Huffman RG, Leduc A, Wichmann C, Di Gioia M, Borriello F, Specht H, Derks J, Khan S, Khoury L, Emmott E, Petelski AA, Perlman DH, Cox J, Zanoni I, Slavov N. 2023. Prioritized mass spectrometry increases the depth, sensitivity and data completeness of single-cell proteomics. Nat Methods 20:714–722.
81. Teoh CP, Lavin P, Yusof NA, González-Aravena M, Najimudin N, Cheah YK, Wong CMVL. 2023. Transcriptomics analysis provides insights into the heat adaptation strategies of an Antarctic bacterium, *Cryobacterium* sp. SO1. Polar Biol 46:185–197.
82. Wang H, Zhang Y, Bartlett DH, Xiao X. 2021. Transcriptomic analysis reveals common adaptation mechanisms under different stresses for moderately piezophilic bacteria. Microb Ecol 81:617–629.
83. McNulty R, Sritharan D, Pahng SH, Meisch JP, Liu S, Brennan MA, Saxer G, Hormoz S, Rosenthal AZ. 2023. Probe-based bacterial single-cell RNA sequencing predicts toxin regulation. Nat Microbiol 8:934–945.
84. Ma P, Amemiya HM, He LL, Gandhi SJ, Nicol R, Bhattacharyya RP, Smillie CS, Hung DT. 2023. Bacterial droplet-based single-cell RNA-seq reveals antibiotic-associated heterogeneous cellular states. Cell 186:877-891.e14.
85. Sánchez-Romero MA, Casadesús J. 2018. Contribution of SPI-1 bistability to *Salmonella enterica* cooperative virulence: insights from single cell analysis. Sci Rep 8:1–11.
86. Beebe DJ, Mensing GA, Walker GM. 2002. Physics and applications of microfluidics in biology. Annu Rev Biomed Eng 4:261–286.
87. Tabeling P. 2023. 'Introduction', Introduction to microfluidics, 2nd edn, p 1–47 (Oxford, 2023; online edn, Oxford Academic), Oxford University Press.
88. McDonald JC, Duffy DC, Anderson JR, Chiu DT, Wu H, Schueller OJA, Whitesides GM. 2000. Fabrication of microfluidic systems in poly(dimethylsiloxane). Electrophoresis 21:27–40.
89. Qin D, Xia Y, Whitesides GM. 2010. Soft lithography for micro- and nanoscale patterning. Nat Protoc 5:491–502.
90. Prabhakar P, Sen RK, Dwivedi N, Khan R, Solanki PR, Srivastava AK, Dhand C. 2021. 3D-Printed microfluidics and potential biomedical applications. Front Nanotechnol 3:1–16.
91. Juang YJ, Chiu YJ. 2022. Fabrication of polymer microfluidics: An overview. Polymers (Basel) 14:1–18.
92. Garmasukis R, Hackl C, Charvat A, Mayr SG, Abel B. 2023. Rapid prototyping of microfluidic chips enabling controlled biotechnology applications in microspace. Curr Opin Biotechnol 81:102948.
93. Pérez-Rodríguez S, García-Aznar JM, Gonzalo-Asensio J. 2022. Microfluidic devices for studying bacterial taxis, drug testing and biofilm formation. Microb Biotechnol 15:395–414.
94. Yuan L, Straub H, Shishaeva L, Ren Q. 2023. Microfluidics for biofilm studies. Annu Rev Anal Chem 16:139–159.
95. Long Z, Nugent E, Javer A, Cicuta P, Sclavi B, Cosentino Lagomarsino M, Dorfman KD. 2013. Microfluidic chemostat for measuring single cell dynamics in bacteria. Lab Chip 13:947–954.
96. Asayesh F, Zarabadi MP, Aznaveh NB, Greener J. 2018. Microfluidic flow confinement to avoid chemotaxis-based upstream growth in a biofilm flow cell reactor. Anal Methods 10:4579–4587.
97. Straub H, Eberl L, Zinn M, Rossi RM, Maniura-Weber K, Ren Q. 2020. A microfluidic platform for in situ investigation of biofilm formation and its treatment under controlled conditions. J Nanobiotechnology 18:1–12.
98. Kim KP, Kim YG, Choi CH, Kim HE, Lee SH, Chang WS, Lee CS. 2010. *In situ* monitoring of antibiotic susceptibility of bacterial biofilms in a microfluidic device. Lab Chip 10:3296–3299.
99. Tang PC, Eriksson O, Sjögren J, Fatsis-Kavalopoulos N, Kreuger J, Andersson DI. 2022. A microfluidic chip for studies of the dynamics of antibiotic resistance selection in bacterial biofilms. Front Cell Infect Microbiol 12:1–14.
100. Bae AJ, Beta C, Bodenschatz E. 2009. Rapid switching of chemical signals in microfluidic devices. Lab Chip 9:3059–3065.
101. Westerwalbesloh C, Grünberger A, Stute B, Weber S, Wiechert W, Kohlheyer D, Von Lieres E. 2015. Modeling and CFD simulation of nutrient distribution in picoliter bioreactors for bacterial growth studies on single-cell level. Lab Chip 15:4177–4186.
102. Zoheir AE, Stolle C, Rabe KS. 2024. Microfluidics for adaptation of microorganisms to stress: design and application. Appl Microbiol Biotechnol 108(1):162.
103. Rochex A, Godon JJ, Bernet N, Escudié R. 2008. Role of shear stress on composition, diversity and dynamics of biofilm bacterial communities. Water Res 42:4915–4922.

104. Wilking JN, Angelini TE, Seminara A, Brenner MP, Weitz DA. 2011. Biofilms as complex fluids. MRS Bull 36:385–391.
105. Tsagkari E, Connelly S, Liu Z, McBride A, Sloan WT. 2022. The role of shear dynamics in biofilm formation. NPJ Biofilms Microbiomes 8(1):33.
106. Park A, Jeong HH, Lee J, Kim KP, Lee CS. 2011. Effect of shear stress on the formation of bacterial biofilm in a microfluidic channel. Biochip J 5:236–241.
107. Lambert G, Kussel E. 2014. Memory and fitness optimization of bacteria under fluctuating environments. PLoS Genet 10(9):e1004556
108. Nguyen J, Fernandez V, Pontrelli S, Sauer U, Ackermann M, Stocker R. 2021. A distinct growth physiology enhances bacterial growth under rapid nutrient fluctuations. Nat Commun 12:3662.
109. Kaiser M, Jug F, Julou T, Deshpande S, Pfohl T, Silander OK, Myers G, van Nimwegen E. 2018. Monitoring single-cell gene regulation under dynamically controllable conditions with integrated microfluidics and software. Nat Commun 9:212.
110. Fenchel T. 2002. Microbial behavior in a heterogeneous world. Science 296(5570):1068-71.
111. Donaldson GP, Lee SM, Mazmanian SK. 2016. Gut biogeography of the bacterial microbiota. Nat Rev Microbiol 14:20–32.
112. Motta JP, Wallace JL, Buret AG, Deraison C, Vergnolle N. 2021. Gastrointestinal biofilms in health and disease. Nat Rev Gastroenterol Hepatol 18:314–334.
113. Karita Y, Limmer DT, Hallatschek O. 2022. Scale-dependent tipping points of bacterial colonization resistance. Proc Natl Acad Sci U S A 119:1–7.
114. Postek W, Staśkiewicz K, Lilja E, Wacław B. 2024. Substrate geometry affects population dynamics in a bacterial biofilm. Proc Natl Acad Sci 121:e2315361121.
115. Kurz DL, Secchi E, Carrillo FJ, Bourg IC, Stocker R, Jimenez-Martine J. 2022. Competition between growth and shear stress drives intermittency in preferential flow paths in porous medium biofilms. Proc Natl Acad Sci U S A 119:1–10.
116. Zoheir AE, Meisch L, Martín MV, Bickmann C, Kiselev A, Lenk F, Kaster AK, Rabe KS, Niemeyer CM. 2022. Macroporous silicone chips for decoding microbial dark matter in environmental microbiomes. ACS Appl Mater Interfaces 14(44):49592–603.
117. Scheidweiler D, Bordoloi AD, Jiao W, Sentchilo V, Bollani M, Chhun A, Engel P, de Anna P. 2024. Spatial structure, chemotaxis and quorum sensing shape bacterial biomass accumulation in complex porous media. Nat Commun 15:1–12.
118. Grünberger A, Probst C, Helfrich S, Nanda A, Stute B, Wiechert W, von Lieres E, Nöh K, Frunzke J, Kohlheyer D. 2015. Spatiotemporal microbial single-cell analysis using a high-throughput microfluidics cultivation platform. Cytom Part A 87:1101–1115.
119. Scheidweiler D, Miele F, Peter H, Battin TJ, De Anna P. 2020. Trait-specific dispersal of bacteria in heterogeneous porous environments: From pore to porous medium scale. J R Soc Interface 17(164):20200046.
120. Nadell CD, Xavier JB, Foster KR. 2008. The sociobiology of biofilms. FEMS Microbiol Rev 33:206–224.
121. Hallatschek O, Nelson DR. 2010. Life at the front of an expanding population. Evolution 64(1):193-206.
122. Kayser J, Schreck CF, Yu Q, Gralka M, Hallatschek O. 2018. Emergence of evolutionary driving forces in pattern-forming microbial populations. Philos Trans R Soc Lond B Biol Sci 373(1747):20170106.
123. Ma T, Rothschild J, Halabeya F, Zilman A, Milstein JN. 2024. Mechanics limits ecological diversity and promotes heterogeneity in confined bacterial communities. Proc Natl Acad Sci U S A 121:1–11.
124. Hennes M, Bender N, Cronenberg T, Welker A, Maier B. 2023. Collective polarization dynamics in bacterial colonies signify the occurrence of distinct subpopulations. PLoS Biol 21:1–20.
125. Wang P, Robert L, Pelletier J, Dang WL, Taddei F, Wright A, Jun S. 2010. Robust growth of *Escherichia coli*. Curr Biol 20:1099–1103.
126. Grünberger A, Wiechert W, Kohlheyer D. 2014. Single-cell microfluidics: Opportunity for bioprocess development. Curr Opin Biotechnol 29:15–23.
127. Kampf J, Gerwig J, Kruse K, Cleverley R, Dormeyer M, Grünberger A, Kohlheyer D, Commichau FM, Lewis RJ, Stülke J. 2018. Selective pressure for biofilm formation in *Bacillus subtilis*: Differential effect of mutations in the master regulator sinR on bistability. mBio 9(5):e01464-18.

128. Hansen SH, Kabbeck T, Radtke CP, Krause S, Krolitzki E, Peschke T, Gasmi J, Rabe KS, Wagner M, Horn H, Hubbuch J, Gescher J, Niemeyer CM. 2019. Machine-assisted cultivation and analysis of biofilms. Sci Rep 9:8933.
129. Klein E, Weiler J, Wagner M, Čelikić M, Niemeyer CM, Horn H, Gescher J. 2022. Enrichment of phosphate-accumulating organisms (PAOs) in a microfluidic model biofilm system by mimicking a typical aerobic granular sludge feast/famine regime. Appl Microbiol Biotechnol 106:1313–1324.
130. Drescher K, Shen Y, Bassler BL, Stone HA. 2013. Biofilm streamers cause catastrophic disruption of flow with consequences for environmental and medical systems. Proc Natl Acad Sci U S A 110:4345–4350.
131. Ackermann M. 2015. A functional perspective on phenotypic heterogeneity in microorganisms. Nat Rev Microbiol 13:497–508.
132. Berlanga M, Guerrero R. 2016. Living together in biofilms: The microbial cell factory and its biotechnological implications. Microb Cell Fact 15:1–11.
133. Westermann AJ, Vogel J. 2021. Cross-species RNA-seq for deciphering host–microbe interactions. Nat Rev Genet 22:361–378.
134. Jovic D, Liang X, Zeng H, Lin L, Xu F, Luo Y. 2022. Single-cell RNA sequencing technologies and applications: A brief overview. Clin Transl Med 12(3):e694.
135. Jenniches L, Michaux C, Popella L, Reichardt S, Vogel J, Westermann AJ, Barquist L. 2024. Improved RNA stability estimation through Bayesian modeling reveals most *Salmonella* transcripts have subminute half-lives. Proc Natl Acad Sci U S A 121:1–28.
136. Steglich C, Lindell D, Futschik M, Rector T, Steen R, Chisholm SW. 2010. Short RNA half-lives in the slow-growing marine cyanobacterium *Prochlorococcus*. Genome Biol 11(5):R54.
137. Dar D, Sorek R. 2018. Extensive reshaping of bacterial operons by programmed mRNA decay. PLoS Genet 14:1–19.
138. Zocher S, McCloskey A, Karasinsky A, Schulte R, Friedrich U, Lesche M, Rund N, Gage FH, Hetzer MW, Toda T. 2024. Lifelong persistence of nuclear RNAs in the mouse brain. Science 384(6691):53-59.
139. Homberger C, Barquist L, Vogel J. 2022. Ushering in a new era of single-cell transcriptomics in bacteria. MicroLife 3:1–9.
140. Mariscal V, Herrero A, Flores E. 2007. Continuous periplasm in a filamentous, heterocyst-forming cyanobacterium. Mol Microbiol 65:1139–1145.
141. Weiss GL, Kieninger A-K, Maldener I, Forchhammer K, Pilhofer M. 2019. Structure and function of a bacterial gap junction analog. Cell 178:374-384.e15.
142. Ermakova M, Allahverdiyeva Y. 2015. Isolation of heterocysts from *Anabaena* sp. PCC 7120. Bio-protocol 5:e1456.
143. Haselkorn R. 1978. Heterocysts. Annu Rev Plant Biol 29:319–344.
144. Park J-J, Lechno-Yossef S, Wolk CP, Vieille C. 2013. Cell-specific gene expression in *Anabaena variabilis* grown phototrophically, mixotrophically, and heterotrophically. BMC Genomics 14:759.
145. Zhao A, Sun J, Liu Y. 2023. Understanding bacterial biofilms: From definition to treatment strategies. Front Cell Infect Microbiol 13:1–23.
146. Jo J, Price-Whelan A, Dietrich LEP. 2022. Gradients and consequences of heterogeneity in biofilms. Nat Rev Microbiol 20(10):593-607.
147. Sauer K, Stoodley P, Goeres DM, Hall-Stoodley L, Burmølle M, Stewart PS, Bjarnsholt T. 2022. The biofilm life cycle: expanding the conceptual model of biofilm formation. Nat Rev Microbiol 20:608–620.
148. Rosenberg AB, Roco CM, Muscat RA, Kuchina A, Sample P, Yao Z, Graybuck LT, Peeler DJ, Mukherjee S, Chen W, Pun SH, Sellers DL, Tasic B, Seelig G. 2018. Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding. Science 360:176–182.
149. Blattman SB, Jiang W, Oikonomou P, Tavazoie S. 2020. Prokaryotic single-cell RNA sequencing by in situ combinatorial indexing. Nat Microbiol 5:1192–1201.
150. Kuchina A, Brettner LM, Paleologu L, Roco CM, Rosenberg AB, Carignano A, Kibler R, Hirano M, DePaolo RW, Seelig G. 2021. Microbial single-cell RNA sequencing by split-pool barcoding. Science 371(6531):eaba5257.
151. Pu Y, Yan X, Liao H, Wang C, Huang C, Zhang W, Guo C. 2024. An advanced bacterial single-cell RNA-seq reveals biofilm heterogeneity. eLife13:RP97543

152. Pountain AW, Jiang P, Yao T, Homaee E, Guan Y, McDonald KJC, Podkowik M, Shopsin B, Torres VJ, Golding I, Yanai I. 2024. Transcription–replication interactions reveal bacterial genome regulation. Nature 626:661–669.
153. Sheng K, Cao W, Niu Y, Deng Q, Zong C. 2017. Effective detection of variation in single-cell transcriptomes using MATQ-seq. Nat Methods 14:267–270.
154. Imdahl F, Vafadarnejad E, Homberger C, Saliba AE, Vogel J. 2020. Single-cell RNA-sequencing reports growth-condition-specific global transcriptomes of individual bacteria. Nat Microbiol 5:1202–1206.
155. Homberger C, Hayward RJ, Barquist L. 2023. Improved bacterial single-cell RNA-seq through automated MATQ-seq and Cas9-based removal of rRNA reads. mBio 14(2):e0355722.
156. Wang B, Lin AE, Yuan J, Novak KE, Koch MD, Wingreen NS, Adamson B, Gitai Z. 2023. Single-cell massively-parallel multiplexed microbial sequencing (M3-seq) identifies rare bacterial populations and profiles phage infection. Nat Microbiol 8:1846–1862.
157. Dar D, Dar N, Cai L, Newman DK. 2021. Spatial transcriptomics of planktonic and sessile bacterial populations at single-cell resolution. Science 373(6556):eabi4882.
158. Lignell A, Kerosuo L, Streichan SJ, Cai L, Bronner ME. 2017. Identification of a neural crest stem cell niche by Spatial Genomic Analysis. Nat Commun 8:1830.
159. Seminara A, Angelini TE, Wilking JN, Vlamakis H, Ebrahim S, Kolter R, Weitz DA, Brenner MP. 2012. Osmotic spreading of *Bacillus subtilis* biofilms driven by an extracellular matrix. Proc Natl Acad Sci U S A 109:1116–1121.
160. Yan J, Fei C, Mao S, Moreau A, Wingreen NS, Košmrlj A, Stone HA, Bassler BL. 2019. Mechanical instability and interfacial energy drive biofilm morphogenesis. eLife 8:e43920.
161. Pönisch W, Eckenrode KB, Alzurqa K, Nasrollahi H, Weber C, Zaburdaev V, Biais N. 2018. Pili mediated intercellular forces shape heterogeneous bacterial microcolonies prior to multicellular differentiation. Sci Rep 8:1–10.
162. Ido N, Lybman A, Hayet S, Azulay DN, Ghrayeb M, Liddawieh S, Chai L. 2020. *Bacillus subtilis* biofilms characterized as hydrogels. Insights on water uptake and water binding in biofilms. Soft Matter 16(26):6180-6190.
163. Azulay DN, Spaeker O, Ghrayeb M, Wilsch-Bräuninger M, Scoppola E, Burghammer M, Zizak I, Bertinetti L, Politi Y, Chai L. 2021. Multiscale X-ray study of *Bacillus subtilis* biofilms reveals interlinked structural hierarchy and elemental heterogeneity. Proc Natl Acad Sci 119:e2118107119.
164. Goodwin K, Nelson CM. 2021. Mechanics of Development. Dev Cell 56:240–250.
165. Claessen D, Rozen DE, Kuipers OP, Søgaard-Andersen L, Van Wezel GP. 2014. Bacterial solutions to multicellularity: A tale of biofilms, filaments and fruiting bodies. Nat Rev Microbiol 12:115–124.
166. Watnick P, Kolter R. 2000. Biofilm, city of microbes. J Bacteriol 182:2675–2679.
167. Gloag ES, Fabbri S, Wozniak DJ, Stoodley P. 2020. Biofilm mechanics: Implications in infection and survival. Biofilm 2:100017.
168. Flemming HC, van Hullebusch ED, Neu TR, Nielsen PH, Seviour T, Stoodley P, Wingender J, Wuertz S. 2023. The biofilm matrix: multitasking in a shared space. Nat Rev Microbiol 21:70–86.
169. Fong JCN, Rogers A, Michael AK, Parsley NC, Cornell WC, Lin YC, Singh PK, Hartmann R, Drescher K, Vinogradov E, Dietrich LEP, Partch CL, Yildiz FH. 2017. Structural dynamics of RbmA governs plasticity of Vibrio cholerae biofilms. eLife 6:e26163.
170. Hou J, Wang C, Rozenbaum RT, Gusnaniar N, de Jong ED, Woudstra W, Geertsema-Doornbusch GI, Atema-Smit J, Sjollema J, Ren Y, Busscher HJ, van der Mei HC. 2019. Bacterial density and biofilm structure determined by optical coherence tomography. Sci Rep 9:9794.
171. Charlton SGV, White MA, Jana S, Eland LE, Jayathilake PG, Burgess JG, Chen J, Wipat A, Curtis TP. 2019. Regulating, measuring, and modeling the viscoelasticity of bacterial biofilms. J Bacteriol 201(18):e00101-19.
172. Guélon T, Mathias JD, Stoodley P. 2011. Advances in biofilm mechanics. In: Flemming HC, Wingender J, Szewzyk U. (eds) Biofilm Highlights. Springer Series on Biofilms, vol 5. Springer, Berlin, Heidelberg.
173. Geisel S, Secchi E, Vermant J. 2022. Experimental challenges in determining the rheological properties of bacterial biofilms. Interface Focus 12(6):20220032.
174. Jana S, Charlton SG V, Eland LE, Burgess JG, Wipat A, Curtis TP, Chen J. 2020. Nonlinear rheological characteristics of single species bacterial biofilms. NPJ Biofilms Microbiomes 6(1):19.

175. Kesel S, Grumbein S, Gümperlein I, Tallawi M, Marel AK, Lieleg O, Opitz M. 2016. Direct comparison of physical properties of *Bacillus subtilis* NCIB 3610 and B-1 biofilms. Appl Environ Microbiol 82:2424–2432.
176. Ohmura T, Skinner DJ, Neuhaus K, Choi GPT, Dunkel J, Drescher K. 2024. *In Vivo* microrheology reveals local elastic and plastic responses inside 3D bacterial biofilms. Adv Mater 36(29):e2314059.
177. Kennedy BF, Wijesinghe P, Sampson DD. 2017. The emergence of optical elastography in biomedicine. Nat Photonics 11:215–221.
178. Jordan J, Jaitner N, Meyer T, Brahmè L, Ghrayeb M, Köppke J, Chandia SK, Zaburdaev V, Chai L, Tzschätzsch H, Mura J, Hagemann AIH, Braun J, Sack I. 2023. Optical time-harmonic elastography for multiscale stiffness mapping across the phylogenetic tree. arXiv https://doi.org/10.48550/arXiv.2312.07380.
179. Cotte M, Gonzalez V, Vanmeert F, Monico L, Dejoie C, Burghammer M, Huder L, de Nolf W, Fisher S, Fazlic I, Chauffeton C, Wallez G, Jiménez N, Albert-Tortosa F, Salvadó N, Possenti E, Colombo C, Ghirardello M, Comelli D, Clerici EA, Vivani R, Romani A, Costantino C, Janssens K, Taniguchi Y, McCarthy J, Reichert H, Susini J. 2022. The “Historical Materials BAG”: a new facilitated access to synchrotron x-ray diffraction analyses for cultural heritage materials at the European Synchrotron Radiation Facility. Molecules 27:6.
180. Riekel C, Burghammer M, Davies R. 2010. Progress in micro- and nano-diffraction at the ESRF ID13 beamline. IOP Conf Ser Mater Sci Eng 14:012013.
181. Birjiniuk A, Billings N, Nance E, Hanes J, Ribbeck K, Doyle PS. 2014. Single particle tracking reveals spatial and dynamic organization of the *Escherichia coli* biofilm matrix. New J Phys 16:1–13.
182. Chew SC, Kundukad B, Seviour T, Van der Maarel JRC, Yang L, Rice SA, Doyle P, Kjelleberg S. 2014. Dynamic remodeling of microbial biofilms by functionally distinct exopolysaccharides. MBio 5:1–11.
183. Rahman MU, Fleming DF, Wang L, Rumbaugh KP, Gordon VD, Christopher GF. 2022. Microrheology of *Pseudomonas aeruginosa* biofilms grown in wound beds. NPJ Biofilms Microbiomes 8:1–9.
184. Galy O, Latour-Lambert P, Zrelli K, Ghigo JM, Beloin C, Henry N. 2012. Mapping of bacterial biofilm local mechanics by magnetic microparticle actuation. Biophys J 103:1400–1408.
185. Zrelli K, Galy O, Latour-Lambert P, Kirwan L, Ghigo JM, Beloin C, Henry N. 2013. Bacterial biofilm mechanical properties persist upon antibiotic treatment and survive cell death. New J Phys 15:1–17.
186. Hart JW, Waigh TA, Lu JR, Roberts IS. 2019. Microrheology and spatial heterogeneity of *Staphylococcus aureus* biofilms modulated by hydrodynamic shear and biofilm-degrading enzymes. Langmuir 35:3553–3561.
187. Wilking JN, Zaburdaev V, De Volder M, Losick R, Brenner MP, Weitz DA. 2012. Liquid transport facilitated by channels in *Bacillus subtilis* biofilms. Proc Natl Acad Sci U S A 110:848–852.
188. Lowenstam HA. 1981. Minerals formed by organisms. Science 211(4487):1126-31.
189. Addadi L, Weiner S. 2014. Biomineralization: Mineral formation by organisms. Phys Scr 89: 098003.
190. Eggebrecht AT, Ferradal SL, Robichaux-Viehoever A, Hassanpour MS, Dehghani H, Snyder AZ, Hershey T, Culver JP. 2014. Mapping distributed brain function and networks with diffuse optical tomography. Nat Photonics 8:448–454.
191. López-Gálvez J, Schiessl K, Besmer MD, Bruckmann C, Harms H, Müller S. 2023. Development of an automated online flow cytometry method to quantify cell density and fingerprint bacterial communities. Cells 12(12):1559.

Supplementary Information

SI MOVIE 1. Cultivation of *Priestia megaterium* DSM 90 in batch culture on DSM 1 medium at room temperature for 28.5 h. Samples were harvested and fixated using a standard formaldehyde/ethanol procedure (see standardized methods in: (59)), and subsequently, the cellular DNA was stained with 0.24 µM DAPI (191) and cells analyzed

using the BD Influx v7 Cell Sorter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The blue 488 nm Sapphire OPS laser (400 mW) was applied for forward scattering (FSC) and a 355 nm Genesis OPS laser (100 mW) was applied for DAPI excitation. The pictures were taken by using AxioScope A1 fluorescence microscope, equipped with a Zeiss Axiocam MRm camera (Carl Zeiss Jena, Germany). Left above: Movie based on samples taken from the growth curve and measured by flow cytometry as DAPI vs. FSC 2D plots. 200,000 cells per sample were measured. Each point represents one cell. The color highlights the increasing number of cells in the virtual z-axis, with the red color indicating the highest number of cells per bin. The movie shows the typical phenotypic distributions during logarithmic growth, which changes rapidly. Different cell types developed, including vegetative cells (veg_1n – veg_4n), which are cells with duplicate chromosome copies. The ‘replicating cells’ are those that are most dominant in the log phase of growth and contain multiple copies of finished and unfinished replicated chromosomes, please see also (65). The strain produced different types of spores (sp_1 – sp_6). Some of the cells were less stained with DAPI for unknown reasons, but differed in scattering and intensity of the dye fluorescence: unknown cells uc_1 – uc_4. Right above: growth curve, measured by OD over time. The moving orange dot highlights the sampling and is directly connected to the 2D-plots appearing on the left. Left below: a gate template was created according to the subpopulations that develop during growth. These subpopulations provide information about changes in the number of cells per gate over time and also enable sorting decisions to be made using FACS. In this case, several gates were sorted and subjected to microscopy. Right below: Phase contrast images of sorted cells from gates that are replicating: veg_1, veg_2, ‘replicating cells’; and from gates in which spores have formed: sp_1 – sp_6.

Author Bios:

Suyen Solange Espinoza Miranda is a postdoctoral scientist at the University of Würzburg. She studied Biochemistry at John Brown University followed by working for five years as a research scientist in Nicaragua. Suyen completed her PhD in Biology with focus in Infectious diseases from Boston College (USA) in 2023 where she specialized in the study of pneumococcal biofilms. Since 2023, she has been designing and optimizing single-cell RNA sequencing protocols in bacteria.

Gorkhmaz Abbaszade is a postdoctoral researcher at the Helmholtz Centre for Environmental Research – UFZ in Leipzig, Germany. He pursued his studies in Ecology and Soil Sciences while simultaneously working as a junior researcher in the Radiobiology Department at the Azerbaijan National Academy of Sciences. Following the completion of his Ph.D. in Environmental Microbiology from Eötvös Loránd University in Hungary, he continued his research in bioremediation, focusing on the restoration of soils contaminated with toxic elements as a postdoctoral fellow. Since 2023, he has been a member of the Flow Cytometry Group at the Helmholtz Centre, where his research focused on microbial population heterogeneity and single-cell analysis.

Wolfgang R. Hess is professor of Genetics and Experimental Bioinformatics at the University of Freiburg, Germany. He graduated from the Humboldt University in Berlin, Germany, and was a postdoctoral fellow or visiting guest scientist at the Friedrich Miescher Institute in Basel, Switzerland, at the CNRS in Roscoff, France, and at the MIT in Boston, U.S. He was the funding director of the Ocean Genome Legacy Foundation affiliated with New England Biolabs in Beverly, U.S. His laboratory focuses on the molecular biology of cyanobacteria and other photosynthetic organisms and their functions in the environment and in biotechnology. Current research activities are centered on the analysis of regulatory RNAs and RNA binding proteins, small protein functions, epigenetic modifications and cell differentiation processes in bacteria.

Knut Drescher is Associate Professor of Biophysics and Microbiology at the Biozentrum of the University of Basel. He studied physics at the University of Oxford, before completing a PhD in biophysics at the University of Cambridge in 2011. He became interested in microbiology and bacterial biofilms during his postdoc at Princeton University from 2011-2014. In 2014, Knut Drescher started his own lab as a Max Planck Research Group Leader at the Max Planck Institute for Terrestrial Microbiology in Marburg, where he focused on combining methods from physics and molecular biology to understand the development and function of bacterial biofilms. In 2015, Knut Drescher also became a professor in the physics department at the University of Marburg. In 2021, his lab moved to the Biozentrum at the University of Basel, and continues to combine methods from physics and molecular biology to study bacterial biofilms.

Antoine-Emmanuel Saliba is an Associate Professor at the University of Würzburg and a group leader at the Helmholtz Institute for RNA-based Infection Research (HIRI). He studied biochemical engineering at INSA Toulouse and earned his PhD at the Institut Curie, where he developed microfluidic systems for analyzing rare cancer cells at the single-cell level. After a postdoctoral fellowship at the European Molecular Biology

Laboratory (EMBL) in Heidelberg, where he worked on protein-lipid interactions, he joined Jörg Vogel's lab in Würzburg. There, he pioneered single-cell RNA sequencing to study *Salmonella enterica* infections. Since 2017, he has led the Single-Cell Analysis group at HIRI, advancing technologies to study host-pathogen interactions. In 2023, he was appointed to a W2 professorship at the University of Würzburg's Faculty of Medicine.

Vasily Zaburdaev is a professor of "Mathematics in Life Sciences" at the Friedrich-Alexander-Universität Erlangen-Nürnberg. He received his PhD in Physics in 2004 at the Russian Research Center "Kurchatov Institute" in Moscow and after three postdoctoral stays at the MPI for Dynamics and Self-Organisation in Goettingen (2004-07), TU Berlin (2007-09), and at Harvard (2009-11) he became a group leader at the MPI for the Physics of Complex Systems in Dresden, Germany in 2011. In 2018 he became a chair (full professor) of Mathematics in Life Sciences at the Friedrich-Alexander-University Erlangen-Nuernberg. He is also a scientific board member of the recently founded Max-Planck-Zentrum für Physik und Medizin in Erlangen. His group develops theoretical models to understand complex biological phenomena and their implications in disease. The group brings expertise in theoretical biophysics, statistical physics and numerical methods and works in close collaboration with experimental groups.

Liraz Chai is an Associate Professor of Chemistry at the Hebrew University of Jerusalem. She holds a Ph.D. degree in Chemistry from the Weizmann Institute of Science (WIS, 2007), where she studied the intermolecular interactions between polymers- and charge-bearing surfaces. Following a one year postdoctoral research at the WIS (2008), she switched gears to microbiology in a second postdoctoral study at Harvard University (2009-2013), where she studied bacterial biofilms. Liraz joined the Hebrew university as Assistant Professor in 2014, and was promoted to Associate Prof. in 2022. Her research combines Biochemistry, Soft Matter, Biophysics and Microbiology - all applied to an interdisciplinary research of bacterial biofilms. Her major interest in biofilms include the properties and assembly of extracellular matrix biopolymers, as well extracellular matrix - associated biomineralization processes.

Klaus Dreisewerd is a Professor of Biophysics at the University of Münster, Germany. He graduated under the supervision of the MALDI pioneers Profs. Franz Hillenkamp and Michael Karas, before moving to the Free University of Amsterdam in 1994 to co-establish the MALDI technique for one of the early single-cell mass spectrometry studies as a postdoc, then with a focus on brain neurons. Since his return to Münster University in 1997, he continues to work on methodological and instrumental advancements of the MALDI technique, with a particular interest in the physical and physicochemical fundamentals of the laser-based method. A strong application-driven research focus of his group is on the advancement of highly-resolved MALDI imaging of biological tissues including eukaryotic and microbial systems and its coupling with correlative microscopy. The team collaborates closely with numerous partners, both from academia and industry and recently introduced MALDI-2 and transmission-mode t-MALDI-2 mass spec imaging.

Alexander Grünberger is professor of “Microsystems in Bioprocess engineering at the Karlsruhe Institute of Technology (KIT), Germany. He received his Dipl.-Ing. (2010) from KIT and his Ph.D. (2015) from RWTH Aachen University in Germany. He underwent his postdoctoral training under the supervision of Prof. Wolfgang Wiechert at the Research Center Jülich in Germany. After being Junior Professor (W1) and Associate Professor (W2) in biotechnology and bioprocess engineering at Bielefeld University, he became Full Professor (W3) in “Microsystems for Bioprocess engineering” at KIT. His laboratory focuses on the development and application of novel microfluidic tools for biotechnology and bioprocess engineering. Current research activities focus on the development and application of microfluidic single-cell cultivation tools and the understanding of microbial phenotypic heterogeneity in the context of bioprocesses.

Christian Westendorf is a research scientist at the Peter Debye Institute for Soft Matter Physics at Leipzig University. He studied biology at the University of Rostock and graduated in physics in 2012 at the Max Planck Institute for Dynamics and Self-Organization (MPIDS) and Göttingen University, respectively. From 2013 to 2021 he worked on biophysics of slime molds and complex flow patterns in the mammalian brain during research stays at Graz University and MPIDS Göttingen. In 2021 he joined the lab of Oskar Hallatschek at UC Berkeley and from 2022 onwards he works at Leipzig University on the biophysics of bacterial evolution.

Susann Müller received her diploma and PhD in biochemistry from the Martin Luther University Halle-Wittenberg, Germany. In 2003 she habilitated in microbiology at the Technical University of Dresden, Germany, and in 2011 she received an apl. Professorship at the University of Leipzig. She was president of the German Society for Flow Cytometry from 2008 to 2010. Her research at the Helmholtz Centre for Environmental Research - UFZ Leipzig focuses on the ecology of microbial communities in natural and managed systems using high-throughput technologies for single cell analysis including cell sorting. Her current work is dedicated to elucidating ecological mechanisms that control microbial communities, with a focus on situations that stabilize communities in structure and function.

Thorsten Mascher is a Professor of General Microbiology at the Technische Universität Dresden, Germany. He received his PhD from the Universität Kaiserslautern (Germany) in 2001. After a postdoctoral stay at Cornell University (2002-2003), he returned to Germany as a research associate to start his own junior research group at the Georg-August Universität Göttingen. In 2008, he accepted the position of an independent research group leader at the Karlsruhe Institute of Technology. A year later, he was appointed as Professor of Synthetic Biology at the Ludwig-Maximilians-Universität München. Since 2022, he is Dean of the Faculty of Biology at the Technische Universität Dresden and coordinator of the DFG-funded priority program SPP2389 “Emergent Functions of Bacterial Multicellularity”. Current research topics include the role of cannibalism toxins in multicellular differentiation of *Bacillus subtilis*, the regulation of their biosynthesis, and functionalizing the endospore of *B. subtilis* for SynBio applications, particularly in the context of engineered living materials.