Analyzing Microbial Population Heterogeneity—Expanding the Toolbox of Microfluidic Single-Cell Cultivations

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Abstract

Recent research on population heterogeneity revealed fascinating insights into microbial behavior. In particular emerging single-cell technologies, image-based microfluidics lab-on-chip systems generate insights with spatio-temporal resolution, which are inaccessible with conventional tools. This review reports recent developments and applications of microfluidic single-cell cultivation technology, highlighting fields of broad interest such as growth, gene expression and antibiotic resistance and susceptibility. Combining advanced microfluidic single-cell cultivation technology for environmental control with automated time-lapse imaging as well as smart computational image analysis offers tremendous potential for novel investigation at the single-cell level. We propose on-chip control of parameters like temperature, gas supply, pressure or a change in cultivation mode providing a versatile technology platform to mimic more complex and natural habitats. Digital analysis of the acquired images is a requirement for the extraction of biological knowledge and statistically reliable results demand for robust and automated solutions. Focusing on microbial cultivations, we compare prominent software systems that emerged during the last decade, discussing their applicability, opportunities and limitations. Next-generation microfluidic devices with a high degree of environmental control combined with time-lapse imaging and automated image analysis will be highly inspiring and beneficial for fruitful interdisciplinary cooperation between microbiologists and microfluidic engineers and image analysts in the field of microbial single-cell analysis.

Introduction

Population heterogeneity

With the introduction of sensitive analysis tools, research on microbial population heterogeneity has increased tremendously over the last years [1,2]. Many fascinating investigations indicate that cell-to-cell heterogeneity, often triggered by internal molecular noise in single cells or external fluctuations in the microenvironment, does highly affect the efficiency of the entire colony regarding, growth, survival, adaption, sporulation, infection, biofilm formation, and so on [3–5]. Most likely, the majority of phenomena on population heterogeneity have evolved under dynamic and spatially more complex environmental settings than in conventional shake flasks or agar plates. Even further, intracellular factors triggering phenotypic heterogeneity, cellular interactions and environmental dynamics form a complex and mostly yet unexplored orchestra, for which we urgently need more advanced cultivation and single-cell analysis tools.

Analysis portfolio

Unraveling microbial population dynamics often involves the whole portfolio of laboratory routines and technologies. Thereby, also fluorescence-activated cell sorting (FACS) is frequently applied to shed light

on phenotypic distributions. Since most physiological cell states do not exhibit a directly visible phenotype, FACS is mostly combined with genetically encoded reporters, transforming intracellular signals into optically detectable fluorescence readout. However, as the integration of fluorescent proteins may impact the cellular physiology, has to be carefully elaborated and is often limited due to spectral overlap of the chromophores [6,7]. Initial cell staining with fluorescent markers and monitoring the subsequent signal dilution due to population growth can also be a suitable technique when combined with FACS, to distinguish between different growth phenotypes [8]. Special chromophores, which initiate fluorescence after being metabolically transformed [9–11], and FRET sensors [12] enlarge the toolbox to disclose phenotypic heterogeneity. Although of tremendous throughput, FACS analyses provide only snapshot insights into microbial populations and unfavorable repetitive sampling during temporal investigations may be necessary. State-of-the-art methods can be suitable for many investigations; however, at some point, researchers may face the challenge to perform more elaborate assays allowing them to precisely monitor single microbes with spatial and temporal resolution, growing and interacting in a well-defined culture environment in which the researcher can precisely trigger external signals. In this testbed, image streams provide direct, unraveled insights about intrinsic or environmental cues.

Microbial single-cell imaging

Remarkably, early single-cell studies on microbial growth applying classical bright-field microscopy date back more than 100 years. Barber [13] developed an isolation apparatus to dilute tiny microfluidic media droplets until single cells were encapsulated. He then performed single-cell growth analysis of *Escherichia coli* under different temperatures by manually observing and sketching single-cell growth over time. For many decades, microbial analysis by classical microscopy was limited on cell morphology, growth and division. However, more recently and with the increasing demand to unravel populations at the single-cell level, advanced and automated time-lapse imaging (fluorescence) microscopes were further developed and applied. In the last decade, investigations were reported, in which automated time-lapse imaging delivered important puzzle pieces helping to understand the complex mechanisms behind population heterogeneity.

For high-resolution microscopy, single-cell studies typically require well-organized cell growth on thin glass surfaces. In cell culture, eukaryotic cells are often cultivated in multiwell plates, forming a monolayer cell tissue at the bottom, ideal for time-lapse microscopy. In microbiology, however, simple live-cell assays often involve manually prepared agarose pads, having the size of a postage stamp, on which bacteria are inoculated and sandwiched between the agarose and a microscopy cover slip [14]. Agarose pads can be assembled and applied in any microbiology laboratory; however, they suffer from minimal environmental control and cell growth is not restricted to cell monolayers, especially when longer cultivations are performed. In contrast microfluidic cultivation setups facilitating well-arranged cell growth in monolayers at the same time featuring robust environmental control are becoming more popular [15–17]. Microfluidic devices that enable control over cell inoculation, growth and medium supply, however, require more advanced fabrication and are more laborious.

State-of-the-art microfluidics

Various complementary microfluidics to reliably trap and cultivate bacteria have been introduced over the last years [18–21]. Among few, we have specialized on microfluidic cultivation compartments accommodating single cells in lanes or microcolonies. Various organisms, such as bacteria, yeast, algae, fungi, microbial consortia, and others, can be cultivated in well-defined cellular monolayers by matching the compartment height to the cell size [22–26]. Hundreds of micrometer-sized cultivation compartments (picoliter volumes) are connected to a network of supply and control channels inside a single device accommodating cell numbers of up to several ten thousands. By continuous media perfusion at the nL/min range, cultivations can be performed under homogeneous and constant environmental conditions, nutrients can be supplied in access, limiting conditions can be precisely adjusted, and moreover, rapid media changes can be performed. Independent from the technical equipment, molecular microbiology laboratories often lack the technical expertise required for the operation of advanced microfluidic cultivation setups equipped with time-lapse microscopy.

Content

This review highlights

1. microbial population heterogeneity research implementing microfluidic single-cell cultivation and time-lapse imaging to derive otherwise inaccessible knowledge,
2. present and next-generation microfluidic environmental sensing and control in single-cell cultivation and potentially new research applications,
3. computational image analysis approaches and data analysis tools in microbial single-cell analysis, and
4. a prospective outlook on next generation microfluidic cultivations for dynamic environments including smart online image analysis and feedback regulations.

This review is intended for researchers in microbiology and biotechnology to draw the interest to new culturing procedures at the single-cell level and interdisciplinary cooperation with technology-experienced groups.

Microbial Microfluidic Single-Cell Analysis

Microfluidic single-cell cultivations offer tremendous potential for research on population heterogeneity. Special structure designs, cultivation modes and methods in data acquisition and analysis can be applied in a modular manner offering outstanding insights into population dynamics, usually masked by bulk measurements. Long-term cultivations, massive parallelization on-chip and subsequent single-cell analysis, however, come with a great quantity of image data. Its meaningful analysis is presently known to be the bottleneck of most microfluidic single-cell analyses, although a variety of customized analysis tools are available. The following chapter highlights recent studies on population heterogeneity regarding bacterial growth and division, gene expression, resistance and persistence in which microfluidic cultivations and image-based single-cell analysis were successfully applied (summarized in Table 1).

Growth

Cellular growth is the directly observable result of all the complex underlying biological regulation mechanisms acting inside cells and is a major research field in which microfluidic single-cell cultivations play an essential role. During microscopy, ordinary phase-contrast imaging directly delivers valuable data on cell morphology, lineages and growth rates with spatio-temporal resolution, without the requirement for laborious genetic modifications or fluorescent staining procedures. In addition, simply constant media perfusion during microfluidic cultivations facilitates environmental control enabling growth under constant concentrations and limiting conditions over long-term periods.

One widely recognized application example is cell-size regulation. So far microbial cell-size regulation in various species was presumed to follow either of two general paradigms: The *sizer*-paradigm states that individual cells have to reach a specific volume until cell division is initiated. The *timer*-paradigm, on the other hand, defines a specific time between birth and division as the determinant of proliferation.

Table 1. Overview of the applications of microfluidic cultivation devices and corresponding analysis tools

Organism	Subject	Microfluidic device/analysis tool	
E. coli	Comprehensive mapping of the chromosome replication cycle and the cell division process	Monolayer growth chamber [27]/custom-written in MATLAB	[28]
	Heterogeneous on- and rapid off-switch of catabolic gene expression in response to nutrient availability and depletion	Poly-L-Lysine (PLL) coated µ-slide VI (ibidi GmbH, Germany)/CellEvaluator	[29]
	Metabolization of competing substrates measured by gene expression of their utilization systems and corresponding growth rates		[30]
	Quantification of protein production in persistent bacteria, displaying a newly observed state of constant activity in non-growing cells	Thin layer of PDMS patterned with narrow grooves on transparent membrane [31]/custom tool in MATLAB, ImageJ	[32]
	Resuscitation from the persistent state by an accelerated pyruvate uptake	Monolayer growth chamber [33]/ImageJ	[34]
E. coli, *B. subtilis*	Maintenance of cell size homeostasis according to the *adder*-principle	MM [35]/custom high-throughput analysis tool	[36]
B. subtilis	Subpopulation dynamics, emergence and interactions	MM [35]/Schnitzcells [14]	[37]
E. coli, *C. glutamicum*, *C. glutamicum*	Experimental data serving as basis for mathematical models		[30] [38] [39]
C. glutamicum	Heterogeneity in production efficiency	Monolayer growth chamber [33]/manual analysis	[40]
Camp. jejuni	Heterogeneity in metabolic activity	Coated *polycarbonate membrane* with 0.4 µm pores between polystyrene channels/manual analysis	[41]
Reference strains, clinical samples	Susceptibility of bacteria causing urinary tract infections in 30 min	MM [35]/custom MATLAB tool	[42]
Various	Co-cultivation, cross-feeding, horizontal gene transfer between spatially separated but interacting strains	Monolayer growth chamber connected by nanochannels [43]/ImageJ	[43]
	Heterogeneity in gene expression patterns	Various	[44]
	Cell enrichment and modular detection methods for antimicrobial compounds, susceptibility test with reporter cells	PDMS-based custom-made, manufactured by LioniX BV (the Netherlands) droplet-cultivation/ImageJ	[45]

Averaged data of entire populations derived by conventional analytics are unable to precisely resolve details on cell-size control [46–49]. Taheri-Araghi *et al*. [36] investigated cell-size control and homeostasis at the single-cell level of *E. coli* and *Bacillus subtilis* under highly defined growth conditions. They applied the microfluidic mother-machine cultivation device [35] (MM) (Fig. 1) with constant medium perfusion. Approximately 20 phase-contrast time-lapse images per cell generation time were acquired, representing an adequate temporal resolution for single-cell growth analysis. To cope with the resulting large data amounts, custom high-throughput analysis software was tailored to analyze movies recorded from the MM cultivations. The study revealed a new strategy of cell size control, in which the investigated cells add a constant cell volume within their division cycle, independent from their birth size or generation time. This newly introduced *adder*-principle agrees with formerly investigated principles of cell homeostasis in a range of bacteria [36,50–52] and is in line with the incremental model by Amir [46]. The application of microfluidics in single-cell analysis also enabled insights into how homeostasis is maintained on a single-cell and population level, and additionally revealed an intrinsic variability of individual cells under constant environmental conditions [36].

The implementation of fluorescent reporters allows further insights into phenotypic cell-to-cell heterogeneity beyond what is possible with mere phase-

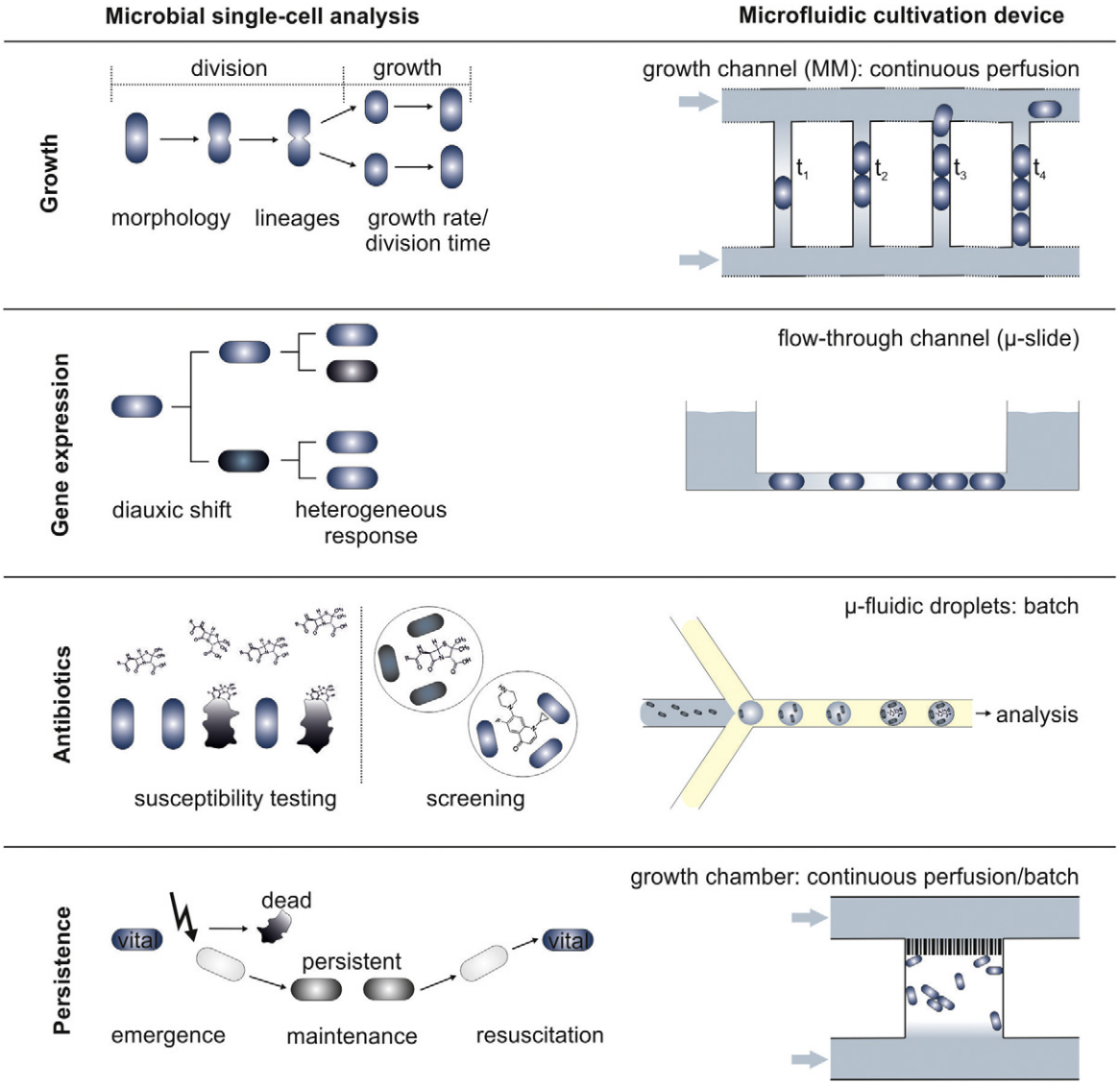

Fig. 1. Significant advances in microfluidic cultivation applications. Microbial single-cell analysis gave unique insights into microbial heterogeneity within growth behavior, gene expression, and response to antibiotics and in the persistent state. For research on these subjects, a variety of microfluidic cultivation devices with different features were applied (not drawn to scale).

contrast imaging. Wallden *et al*. [28] applied genetically encoded sensors and followed up on the work of Taheri-Araghi *et al*. [36]. The authors established a comprehensive map of the chromosome replication cycle and the cell division process in *E. coli*. In order to acquire single-cell data, they applied a microfluidic cultivation device reported earlier by Ullman *et al*. [27]. This cultivation device incorporates monolayer growth chambers for the cultivation of planar microcolonies under constant medium perfusion (Fig. 1). A custom image analysis tool delivered cell sizes, tracking and linkage between mother and daughter cells. The study disclosed not only molecular mechanisms of the *adder*-principle but also reasons for deviations from it. It was found that cell replication is initiated once a defined cell-volume-per-chromosome ratio is formed which is independent of the primary birth volume and growth rate. In addition, a single-cell version of the Cooper–Helmstetter model [53] was established that is able to describe the *adder*-principle in slow- and fast-growing cells [28]. It supports that the *adder*-principle is dependent on growth conditions and independent of birth size. Deviations from the principle were found in slowly growing cells, where replication and division are initiated and finished in one generation, excluding the occurrences of multiple replication sites. The study concluded that the cell division is limited by a number of produced components that is only reached at a minimally required cell size [28].

As shown, microfluidic single-cell cultivation was able to shed light on fundamental insights in microbial growth that was previously not accessible. Data extracted from images serve as the basis for mathematical or statistical models used for hypothesis testing, unraveling cellular mechanisms, deepening our understanding or suggesting new experiments, not limited to the context of growth and division. For instance, exploiting the high parallelism of microfluidic chip devices, Helfrich *et al*. could derive a detailed state-transition model describing spontaneous prophage induction in *Corynebacterium glutamicum*, a rare event that is otherwise covered by biological noise [38]. Besides investigating intracellular mechanisms, microfluidics combined with computational modeling also advanced our understanding of molecular processes by which cells interact and sense their environment [30,38,39].

Gene expression

Many phenotypes in population heterogeneity are not directly visible and require further approaches transforming intracellular signals into a detectable readout. With the implementation of genetically encoded fluorescence reporter systems, the number of relevant markers and measurable parameters in bacterial populations and single cells was greatly increased, covering production efficiency [40], metabolic activity [41] or gene expression patterns [44]. In most cases, the introduction of chromosomal reporters can be mandatory for bacterial systems as it has been shown by Glaeser and Heermann [54]. In comparison, plasmid-based reporters are often not useful as they mimic heterogeneity due to uneven plasmid distribution among a bacterial population.

Fluorescence reporters are frequently implemented as a feedback marker for extrinsically induced heterogeneity, commonly indicating phenotypic switches as a response to fluctuating environments. Fast and efficient cellular adaptions are crucial for bacteria to survive naturally occurring variations in, for example, temperature, water and nutrient availability. Emulating environmental dynamics is hardly possible in conventional cultivation systems, besides some special instrumentation such as by-pass bioreactors [55]. In contrast, microfluidic cultivation devices facilitate small scales, high-flow rates of medium and fast mass transfer enabling control of the cell's microenvironment. Fritz *et al*. [29] induced sudden up- and downshifts of arabinose supplemented to the continuous media stream to investigate the expression kinetics of the associated degradation pathway operon in *E. coli*, when cultivated in commercially available microfluidic chambers (μ-slide VI; Ibidi, Germany, compare Fig. 1). Data analysis was performed applying the ImageJ plugin "Cell-Evaluator," enabling segmentation and tracking of single cells. Subsequently, cell numbers and single-cell fluorescence can be followed in time [56], deriving growth rates and gene expression. It was found that the induction of gene expression was highly liable to variations in time delay, especially in lower nutrient concentrations. In contrast to that, the turn-off happened rapidly with low variations after intracellular arabinose depletion, presumably based on a nonlinear positive feedback of the arabinose system regulation [29].

An extended analysis on metabolic switching was performed by Westermayer *et al*. [30]. They experimentally validated present theoretical assumption by deriving novel data from microfluidic single-cell cultivations. The study focused on the phosphotransferase system in *E. coli* in which the utilization of sugar modules requires phosphoryl flux from general phosphotransferase system proteins. So far, detailed uptake responses of this system for more than one sugar have not been investigated. Therefore, the authors analyzed the metabolic competition between *N*-acetyl-glucosamine and sorbitol at various concentrations, by measuring the expression of their respective utilization systems, visualized by implemented fluorescent reporters and corresponding growth rates. The results suggest a sequential uptake of the provided sugar, with a preference for *N*-acetyl-glucosamine and a co-expression of both sugars' utilization systems simultaneously. Under non-limiting phosphate flux, a decoupling of both utilization systems justifies the co-expression, while a low phosphate flux causes carbon uptake limitation. Under environmental

fluctuations, with respect to the sugar source, the cells adapt to the available sugar, as well as the underlying utilization system in a dynamic way [30]. Fluctuating conditions were precisely implemented in a microfluidic flow channel in which cells were immobilized on the channel surface. This way fast medium transitions and subsequent homogeneous and constant cultivation states were achieved. Fluorescence markers indicated expression levels, which served for a metabolic expression-state phase diagram. Although quantitative measurements were not performed yet, qualitative analyses revealed a high heterogeneity on induction delay, as previously reported for arabinose by Fritz *et al.* [29], which would not have been possible in conventional bulk measurements.

Regarding population dynamics upon phenotypic switching, Rosenthal *et al.* [37] studied the emergence of distinct subpopulations. They were in particular interested in how metabolic specialization in clonal populations is achieved. The authors applied the MM device [35] with spent media taken from batch growth cultures and monitored the response of exponentially growing *B. subtilis* cultures. Those divided into subpopulations, showing distinctly different metabolic activities in form of acetate secretion. The subsequent accumulation of acetate in turn activates another subset of cells to express genes for the production of acetoin. With time, the non-toxic acetoin then replaces acetate in the medium. The long-term observation revealed the spatially separated metabolic switches appeared in response to environmental changes of the acetate concentration [37].

A precise and comprehensive characterization of the metabolic state of such subpopulations has not yet been accomplished in single-cell analysis. So far only targeted and thereby labeled metabolites and proteins are detectable, whereas the behavior of non-targeted subjects remains unseen. Anyhow, the results show a metabolic specialization of isogenic populations in response to dynamic environments. Such findings are highly relevant in evolutionary biology, where metabolic switching might be an alternative to definite evolution into inflexible specialized cells. Even further, industrial fermentation may profit from such knowledge on how bacteria manage multiple carbon sources to increase the efficiency of production strains developed by metabolic engineering [37].

The aspect of inter-population signaling discussed by Rosenthal *et al.* [37] has been a broad field of interest in various studies applying microfluidic single-cell cultivation. Especially in phenomena related to quorum sensing and bet hedging, for example, auto-inducer [57] or heterogeneous virulence gene expression [58] has been discovered. A rather new development in microfluidic cultivation platforms enables the observation of microbial consortia. Interacting strains can be spatially separated on two opposite sides of a nanochannel barrier. So far the design was used to study co-cultivation, cross-feeding and horizontal gene transfer across the connecting nanochannels [43].

Antibiotics

Microfluidic single-cell analyses are highly favorable when investigating the susceptibility of microbes toward novel antimicrobial substances and their biotechnological production. Especially in clinical environments and intensive livestock farming, multi-resistant strains cause great health risks for patients, animals and consumers. Discovering new antibiotics and susceptibility screening could highly benefit from the opportunity of high-throughput and direct observation of growth responses inside microfluidic cultivation devices. Phenotypic variation caused by non-genetic mechanisms is inherently difficult to study because of the transient nature of persistent states, calling for temporally resolved analysis of individual cells and their progeny.

In microfluidic droplets, Mahler *et al.* [45] were able to screen for synthesized antibiotics by the co-cultivation of two species simultaneously. Their comprehensive approach covered single-cell and spore cultivation as well as the enrichment of antimicrobial compounds for modular detection methods. In addition, they developed a high-throughput droplet based screening platform for whole-cell assays in which reporter cells were injected into the cultivation droplets together with the producer cells, enabling susceptibility testing. For metabolite analysis, the authors implemented mass spectrometry enabling the detection of metabolites inside single droplets. Droplet cultivations are highly favorable for high-throughput screening among microfluidic cultivation techniques. However, they suffer from poor environmental control due to fixed volumes and low spatio-temporal single-cell resolution as cells are freely suspended inside the volume.

To overcome these limitations, Baltekin *et al.* applied a microfluidic MM device [35] to monitor fast growth response of bacteria causing urinary tract infections upon the perfusion with antibiotics in about 30 min [42]. Growth under cell stress of many individual cells was investigated. The direct observation enabled short-time analyses, directly confined to the biological response time of the bacteria themselves [42]. Bacteria arranged in well-defined lanes inside the MM enabled the continuous observation and exposure to a number of different antibiotics on the same clinical sample without the necessity of laborious cell enrichment, reloading or resampling. The presented technology platform for "fast antibiotic susceptibility tests" stands thereby uniquely for a rapid susceptibility detection that could not be achieved applying averaging bulk methods. In addition, the risk of contamination inside the MM is low, since conspicuous morphology or growth rates can be easily detected at the single-cell level. Even polymicrobial infections with diverse antibiotic resistance

could be observed in a heterogeneous growth rate distribution in response to antibiotic infusion [42].

Viable but non-culturable cells

Related to identification of new antimicrobial substances and their efficiency is the occurrence of persister cells. Persistent cells do not refer to certain strains, but retain a specific metabolic state in which cells temporary maintain low levels of metabolism and typically no growth, a strategy associated with the bet hedging phenomenon. Such phenotypes have long been discussed but are impossible to detect by bulk measurements or classical cultivations and have first been experimentally proven in a microfluidic analysis format of cells upon the treatment with antibiotics [31]. The phenotypic switch to a persistent cell is rather individual and can be caused by intercellular communication [59] or environmental influences [60]. In order to artificially initiate and detect cells in persistent state, cells can be exposed to starvation, environmental stress or antibiotics.

Gefen *et al*. [32] were interested in a deeper understanding of the persistent state and visualized protein production in bacterial populations, which were maintained as persisters displaying a newly observed state of constant activity in non-growing cells. The maintenance of a predominantly persistent population was realized by starvation of *E. coli* in a microfluidic flow channel, while the formation of fluorescent proteins was measured over several days. The experimental approach can be adapted to various applications and is of major interest in single-cell research, since in their native environment, bacteria often remain in stationary growth phases for extended periods of time. The presented results show a decrease of protein production upon entry into growth arrest and thereafter relatively constant production rates over several days. The aspect of such long-term observation with a low input of medium and temperature maintenance under strict environmental control enabled the investigation of this exceptional metabolic state.

In complementary research on persister cells, Vilhena *et al*. [34] applied microfluidic growth chambers to study the recovery from this metabolic state, a process called resuscitation. They applied cold stress for 120 days to initiate persistent cells of the well-known strain *E. coli* K12. During microfluidic single-cell analysis, cells were reactivated from the viable but non-culturable (VBNC) state by an accelerated pyruvate uptake through the induced high-affinity transporter BtsT. The microfluidic application provided reactivation kinetics of VBNC over time, supporting their results on a population-level. In addition, the single-cell observation excluded contamination to be the cause of regrowth of VBNCs and determined the exact number of VBNCs compared to non-viable cells what was formerly not achievable by FACS.

Environmental Control in Microfluidic Single-Cell Cultivations

Microbial single-cell analyses applying microfluidics highly benefit from accurate environmental control of nano- to picoliter cultivation volumes [61], due to high-flow rates of medium, small cultivation volumes, high surface to volume ratios and small length scales. The majority of present microfluidic cultivation devices are operated under continuous media perfusion ensuring stable environmental conditions. This allows observing conspicuous phenotypes over long time periods under homogenous conditions without inducing potentially interfering external noise.

An example for such a state-of-the-art setup suitable for many microbial single-cell analyses under continuous medium supply was introduced by Grünberger *et al*. [62]. The cultivation device incorporates a polydimethylsiloxane (PDMS) chip with imprinted fluidic structures including supply channels of 10 µm height and cultivation chambers with a height matching the cellular diameter. The fluidic structures are irreversibly closed by bonding a 170 µm thick glass slide to the PDMS chip. PDMS was chosen for its biocompatibility and simple fabrication process [63]. Automated syringe pumps connected by silicone tubing to the microfluidic chip ensure continuous supply of fresh medium. The chip is mounted on an inverted microscope equipped with temperature incubator. During cultivation, automated (fluorescence) time-lapse microscopy is carried out. Cultivations can be performed under continuous medium supply, and medium changes can be realized by actuating external valves and pumps.

Nevertheless, many genetic circuits, molecular regulations and phenotypic heterogeneity evolved allowing cells to cope with dynamic and spatially complex environments including environmental fluctuations, chemical gradients and geometrical constraints. Considering natural microbial habitats in their tremendous diversity, it becomes clear that short-term environmental fluctuations and gradients occur frequently, which is also true for most conventional laboratory cultivations [55,64]. Novel microfluidic single-cell cultivation devices allowing to mimic natural and dynamic environments necessitate more sophisticated (online) environmental sensing and control at the micrometer scale (Fig. 2). However, the vast majority of sensors and actuators normally implemented in shake flasks or bioreactors cannot be scaled down to monitor picoliter environments. Instead, novel bottom-up approaches have to be identified and implemented. In the following, relevant environmental parameters, as well as appropriate sensing and control methods for the application in next-generation microfluidic cultivation devices are discussed.

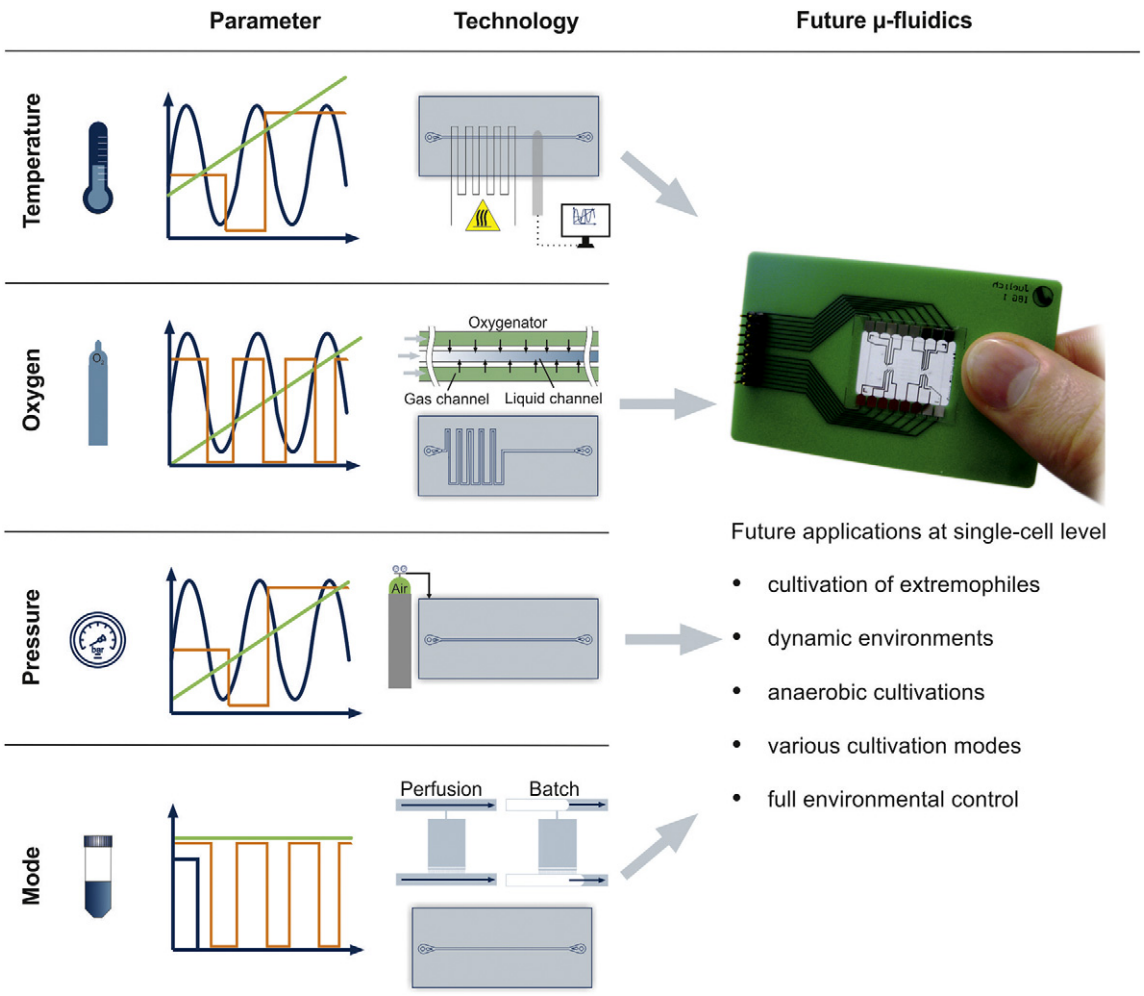

Fig. 2. Integrated environmental control and future applications in microfluidic cultivation devices. Presented are the various parameters and their dynamic control patterns over time (graph). Existing and future technologies and equipment may lead to integrated µ-fluidic devices, enabling new applications.

Temperature

Since all biochemical reactions are temperature depended, accurate temperature control is essential in microbial cultivations and analyses. Most common laboratory strains are typically cultivated at constant temperature between 20 and 37 °C, although microbial life occurs at a much broader temperature range. Thermo- and psychrophilic microorganisms accommodate ecological niches with temperatures between 113 °C [65] and −15 °C [66].

Maintaining a constant temperature in conventional laboratory shake-flask or bioreactor cultivations is state of the art. However, cultivations with temperature shifts and fluctuations require sophisticated technical control and dynamics are mostly limited by large volumes with low surface-to-volume ratio.

Also, microfluidic cultivations are commonly performed inside large microscope incubators, in which the temperature is typically measured by sensors placed in close proximity to the chip and regulated by the heat in-flow. This way the cultivation chip and major optical parts of the microscope reach nearly identical temperature reducing optical distortion during microscopy. Moreover, advanced focus-correction mechanisms compensate for thermal drifts induced by temperature inaccuracy. Typically, microscope incubators require preheating of up to several hours due to the large volume and microscope mass restricting temperature dynamics and ranges. Even temperature shifts over several hours are challenging since focus-correction systems usually compensate for thermal drift in a narrow temperature range only, resulting in focus loss over time and the requirement for manual correction. For high-temperature analysis of thermophilic organisms, even specially equipped microscopes are available, which can be operated at temperatures up to 110 °C [67].

During cultivation, identical temperature inside the incubator and the microfluidic chip are commonly assumed. However, continuous in-flow of fresh medium placed outside at room temperature and heat dissipation can induce local temperature deviations inside the cultivation media. In contrast, miniaturization and integration of temperature control on-chip offers tremendous potential for cultivations under highly defined temperature settings and dynamics. Incorporated micro-heaters enable local, precise and fast temperature modulations due to low masses and small liquid volumes. Even temperature gradients can be established under continuous medium flow on-chip.

In contrast to bench-scale system, at micrometer dimensions, highly innovative approaches for heating and cooling become possible. Some of them have been demonstrated in microfluidic devices for various life sciences. These temperature control methods utilize, for example, the integration of thin-film metal resistors serving as temperature sensors and micro-heaters [68], the infusion of Joule-heated ionic liquids in adjacent support channels [69], the application of infrared radiation for localized and short heating [70], the implementation of Peltier thermoelectric elements on top and below the microfluidic chips enabling heating and cooling [71] and the utilization of chemical or physical processes such as evaporation for cooling and exothermic reactions for heating [72]. In addition, also pre-heated or pre-cooled liquids can be infused into the microfluidic channels. Achievable temperatures then range between −3 and 120 °C, but extreme temperatures are only required in specialized applications such as PCR, chemical synthesis or cryoconservation [73]. Recently, Peng *et al*. [74] reported a microfluidic device, which supports precise microscale temperature control. Therefore, an active cooling system with pre-cooled calcium chloride and an integrated conductive gold-wire microheater were utilized for fast (10 s) and precise temperature changes in a range between 2 and 37 °C. For a more comprehensive review on temperature control in microfluidics, the reader is referred to Miralles *et al*. [73].

Considering the broad portfolio of microfluidic temperature control methods, it becomes clear that the method of choice for integration in microbial single-cell analysis highly depends on the desired application and studied organism. Obviously, on-chip temperature control comes along with the requirement for more advanced chip fabrication and device control, however, in turn offers unique possibilities for complex and dynamic temperature settings during cultivation: Cells can be exposed to sudden temperature changes, growth rate *versus* temperature functions can be derived from a single experiment by exposing cells to temperature profiles and continuous flow and recording direct growth response, cellular migration along thermal gradients can be investigated, and even thermophilic microbes can be cultivated under high-temperature dynamics on conventional microscopes. For example, significant phenotypic heterogeneity was found in yeast when exposed to harsh temperature changes impacting adaption and related gene expression of stress proteins [75]. Furthermore, Gupta *et al*. [76] observed phenotypic heterogeneity in the morphology of *E. coli* upon temperature increase.

Oxygen

Dissolved oxygen plays an important role in microbiological cultivations and bioprocesses. Oxygen transfer during aerobic cultivations is simply controlled by shaking of cultivation flasks or by injecting air into the bioreactor, methods that cannot be scaled down to single-cell dimensions. In particular in anaerobic microbiology, sample preparation, medium deoxygenation and cultivations are quite laborious and have to be performed under a special atmosphere in the absence of oxygen.

Most single-cell analysis research has therefore focused on cultivations under aerobic conditions due to its reduced technical efforts. In microfluidic cultivation devices, aerobic conditions basically rely on the oxygen supplied by the continuous influx of fresh medium and often diffusion of molecular oxygen through a gas-permeable chip material, for example, PDMS. Due to various technical challenges, cultivations under controlled anaerobic or reduced oxygen conditions combined with single-cell resolution are still in their early development. Nevertheless, anaerobic organisms play an important role in microbiology, evolution biology, and industrial biotechnology and single-cell investigations are highly desirable.

Various methods for on-chip oxygen control were recently reviewed by Brennan *et al*. [77]. Microfluidic oxygen control can be implemented on-chip, for example, by a multi-layered structure and adjacent channels for the separate transport of gas and fluid [78]. Molecular diffusion of gas is then realized across a permeable membrane (mostly PDMS) facilitating online gas exchange under continuous medium flow. The gas flow can be controlled in a highly dynamic way and mixed on demand in upstream microfluidic mixers or simply conventional T-junctions.

The application of adjacent gas-fluid exchange channels was characterized in several publications [79,80]. Kim *et al*. [81] implemented the so-called "oxygenator" and proposed a design framework for on-chip gas control. Lam *et al*. [82] implemented the oxygenator and demonstrated the cultivation of aerobic and anaerobic bacteria as well as mammalian cells. In addition, they incorporated a gradient mixer enabling the online mixing resulting in various steady-state oxygen concentrations inside a single chip simultaneously. However, studies by Diaz *et al*. indicate that dense cultures of the obligate anaerobic organism

Fusobacterium nucleateum, which were incubated anaerobically before, are able to grow under varying oxygen levels, indicating that the requirements for strict anaerobic conditions, without further isolation, were probably not met by Lam *et al*. [83,84].

On the one hand, PDMS facilitates gas exchange inside the microfluidic oxygenator, but on the other hand, molecular oxygen from the surrounding atmosphere can often freely intrude the cultivation channels across the entire chip layer. Thus, several features have to be implemented minimizing undesirable O_2 diffusion. For example, Fievet *et al*. [85] reported the cultivation of the obligate anaerobe *Desulfovibrio vulgaris* Hildenborough inside a agarose device based on the work described earlier by Ducret *et al*. [86]. Fievet *et al*. used simple agarose pads for microbial cultivations covered with a poly (methyl methacrylate) gasket serving as anaerobic micro-incubator shielding the cultivation from the surrounding atmosphere. However, agarose pads enable minimal environmental control and cells often grow in multiple layers.

Online gas control requires additionally on-chip gas detection and ideally image-based monitoring. Since conventional sensors are mostly not applicable at micrometer scales, often oxygen-sensitive fluorescent dyes are implemented as sensor layers as described by Ungerböck *et al*. [87]. For a more detailed review on oxygen sensing and optical sensors, the reader is referred to the review by Gruber *et al*. [88].

Microfluidics carry great potential for on-chip gas control and the cultivation of anaerobic organisms. On-chip solutions for oxygen control enable efficient gas transfer rates due to the small volumes, high-flow rates and efficient mass transfer, enabling constant and homogenous conditions but also steady-state gradients and oxygen dynamics, cultivations under highly dynamic oxygen environments and gradients. In anaerobic cultivations, spores can be directly inoculated into the microfluidic chip without the need for severe precautions, and then oxygen is reduced enabling anaerobic cell growth.

Cultivation mode

Microbial cultivations and biotechnological processes can be performed in various cultivation modes, for example, batch and chemostat. Most microfluidic cultivation systems feature continuous medium perfusion and, therefore, offer the advantage of well-defined environments by a constant supply of nutrients and removal of by-products [33,89,90]. This operation mode typically resembles a chemostat. In contrast, during batch cultivations inside fixed volumes, the consumption of nutrients and the accumulation of by-products impact single-cell metabolism and growth. Furthermore, the majority of cultivations are performed in batch reactors and recent single-cell studies clearly indicate that heterogeneous populations consisting of, for example, fast and slow growing, or non-growing cells, may contribute to the observed growth patterns.

Performing batch cultivations in conventional scale is usually straightforward. However, in microfluidics with fluid volumes of few picoliter and fluid layers of few hundred nanometers thickness only, batch cultivations become quite challenging, in particular when high single-cell resolution is desired. Many technological aspects should be carefully considered during batch-chip development, for example, sufficient sealing of the small cultivation volume preventing fluid evaporation and molecular diffusion into and out of the chip material. At very low volumes and large surfaces, even glass itself may impact the local pH to some extent due to the dissociation of protons. Volume, cell adhesion, surfaces, mass transport, illumination, temperature, cell alignment and inoculation are other important parameters.

Droplet microfluidics (Fig. 1) can be applied for single-cell studies in fixed volumes, offering high-throughput and the ability to perform fluid manipulations such as droplet storage, sorting and fusion. Droplets facilitate batch cultivations in fixed nano- to picoliter volumes and low cell numbers, and quantitative analytics by mass spectrometry are possible. Although in droplets single cells are freely suspended, precise single-cell identification and tracking is hardly possible.

Recently, we have developed a microfluidic PDMS-based device for batch cultivations inside cultivation chambers of 5 pL [91]. In our device, cell cultivation is performed in monolayer growth chambers (1 μm in height), which makes the device ideally suited for time-lapse imaging. The chambers can be isolated by replacing the aqueous media inside the supply channels with humidified air, and several measures were implemented to minimize fluid evaporation. We successfully demonstrated repetitive batch cultivations and analyzed *E. coli* MG1655 on various substrate concentrations observing different growth, morphology and cell number during the distinct batch phases.

Specific applications in microbial single-cell analysis require appropriate fluid control. Microfluidic single-cell analysis under continuous media perfusion enables constant conditions and the accurate correlation of external triggers and cellular consequences. Cultivations can also be performed under limiting conditions. However, under continuous flow, molecules secreted by the cells are continuously washed out restricting the range of applications. In contrast, microfluidic single-cell cultivations in fixed volumes under batch mode enable the accumulation of products and metabolites in small volumes, the transient depletion of nutrients and cellular-induced environmental changes. Such environmental setting carries great potential for microbial single-cell analysis studies in the field of, for example, starvation [33,92], cannibalism [93,94], quorum sensing [3,57] and quantitative mass balancing [38].

Pressure control

Extremophilic organisms can withstand severe environmental conditions such as high temperature, strong water activity, extreme pH, high salt concentration and high pressure [95]. Microbial life occurs at the deep sea quite frequently and piezophilic or barophilic organisms were found at 11,000 m below surface level. This depth corresponds to a pressure of 110 MPa. At laboratory conditions, these organisms required at least 50 MPa for growth [96]. High-pressure cultivations at laboratory scale require specialized equipment and numerous safety measures. In contrast, microfluidics enable relatively safe cultivations under high-pressure conditions or pressure dynamics due to small volumes and small resulting forces.

Typically, rigid material (e.g., glass), silicon or special polymers (e.g., "Ostemer® 322 Crystal Clear") are mandatory for high-pressure application in microfluidics, rather than the soft PDMS. Glass and silicon processing, however, requires elaborate cleanroom techniques and hazardous chemicals. In high-pressure chemistry, microfluidic devices made from glass were operated at extreme pressures of up to several hundred bar [97]. Martin *et al.* [98] used off-stoichiometry thiol-enes (OSTE+) in order to develop a high pressure-resistant microfluidic device, based on the work of Carlborg *et al.* [99]. They showed that the device was able to withstand pressures of at least 20 MPa of carbon dioxide flow. The fabrication method for OSTEMER polymer devices is comparable to PDMS processing.

The implementation of high-pressure cultivation in single-cell studies is of major interest for deep sea microbiology and enables laboratory investigations that have not been possible so far. Even non-barophilic organisms like *E. coli* adapt growth and morphology upon pressure changes as experimentally demonstrated by Kumar and Libchaber [100]. Furthermore, it was found that dynamic pressure conditions induce a high number of heat and cold shock proteins and heterogeneity simultaneously [101].

Time-Lapse Microscopy and Image Analysis

As unavoidable consequence of the opportunities offered by microfluidics—long-term cultivations and massive parallelization on-chip—high data volumes in the gigabyte (GB) to terabyte (TB) range per experiment are produced. Typical image sequences comprise thousands of images, each containing hundreds of single cells, rendering manual processing a hopeless endeavor. Here, clearly, computer-assisted automated approaches are required for data management and objective feature extraction. In this context, a variety of general and specific analysis tools is available. The landscape of existing solutions is as diverse as the microfluidic setups, which complicates selecting "the right" tool that is fit-for-purpose. This section gives an overview of recent developments in the field of microbial bioimage analysis, with focus on the downstream handling and processing of the acquired image data.

Image acquisition and handling

Research microscopes are usually equipped with vendor-specific software solutions for computerized control that allow for standard automation and control tasks (i.e., scheduled acquisitions). Although such solutions provide rudimentary macro scripting support to automate experiments beyond standard application, depending on the flexibility of control or peripheral device support desired, such solutions quickly reach their limits. In this situation, alternate microscopic control software is desired. Outstanding in this area is the open-source μManager project, which is continuously developed since 2007 [102,103].

Since microfluidic time-lapse experiments quickly run into hundreds of GB, sustainable data storage and accession becomes important. In particular, for the purpose of making the data accessible and reusable, additional metadata have to be captured and stored together with the primary image data. Here, the OMERO image data management platform [104] represents the state-of-the-art solution for structured storage, maintenance and remote data access [105], while also other less popular solutions exist such as BisQue [106]. Recently, the structured metadata entry method OMERO.forms [107] was developed to design metadata templates and ease the data annotation process.

In view of sharing and reusing data, care should be taken that the stored image data are accessible, ideally without relying on the use of (often costly) microscope vendor software. Nevertheless, proprietary image formats are still the rule. To facilitate data usability in open image analysis software, the Bio-Formats project was initiated [108]. The Bio-Formats library encompasses format readers for over 150 formats, many of them proprietary and vendor-specific. OMERO and Bio-Formats are developed within the consortial framework of the OME (Open Microscopy Environment, www.openmicroscopy.org), which strives to develop open specifications and tools for performant data access. Independent of the data management solution, adherence to good practices of data annotation and archiving is essential for long-term re-usability of the data.

Image analysis

Once being acquired in a microfluidic time-lapse experiment, the image data have to be analyzed to

extract biological results. This typically includes the detection and segmentation of cells and tracking their development through the time-lapse sequence. Hereby, the image analysis often follows a sequential procedure as illustrated in Fig. 3: (i) image preprocessing, such as brightness/contrast normalization, registration to remove x/y shifts in the temporal domain and cropping to a region of interest; (ii) segmentation/classification, to detect desired objects, that is, cells; (iii) tracking, to follow the single cells over time to deduce biological information about their behavior; and (iv) post-processing the readout and prepare for interpretation. Automated image analysis is a challenging part of microfluidic single-cell analysis, often becoming a severe bottleneck when robust software tools are lacking. Unfortunately, there is no one-fits-all solution adequate for all scenarios. Instead a zoo of analysis tools has emerged over the years. Depending on the investigated problem, user capabilities or requirements, analysis pipelines can be tailored using existing frameworks or available tools can be used.

Frameworks for bioimage analysis

As a framework, we define comprehensive, multifunctional software platforms that are scaffolds for the on-demand assembly of analysis workflows. Most important in the field of scientific bioimage processing is the open-source tool ImageJ, with over 10,000 citations on Google Scholar [113]. ImageJ was developed as a cross-platform Java tool first released in 1997. ImageJ provides a core set of interactive functionalities, an extensible plugin architecture and automated macro-scripting capabilities. Ten years later, the Fiji (Fiji is just ImageJ) [114] distribution of ImageJ was released, joining ImageJ with a collection of ready-to-use plugins. This bundle evolved into a versatile plugin collection, which is now the de facto ImageJ installation standard in the bioimaging field. In particular, Fiji plugins address the complete spectrum of tasks in time-lapse image analysis: preprocessing, registration (StackReg, [115]), segmentation (trainable Weka [116]) and tracking (TrackMate [117]). Using plugins, ImageJ can directly read image data from and store results back to OMERO.

Another powerful general-purpose bioimage analysis platform is Icy [118], comprising a variety of user-developed plugins (including access to ImageJ) and convenient graphical workflow development. A general biomedical image processing tool designed for biologists is CellProfiler, which contains various segmentation and processing approaches, which is popular in biomedical research [119–121]. A particular strength of CellProfiler is its large support community and continuous development, enriching the software with modern techniques, such as deep learning. CellProfiler has a computationally efficient core written in Python. Focusing solely on the segmentation task, ilastik [122] allows the user to interactively train machine learning classifiers to segment and classify image regions. To further extract useful biological metrics, ilastik can be coupled with other processing tools, such as self-built programs, ImageJ or CellProfiler. Further important general processing frameworks are the libraries

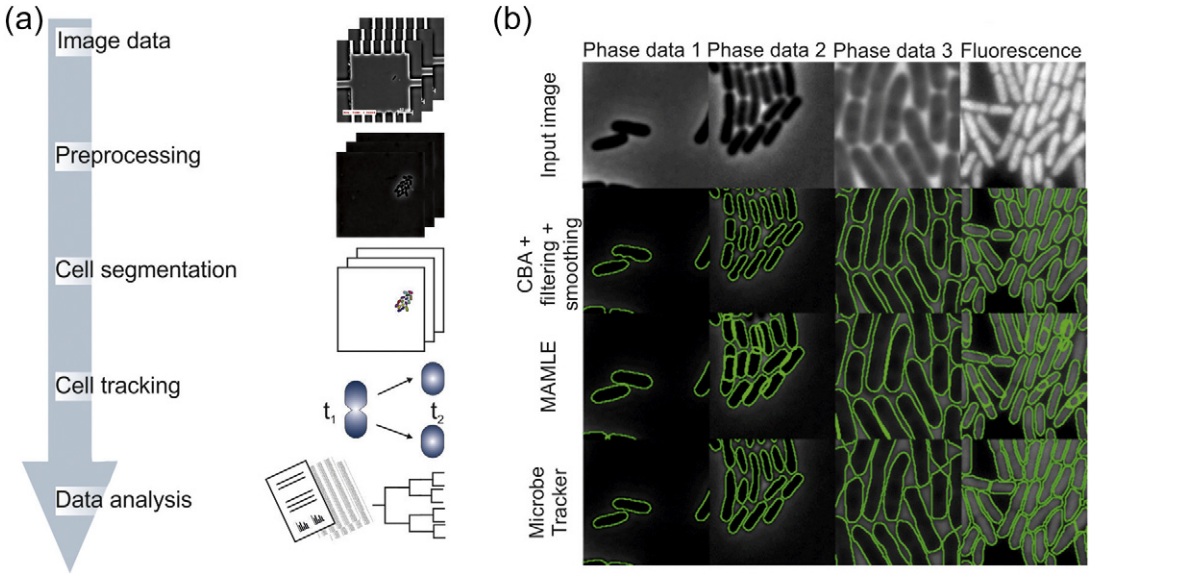

Fig. 3. General workflow of bioimage analysis. (A) Several steps are needed to extract knowledge from the images: preprocessing (quality control, denoising, registration); cell detection and segmentation; tracking of cells across frames; feature quantification and visualization (modified from Grünberger *et al.* [109]). (B) Example segmentations of rod-shaped bacteria using three different tools, CBA [110], MAMLE [111] and MicrobeTracker [112] (top down) (Copyright © IEEE. All rights reserved. Reprinted, with permission, from Sadanandan *et al.* [110]).

Table 2. Overview of ready-to-use tools for microbial time-lapse image data

Tool	Growth	Programming language	GUI	Pre-processing	Segmentation	Tracking	Result export	Remarks	Year	Citations[c]	Reference	URL
MoMA[a]	1D	Java	✓	✓	✓ (ML-based)	✓ (global)	✓ (CSV)	Joint segmentation/ tracking	2018	5	[125]	https://github.com/fjug/MoMA
Molyso	1D	Python	✗	✓	✓ (conventional)	✓ (greedy optimization)	✓ (CSV)	Aimed at high-throughput processing	2016	5	[126]	https://github.com/modsim/molyso
Schnitzcells	2D	Matlab, C++	✓	✗	✓ (conventional)	✓ (Thin-plate-spline)	✓ (Matlab MAT)	Control via Matlab commands	2012	138	[14]	http://easerver.caltech.edu/wordpress/schnitzcells/
TLM-Tracker	2D	Matlab	✓	✓	✓ (conventional, many)	✓ (overlapping boxes)	✓ (CSV, Excel)	Streamlined GUI	2012	21	[127]	http://www.tlmtracker.tu-bs.de
MicrobeTracker[b]	2D	Matlab, C++	✓	✓	✓ (conventional)	✓ (overlaying)	✓ (CSV, Excel)	Shape fitting, sub-pixel resolution	2011	271	[112]	http://www.microbetracker.org
Oufti	2D	Matlab, C++	✓	✓	✓ (conventional)	✓ (overlaying)	✓ (CSV, Excel)	MicrobeTracker successor, higher throughput	2016	67	[128]	http://www.oufti.org/
BactImAs	2D	Java	✓	✗	✓ (conventional, optimized for *M. smegmatis*)	✓	✓ (CSV, SQLite)	ImageJ plugin	2014	5	[129]	http://homer.zpr.fer.hr/BactImAS/
SuperSegger	2D	Matlab	✓	✓	✓ (conventional + ML-based merging)	✓ (greedy optimization)	✓ (Matlab MAT)		2016	26	[130]	http://mtshasta.phys.washington.edu/website/SuperSegger.php
CBA	2D	Python	✗	✗	✓ (CBA)	✗	✗	Segmentation tool	2016	12	[110]	https://bitbucket.org/sajithks/cba
CellShape	2D	Python	✓	✗	✓ (level-line based)	✗	✓ (Visualization)	For fluorescence data,	2016	1	[131]	http://wwwuser.cnb.csic.es/~synbio/?avada_portfolio = cell-shape
MicrobeJ	2D	Java	✓	✗	✓ (conventional, many to choose)	✓ (nearest neighbor)	✓ (CSV, internal visualizations)	ImageJ plugin, versatile framework-like tool	2016	84	[132]	https://www.microbej.com
BacStalk	2D	Matlab	✓	✓	✓ (conventional)	✓ (overlaying)	✓ (Visualization, Excel)	Aimed at analysis of stalks/buds	2018	–	[133]	http://drescherlab.org/bacstalk

[a] Needs a license for the LP solver Gurobi.
[b] Needs a MATLAB license.
[c] According to Web of Science (as of 04–2019).

OpenCV [123] and VIGRA ("Vision with Generic Algorithms") [124], which are attractive for developers.

Software tools for microbial time-lapse image data analysis

Besides general purpose software frameworks, countless more or less ad hoc approaches have been developed as Fiji/ImageJ plugins, Python or MATLAB applications, and new ones appear regularly. This section surveys more prominent ready-to-use analysis tools for microbial time-lapse image data for applications with rod-shaped bacteria, listed in Table 2.

The choice of analysis software is highly dependent on the cultivation modalities. Specialized software tools are available as standalone programs: For MM cultivation systems (1D growth), for example, MoMA [125] and molyso [126]. A greater variety of software exists for microbes growing in 2D microcolonies, as this is the case in many microfluidic setups or simpler approaches like agarose pads. Many approaches use MATLAB and are equipped with convenient graphical user interfaces (GUIs). Usually, the data need to be prepared precisely to the tool's specifications. Pre-processing, such as registration, background correction or cropping is often included. The core functionalities covered by most tools are segmentation and tracking, typically following conventional image processing approaches. Advances in computer vision using artificial neural networks, especially the special type of deep convolutional neuronal networks [134], currently push a new wave of bioimage analysis tools that keep promise to outperform traditional approaches that rely on hand-engineered visual features. The segmentation performance is crucial for cell tracking where most tools employ greedy approaches, that is, pairwise matching cells between consecutive frames, with the noteworthy exception of MoMA [125] being the only tool seeking a tracking solution, which is optimal across the whole sequence. Typically, results are provided in tabular formats (like CSV or Excel), while some tools offer additional domain-specific visualization capabilities.

Experienced users of microfluidics are aware of the fact that density, diversity and noise in images and the analysis procedure contain many error sources and unsupervised derived biological insights have to be treated with caution. Unfortunately, only few authors explicitly mention the limitations of their analysis tools with regard to focus loss [30] or the necessity of manual post-processing and correction wherever segmentation fails [135]. Following the lead of biomedical imaging [136], collecting and curating benchmark data sets in a community effort to encourage comparability challenges of developed tools and algorithms is desirable to improve the status quo.

At the current state, what is a practical strategy for the less experienced? As a rule of thumb, experimenters could check if specialized software packages for their task exist, whether the tools listed in this review, as found by search in publication databases or specific repositories like the Bioimage Informatics Search Engine (BISE, www.biii.eu), and try them from new to old. If the analysis errors are only small, reconfiguring and slightly extending the software might lead to satisfactory results. If no specialized software exists, the aforementioned frameworks provide experimenters with a toolbox, which can be combined and used at varying levels of automation. In general, the subtleties of image analysis should be kept in mind already in the planning phase of the experiment. Acquisition parameters should be optimized, as far as possible to facilitate the analysis processes.

Cell tracking and visualization

The final step before interpretation is tracking, that is, finding the correct association between cells in consecutive time-lapse frames, and visualization of the extracted spatio-temporal biological measures such as elongation rates. For single cultivations, these are collected in lineage trees, which can, for example, be generated using TrackMate [117]. The tracking step is particularly challenging at low imaging frequency and distinct cell movement. On the other hand, the imaging frequency needs careful adjustment regarding expected growth rate, cellular migration, imaged positions and possible phototoxicity. Therefore, an appropriate choice is always subject to trade-offs. When frame rates are insufficient, cell tracking becomes a guessing game even for experts. Theorell *et al.* [137] tackled this challenge implementing a Bayesian particle filter overcoming the need to produce a single lineage from which single-cell measure are extracted. Instead a forest of lineages is produced, from which in a statistical procedure the desired single-cell measures including the tracking-induced error bars are deduced. Although computationally intense, this approach allowed the fully automated derivation of single-cell measures for *C. glutamicum* cultivated in 2D microfluidic growth chambers. In a different approach, Hattab *et al.* [138] tackled the challenges of cell tracking by tracing cell groups, which are recognized as patches, eradicating the need to first identify single cells. In this way, spatial and temporal coherence of patches with common ancestry could be detected. The clustering method grouped particles by trajectories, fluorescence characteristics and spatial closeness, reflecting the development of different subpopulations and their mergence, while neglecting the characteristics of single-cell analysis [138].

Concluding Remarks

Microfluidic single-cell cultivation systems, accompanied with image analysis techniques, have made

enormous impact on advancing our understanding about molecular mechanisms acting in cells. Novel insights into microbial behavior and heterogeneity at the single-cell level have been demonstrated in several studies ranging from single-cell growth studies to complex microbial division of labor. In most of these investigations, very simple agarose cultivation pads with limited environmental control or basic microfluidic cultivation devices were utilized. Assessing the rich palette of microfluidic tools, which have been developed over the last two decades, it becomes clear that present microbial single-cell analysis using microfluidics is still in its infancy, yet has wide future prospects. Single-cell research will highly benefit from a more advanced, next generation of microfluidic cultivation devices. With the implementation of on-chip functionality, the cultivation environment can be controlled precisely and, if desired, in a dynamic way, accurately resembling complex natural or biotechnological habitats. However, technology integration has to be performed carefully without losing sight on practical applicability in microbiology laboratories. In the realm of image analysis, the most immediate direction to take is the consolidation, optimization and generalization of existing tools, so that their potential can be fully exploited. An automated image processing and analysis, robust toward noise, that can be operated online and in real-time is highly desirable. First encouraging approaches exist but are highly confined to specialized scenarios [125]. In this context, most noteworthy, are deep learning approaches that show high promises in becoming a standard building block of automated image analysis pipelines. Still such learning approaches require tremendous amounts of annotated data to achieve high-quality results, which currently slows down their practical implementation. Once online analysis is in place, direct image-based feedback-regulated experimentation is the logical next step. In particular dynamically controlled environments benefit industrial applications, since growth–production relations can be monitored and regulated precisely. In this review, we specialized on microfluidic single-cell cultivations and image analysis, which has proven to be a very fruitful interdisciplinary cooperation. Here, this division of labor is beneficial strategy for successful and complex fundamental research in the field of phenotypic population heterogeneity, while at the same time pushing the single-cell cultivation technology and image analysis algorithms to new boundaries.

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Abbreviations used:
FACS, fluorescence-activated cell sorting; VBNC, viable but non-culturable; PDMS, polydimethylsiloxane.

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