Microfluidic single-cell analytics

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Contents

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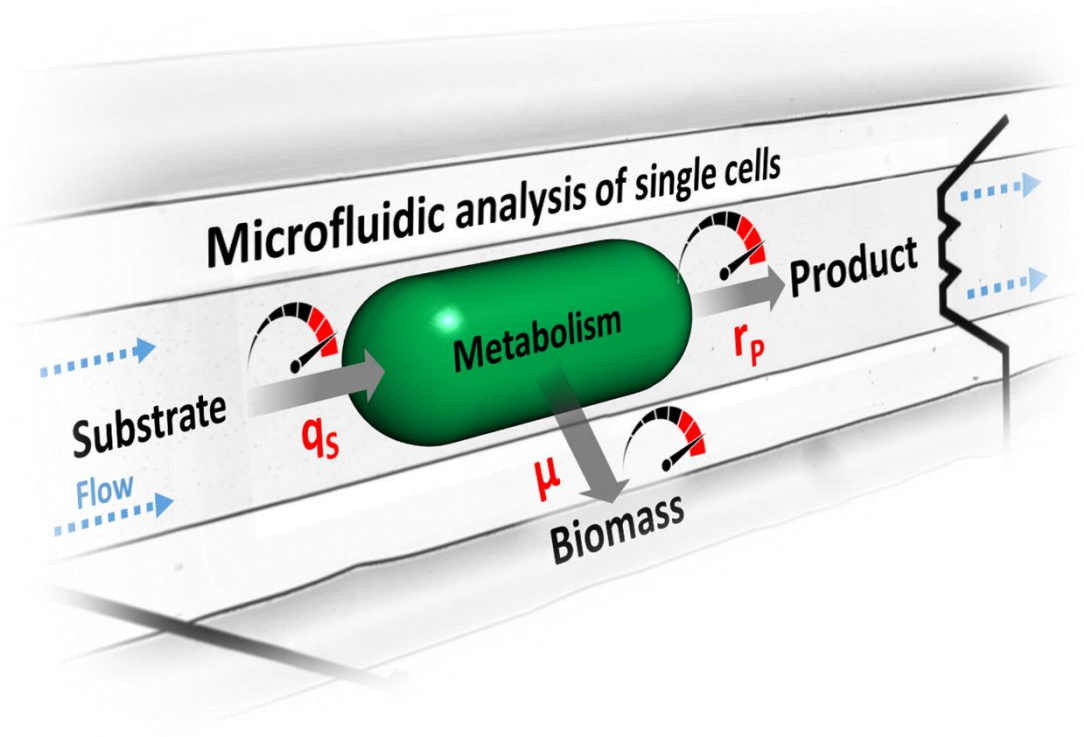


Abstract

What is the impact of cellular heterogeneity on process performance? How do individual cells contribute to averaged process productivity? Single-cell analysis is a key technology for answering such key questions of biotechnology, beyond bulky measurements with populations. The analysis of cellular individuality, its origins, and the dependency of process performance on cellular heterogeneity has tremendous potential for optimizing biotechnological processes in terms of metabolic, reaction, and process engineering. Microfluidics offer unmatched environmental control of the cellular environment and allow massively parallelized cultivation of single cells. However, the analytical accessibility to a cell´s physiology is of crucial importance for obtaining the desired information on the single-cell production phenotype. Highly sensitive analytics are required to detect and quantify the minute amounts of target analytes and small physiological changes in a single cell. For their application to biotechnological questions, single-cell analytics must evolve towards the measurement of kinetics and specific rates of the smallest catalytic unit, the single cell. In this chapter, we focus on an introduction to the latest single-cell analytics and their application for obtaining physiological parameters in a biotechnological context from single cells. We present and discuss recent advancements in single-cell analytics that enable the analysis of cell-specific growth, uptake, and production kinetics, as well as the gene expression and regulatory mechanisms at a single-cell level.

Introduction

Cells are used as living catalysts for the efficient production of chemicals and energy carriers [1,2]. In whole-cell biocatalysis, the efficiency of the catalytic conversion of a substrate to the desired product is a result of cell physiology [3,4]. At the population level, the performance of microbial biocatalysts is typically determined by analyzing kinetic parameters in physiological key experiments, where substrate uptake and production rates are used to quantify cell physiology in terms of growth rate, production, gene expression, and regulation (see Figure 1) [5,6].

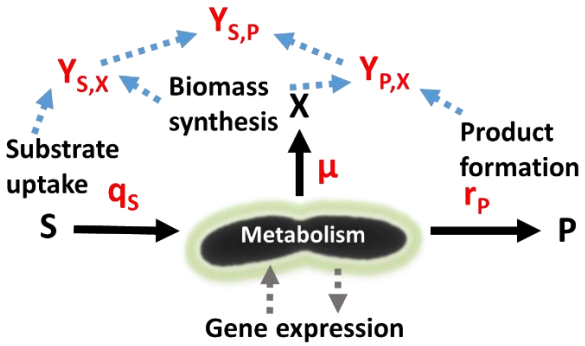


Figure 1: Quantitative physiology and performance characterization of whole-cell biocatalysts in biotechnology based on cell kinetics and yield coefficients.

With advanced analytical concepts, such as transcriptome and proteome profiling, as well as metabolic flux analysis, these links can be further refined to obtain a global picture of biocatalyst physiology and to establish a systems-level understanding of the functioning of living cells that serve as catalytic units [7-9]. This holistic approach for the analytical dissection of producer cell physiology is often termed systems biotechnology. This systems-level approach relies, however, on averaged data from populations and does not consider the individual cell dynamics and heterogeneities due to the lack of true quantitative data on the physiology of single cells [10]. It is therefore of utmost interest to make use of the latest analytical concepts to bring single-cell analysis to the next level for realizing single cell-based system biotechnology.

The current situation underlines the increasing discrepancy between the rapid evolution of microfluidic cultivation and the lack of analytical concepts for microfluidics and single cells [11]. However, this is not without reason – single-cell analysis poses tremendous analytical challenges in terms of dimension, analyte amounts, and resulting concentrations. These challenges can be exemplified with the corresponding numbers in terms of cell size, volume, and single-cell specific uptake rates and productivities, as well as product and biomass yield coefficients of microbial biocatalysts (see Figure 2).

Cell	Diameter	Volume	Wet weight	Dry weight	μ_{max}	q_S	r_P
 *Escherichia coli*	2.5 x 0.8 µm	1-2 fL	0.6-1 pg	0.2-0.3 pg	0.1-0.77 h^{-1}	1-10 fmol $cell^{-1}$ h^{-1}	0.1-5 fmol $cell^{-1}$ h^{-1}
 *Synechocystis* sp. PCC6803	2-3 µm	5-15 fL	12-15 pg	4-5 pg	0.01-0.13 h^{-1}	150 fmol CO_2 $cell^{-1}$ h^{-1}	0.01-0.3 fmol $cell^{-1}$ h^{-1}
 *Saccharomyces cervisiae*	3-6 µm	20-100 fL	80-100 pg	25-35 pg	0.05-0.45 h^{-1}	1-10 fmol $cell^{-1}$ h^{-1}	0.01-0.3 fmol $cell^{-1}$ h^{-1}

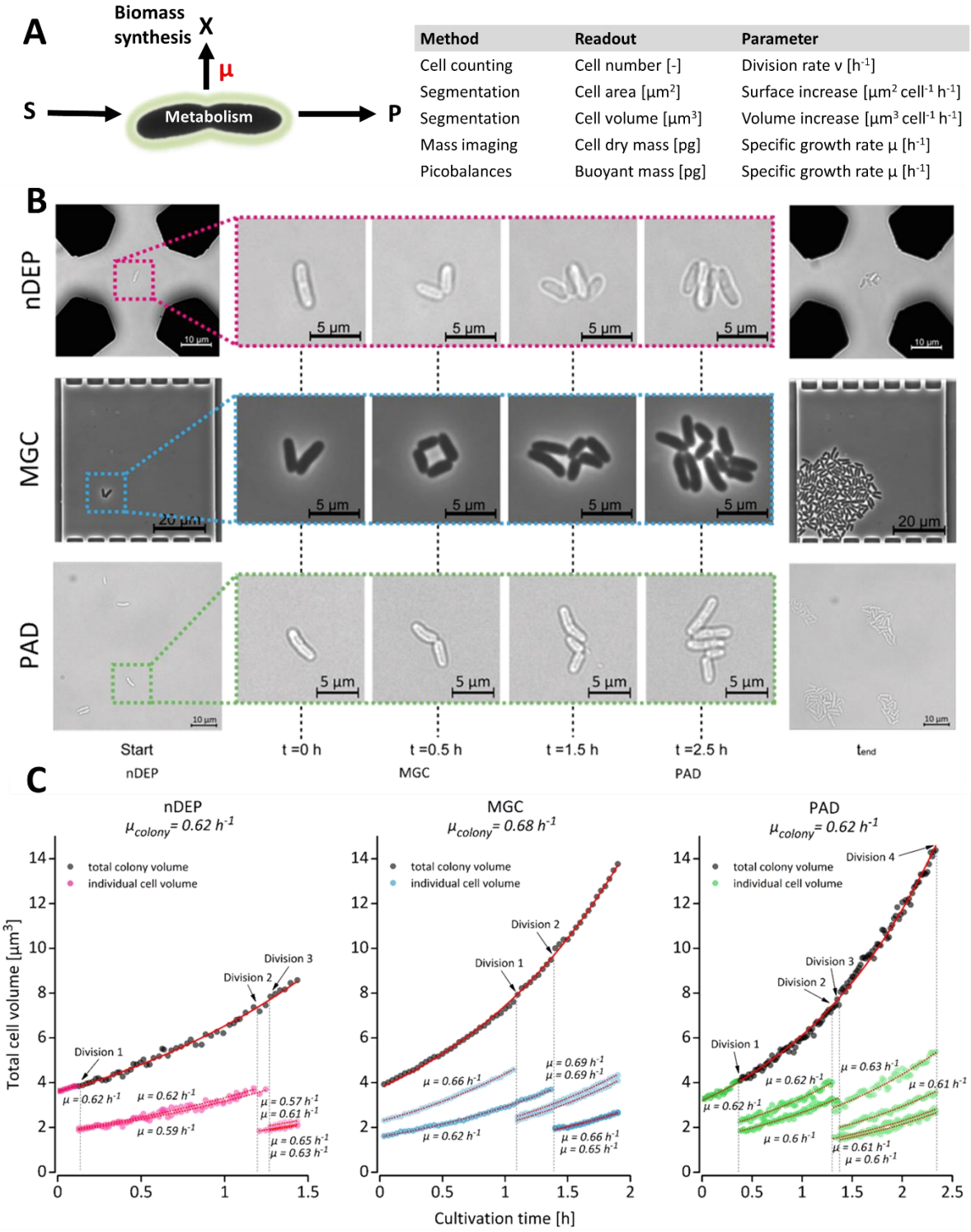
Figure 2: Key numbers for analyzing the physiology of whole-cell biocatalysts at the single-cell level. The numbers are given for cell diameter, cell volume, cell wet weight, cell dry weight, specific growth rates µ, specific substrate uptake rate q_S, and product formation rates r_P of *Escherichia coli*, *Synechocystis* sp. PCC6803 and *Saccharomyces cerevisiae* [12-18].

As can be deduced from these key figures, the analytical and conceptual challenges for quantitatively analyzing these biotechnologically relevant parameters at a single-cell level are tremendous [13,10,19]. This is why, microscopic technologies, such as time-lapse microscopy in combination fluorescent markers or biosensors, are still the analytical tool of choice for obtaining quantitative and time-resolved data of single cells. Optical analytics and imaging technologies are relatively simple to use and can be sensitive down to the single-molecule level with technologies such as super-resolution microscopy [20,21]. Even with standard microscopy equipment, smaller cell types, such as coccoid bacteria, can be easily visualized. Cell dynamics and heterogeneities in terms of growth, morphology, gene expression, or regulation can be assessed via fluorescence time-lapse microscopy imaging [22-25]. However, obtaining truly quantitative data with absolute numbers is still difficult via imaging. Even simple global physiological parameters, such as specific growth rates and biomass cannot be easily deduced from microscopy, although growth is one the key parameters when it comes to the characterization of cells in technical processes [26-29]. Yet, data on growth kinetics and biomass formation provide the basis for the holistic description of whole-cell biocatalysts at a single-cell level. This lack of analytical concepts for assessing producer cells at the microscale is the reason why microfluidic single-cell analysis is given little consideration in biotechnology [11]. Rendering microfluidic analysis more meaningful for biotechnology hence starts with making growth kinetics available at the single-cell level. In the following section, we describe fundamental technologies beyond visualization that enable us to quantitatively assess growth and biomass with high accuracy of single microbes in microfluidic bioreactors.

Growth analysis of single cells

Cell growth is a pivotal descriptor for global cell physiology. The kinetics of biomass formation, the specific growth rate µ, provides information about cellular fitness and the functional state of the cell. Many cellular parameters, such as plasmid copy number, mRNA and ribosome abundance, protein

synthesis, and hence cell productivity are tightly linked to the specific growth rate of a cell [30-32]. In steady-state growth, the environmental influences are directly manifested in the growth rate itself and directly reflect the impact of extracellular conditions on the cellular machinery and its efficiency for performing catalytic conversions [33]. The power of single-cell growth analysis for answering questions of biotechnological relevance has been demonstrated in countless studies. These studies investigated fundamental characteristics of microbial growth that are indispensable for optimizing cell performance and efficiency in a technical context. The topics investigated encompass compensation auxotrophy in mixed-species microcultures [34], cell aging in yeast [35] and bacteria [36], linking growth rate and extracellular environment [37], linking growth kinetics and gene expression [38] growth dynamics upon nutrient shifts [39] and the impact of spatial confinement in cell growth [40]. In nature, heterogeneity in growth across an isogenic population is a simple, but most effective measure to cope with environmental changes or threatening conditions such as the presence of antibiotics [41-46]. Determining specific growth rates of single cells, its dynamics, and heterogeneities is hence of utmost interest for understanding the physiological structure of a productive population under distinct growth or process conditions (see Figure 3).



Method	Readout	Parameter
Cell counting	Cell number [-]	Division rate ν [h^{-1}]
Segmentation	Cell area [μm^2]	Surface increase [μm^2 $cell^{-1}$ h^{-1}]
Segmentation	Cell volume [μm^3]	Volume increase [μm^3 $cell^{-1}$ h^{-1}]
Mass imaging	Cell dry mass [pg]	Specific growth rate μ [h^{-1}]
Picobalances	Buoyant mass [pg]	Specific growth rate μ [h^{-1}]

Figure 3: A) Analytical methods, readouts, and deducible kinetic parameters for growth rate analysis at a single-cell level. **B)** Time-lapse imaging of single *C. glutamicum* cells grown in different microfluidic cultivation systems. **C)** Image-based analysis of single cell-specific volumetric growth rates in the different microfluidic cultivation systems [47] - Published by The Royal Society of Chemistry .

For determining growth at the level of populations, the experimenter can rely on numerous standardized methods, such as optical density measurements, weighing of wet and dry biomass, manual or automated cell counting with a microscope, a flow cytometer, or Coulter counter devices

[29,48,49]. With knowledge on cell or biomass concentration, important performance descriptors such as uptake and synthesis rates can be specified and normalized to the respective concentration measure, allowing for an absolute and laboratory-independent evaluation of the catalytic efficiency of populations [28]. As can be inferred from this information, proper analytics for growth and mass profiling are a basal prerequisite for characterizing single cells in a biotechnology context. In the following, we will discuss current state-of-the-art analytics for single-cell growth and biomass analysis and review cutting-edge methods that hold the potential for becoming standard methods for the precise determination of cell wet and dry weights in future.

Cell counting, morphometrics, and segmentation

Microscopy is the simplest, but also one of the most powerful methods for analyzing single cells and their physiology. Quantitative morphometric analyses of single microbes have been established more than a century ago, but almost vanished during the last decades [50-53]. It was only with the introduction of automated time-lapse microscopy and powerful image processing routines that made microscopy the measure of choice for single-cell analysis. Enabled by narrow microfluidic structures that force the cells to grow in monolayers or even as separated single cells, a large number of cells can optically be analyzed without artifacts arising from cell overlapping [54,36,55]. Arguably, the simplest measure to determine cellular growth kinetics at the microscale is cell counting [56-58]. Recording cell-division kinetics enables the experimenter to normalize physiological parameters, such as induction or adaption kinetics, to obtain cell-specific values [24]. Tracking cell divisions in bacteria can be performed with the bare eye and represents an excellent measure to compare division kinetics at the microscale with the increase in cell numbers of a population [59,60]. Only such comparative studies reveal environmental effects on growth that remain hidden at a population scale. In a significant exemplary study, Unthan *et al.* used manual cell counting in micropopulations of *Corynebacterium glutamicum* cells and revealed that the cells divided much faster in the microfluidic perfusion environment as compared to division kinetics of populations, although the same defined growth medium was used [26]. This observation was the kick-off for a systems-level study that disclosed the reasons for the observed elevated growth rates at the microscale. Based on single cell-cultivations, bioreactor experiments at extremely low cell densities, as well as transcriptomics, metabolomics, and integrative *in silico* analysis, it was disclosed that protocatechuic acid was utilized as a hidden co-substrate that drove *C. glutamicum* cells to higher specific growth rates than ever observed before in minimal CGXII medium [26]. Cell counting can also be used to assess single-cell growth of more uncommon cell types. Helingwerf *et al.* applied cell counting of phototrophic *Synechocystis* sp. PCC6803 for determining growth in massively parallelized droplet cultivations. In conjunction with an enzyme-based assay for the quantification of lactate in the individual droplets, an enrichment of high-producing lactate forming strains could be realized [61].

Determining growth kinetics via cell counting assumes that all observed cells are similar in length and volume [62]. While this is often true for balanced growth under steady-state conditions, environmental fluctuations and stress in production setups often entail a diversification in cell size, which has to be considered when calculating growth kinetics from cell counts [63,64]. For many investigations, such as *in silico* models of growth in populations based on single-cell data or analyses that focus on growth kinetics in between two cell divisions, the need for data on growth kinetics of individual cells arises. Here, morphometric analysis is the measure of choice. Quantitative measurements of cell dimensions, such as cell area, length volumes are more precise than cell counting and can be better compared to optical density measurements or cell dry matter determination performed at the population scale. Following the dynamics of individual cell geometry, such as the cell projection area or cell volumes calculated from cell dimension, enable us to quantify the growth of individual cells, even between two cell divisions or budding events [65]. Moreover, this type of image-based growth analysis can also account for cell proliferation mechanisms other than binary fission, such as budding in yeast or asymmetric cell division [62]. The most straightforward approach to determine the growth of individual cells between division or budding events is to measure the area and volume of cells via manual segmentation from microscopic images. Several studies demonstrated the applicability and use of this method for determining specific growth rates and their heterogeneities of single microbes and comparing the obtained values to populations [27,37,66]. With the assumption that the density of the cells remains constant at balanced growth, the cell volume is a suitable proxy for cell mass and can be directly compared to cell dry matter concentrations in lab-scale cultivations. Manual cell volume approximation has been shown to deliver solid results with several distinct microfluidic bioreactor concepts such as microfluidic monolayer growth chambers and cell traps based on negative dielectrophoresis [27,66,37]. A striking insight of microfluidic growth analyses was that the volumetric growth rates of single cells consistently exceeded population growth rates by up to 50% and demonstrated the biological potential in terms of maximal possible growth rates [37]. Realizing such high growth rates at the bulk scale might improve biocatalyst formation and averaged volumetric productivities in bioprocesses. Morphometric analyses revealed that division rates, division angles, and division symmetry of cells were influenced by the specific microfluidic habitat. These results suggest a careful choice of the microfluidic cultivation format to avoid artifacts stemming from the respective microenvironments. One of the most striking studies of the past years on single-cell growth revealed the basic laws of bacterial size control in *Escherichia coli* [67]. Taheri-Araghi *et al.* monitored the cell length during the cell cycle of individual *E. coli* cells upon the shift of growth media. Based on the imaged cell length, the authors calculated cell volumes and found that the average cell volume scales exponentially with DNA replication and growth rate. However, in such high-throughput growth

experiments, manual determination of cell dimension is virtually impossible and demands automated cell segmentation algorithms.

Manual image processing is tedious and time-consuming. When the number of observed cells is high or cell reproduction has to be tracked in colonies of hundreds of single cells, manual cell counting is not a viable option anymore. With advanced image processing algorithms, automated cell segmentation can be conveniently performed at high-throughput [63,64]. However, due to the huge variety of microbial morphology within isogenic populations and across different microbial strains, error rates of segmentation algorithms can be high and require extensive adaption of the segmentation codes to the strain of interest [68,69]. Next to morphological challenges, segmentation algorithms have to robust against poor image quality, out of focus images, overlapping of neighboring and noise [68].

Available image segmentation algorithms such as Schnitzcells, Oufti, or MicrobeTracker are optimized for tracking certain types of microbes [70-72]. It is not of surprise that the detection of cell boundaries and the corresponding morphological traits has been adapted to rod-shaped bacteria such as *E. coli, C. glutamicum, Bacillus subtilis, or Pseudomonas sp.* and other commonly used model strains [70,22]. As the natural variety of cell morphology is overwhelming, the growth of many uncommon microorganisms cannot be quantified out-of-the-box with available software packages. However, recent image analysis algorithms such as Oufti are tackling this problem and offer extensive customization options to segment cells with uncommon or even irregular shapes [73,74]. Oufti allows the quantification of various cell morphologies, irregular shapes, and even the identification of individual cells that form confluent monolayers by using powerful and flexible segmentation algorithms for high-content imaging. The algorithm includes for example mathematical routines for the identification of differential growth behavior among single cells such significantly slower or faster growth of cells. MicrobeJ is another recent image-processing framework for extracting grey-value, cell dimension, and morphological routines, as well as subcellular analysis of fluorescence localization from microscopy images [75]. A powerful code for data integrity validation has been integrated as well. Among the wealth of image analysis algorithms, highly customized solutions exist as well. An important example constitutes the tool Molyso, which has been specifically developed to extract growth data from mother machine time-lapse [76].

In general, this is only a small excerpt from the many image analysis tools available. It is recommended to cross-check the available tools for specific scientific strains, scientific questions, and experimental setups. The above-described algorithms pose universally applicable tools that enable automated high-throughput analyses of single-cell traits from images and are invaluable for processing the massive data amounts from time-lapse experiments. However, automated image analysis algorithms are error-

prone and supervision of segmentation results is still indispensable. As a possible remedy, deep-learning and AI-based algorithms might allow higher accuracy and handling of challenging image sets for determining growth kinetics at the single-cell level.

Mass imaging

Mass imaging has the potential to become the next evolutionary stage in single-cell growth analysis [11]. In contrast to the extraction of spatial data from images for growth analysis, novel phase imaging concepts promise the fast, accurate, and non-invasive optical profiling of single-cell dry weights with sub-pg resolution [77-80]. Tracking growth at such resolution is the only measure to accurately analyze specific growth rates, as the growth rate is defined as the mass increase in a given biological system over time. Time-resolved data on cell mass enables us to directly normalize physiological parameters to single-cell dry matter and render them comparable to population data by that [62]. Mass imaging is based on interferometry and quantitative phase microscopy. By measuring the phase shift of light that passes a cell, the refractive index of the cell can be determined and related to cell mass. While mass imaging has been extensively applied to profile growth and density of mammalian cells, corresponding examples for bacteria or yeast are rare [81-83]. Nevertheless, mass imaging enabled profiling mass and calculating specific growth rates of individual *E. coli* cells [84]. In this study, significant heterogeneity in terms of cell mass increase was observed and demonstrated for the first time the contribution of individual bacteria growth to the macroscopic growth of populations. Two studies performed density mapping of individual *E. coli* cells with a lateral resolution of 90 nm by integrating super resolution microscopy and phase microscopy for [85] or visualized subcellular structure via tomography [86]. As can be seen, mass imaging is not widespread in microfluidic single-cell analysis for biotechnology, despite its huge potential for unraveling process-relevant growth mechanisms and heterogeneities at the smallest possible scale. Nonetheless, we are convinced that this will change in the future, as mass imaging technologies are universally applicable, compatible with other modalities such as fluorescence microscopy and require merely the upgrade of a time-lapse microscope with a simple camera. By now, many companies offer commercialized calibration-free mass imaging solutions that are sufficiently sensitive and accurate to profile mass and growth kinetics at the single microbe-level.

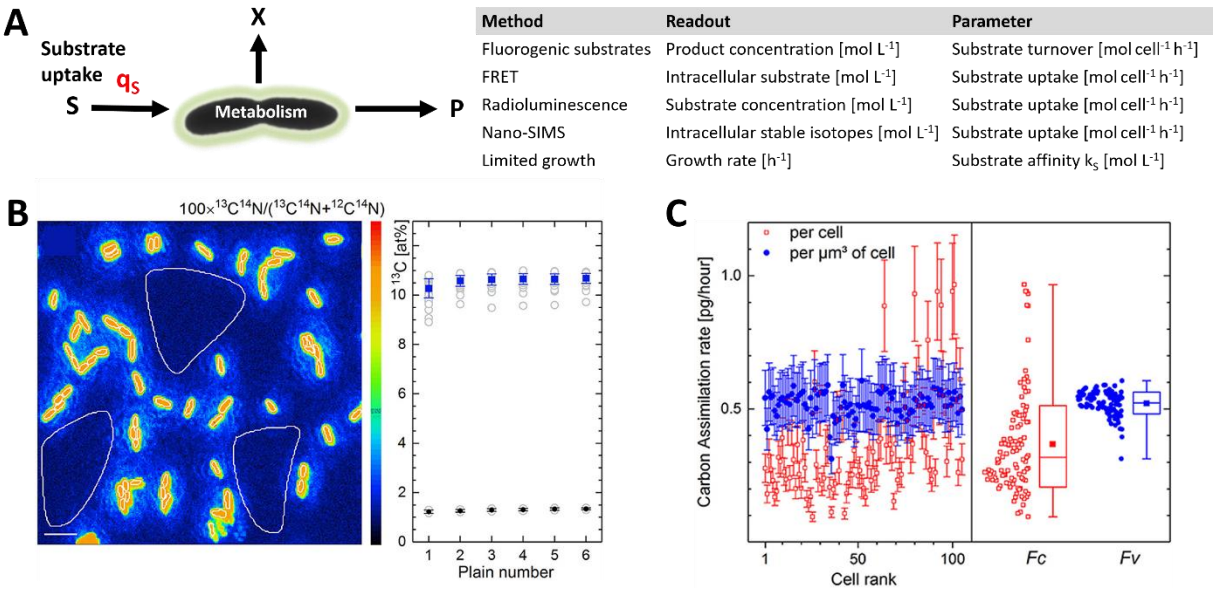
Picobalances

Microfluidic resonators based on dynamic cantilevers can be used as picobalances for weighing single cells and enables us to measure the buoyant mass of a cell with extreme precision [87,88]. Sophisticated resonator designs exist that swing in a vacuum and can be perfused with cell suspension [89,90]. The passing cells influence the cantilever resonance frequency and allow to measure cell mass with a resolution in the low fg-range, which makes the analytical concepts applicable to even the smallest know types of microbial cells [91]. Microfluidic resonators have been applied for the detection of single *E. coli* already two centuries ago [92]. As the cells are suspended, this microfluidic resonator

enables the investigation of physicochemical perturbations on cell mass and growth [93,94]. Due to the high precision, correlations between growth rate and cell mass could be revealed [89]. A comparison between mass and volume growth kinetics in single yeast cells was achieved by combining a Coulter Counter with a microfluidic resonator [95]. Mass and density profiles of viable, stressed, and dead *E. coli* cells could be investigated with resonator structures at high throughput for the first time and demonstrated that dead cells have a larger density, but a lower cell mass [96]. Suspended microfluidic resonators were used to determine the weight of single marine bacteria and these results from the microscale were used to estimate the total amount of marine biomass on earth for calculating global oceanic carbon fluxes [93]. The examples demonstrate the usefulness of picobalances for fundamental research. However, questions of biotechnological interest have not been approached with picobalances yet. This is mostly due to the complicated and extensive microfabrication that is needed. Moreover, combining resonators with other imaging techniques might be difficult. It can be stated that microfluidic resonators are well suited for high precision measurements of single-cell mass, but always involves a trade-off between the time-period of tracking and the number of cells that can be tracked.

Substrate Uptake

Next to growth, the kinetics of substrate utilization, namely the specific substrate uptake rate q_S, is of utmost interest when analyzing the performance of whole-cell biocatalysts. Bioprocesses are often controlled by limited substrate feeding to prevent the formation of bioproducts or limit growth [97-100]. Moreover, specific yield coefficients of biomass on the substrate can be calculated with on q_S, and the specific growth rate μ. Measuring specific substrate uptake is therefore of mandatory to identify the efficiencies of substrate to biomass and product conversions and its heterogeneities at a single-cell level (see Figure 4).



Method	Readout	Parameter
Fluorogenic substrates	Product concentration [mol L^{-1}]	Substrate turnover [mol $cell^{-1}$ h^{-1}]
FRET	Intracellular substrate [mol L^{-1}]	Substrate uptake [mol $cell^{-1}$ h^{-1}]
Radioluminescence	Substrate concentration [mol L^{-1}]	Substrate uptake [mol $cell^{-1}$ h^{-1}]
Nano-SIMS	Intracellular stable isotopes [mol L^{-1}]	Substrate uptake [mol $cell^{-1}$ h^{-1}]
Limited growth	Growth rate [h^{-1}]	Substrate affinity k_s [mol L^{-1}]

Figure 4: A) Analytical methods, readouts, and deducible kinetic parameters for substrate uptake rates at a single-cell level. **B)** NanoSIMS-based chemical microscopy of carbon and nitrogen stable isotope ratios in single cells . **C)** Cell-volume- and cell-specific carbon uptake rates of single *P. putida* cells calculated from isotope ratios [101].

Fluorescence analysis

To date, quantitative studies of substrate uptake in single cells mostly rely on specific labels, such as fluorescence, stable isotopes, or radiolabels [102-105]. Label-free analytical concepts for measuring uptake in single cells comprise genetically encoded fluorescence biosensors, mostly basing on intracellular and extracellular substrate-sensitive Förster resonance electron transfer (FRET) probes, transcriptional reporters or specific binding motifs of fluorescent proteins have been utilized to study substrate uptake [106-109]. However, most of the published studies on substrate uptake in single microbes can be found in the field of environmental microbiology for characterizing carbon, phosphorous, and nitrogen assimilation processes in natural environments [110,101,111]. Studies focusing specifically on single-cell substrate uptake for tackling biotechnological questions are still rare. Nevertheless, promising analytical concepts have been developed and will be discussed in the following.

One of the simplest concepts for following substrate uptake in live single cells is to use fluorescently labeled substrate conjugates. Hehemann *et al.* used fluorescent glycan-conjugates to image and quantify its uptake in intestinal bacteria [103]. Another study by Straeuber *et al.* used N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino (NBD)-labelled toluene to visualize its incorporation into different live *Pseudomonas* strains and *E. coli* [112]. However, the uptake data obtained with such modified substrates have to be carefully evaluated, as the chemical changes might lead to significant differences in transmembrane transport kinetics V_{max} and K_S in comparison to the unmodified compounds. Natarajan *et al.* demonstrated a noteworthy concept for quantifying glucose uptake in single *E. coli*

cells [113,114]. By exploiting the competitive inhibition of the fluorescent glucose analog 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) on glucose uptake, the authors used flow cytometry measure specific uptake rates in single cells from populations grown in chemostat cultures. This elegant concept for quantifying cell-specific substrate uptake rates shows that it is not compulsory to label the substrate of interest but exploit the competition of a fluorescent analog at the transport porin. However, following the temporal dynamics of glucose incorporation in specific single cells is not possible with this method due to the snapshot character of flow cytometer analyses. It rather exploits the characteristics of the uptake mechanism and its kinetics for indirectly determining glucose uptake. Due to the need for fast medium exchange and recording of fluorescence increase, this concept has never been transferred to microfluidic cultivations.

Next to fluorescence labeling, quantifying the intracellular accumulation of radio-labeled ^{18}F-fluorodeoxyglucose is a common method to approximate glucose uptake in single cells [102,104,115]. Using radioluminescence microscopy, which can be integrated into common light microscopes, multimodal analysis of single-cell substrate incorporation and other physiological parameters such as growth is possible.

FRET-based sensors, exploiting conformational changes or energetic interactions of two fluorescent proteins by binding of a substrate molecule, are useful for monitoring intracellular substrate concentrations in individual cells [116-118]. The uptake of several different hydrocarbons by living microbes could be realized with genetically-encoded FRET-probes. A highly responsive CFP/YFP-based FRET sensor was demonstrated to facilitate the visualization of intracellular glucose accumulation in *Saccharomyces cerevisiae* uptake mutants [119]. Based on a similar FRET design, maltose uptake in single *S. cerevisiae* cells could be quantified [108]. Visualization of arabinose and maltose influx was also demonstrated for single *E. coli* cells [120]. Purified FRET-probes can be also used *ex vivo*. Purified FRET-based glucose biosensors could be applied to measure extracellular glucose concentrations in an *E. coli* culture [107]. Although this concept has not been implemented in microfluidic cultivation devices, it has the potential to enable substrate measurements in microbioreactors in the future.

Other genetic elements, such as transcriptional regulators can be exploited as indirect reporters for the capacity of single cells to process and take up nutrients. Such transcriptional reporters were applied to control GFP expression in *E. coli* cultures [109]. The obtained results indicated the heterogeneous expression of genes involved in glucose uptake.

Mass spectrometry

Label-free mass spectrometry-based methods such as NanoSIMS can be used to study the assimilation of isotope-labeled substrates into microbial cells. Nikolic *et al.* applied ^{13}C- and ^{2}H-labelled glucose to characterize glucose uptake in a clonal *E. coli* population via Nano-SIMS analysis [121]. This method

enabled to identify the magnitude of metabolic heterogeneity in terms of glucose uptake in the cultures, Glucose uptake rates did not correlate with gene expression profiles. Furthermore, the experimental results suggest a metabolic specialization of subpopulations in terms of sugar metabolism. The applications of NanoSIMS are manifold in single-cell analysis and allows assessing phenomena that are not analytically accessible otherwise. In mixed-species systems, NanoSIMS could be used to the nutrient transfer between fungi and bacteria [122]. Despite its sensitivity and spatial resolution, it is difficult to obtain quantitative data on cell-specific uptake kinetics [123]. Nevertheless, Stryhanyuk *et al.* succeeded in determining cell-specific glucose uptake rates of *Pseudomonas* cells based on a comprehensive mathematical framework [101]. Unfortunately, SIMS analysis destroys the cell during analysis and does not enable to follow individual cell dynamics in glucose uptake.

Inferring kinetic constants of substrate uptake

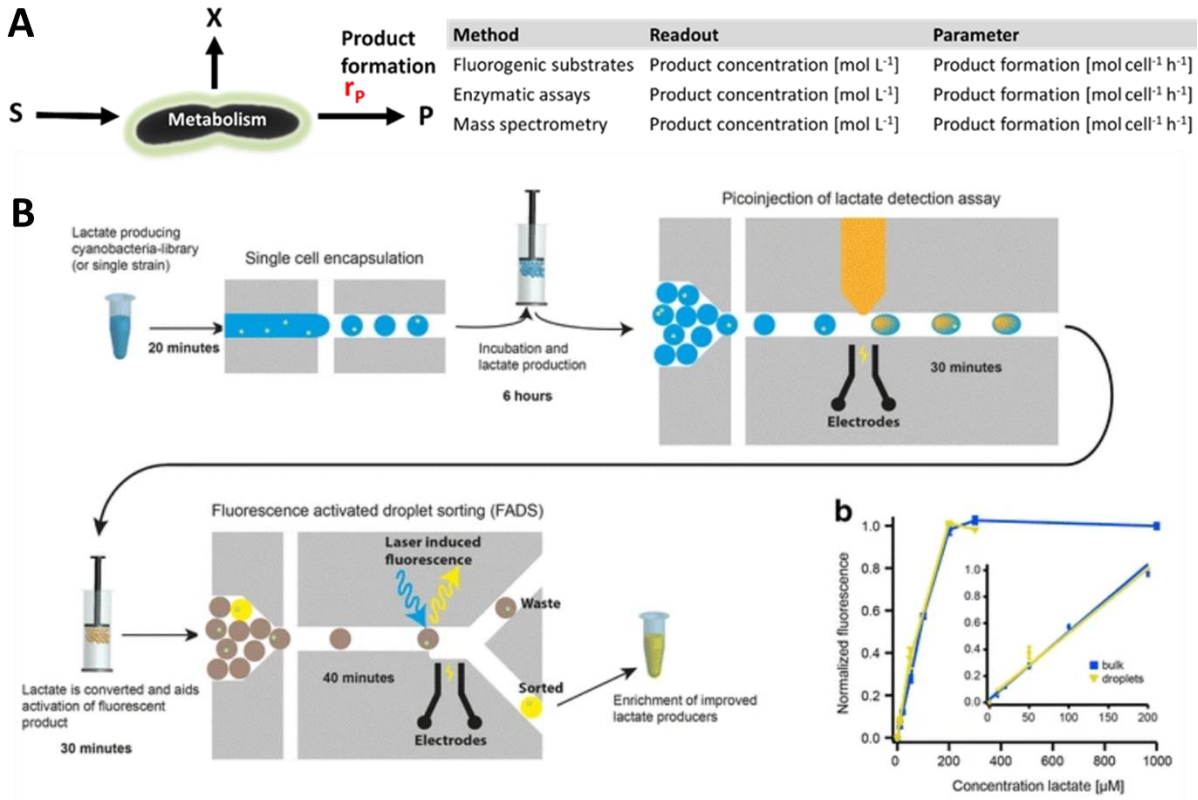
Kinetic parameters such as the affinity of transporter enzymes towards the substrate govern the cellular uptake. To date, there is only a little knowledge about whole-cell kinetics and its heterogeneity. A notable example that demonstrates how substrate affinities of microbial cells could be determined in microfluidics was recently presented by Lindemann *et al.* [124]. The authors applied carbon-limiting conditions in perfusion microfluidics and quantified growth of *C. glutamicum* cells and its heterogeneity in response to the extracellular substrate availability. At extremely limiting carbon conditions, it was found that the variability in cell-specific division times increased significantly. These results suggest a strong individuality among isogeneic microbes in terms of glucose uptake and metabolization. Moreover, the authors could approximate K_S values from single-cell cultivations for the first time.

The presented studies impressively demonstrate the significant advancements of analytical technologies for quantifying substrate uptake at a single-cell level. It must be stated, however, that there is no universally applicable analytical concept available for quantifying substrate utilization in single cells. Rather, it has to be decided depending on the biological questions which the analytical method can be applied to for obtaining meaningful data. For the future, advances in single-cell mass spectrometry might deliver remedies for the current situation and enable us to determine substrate concentrations in microfluidic bioreactors and cell-specific uptake rates with high accuracy.

Product formation

The efficiency and kinetics of product formation are the most important performance parameters in bioprocesses. Maximizing the specific product formation rate r_P of the whole-cell biocatalyst is the central goal of strain and process engineering endeavors (see Figure 5). But what is the effect of physiological heterogeneity and cell dynamics with regards to individual product formation on the performance of a process? To date, many indications exist that suggest a significant influence of

phenotypic heterogeneity on the productivity of a process. Understanding the activity structure of population-based on single cell-specific performance analyses is important and might lead to novel engineering targets for process improvement in the future [125].



Method	Readout	Parameter
Fluorogenic substrates	Product concentration [mol L^{-1}]	Product formation [mol $cell^{-1}$ h^{-1}]
Enzymatic assays	Product concentration [mol L^{-1}]	Product formation [mol $cell^{-1}$ h^{-1}]
Mass spectrometry	Product concentration [mol L^{-1}]	Product formation [mol $cell^{-1}$ h^{-1}]

Figure 5: A) Analytical methods, readouts, and deducible kinetic parameters for product formation rates at a single-cell level. **B)** Quantifying lactate production by phototrophic cyanobacteria cultivated in microfluidic droplets via a pico-injected enzymatic assay [61].

Fluorescence analysis

Analytical concepts have been developed to analyze productivity in microfluidic cultivation experiments. As extreme sensitivity is required to accomplish quantitative product analysis at a single cell-level, optical methods are dominating and often the measure of choice [12]. A recently published key study impressively demonstrated the massive effect of phenotypic heterogeneity on the output of a productive process [126]. Xiao *et al.* applied fluorescence staining to visualize and quantify free fatty acid and tyrosine production in isogenic *E. coli* mutants. Their investigations confirmed the existence of high and low producer cell variants and showed that only 15% of the cell population was responsible for more than 50% of product formation. Based on this knowledge, a molecular population control strategy was developed and implemented, which led to a significantly enhanced productivity of the controlled populations. However, simple fluorescence staining methods cannot be used to cover the wealth of microbial products.

Another prevalent method to determine productivities at a single-cell level is the application of fluorogenic substrates [127]. A wealth of non-fluorescent substrate compounds exist that turn fluorescent upon microbial conversion. The class of fluorogenic substrates is restricted to the detection of hydrolytic activity, by amylases, cellulases, xylanases, lysozyme, and phosphatases. One of the most widely applied and most sensitive substances of this class are fluorogenic β-galactosidase substrates such as fluorescein di-β-D-galactopyranoside (FDG).

Next to the application of fluorogenic substrates, relative intracellular product levels can also be visualized by exploiting titratable regulatory circuits that control the expression of a fluorescent indicator gene in response to metabolite abundance. A prominent example was demonstrated by Binder *et al.* who coupled the concentration-dependent expression of fluorescent genes to transcription factors in *E. coli* and *C. glutamicum* [128]. Based on this concept, transcription factor-based product sensors for several different amino acids were established and validated. In a follow-up study, t l-lysine, l-arginine, and l-histidine sensors were applied for rerouting metabolic fluxes towards the desired products in *C. glutamicum* [129]. Although this approach does not allow determining absolute cytosolic product concentrations or even production at a single-cell level, it is a valuable tool for strain improvement and mutant-screening using microfluidics. In the future, such intracellular sensors might be calibrated to determine absolute cytosolic product concentrations or even product formation kinetics.

Some examples also demonstrate the direct visualization of cell products via antibody assays in microfluidic cultivations. In pioneering studies, Love and coworkers implemented microarray technology to microfluidics to quantify secreted protein of single or few *Pichia pastoris* cells cultivated in nanowells [130,131]. For protein quantification, a glass slide was functionalized with an antibody specific to a human FC fragment and was bonded to the nanowell array. After a specific incubation time, the glass slide was removed and bound protein was quantified via fluorescence microscopy. With this concept, the authors were able to determine volumetric protein secretion rates in the nanowells. Based on this technology, the authors could disclose a stochastic protein secretion in single yeast cells [130]. A comprehensive follow-up study revealed that the secretory capacity of single yeast cells is the productivity-determining bottleneck in the production of heterologous protein in *P. pastoris* [131].

It was also demonstrated that enzymatic assays can be used in microfluidic cultivation devices for product quantification. Hammar *et al.* performed the on-chip analysis of lactate production by phototrophic *Synechocystis* sp. PCC6803 cells cultivated in microdroplets [61]. The produced lactate served as a substrate for a subsequent enzymatic reaction that yielded a fluorescent product. The enzyme assay solution was pico-injected into the microdroplets after cell incubation. Based on the intensity of the fluorescent signal, the droplets were sorted and subcultivation to yield a population

with improved lactate-production characteristics. Next to this prominent study, other concepts have been developed to quantify products such as antibodies in microfluidics, but these mostly focus on the analysis of yeast, mammalian cells, or enzyme mutants [132-136].

Mass spectrometry

Modern mass spectrometry is sensitive enough to detect and quantify products from single whole-cell biocatalysts. The power of single-cell mass spectrometry for analyzing mammalian cell systems was demonstrated already, but for the analysis of microbes and their catalytic products, mass spectrometry is still in its infancy [137-139]. However, several key studies recently illustrated the power of mass spectrometry analysis for productivity analyses at the single-cell level and will be reviewed and discussed in the following.

Electrospray Ionization- Fourier Transform Ion Cyclotron (ESI-FTICR) mass spectrometry coupled to microfluidic cell cultivation enabled for the first time to detect and quantify the productivity of microbial cell factories at a single cell-level without the need for labeling [140]. In this key study, a few living L-lysine producing *C. glutamicum* were trapped via negative dielectrophoresis with the Envirostat microfluidic single-cell bioreactor [141,66,142]. The cell supernatant was continuously sampled in chip-coupled fused-silica capillaries and analyzed via nanospray-ESI-FTICR mass spectrometry. The produced lysine was accurately quantified by spiking the cell medium with a stable isotope-labeled internal standard. Cell-specific L-lysine production rates r_P ranged from 2 -20 $fmol^{-1}$ cell h^{-1}. Despite the analytical power of mass spectrometry, this study is the first example of how specific product formation rates in microbes can be obtained from microfluidic single cell-experiments. Ion suppression caused by the high salt cargo of standard growth and production media for microbes were recently identified as the key reason for this lack of successful studies. The development of a volatile, ammonium salt-based reaction medium, that was causing low ion suppression, but enabled high cellular activity was the key to success for realizing single-cell product analysis via mass spectrometry [140]. Microfluidics interfaced with ultrasensitive label-free mass spectrometry might become one of the key concepts for unraveling strain productivity and its heterogeneity based on single-cell data.

Next to FTICR-MS, a Chip-MS interface based in droplet microarrays and subsequent ionization via Matrix-assisted Laser Desorption Ionization (MALDI) was demonstrated the multi-modal analysis of protein secretion and enzymatic activity with only 50-100 living *Komagataella phaffii* cells [143,144]. Via MADLI-MS and the application of a fluorogenic substrate, the multistep conversion of phytic acid by secreted phytase could be monitored with several modalities [143]. The approach was further refined and even allowed the separation of yeast cells and droplet supernatant for subcultivation of the analyzed micropopulations based on the results obtained from the multimodal analysis of the secreted enzyme [144]. Noteworthy, these studies were also enabled by the application of volatile salt

buffers as reaction media [140]. The developed analytical concept has a broad range of applications and can be adapted to on-chip microfluidic droplet cultivations, but also interfaced with any other microbioreactor concepts, such as perfusion reactors.

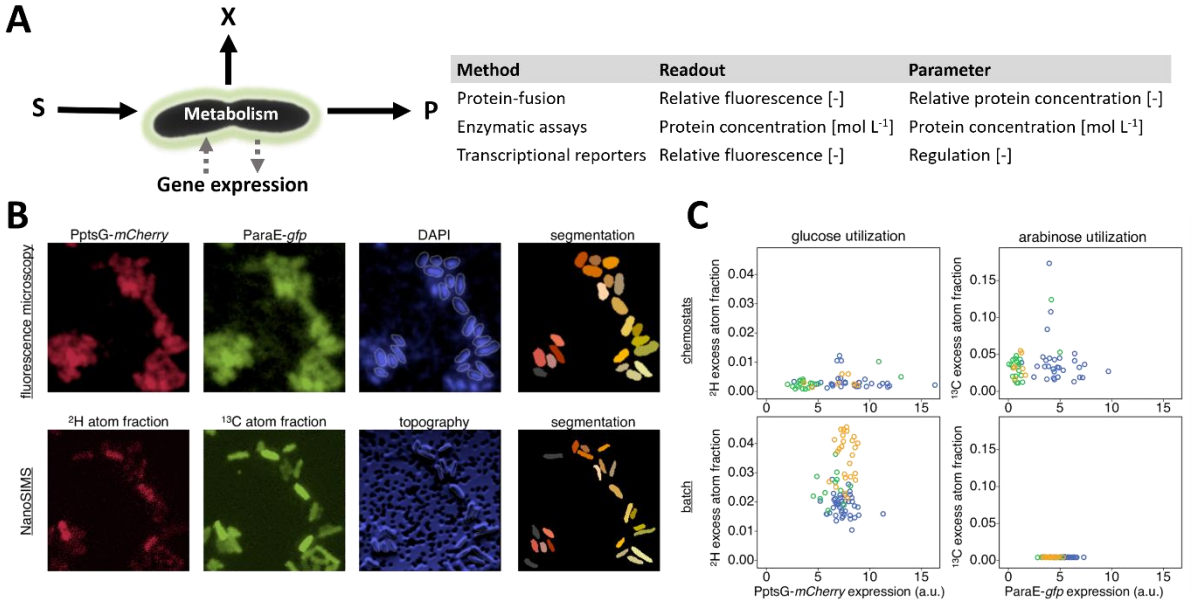
The above described high-density droplet arrays for interfacing microfluidics and mass spectrometry were designed to aliquot droplets of solutions or cell suspensions. With this concept, high-throughput analyses of intracellular metabolites in single *S. cerevisiae* cells were realized with a detection limit down to 10 fmol total analyte amount [145,146]. Intrinsic heterogeneity in terms of relative intracellular metabolite concentration could be revealed, that correlated with cell size, cell age, or cell cycle stage. Based on these intracellular levels of the glycolytic metabolite fructose-1,6-bisphosphate, two distinct metabolic phenotypes could be identified [145]. Another study intensified the application of the high-density droplet arrays and disclosed that yeast cells exhibited a more active pentose phosphate pathway upon perturbation of glycolysis [146]. The authors used ^{13}C-labelled glucose to infer the pathway activity of single cells via MALDI-MS. The pioneering studies demonstrate how the analysis of metabolic fluxes can be accomplished with single cells and mark the first steps towards systems biotechnology with single microbial cells.

Product analysis and quantification for determining synthesis kinetics of single cells are close. Optical methods, basing on fluorescence readouts, cover a broad range of important microbial products and concepts like novel FRET sensors for product measurements in microfluidic cultivations are likely to emerge. With the latest developments in mass spectrometry, a universal and label-free analytical concept for detecting tiniest product amounts comes into reach. However, this requires future research in terms of microfluidic interfacing and media design [11]. With the discussed technologies, cellular heterogeneities and its manifestation in the catalytic efficiencies are now accessible and will lead to the development of novel strategies for strain development and process engineering. Indeed, this might enable us to perform systems-level studies with the cell as the minimal unit of biotechnological processes.

Gene expression, protein synthesis, and regulation

Fluorescence analysis of individual microbes re-awakened the field of microfluidic single-cell analysis a decade ago. Cell-to-cell differences in gene expression were revealed by the application of fluorescent proteins and microscopy and the results pointed to significant functional heterogeneity in isogenic populations (see Figure 6) [147]. Many comprehensive studies followed that elucidated the fundamental concepts of stochasticity and noise in gene expression [148-152]. The excellent control of extracellular conditions in microfluidics enabled to link observations of gene expression with environmental cues and fluctuations [151,153,154]. With the ever-increasing sensitivity of analytical technologies, it became even possible to track gene expression at the level of single molecules [155].

In biotechnological processes, the content of catalytically active enzyme comprises important parameters for the activity of whole-cell biocatalysts. On a population level, it is therefore a common procedure to characterize the expression of key enzymes for a target catalytic conversion.



Method	Readout	Parameter
Protein-fusion	Relative fluorescence [-]	Relative protein concentration [-]
Enzymatic assays	Protein concentration [mol L^{-1}]	Protein concentration [mol L^{-1}]
Transcriptional reporters	Relative fluorescence [-]	Regulation [-]

Figure 6: A) Analytical methods, readouts, and parameters for gene expression and regulation at a single-cell level. **B)** Measuring heterogeneity in gene expressions of the sugar metabolism in single *E. colis* cells via the expression of dual fluorescent reporter genes. Next to the synthesis of reporter proteins, carbon uptake of the cells were analyzed via nanoSIMS. **C)** Correlation between stable isotope incorporation and expression of two genes involved in sugr metabolism [121].

Analyses of gene expression can be performed at a single-cell level, mostly by molecular fusion of reporter genes, coding for fluorescent proteins, to the gene of interest [156,157]. With microfluidic cultivation, novel insight into the general mechanisms of gene expression could be obtained by the application of such fusion constructs. However, a fluorescent reporter can also be just simply put under the control of a certain regulatory element such as a promoter to study its functioning. Gefen *et al.* analyzed gene expression kinetics and magnitudes in single starving *E. coli* cells [158]. The microfluidic chip was connected to a shake flask batch culture to establish identical growth conditions in the flask and in on-chip cultures. Upon reaching the stationary growth phase, the majority of cells stopped growing due to the lack of carbon source, while approximately 7% of the cells lysed. Inducing the expression of genes coding for fluorescent proteins, it was found that the starving cells still maintained their capability to synthesize protein *de novo* for several days. It could be hence proven that the synthesis activity of *E. coli* in the stationary growth phase is maintained for longer periods of starvation. The obtained results also suggest that cells can be metabolically active, despite the absence of growth. However, this is only one prominent example of how gene expression analysis with fluorescent reporters can be accomplished. The use of fluorescent proteins and microscopy for probing cellular behavior, promoter activity, protein localization, gene expression dynamics, and many other cellular

parameters is certainly the most widespread method for analyzing single cells in microfluidics [159-164]. As an extensive discussion of these applications would exceed the scope of this chapter, we refer to key review papers on this topic [71,165,166].

Gene expression analysis via fluorescent reporter enzyme gives access to relative protein amounts inside the cell. Absolute enzyme levels are usually determined via mass spectrometry-based proteomics, but proteomics with single microbes are difficult to perform due to the low number of enzyme copies inside a cell [12]. The amount of a target enzyme in *E. coli* cells could be quantified via a microscale enzyme-linked immunosorbent assay (ELISA) and the corresponding fluorescence read-out [167]. For this, individual cells were trapped hydrodynamically in sealable fluidic microchambers. Upon cell trapping, the chambers were closed and the target enzyme β-galactosidase was liberated via on-chip cell lysis. The free enzyme was bound to immobilized antibodies. The enzyme quantity was then determined by the addition of the fluorogenic substrate fluorescein di-β-D-galactopyranoside (FDG), which was hydrolyzed to fluorescein and galactose. With this method, enzyme copy numbers as low as 200 copies per cell could be detected. The authors found that the abundance of β-galactosidase was variable among individual *E. coli* cells and depended on the extracellular cultivation conditions, proving proteome heterogeneity in isogenic populations.

Microfluidic single-cell analysis can also be used to unravel regulatory mechanisms that are hidden behind averaged values of populations. The analysis of carbon-catabolite repression in the yeast *Ogataea polymorpha* (formerly known as *Hansenula polymorpha*) at a single cell-level disclosed that threshold glucose concentrations for promoter repression differed up to four orders of magnitude at the microscale compared to population experiments [168]. The authors simply put the gene expression of a GFP under the control of the *MOX* promoter to unravel these intriguing insights into promoter repression. Optimized carbon-limited fed-batch strategies for increasing the productivity of the *MOX* promoter system could be derived from the microfluidic single-cell experiments.

As can be seen, the analysis of gene expression and its regulation at a single cell level can contribute significantly to the improvement of bioprocesses and microbial cell factories via rational genetic or process modifications.

Analytical pitfalls in microfluidic single-cell analysis

Many analytical pitfalls have to be considered when analyzing cellular behavior at a microscale. Bias arising from the analytical method can result in biological artifacts that lead to misinterpretation of the obtained results. The microfluidic cultivation habitat, including the physical laws at the microscale and the high surface-to-volume ratios, constitute the most important sources of technical bias in microfluidic single-cell analysis [67,169,170,47]. It is therefore important to perform suitable control

experiments to ensure that the physiological state of the cells to be analyzed is not a result of the cultivation environment.

As discussed before, optical methods are the most widespread analytical technologies for investigating the behavior of single microbes. Optical analyses are generally seen as non-invasive, but can have tremendous impact on cellular physiology. Although optical analysis technologies are mechanically non-invasive, illumination transfers energy to the cells. Light-induced phototoxic effects can severely affect the physiology of the cells, mostly by the formation of reactive oxygen species (ROS) or radicals [171,172]. Such photochemical-induced toxicity can be even caused by standard white illumination for brightfield imaging [173,174] Photo-induced physiological effects inversely scale with the UV light contents of the white-light source. By using filters or LED-illumination with defined spectra, UV-induced effects on physiology can be minimized or even circumvented.

While phototoxicity can be critical during white-light illumination, it is mandatory to study the effects of phototoxicity during fluorescence imaging. As the excitation light for fluorescence analysis is typical of high intensity, the physiology of microbes can be strongly influenced by fluorescence excitation [175]. A negative correlation was found between the dose of excitation light at 488 nm (typical wavelength for GFP-excitation) and doubling times in single *E. coli* cells[176]. Minimizing of phototoxicity during fluorescence imaging involves a reduction of exposure times and excitation. Comprehensive guidelines for optimal experimental design for fluorescence imaging have been published [177,178]. The basic principles for avoiding the technical bias of fluorescence imaging can be also applied for microbes, although most work bases on cell cultures.

Protein synthesis and degradation dynamics have to considered when using genetically-fluorescent probes for the visualization of dynamic processes in single cells [179]. This included maturation times of the fluorescent proteins, as well as their extended cytosolic half-life of often more than 24 h [180]. It is advisable to apply fast maturating mutants of fluorescent proteins and, if necessary, to add a proteasome degradation tag for decreasing the protein's half-life [181].

When using chemical dyes for fluorescence imaging, the experimenter has to consider that these compounds can intercalate DNA or alter the properties of the stained molecules [182]. These aspects have to be considered and its effects should be properly characterized via control experiments to ensure the analysis of undisturbed single-cell physiology.

Conclusion

The analytical concepts for microfluidic single-cell analysis now enable measuring and quantifying the physiology and the underlying cellular parameters of whole-cell biocatalysts at the level of individual cells. Advanced analytics, such as optical imaging technologies and mass spectrometry, matured and

give access to the kinetics of biomass and product formation, as well as substrate uptake. With knowledge on cell-specific μ, $q_{S,}$ and r_P, mass and energy balances of single cells can be established to uncover the catalytic landscape of cellular performance and efficiency. Based on such kinetic single-cell data, we will learn about the role of individual phenotypes and their contribution to the output of the bioprocesses. In combination with powerful microfluidic cultivation concepts, single-cell analytics will uncover hidden links between environmental conditions and individual cell performance that are blurred by averaged values from populations. Novel engineering targets for metabolic, reaction, and process engineering will be derived from data on single-cell physiology.

References

1. Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B (2001) Industrial biocatalysis today and tomorrow. Nature 409 (6817):258-268.

2. Schrewe M, Julsing MK, Buhler B, Schmid A (2013) Whole-cell biocatalysis for selective and productive C-O functional group introduction and modification. Chemical Society reviews 42 (15):6346-6377.

3. Blank LM, Ebert BE, Buhler B, Schmid A (2008) Metabolic capacity estimation of *Escherichia coli* as a platform for redox biocatalysis: constraint-based modeling and experimental verification. Biotechnology and bioengineering 100 (6):1050-1065.

4. Olaofe OA, Fenner CJ, Gudiminchi RK, Smit MS, Harrison ST (2013) The influence of microbial physiology on biocatalyst activity and efficiency in the terminal hydroxylation of n-octane using Escherichia coli expressing the alkane hydroxylase, CYP153A6. Microb Cell Fact 12:8.

5. Litsios A, Ortega AD, Wit EC, Heinemann M (2018) Metabolic-flux dependent regulation of microbial physiology. Curr Opin Microbiol 42:71-78.

6. Davies J (2002) Re-birth of microbial physiology. Environ Microbiol 4 (1):6.

7. Royle K, Kontoravdi C (2013) A systems biology approach to optimising hosts for industrial protein production. Biotechnol Lett 35 (12):1961-1969.

8. Schuetz R, Zamboni N, Zampieri M, Heinemann M, Sauer U (2012) Multidimensional optimality of microbial metabolism. Science 336 (6081):601-604.

9. Lee SY, Lee DY, Kim TY (2005) Systems biotechnology for strain improvement. Trends Biotechnol 23 (7):349-358.

10. Fritzsch FS, Dusny C, Frick O, Schmid A (2012) Single-cell analysis in biotechnology, systems biology, and biocatalysis. Annu Rev Chem Biomol Eng 3 (1):129-155.

11. Dusny C, Grunberger A (2019) Microfluidic single-cell analysis in biotechnology: from monitoring towards understanding. Curr Opin Biotechnol 63:26-33.

12. Kortmann H, Blank LM, Schmid A (2011) Single cell analytics: An overview. Advances in Biochemical Engineering/Biotechnology 124:99-122.

13. Schmid A, Kortmann H, Dittrich PS, Blank LM (2010) Chemical and biological single cell analysis. Curr Opin Biotechnol 21 (1):12-20.

14. Reshes G, Vanounou S, Fishov I, Feingold M (2008) Cell shape dynamics in *Escherichia coli*. Biophys J 94 (1):251-264.

15. Boyd ES, Leavitt WD, Geesey GG (2009) CO_2 uptake and fixation by a thermoacidophilic microbial community attached to precipitated sulfur in a geothermal spring. Appl Environ Microb 75 (13):4289-4296.

16. Zakhartsev M, Reuss M (2018) Cell size and morphological properties of yeast *Saccharomyces cerevisiae* in relation to growth temperature. FEMS Yeast Res 18 (6).

17. Zavrel T, Ocenasova P, Cerveny J (2017) Phenotypic characterization of *Synechocystis* sp. PCC 6803 substrains reveals differences in sensitivity to abiotic stress. PLoS One 12 (12):e0189130.

18. Milo R, Jorgensen P, Moran U, Weber G, Springer M (2010) BioNumbers-the database of key numbers in molecular and cell biology. Nucleic Acids Res 38:D750-D753.

19. Dittrich P, Jakubowski N (2014) Current trends in single cell analysis. Anal Bioanal Chem 406 (27):6957-6961.

20. Lubeck E, Cai L (2012) Single-cell systems biology by super-resolution imaging and combinatorial labeling. Nat Methods 9 (7):743-748.

21. Altinoglu I, Merrifield CJ, Yamaichi Y (2019) Single molecule super-resolution imaging of bacterial cell pole proteins with high-throughput quantitative analysis pipeline. Sci Rep 9 (1):6680.

22. Young JW, Locke JCW, Altinok A, Rosenfeld N, Bacarian T, Swain PS, Mjolsness E, Elowitz MB (2012) Measuring single-cell gene expression dynamics in bacteria using fluorescence time-lapse microscopy. Nat Protoc 7 (1):80-88.

23. Locke JC, Elowitz MB (2009) Using movies to analyse gene circuit dynamics in single cells. Nature reviews Microbiology 7 (5):383-392.

24. Young JW, Locke JC, Elowitz MB (2013) Rate of environmental change determines stress response specificity. Proc Natl Acad Sci U S A 110 (10):4140-4145.

25. Locke JCW, Young JW, Fontes M, Jimenez MJH, Elowitz MB (2011) Stochastic pulse regulation in bacterial stress response. Science 334 (6054):366-369.

26. Unthan S, Gruenberger A, van Ooyen J, Gaetgens J, Heinrich J, Paczia N, Wiechert W, Kohlheyer D, Noack S (2014) Beyond growth rate 0.6: What drives *Corynebacterium glutamicum* to higher growth rates in defined medium. Biotechnology and bioengineering 111 (2):359-371.

27. Dusny C, Grunberger A, Probst C, Wiechert W, Kohlheyer D, Schmid A (2015) Technical bias of microcultivation environments on single-cell physiology. Lab on a chip 15 (8):1822-1834.

28. Dusny C, Schmid A (2015) Challenging biological limits with microfluidic single cell analysis. Microbial biotechnology 8 (1):23-25.

29. Dusny C, Schmid A (2015) Microfluidic single-cell analysis links boundary environments and individual microbial phenotypes. Environmental Microbiology 17 (6):1839-1856.

30. Klumpp S, Hwa T (2014) Bacterial growth: Global effects on gene expression, growth feedback and proteome partition. Curr Opin Biotech 28:96-102.

31. Klumpp S (2011) Growth-rate dependence reveals design principles of plasmid copy number control. PLoS One 6 (5):e20403.

32. Ullman G, Wallden M, Marklund EG, Mahmutovic A, Razinkov I, Elf J (2013) High-throughput gene expression analysis at the level of single proteins using a microfluidic turbidostat and automated cell tracking. Philos T R Soc B 368 (1611).

33. Klumpp S, Zhang ZG, Hwa T (2009) Growth rate-dependent global effects on gene expression in bacteria. Cell 139 (7):1366-1375.

34. Moffitt JR, Lee JB, Cluzel P (2012) The single-cell chemostat: an agarose-based, microfluidic device for high-throughput, single-cell studies of bacteria and bacterial communities. Lab on a chip 12 (8):1487-1494.

35. Lee SS, Avalos Vizcarra I, Huberts DH, Lee LP, Heinemann M (2012) Whole lifespan microscopic observation of budding yeast aging through a microfluidic dissection platform. Proc Natl Acad Sci U S A 109 (13):4916-4920.

36. Wang P, Robert L, Pelletier J, Dang WL, Taddei F, Wright A, Jun S (2010) Robust growth of *Escherichia coli*. Curr Biol 20 (12):1099-1103.

37. Dusny C, Fritzsch FS, Frick O, Schmid A (2012) Isolated microbial single cells and resulting micropopulations grow faster in controlled environments. Appl Environ Microbiol 78 (19):7132-7136.

38. Sweedler JV, Arriaga EA (2007) Single cell analysis. Anal Bioanal Chem 387 (1):1-2.

39. Boulineau S, Tostevin F, Kiviet DJ, ten Wolde PR, Nghe P, Tans SJ (2013) Single-cell dynamics reveals sustained growth during diauxic shifts. PLoS One 8 (4):1-9.

40. Mannik J, Wu F, Hol FJ, Bisicchia P, Sherratt DJ, Keymer JE, Dekker C (2012) Robustness and accuracy of cell division in *Escherichia coli* in diverse cell shapes. Proc Natl Acad Sci U S A 109 (18):6957-6962.

41. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S (2004) Bacterial persistence as a phenotypic switch. Science 305 (5690):1622-1625.

42. Evans SN, Ralph PL, Schreiber SJ, Sen A (2013) Stochastic population growth in spatially heterogeneous environments. J Math Biol 66 (3):423-476.

43. Gefen O, Balaban NQ (2009) The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. Fems Microbiol Rev 33 (4):704-717.

44. Gefen O, Gabay C, Mumcuoglu M, Engel G, Balaban NQ (2008) Single-cell protein induction dynamics reveals a period of vulnerability to antibiotics in persister bacteria. Proc Natl Acad Sci U S A 105 (16):6145-6149.

45. Li B, Qiu Y, Glidle A, Cooper J, Shi H, Yin H (2014) Single cell growth rate and morphological dynamics revealing an "opportunistic" persistence. Analyst 139 (13):3305-3313.

46. Lidstrom ME, Konopka MC (2010) The role of physiological heterogeneity in microbial population behavior. Nat Chem Biol 6 (10):705-712.

47. Dusny C, Gruenberger A, Probst C, Wiechert W, Kohlheyer D, Schmid A (2015) Technical bias of microcultivation environments on single-cell physiology. Lab on a chip 15 (8):1822-1834.

48. Bryan AK, Hecht VC, Shen WJ, Payer K, Grover WH, Manalis SR (2014) Measuring single cell mass, volume, and density with dual suspended microchannel resonators. Lab on a chip 14 (3):569-576.

49. Bryan AK, Engler A, Gulati A, Manalis SR (2012) Continuous and long-term volume measurements with a commercial Coulter counter. PLoS One 7 (1):e29866.

50. Schaechter M (2015) A brief history of bacterial growth physiology. Front Microbiol 6:289.

51. Schaechter M, Williamson JP, Hood JR, Jr., Koch AL (1962) Growth, cell and nuclear divisions in some bacteria. Journal of general microbiology 29:421-434.

52. Schaechter M, Maaloe O, Kjeldgaard NO (1958) Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. Journal of general microbiology 19 (3):592-606.

53. Kjeldgaard NO, Maaloe O, Schaechter M (1958) The transition between different physiological states during balanced growth of *Salmonella typhimurium*. Journal of general microbiology 19 (3):607-616.

54. Dal Co A, van Vliet S, Ackermann M (2019) Emergent microscale gradients give rise to metabolic cross-feeding and antibiotic tolerance in clonal bacterial populations. Philos Trans R Soc Lond B Biol Sci 374 (1786):20190080.

55. Gruenberger A, Probst C, Heyer A, Wiechert W, Frunzke J, Kohlheyer D (2013) Microfluidic picoliter bioreactor for microbial single-cell analysis: fabrication, system setup, and operation. Journal of visualized experiments : JoVE (82):50560.

56. Saeki T, Hosokawa M, Lim TK, Harada M, Matsunaga T, Tanaka T (2014) Digital cell counting device integrated with a single-cell array. PLoS One 9 (2):e89011.

57. Lu H, Caen O, Vrignon J, Zonta E, El Harrak Z, Nizard P, Baret JC, Taly V (2017) High throughput single cell counting in droplet-based microfluidics. Sci Rep 7 (1):1366.

58. Peitz I, van Leeuwen R (2010) Single-cell bacteria growth monitoring by automated DEP-facilitated image analysis. Lab on a chip 10 (21):2944-2951.

59. Grunberger A, Paczia N, Probst C, Schendzielorz G, Eggeling L, Noack S, Wiechert W, Kohlheyer D (2012) A disposable picolitre bioreactor for cultivation and investigation of industrially relevant bacteria on the single cell level. Lab on a chip 12 (11):2060-2068.

60. Grunberger A, van Ooyen J, Paczia N, Rohe P, Schiendzielorz G, Eggeling L, Wiechert W, Kohlheyer D, Noack S (2013) Beyond growth rate 0.6: *Corynebacterium glutamicum* cultivated in highly diluted environments. Biotechnology and bioengineering 110 (1):220-228.

61. Hammar P, Angermayr SA, Sjostrom SL, van der Meer J, Hellingwerf KJ, Hudson EP, Joensson HN (2015) Single-cell screening of photosynthetic growth and lactate production by cyanobacteria. Biotechnol Biofuels 8:193.

62. Dusny C, Schmid A (2015) Microfluidic single-cell analysis links boundary environments and individual microbial phenotypes. Environ Microbiol 17 (6):1839-1856.

63. Probst C, Grunberger A, Wiechert W, Kohlheyer D (2013) Microfluidic growth chambers with optical tweezers for full spatial single-cell control and analysis of evolving microbes. J Microbiol Meth 95 (3):470-476.

64. Probst C, Grunberger A, Wiechert W, Kohlheyer D (2013) Polydimethylsiloxane (PDMS) sub-micron traps for single-cell analysis of bacteria. Micromachines-Basel 4 (4):357-369.

65. Harris LK, Theriot JA (2018) Surface area to volume ratio: A natural variable for bacterial morphogenesis. Trends Microbiol 26 (10):815-832.

66. Rosenthal K, Falke F, Frick O, Dusny C, Schmid A (2015) An inert continuous microreactor for the isolation and analysis of a single microbial cell. Micromachines-Basel 6 (12):1836-1855.

67. Taheri-Araghi S, Bradde S, Sauls JT, Hill NS, Levin PA, Paulsson J, Vergassola M, Jun S (2015) Cell-size control and homeostasis in bacteria. Curr Biol 25 (3):385-391.

68. Wang Z (2019) Cell segmentation for image cytometry: Advances, insufficiencies, and challenges. Cytometry A 95 (7):708-711.

69. Leygeber M, Lindemann D, Sachs CC, Kaganovitch E, Wiechert W, Noh K, Kohlheyer D (2019) Analyzing microbial population heterogeneity-Expanding the toolbox of microfluidic single-cell cultivations. J Mol Biol 431 (23):4569-4588.

70. Sliusarenko O, Heinritz J, Emonet T, Jacobs-Wagner C (2011) High-throughput, subpixel precision analysis of bacterial morphogenesis and intracellular spatio-temporal dynamics. Mol Microbiol 80 (3):612-627.

71. Young JW, Locke JC, Altinok A, Rosenfeld N, Bacarian T, Swain PS, Mjolsness E, Elowitz MB (2012) Measuring single-cell gene expression dynamics in bacteria using fluorescence time-lapse microscopy. Nat Protoc 7 (1):80-88.

72. Garner EC (2011) MicrobeTracker: quantitative image analysis designed for the smallest organisms. Mol Microbiol 80 (3):577-579.

73. Campos M, Surovtsev IV, Kato S, Paintdakhi A, Beltran B, Ebmeier SE, Jacobs-Wagner C (2014) A constant size extension drives bacterial cell size homeostasis. Cell 159 (6):1433-1446.

74. 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 (4):767-777.

75. Ducret A, Quardokus EM, Brun YV (2016) MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. Nat Microbiol 1 (7):16077.

76. Sachs CC, Grunberger A, Helfrich S, Probst C, Wiechert W, Kohlheyer D, Noh K (2016) Image-based single cell profiling: High-throughput processing of mother machine experiments. PLoS One 11 (9):e0163453.

77. Popescu G, Park K, Mir M, Bashir R (2014) New technologies for measuring single cell mass. Lab on a chip 14 (4):646-652.

78. Popescu G (2008) Quantitative phase imaging of nanoscale cell structure and dynamics. Methods in cell biology 90:87-115.

79. Popescu G, Park Y, Lue N, Best-Popescu C, Deflores L, Dasari RR, Feld MS, Badizadegan K (2008) Optical imaging of cell mass and growth dynamics. Am J Physiol Cell Physiol 295 (2):C538-544.

80. Phillips KG, Jacques SL, McCarty OJ (2012) Measurement of single cell refractive index, dry mass, volume, and density using a transillumination microscope. Phys Rev Lett 109 (11):118105.

81. Zangle TA, Chun J, Zhang J, Reed J, Teitell MA (2013) Quantification of biomass and cell motion in human pluripotent stem cell colonies. Biophys J 105 (3):593-601.

82. Chun J, Zangle TA, Kolarova T, Finn RS, Teitell MA, Reed J (2012) Rapidly quantifying drug sensitivity of dispersed and clumped breast cancer cells by mass profiling. Analyst 137 (23):5495-5498.

83. Reed J, Troke JJ, Schmit J, Han S, Teitell MA, Gimzewski JK (2008) Live cell interferometry reveals cellular dynamism during force propagation. ACS Nano 2 (5):841-846.

84. Mir M, Wang Z, Shen Z, Bednarz M, Bashir R, Golding I, Prasanth SG, Popescu G (2011) Optical measurement of cycle-dependent cell growth. Proc Natl Acad Sci U S A 108 (32):13124-13129.

85. Cotte Y, Toy F, Jourdain P, Pavillon N, Boss D, Magistretti P, Marquet P, Depeursinge C (2013) Marker-free phase nanoscopy. Nat Photonics 7 (2):113-117.

86. Mir M, Babacan SD, Bednarz M, Do MN, Golding I, Popescu G (2012) Visualizing *Escherichia coli* sub-cellular structure using sparse deconvolution Spatial Light Interference Tomography. PLoS One 7 (6):e39816.

87. Johnson BN, Mutharasan R (2012) Biosensing using dynamic-mode cantilever sensors: A review. Biosens Bioelectron 32 (1):1-18.

88. Martinez-Martin D, Flaschner G, Gaub B, Martin S, Newton R, Beerli C, Mercer J, Gerber C, Muller DJ (2017) Inertial picobalance reveals fast mass fluctuations in mammalian cells. Nature 550 (7677):500-+.

89. Godin M, Delgado FF, Son SM, Grover WH, Bryan AK, Tzur A, Jorgensen P, Payer K, Grossman AD, Kirschner MW, Manalis SR (2010) Using buoyant mass to measure the growth of single cells. Nat Methods 7 (5):387-390.

90. Godin M, Bryan AK, Burg TP, Babcock K, Manalis SR (2007) Measuring the mass, density, and size of particles and cells using a suspended microchannel resonator. Appl Phys Lett 91 (12):123121.

91. Son S, Tzur A, Weng Y, Jorgensen P, Kim J, Kirschner MW, Manalis SR (2012) Direct observation of mammalian cell growth and size regulation. Nat Methods 9 (9):910-912.

92. Ilic B, Czaplewski D, Zalalutdinov M, Craighead HG, Neuzil P, Campagnolo C, Batt C (2001) Single cell detection with micromechanical oscillators. J Vac Sci Technol B 19 (6):2825-2828.

93. Weng Y, Delgado FF, Son S, Burg TP, Wasserman SC, Manalis SR (2011) Mass sensors with mechanical traps for weighing single cells in different fluids. Lab on a chip 11 (24):4174-4180.

94. Zangle TA, Teitell MA (2014) Live-cell mass profiling: an emerging approach in quantitative biophysics. Nat Methods 11 (12):1221-1228.

95. Bryan AK, Goranov A, Amon A, Manalis SR (2010) Measurement of mass, density, and volume during the cell cycle of yeast. Proc Natl Acad Sci U S A 107 (3):999-1004.

96. Lewis CL, Craig CC, Senecal AG (2014) Mass and density measurements of live and dead Gram-negative and Gram-positive bacterial populations. Appl Environ Microbiol 80 (12):3622-3631.

97. Ackermann M (2015) A functional perspective on phenotypic heterogeneity in microorganisms. Nature reviews Microbiology 13 (8):497-508.

98. Baumann K, Maurer M, Dragosits M, Cos O, Ferrer P, Mattanovich D (2008) Hypoxic fed-batch cultivation of *Pichia pastoris* increases specific and volumetric productivity of recombinant proteins. Biotechnology and bioengineering 100 (1):177-183.

99. Chen BY, You JW, Hsieh YT, Chang JS (2008) Feasibility study of exponential feeding strategy in fed-batch cultures for phenol degradation using *Cupriavidus taiwanensis*. Biochem Eng J 41 (2):175-180.

100. d'Anjou MC, Daugulis AJ (2000) Mixed-feed exponential feeding for fed-batch culture of recombinant methylotrophic yeast. Biotechnol Lett 22 (5):341-346.

101. Stryhanyuk H, Calabrese F, Kummel S, Musat F, Richnow HH, Musat N (2018) Calculation of single cell assimilation rates From SIP-NanoSIMS-derived isotope ratios: A comprehensive approach. Front Microbiol 9:2342.

102. Sengupta D, Mongersun A, Kim TJ, Mongersun K, von Eyben R, Abbyad P, Pratx G (2019) Multiplexed single-cell measurements of FDG uptake and lactate release using droplet microfluidics. Technol Cancer Res T 18.

103. Hehemann JH, Reintjes G, Klassen L, Smith AD, Ndeh D, Arnosti C, Amann R, Abbott DW (2019) Single cell fluorescence imaging of glycan uptake by intestinal bacteria. Isme J 13 (7):1883-1889.

104. Sung Y, Tetrault MA, Takahashi K, Ouyang J, Pratx G, Fakhri GE, Normandin MD (2020) Dependence of fluorodeoxyglucose (FDG) uptake on cell cycle and dry mass: a single-cell study using a multi-modal radiography platform. Sci Rep 10 (1):4280.

105. Achilles J, Muller S, Bley T, Babel W (2004) Affinity of single *S. cerevisiae* cells to 2-NBDglucose under changing substrate concentrations. Cytom Part A 61A (1):88-98.

106. VanEngelenburg SB, Palmer AE (2008) Fluorescent biosensors of protein function. Curr Opin Chem Biol 12 (1):60-65.

107. Otten J, Tenhaef N, Jansen RP, Dobber J, Jungbluth L, Noack S, Oldiges M, Wiechert W, Pohl M (2019) A FRET-based biosensor for the quantification of glucose in culture supernatants of mL scale microbial cultivations. Microbial Cell Factories 18 (1).

108. Fehr M, Frommer WB, Lalonde S (2002) Visualization of maltose uptake in living yeast cells by fluorescent nanosensors. Proc Natl Acad Sci U S A 99 (15):9846-9851.

109. Nikolic N, Barner T, Ackermann M (2013) Analysis of fluorescent reporters indicates heterogeneity in glucose uptake and utilization in clonal bacterial populations. BMC Microbiol 13:258.

110. Musat N, Foster R, Vagner T, Adam B, Kuypers MM (2012) Detecting metabolic activities in single cells, with emphasis on nanoSIMS. Fems Microbiology Reviews 36 (2):486-511.

111. Schoffelen NJ, Mohr W, Ferdelman TG, Littmann S, Duerschlag J, Zubkov MV, Ploug H, Kuypers MMM (2018) Single-cell imaging of phosphorus uptake shows that key harmful algae rely on different phosphorus sources for growth. Sci Rep-Uk 8.

112. Straeuber H, Huebschmann T, Jehmlich N, Schmidt F, von Bergen M, Harms H, Mueller S (2010) NBDT (3-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-3-toluene) - A novel fluorescent dye for studying mechanisms of toluene uptake into vital bacteria. Cytometry Part A : the journal of the International Society for Analytical Cytology 77 (2):113-120.

113. Natarajan A, Srienc F (2000) Glucose uptake rates of single *E. coli* cells grown in glucose-limited chemostat cultures. Journal of microbiological methods 42 (1):87-96.

114. Natarajan A, Srienc F (1999) Dynamics of glucose uptake by single *Escherichia coli* cells. Metab Eng 1 (4):320-333.

115. Heuker M, Sijbesma JWA, Suarez RA, de Jong JR, Boersma HH, Luurtsema G, Elsinga PH, Glaudemans AWJM, van Dam GM, van Dijl JM, Slart RHJA, van Oosten M (2017) In vitro imaging of bacteria using F-18-fluorodeoxyglucose micro positron emission tomography. Sci Rep-Uk 7.

116. Berg J, Hung YP, Yellen G (2009) A genetically encoded fluorescent reporter of ATP:ADP ratio. Nat Methods 6 (2):161-166.

117. Yaginuma H, Kawai S, Tabata KV, Tomiyama K, Kakizuka A, Komatsuzaki T, Noji H, Imamura H (2014) Diversity in ATP concentrations in a single bacterial cell population revealed by quantitative single-cell imaging. Sci Rep-Uk 4:6522.

118. Boersma AJ, Zuhorn IS, Poolman B (2015) A sensor for quantification of macromolecular crowding in living cells. Nat Methods 12 (3):227-229.

119. Ha JS, Song JJ, Lee YM, Kim SJ, Sohn JH, Shin CS, Lee SG (2007) Design and application of highly responsive fluorescence resonance energy transfer biosensors for detection of sugar in living *Saccharomyces cerevisiae* cells. Appl Environ Microbiol 73 (22):7408-7414.

120. Kaper T, Lager I, Looger LL, Chermak D, Frommer WB (2008) Fluorescence resonance energy transfer sensors for quantitative monitoring of pentose and disaccharide accumulation in bacteria. Biotechnol Biofuels 1 (1):11.

121. Nikolic N, Schreiber F, Dal Co A, Kiviet DJ, Bergmiller T, Littmann S, Kuypers MMM, Ackermann M (2017) Cell-to-cell variation and specialization in sugar metabolism in clonal bacterial populations. Plos Genet 13 (12).

122. Worrich A, Stryhanyuk H, Musat N, Konig S, Banitz T, Centler F, Frank K, Thullner M, Harms H, Richnow HH, Miltner A, Kastner M, Wick LY (2017) Mycelium-mediated transfer of water and nutrients stimulates bacterial activity in dry and oligotrophic environments. Nat Commun 8.

123. Kopf SH, McGlynn SE, Green-Saxena A, Guan YB, Newman DK, Orphan VJ (2015) Heavy water and N-15 labelling with NanoSIMS analysis reveals growth rate-dependent metabolic heterogeneity in chemostats. Environmental Microbiology 17 (7):2542-2556.

124. Lindemann D, Westerwalbesloh C, Kohlheyer D, Grunberger A, von Lieres E (2019) Microbial single-cell growth response at defined carbon limiting conditions. Rsc Adv 9 (25):14040-14050.

125. Binder D, Drepper T, Jaeger KE, Delvigne F, Wiechert W, Kohlheyer D, Grunberger A (2017) Homogenizing bacterial cell factories: Analysis and engineering of phenotypic heterogeneity. Metab Eng 42:145-156.

126. Xiao Y, Bowen CH, Liu D, Zhang F (2016) Exploiting nongenetic cell-to-cell variation for enhanced biosynthesis. Nat Chem Biol 12 (5):339-344.

127. Manafi M, Kneifel W, Bascomb S (1991) Fluorogenic and chromogenic substrates used in bacterial diagnostics. Microbiol Rev 55 (3):335-348.

128. Binder S, Schendzielorz G, Stabler N, Krumbach K, Hoffmann K, Bott M, Eggeling L (2012) A high-throughput approach to identify genomic variants of bacterial metabolite producers at the single-cell level. Genome Biol 13 (5):R40.

129. Schendzielorz G, Dippong M, Grunberger A, Kohlheyer D, Yoshida A, Binder S, Nishiyama C, Nishiyama M, Bott M, Eggeling L (2013) Taking control over control: Use of product sensing in single cells to remove flux control at key enzymes in biosynthesis pathways. ACS Synth Biol.

130. Love KR, Panagiotou V, Jiang B, Stadheim TA, Love JC (2010) Integrated single-cell analysis shows *Pichia pastoris* secretes protein stochastically. Biotechnology and bioengineering 106 (2):319-325.

131. Love KR, Politano TJ, Panagiotou V, Jiang B, Stadheim TA, Love JC (2012) Systematic single-cell analysis of *Pichia pastoris* reveals secretory capacity limits productivity. PLoS One 7 (6):e37915.

132. Mazutis L, Gilbert J, Ung WL, Weitz DA, Griffiths AD, Heyman JA (2013) Single-cell analysis and sorting using droplet-based microfluidics. Nat Protoc 8 (5):870-891.

133. Wang BL, Ghaderi A, Zhou H, Agresti J, Weitz DA, Fink GR, Stephanopoulos G (2014) Microfluidic high-throughput culturing of single cells for selection based on extracellular metabolite production or consumption. Nat Biotechnol 32 (5):473-478.

134. El Debs B, Utharala R, Balyasnikova IV, Griffiths AD, Merten CA (2012) Functional single-cell hybridoma screening using droplet-based microfluidics. Proc Natl Acad Sci U S A 109 (29):11570-11575.

135. Sjostrom SL, Bai Y, Huang M, Liu Z, Nielsen J, Joensson HN, Andersson Svahn H (2014) High-throughput screening for industrial enzyme production hosts by droplet microfluidics. Lab on a chip 14 (4):806-813.

136. Prodanovic R, Ung WL, Durdic KI, Fischer R, Weitz DA, Ostafe R (2020) A high-throughput screening system based on droplet microfluidics for glucose oxidase gene libraries. Molecules 25 (10).

137. Amantonico A, Urban PL, Zenobi R (2010) Analytical techniques for single-cell metabolomics: state of the art and trends. Anal Bioanal Chem 398:2493-2504.

138. Heinemann M, Zenobi R (2011) Single cell metabolomics. Curr Opin Biotechnol 22 (1):26-31.

139. Zenobi R (2013) Single-cell metabolomics: analytical and biological perspectives. Science 342 (6163):1201-1211.

140. Dusny C, Lohse M, Reemtsma T, Schmid A, Lechtenfeld OJ (2019) Quantifying a biocatalytic product from a few living microbial cells using microfluidic cultivation coupled to FT-ICR-MS. Anal Chem 91 (11):7012-7018.

141. Fritzsch FS, Rosenthal K, Kampert A, Howitz S, Dusny C, Blank LM, Schmid A (2013) Picoliter nDEP traps enable time-resolved contactless single bacterial cell analysis in controlled microenvironments. Lab on a chip 13 (3):397-408.

142. Kortmann H, Chasanis P, Blank LM, Franzke J, Kenig E, Schmid A (2009) The Envirostat - A new bioreactor concept. Lab on a chip 9 (4):576-585.

143. Haidas D, Bachler S, Kohler M, Blank LM, Zenobi R, Dittrich PS (2019) Microfluidic platform for multimodal analysis of enzyme secretion in nanoliter droplet arrays. Anal Chem 91 (3):2066-2073.

144. Haidas D, Napiorkowska M, Schmitt S, Dittrich PS (2020) Parallel sampling of nanoliter droplet arrays for noninvasive protein analysis in discrete yeast cultivations by MALDI-MS. Anal Chem 92 (5):3810-3818.

145. Ibanez AJ, Fagerer SR, Schmidt AM, Urban PL, Jefimovs K, Geiger P, Dechant R, Heinemann M, Zenobi R (2013) Mass spectrometry-based metabolomics of single yeast cells. Proc Natl Acad Sci USA 110 (22):8790-8794.

146. Urban PL, Schmidt AM, Fagerer SR, Amantonico A, Ibanez A, Jefimovs K, Heinemann M, Zenobi R (2011) Carbon^{13} labelling strategy for studying the ATP metabolism in individual yeast cells by micro-arrays for mass spectrometry. Mol Biosyst 7 (10):2837-2840.

147. Elowitz MB, Levine AJ, Siggia ED, Swain PS (2002) Stochastic gene expression in a single cell. Science 297 (5584):1183-1186.

148. Ozbudak EM, Thattai M, Kurtser I, Grossman AD, van Oudenaarden A (2002) Regulation of noise in the expression of a single gene. Nature genetics 31 (1):69-73.

149. Swain PS, Elowitz MB, Siggia ED (2002) Intrinsic and extrinsic contributions to stochasticity in gene expression. Proc Natl Acad Sci U S A 99 (20):12795-12800.

150. Meng TC, Somani S, Dhar P (2004) Modeling and simulation of biological systems with stochasticity. In silico biology 4 (3):293-309.

151. Thattai M, van Oudenaarden A (2004) Stochastic gene expression in fluctuating environments. Genetics 167 (1):523-530.

152. Kaern M, Elston TC, Blake WJ, Collins JJ (2005) Stochasticity in gene expression: from theories to phenotypes. Nature Rev Genet 6 (6):451-464.

153. Kussell E, Leibler S (2005) Phenotypic diversity, population growth, and information in fluctuating environments. Science 309 (5743):2075-2078.

154. Raser JM, O'Shea EK (2005) Noise in gene expression: origins, consequences, and control. Science 309 (5743):2010-2013.

155. Yu J, Xiao J, Ren XJ, Lao KQ, Xie XS (2006) Probing gene expression in live cells, one protein molecule at a time. Science (New York, NY) 311 (5767):1600-1603.

156. Lindmeyer M, Jahn M, Vorpahl C, Muller S, Schmid A, Buhler B (2015) Variability in subpopulation formation propagates into biocatalytic variability of engineered *Pseudomonas putida* strains. Front Microbiol 6:1042.

157. Nikel PI, Silva-Rocha R, Benedetti I, de Lorenzo V (2014) The private life of environmental bacteria: pollutant biodegradation at the single cell level. Environ Microbiol 16 (3):628-642.

158. Gefen O, Fridman O, Ronin I, Balaban NQ (2014) Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity. Proc Natl Acad Sci U S A 111 (1):556-561.

159. Stricker J, Maddox P, Salmon ED, Erickson HP (2002) Rapid assembly dynamics of the *Escherichia coli* FtsZ-ring demonstrated by fluorescence recovery after photobleaching. Proc Natl Acad Sci U S A 99 (5):3171-3175.

160. Dai J, Yoon SH, Sim HY, Yang YS, Oh TK, Kim JF, Hong JW (2013) Charting microbial phenotypes in multiplex nanoliter batch bioreactors. Anal Chem 85 (12):5892-5899.

161. Sun YQ, Casella S, Fang Y, Huang F, Faulkner M, Barrett S, Liu LN (2016) Light modulates the biosynthesis and organization of cyanobacterial carbon fixation machinery through photosynthetic electron flow. Plant Physiol 171 (1):530-541.

162. Long ZC, Olliver A, Brambilla E, Sclavi B, Lagomarsino MC, Dorfman KD (2014) Measuring bacterial adaptation dynamics at the single-cell level using a microfluidic chemostat and time-lapse fluorescence microscopy. The Analyst 139 (20):5254-5262.

163. Kimmerling RJ, Prakadan SM, Gupta AJ, Calistri NL, Stevens MM, Olcum S, Cermak N, Drake RS, Pelton K, De Smet F, Ligon KL, Shalek AK, Manalis SR (2018) Linking single-cell measurements of mass, growth rate, and gene expression. Genome Biol 19 (1):207.

164. Friedman N, Vardi S, Ronen M, Alon U, Stavans J (2005) Precise temporal modulation in the response of the SOS DNA repair network in individual bacteria. PLoS Biol 3 (7):e238.

165. Bennett MR, Hasty J (2009) Microfluidic devices for measuring gene network dynamics in single cells. Nat Rev Genet 10 (9):628-638.

166. Ma Z, Chu PM, Su Y, Yu Y, Wen H, Fu X, Huang S (2019) Applications of single-cell technology on bacterial analysis. Quantitative Biology 7 (3):171-181.

167. Stratz S, Eyer K, Kurth F, Dittrich PS (2014) On-chip enzyme quantification of single *Escherichia coli b*acteria by immunoassay-based analysis. Anal Chem 86 (24):12375-12381.

168. Dusny C, Schmid A (2016) The *MOX* promoter in *Hansenula polymorpha* is ultrasensitive to glucose-mediated carbon catabolite repression. FEMS Yeast Res 16 (6).

169. Taheri-Araghi S, Brown SD, Sauls JT, McIntosh DB, Jun S (2015) Single-Cell Physiology. Annual review of biophysics 44:123-142.

170. Langdahl BR, Ingvorsen K (1997) Temperature characteristics of bacterial iron solubilisation and C-14 assimilation in naturally exposed sulfide ore material at Citronen fjord, north Greenland (83 degrees N). Fems Microbiol Ecol 23 (4):275-283.

171. Zipfel WR, Williams RM, Webb WW (2003) Nonlinear magic: multiphoton microscopy in the biosciences. Nat Biotechnol 21 (11):1369-1377.

172. Davies MJ (2004) Reactive species formed on proteins exposed to singlet oxygen. Photochem Photobiol Sci 3 (1):17-25.

173. Merbt SN, Stahl DA, Casamayor EO, Marti E, Nicol GW, Prosser JI (2012) Differential photoinhibition of bacterial and archaeal ammonia oxidation. Fems Microbiol Lett 327 (1):41-46.

174. Woodward JR, Cirillo VP, Edmunds LN (1978) Light effects in Yeast - Inhibition by visible light of growth and transport in *Saccharomyces cerevisiae* grown at low temperatures. J Bacteriol 133 (2):692-698.

175. Tinevez JY, Dragavon J, Baba-Aissa L, Roux P, Perret E, Canivet A, Galy V, Shorte S (2012) A quantitative method for measuring phototoxicity of a live cell imaging microscope. Method Enzymol 506:291-309.

176. Jun S, Taheri-Araghi S (2015) Cell-size maintenance: Universal strategy revealed. Trends in Microbiology 23 (1):4-6.

177. Frigault MM, Lacoste J, Swift JL, Brown CM (2009) Live-cell microscopy - tips and tools. J Cell Sci 122 (Pt 6):753-767.

178. Dixit R, Cyr R (2003) Cell damage and reactive oxygen species production induced by fluorescence microscopy: effect on mitosis and guidelines for non-invasive fluorescence microscopy. Plant J 36 (2):280-290.

179. Hebisch E, Knebel J, Landsberg J, Frey E, Leisner M (2013) High variation of fluorescence protein maturation times in closely related *Escherichia coli* strains. PLoS One 8 (10):e75991.

180. Andersen JB, Sternberg C, Poulsen LK, Bjorn SP, Givskov M, Molin S (1998) New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl Environ Microbiol 64 (6):2240-2246.

181. McGinness KE, Baker TA, Sauer RT (2006) Engineering controllable protein degradation. Mol Cell 22 (5):701-707.

182. Terai T, Nagano T (2013) Small-molecule fluorophores and fluorescent probes for bioimaging. Pflugers Arch 465 (3):347-359.