Microfluidic single-cell analysis in biotechnology: From monitoring towards understanding

Christian Dusny[1*] and Alexander Grünberger[2*]

[1] Microbial Single Cell Analysis, Department Solar Materials, Helmholtz-Centre for Environmental Research – UFZ Leipzig, Permoserstr. 15 04318 Leipzig, Germany

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

*Corresponding author:

E-Mail: alexander.gruenberger@uni-bielefeld.de; christian.dusny@ufz.de

Phone: +49-521-106-5289

Running title: Microfluidics: From monitoring to understanding

Keywords: single-cell analysis, single-cell cultivation, microfluidics, single-cell phenotype, single-cell analytics, microbial single cells, bioprocess development, cellular heterogeneity

Highlights:

- Microfluidics have become a powerful tool for studying single-cell behavior
- Single-cell analysis revealed insights into phenotypic heterogeneity
- Microfluidic single-cell analysis is shifting from monitoring towards (quantitative) understanding of cellular mechanisms
- Integrating single-cell analysis into biotechnological workflows allows to evaluate the consequences of single-cell physiology for bioprocesses

Abstract

Our understanding of the microbial cell is based on averaged values from bulks. Microfluidic single-cell analysis holds the promise of understanding bioprocesses from a single cell perspective. But what is needed to measure single-cell physiology and to disclose the consequences of individuality for biotechnology? Current single-cell research is not yet able to provide all the necessary insights, but innovative approaches now emerge that propel the field towards a better understanding of cellular processes via quantitative physiology. Here, we critically review novel single-cell technologies that enable us to control cellular input parameters such as environmental conditions and to measure intracellular processes, as well as groundbreaking approaches that enable for the first time to quantify cellular physiology in terms of non-averaged cell-specific rates and yields. We further discuss how a complementary combination of these technologies can contribute to a more detailed and quantitative understanding of cell behavior in bioprocesses. Finally, we demonstrate how integrating microfluidic single-cell analysis into established population-based experimental workflows might unlock its full potential for biotechnology research in the future.

Introduction

Microbial cells are remarkable systems that coordinate a vast biochemical network for performing complex chemistry [1]. The catalytic wealth of microbial cells has made them a valuable tool for the production of compounds that are difficult to synthesize via chemical routes [2]. Cells represent a multiscale system that spans from molecules to populations and hence requires analyses across all scales for understanding the cellular operating principles [3]. For a long time, cell research was limited to macroscopic analyses of populations, but the advent of microsystems technology and integration into biology in the late '90s heralded a new era of single-cell biotechnology research [4]. The possibility to cultivate cells with microstructured habitats in handy chip formats bestowed us exciting insights into cellular individuality that hitherto remained hidden behind averaged values of populations [5]. Since then, measuring cellular heterogeneity was the major impetus for microfluidic single-cell analysis and produced valuable insight into the origins, mechanisms, and impacts of heterogeneity [6]. However, microfluidic single-cell analysis is much more replete in opportunities for understanding cellular functioning and the underlying mechanism in a biotechnology context than monitoring heterogeneities [7]. Novel technologies for analyzing single microbes are now emerging, allowing the first time to collect quantitative information on cell input and environment, intracellular processes, as well as the cellular output. These technological advancements show that the field has the potential to develop from cell observation towards physiological studies of the minimal biological unit. For biotechnology, these developments are invaluable as a holistic analysis of single-cell performance, including quantitative data on cell-specific rates for substrate uptake, product formation, as well as growth, and their interrelation with environmental conditions and intracellular processes is becoming possible. In this article, we critically review these novel approaches and discuss their opportunities and challenges with several exemplary studies. Furthermore, we explain how directing technology combination, as well as the rational and directed integration of microfluidic and population-based methods into experimental workflows, accelerates the application of microfluidic single-cell analysis (MSCA) in biotechnology.

Microfluidics: from monitoring to understanding

Microfluidic single-cell technologies have been used for observing heterogeneities (Figure 1A) and cell dynamics in microbial populations in terms of growth [8], gene expression [9], cell interactions [10-12], production [13] or regulation [14]. The promise of understanding individuality at the cellular level made MSCA one of the most sought-after technologies of the past two decades [5,15]. With the unification of microbiology and microfluidics, supposedly well-understood physiological phenomena were reassessed. Diauxic growth in *E. coli* was shown to result from individuality in gene regulation and expression instead of collective and concerted cooperative behavior of the whole population [16,17]. By using smart microfluidic designs, cell aging and its physiological consequences could be studied in single bacteria and yeasts for the first time [8,18]. But what can we learn from this in biotechnology? Despite such studies demonstrated how microfluidics can be used to obtain mechanistic insights, single cell research is often focused on binary readouts in form of probability distributions (Figure 1A) instead of determining physiological or molecular parameters quantitatively(Figure 1B).

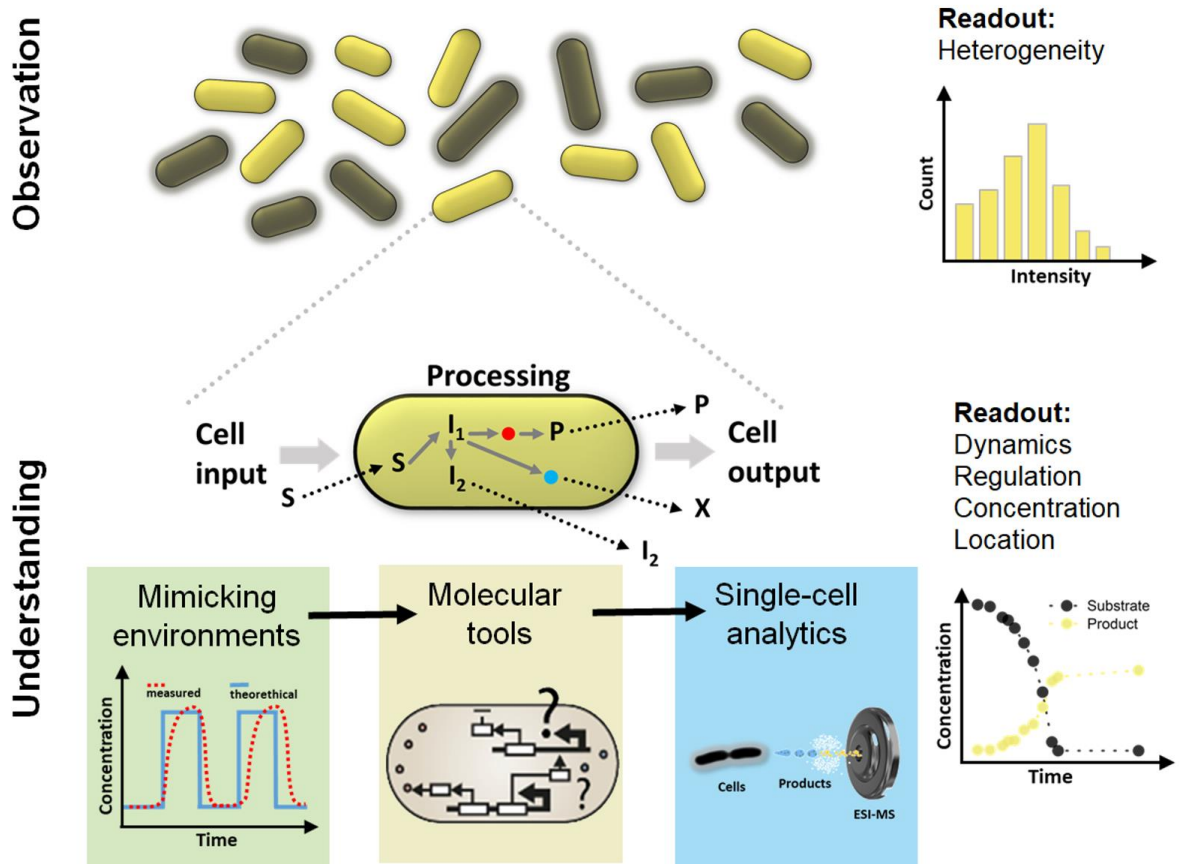

Figure 1: Microfluidic single-cell analysis – From observation to understanding. Microfluidic systems can be used to observe heterogeneity on a single-cell level (upper part). Input-output analyses for understanding condition-dependent single-cell physiology (upper part). Controlling the extracellular environment for analyzing the dependence of cellular processes on external input. Molecular tools such as fluorescent sensors detect and survey intracellular processes. Cellular output and performance of single cells can be accessed via novel analytical technologies.

It is now important to ask how the unique capabilities of microfluidics can advance our understanding of cells, beyond observation (Figure 1B). MSCA features stringent control of the cellular environment that cannot be achieved in reactor or flask setups. Environmental control enables one to define the cell input and uncouple cells from the activity or signaling of other cells. As very low hydraulic residence times can be achieved with microfluidics, it is also possible to emulate sub-second environmental fluctuations that often occur at population scales and study the resulting concentration-dependent effects on cell physiology [19] Next to environmental control, advanced molecular tools enable the visualization, spatial analysis, and quantification of intracellular processes in cells. Several techniques, mostly based on fluorescent reporter compounds, give quantitative access to mutation rates, intracellular metabolite fluxes or spatial gene expression patterns. The output of a cell is one of the most important parameters for applied biotechnology and knowledge on cell-specific rates for production, uptake and growth identifies the contribution of individuals to the macroscopic process output. However, determining kinetics with single cells is one of the most challenging tasks as the amounts of target analytes that are produced by a single cell are minute. However, emerging analytical methods are now capable of measuring catalytic products and substrates at the level of only a few or even single living microbial cells and the development of quantitative sensors for metabolic products progressed enormously.

In the following, we will present some outstanding examples (Figure 2A) that demonstrate the development and application of pioneering tools and technologies towards the next level of MSCA and discuss their relevance for future developments in single-cell analysis for biotechnology.

Environmental control and cell input

Modifying environmental conditions in a controlled manner increases the experimental portfolio of single-cell analysis significantly. However, not only modifying the extracellular environment is of importance, but also the mode of true batch cultivation has to be implemented on the microscale. Kaganovitch *et al.* developed a microfluidic single-cell batch system for the characterization of cell-to-cell heterogeneity under batch growth conditions. They could show that the growth behavior of *E.coli* cells in the microscale batch resembles flask or bioreactor growth profiles [20].

Many cellular processes, such as nutrient uptake, are controlled by extracellular concentrations and substrate affinity. Lindemann and co-workers developed a microfluidic workflow to determine the substrate affinity K_S of single *C. glutamicum* cells to protocatechuic acid by performing mother machine experiments under limiting carbon source conditions [21].

Next to constant conditions, microfluidic systems have been developed to emulate/mimic fluctuating environmental conditions, which predominate in both natural and technical systems.

For example, Kaiser *et al.* investigated gene regulation under oscillating carbon source conditions by rapidly switching between glucose and lactose in 4-hour intervals (Figure 2B-left) [14]. They could show the dynamics of growth and gene expression of single *E. coli* cells upon medium switch (Figure 2B-right).

In comparison, Rojas *et al.* applied short pulses of high salt medium to investigate *E. coli* growth rate conservation under osmotic stress [22]. Bolineau *et al.* performed defined carbon source switches from glucose to lactose and investigated how it affects cellular growth and expression of *lac* genes. Their experiments showed sustained growth of a few single cells upon the diauxic shifts and revealed that the carbon utilization machinery of some cells is readily prepared to utilize lactose as carbon source. [16].

Recently, integrative microfluidic feedback control experiments have become of broader interest. Here, data on cellular output in the form of single-cell fluorescence is fed to a model-based control loop that adjusts the environmental conditions in order to direct gene expression. This technology is a powerful tool to understand and learn about predicting mechanisms of cellular regulation. Uhlendorf *et al.* used a feedback-loop to control gene expression and minimize the effects of expression stochasticity in yeast at both, the population and the single-cell level [23]. A comprehensive overview of feedback control strategies can be found in a recent review by Dunlop and co-workers [24].

Molecular tools - looking at intracellular traits

Progress in biosensor development continuously improves our understanding of intracellular processes. Fluorescence reporters where widely used to unravel cell-to-cell heterogeneity within populations, report of cellular states or tracking molecular mechanisms [25] Especially during the last two years, the portfolio of sensor applications expanded significantly, allowing for improved analysis in spatial and temporal accuracy, metabolic specificity and even the quantification of cellular changes or intracellular concentrations. Si *et al.* developed a functional fluorescently labeled replisome protein to track replication and division cycles at the single-cell level [26].

Van Vliet *et al.* used transcription-based fluorescence reporters in combination with live-cell imaging to investigate spatial gene expression patterns in bacterial colonies [27]. Novel fluorescence reporters enabled to investigate fitness and quantify mutation dynamics at the single-cell level [28-30].

Recently, Monteiro *et al.* [31] reported on a transcription factor-based fluorescent “flux sensor” (Figure 2C-left) that for the first time allows us to visualize and quantify glycolytic fluxes within single yeast cells. With the flux sensor, they identified subpopulations with altered metabolic pathway usage (Figure 2C-right). The principle of sensing metabolic fluxes in single cells might

become a valuable tool to study the consequences of heterogeneous pathway usage on the fermentative capacity of populations.

The latest developments in genetically-encoded FRET biosensors enabled the quantification of extracellular metabolites, such as L-lysine, on the bulk level [32]. Product-sensing FRET sensors are likely to be transferable to the single-cell level and might enable fluorescence-based metabolite quantification for screening single-cell behavior in production processes. Monitoring intracellular levels of target compounds might become accessible by further refinement of FRET biosensors. For a comprehensive overview of quantitative fluorescence imaging we refer to the excellent review article of Okumoto *et al.* [33]

Quantitative analytics for measuring cell output

The most challenging pillar of single-cell analysis is marked by the quantification of cellular performance in terms of cell-specific rates. Missing analytical capabilities often restrict cellular analysis to optical observables based on live-cell imaging. Furthermore, most optical procedures for quantitative measurements rely on highly specific fluorescent dyes or complicated genetically encoded protein probes.

Recent progress in the coupling of microfluidic cultivation to mass spectrometry-based offline analytics (Figure 2D – left) demonstrates the label-free quantification of the metabolic product L-lysine from only a dozen of living *Corynebacterium glutamicum* cells (Figure 2D, right) [34]. Assuming that MSCA-coupled mass spectrometry can be further refined for analyzing single cells via mass spectrometry, not only that arbitrary substrates and products would become quantifiable for determining yields and rates with single cells, but also obtaining structural information on cellular products would come into reach [35-37].

A recent breakthrough in cell output measurements via mass spectrometry was achieved by Haidas *et al.* In an integrated approach, they combined fluorescence analysis with mass spectrometry and used high-throughput microfluidic droplet encapsulation for the quantification and activity analysis of enzyme secretion in yeast [38].

Another elegant method for the quantification of enzyme copy numbers in single cells was developed by Stratz *et al.* [39]. Via on-chip cell lysis and a chip-integrated immunoassay, the intracellular β-galactosidase levels of individual *E. coli* cells could be accurately quantified. Based on cell encapsulation with droplets, Hammar *et al.* linked the lactate producing capacity and growth of phototrophic *Synechocystis* sp. cells via a picoinjected enzyme assay for lactate quantification [40].

Krone *et al.* recently demonstrated an approach for using UV-LIF analysis to determine the enantioselectivity of a hydrolytic reaction with only a few hundred cells cultivated in a microfluidic channel [41].

A

	Study	Approach	Aim
Input	Kaganovitch *et al.*, 2018, [20]	Microfluidic batch cultivations	Growth kinetics of *E.coli* cells on single-cell level
	Lindemann *et al.*, 2019, [21]	Defined carbon limitations	Substrate affinity of *C. glutamicum* of single cells
	Kaiser *et al.*, 2018, [14]	Fluctuating substracte concentrations	Regulation of gene expression on single-cell level
	Boulineau *et al.*, 2013, [16]	Carbon source switch	Single-cell diauxic behaviour of *E. coli*
	Uhlendorf *et al.*, 2012, [23]	Oscillating osmostic pressure	Real-time control of gene expression
Molecular tools	Si *et al.*, 2019, [26]	Tracking growth and cell division	Mechanism of cell-size control
	van Vliet *et al.*, 2018, [27]	Fluoresence based transcriptional reporters	Spatial gene expression in bacterial colonies
	Robert *et al.*, 2018, [29]	Cultivation with fluorescent mutation tag	Mutation dynamics and fitness effects
	Uphof *et al.*, 2018, [30]	Cultivation with fluorescent mutation tag	Real-time imaging of DNA mismatch rate dynamics
	Monteiro *et al.*, 2019, [31]	Fluoresence based flux sensor	Identification of metabolic subpopulations in yeast
Output	Dusny *et al.*, 2019, [34]	Microfluidic cultivation coupled to ESI-MS	Quantification of L-lysin from a few living cells
	Haidas *et al.*, 2018, [38]	Droplet microfluidics coupled to MALDI-MS	Multimodal analysis of enzyme secretion
	Stratz *et al.*, 2014, [39]	Immunoassay based analysis	Quantification of intracellular enzymes in single cells
	Hammer *et al.*, 2015, [40]	Droplet based fluorescent enzyme assay	Population heterogeneity in growth and production
	Krone *et al.*, 2016, [41]	UV-LIV analyss of catalytic products	Identyfying enantioselectivity of biotransformations

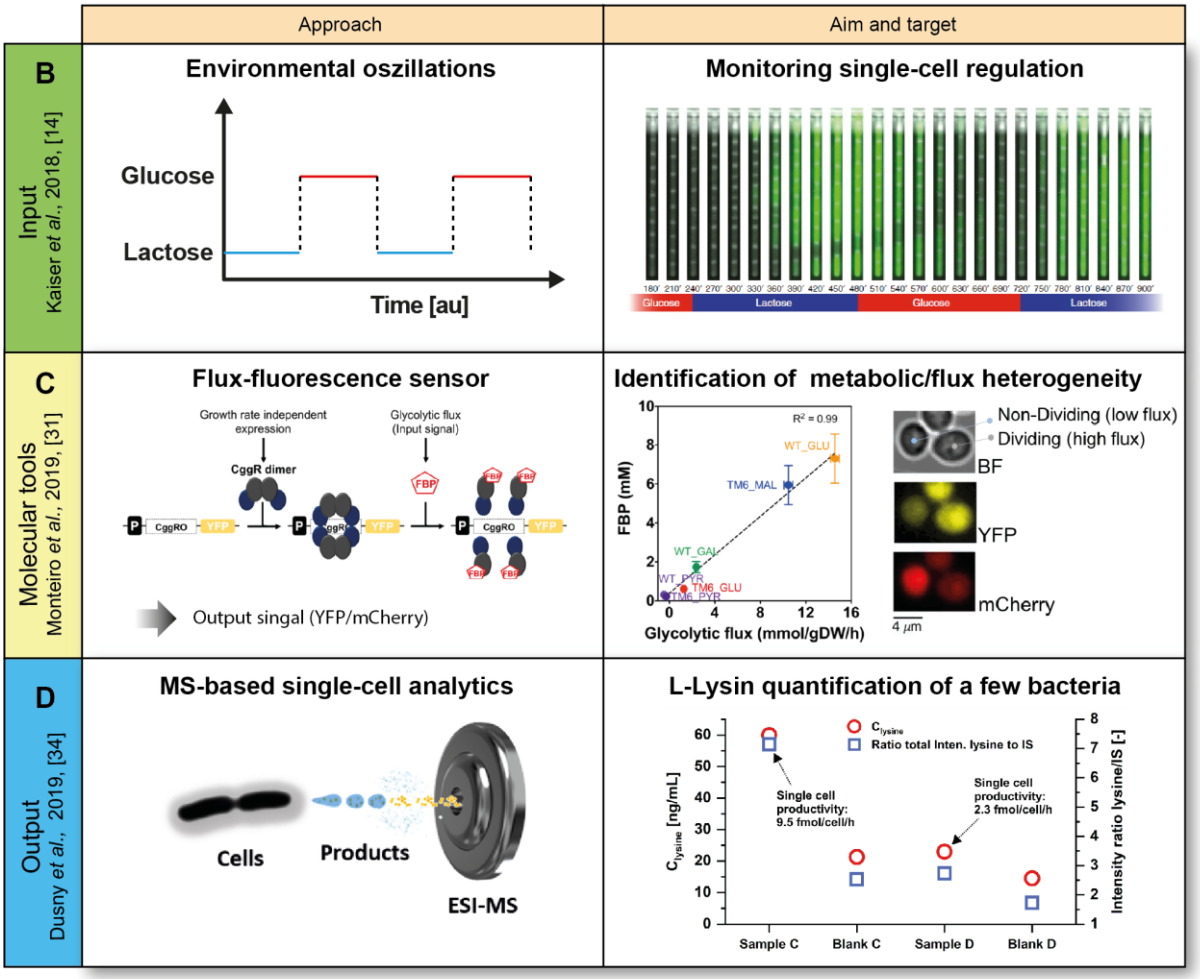

Figure 2: Examples for emerging approaches in input, molecular tools and output for improved understanding of single-cell processes. (A) Examples of recent microfluidic studies that demonstrate control and modification of environmental conditions, development of novel molecular tools for analysis

and visualization of intracellular processes and quantification of different metabolites at the single-cell level. (B-D) representative examples for each category: (B) *Kaiser et al.* used oscillating conditions to monitor single-cell regulation under varying conditions. Images printed with permission from [14]. (C) Monteiro *et al.* used fluorescence flux sensors to determine glycolytic flux of *E.coli* single cells. Images printed with permission from [31]. (D) Micro populations of cells entrapped in droplets were coupled to MS-based analysis and L-Lysine concentration was quantified. Reprinted with permission from [34] Copyright 2019 American Chemical Society.

Integrative and combinatory MSCA concepts enable holistic cell analysis

The examples from the previous sections show how cell input control, molecular tools and cell output measurements in MSCA leads to an improved understanding of biotechnological relevant questions. The integration of such analytical principles can now be used to access cell physiology in an unprecedented level of detail and bridge the gap between monitoring and quantitative understanding of cellular functions at a single-cell level. One strategy is the integration of these tools as functional units in a single-chip format (Figure 3A). However, the development of fully functional and user-friendly lab-on-chip systems is challenging, despite the successful miniaturization of pumps and valves for the creation of complex environmental profiles [42] and first approaches for quantitative on-chip analytics [41]. The so-called “chip-in-a box” or “chip-in-a-lab” solutions offer far more flexibility for applying microfluidics in biotechnology and bioprocess engineering research [43]. We propose to link or even couple microfluidic systems with classical cultivation and analytics workflows instead of merging all functional units within one single chip or device.

A few studies exist that demonstrate the benefits of combining microfluidic single-cell analyses and population experiments for complementary biological measurements. The cross-scale analysis (Figure 3B-left) of carbon-catabolite repression in the yeast *Ogataea polymorpha* revealed that the detectable glucose threshold concentrations for promoter repression differed up to four orders of magnitude across the cultivation scales from populations to single cells [44]. Only by uncoupling single cells with perfusion microfluidics from the metabolic activity of surrounding cells, a quantitative relation between promoter activity and extracellular glucose concentration could be established and revealed the ultrasensitivity of the promoter system to glucose-mediated carbon-catabolite repression (Figure 3D - right). Other cross scale-analyses of growth in *C. glutamicum* revealed higher cell-specific growth rates at the single-cell level, both at complex [45] and minimal medium conditions [46].

Gefen *et al.* directly interlinked micro- and macroscale cultivations (Figure 3C - left) and investigated growth and induction of gene expression in single *E. coli* cells under true batch conditions. To reproduce the cultivation dynamics of population-based batch cultures with

microfluidics, single cells were trapped beneath a nutrient-permeable membrane and continuously perfused with culture supernatant from shake flasks. Growth and gene expression could be tracked individually at the same conditions as in the batch culture. Using this approach, the authors found that cells are able to *de novo* synthesize protein after extended periods of carbon source starvation in the stationary growth phase (Figure 3C - right) [9].

In a multiscale approach (Figure 3D – left), Unthan and co-workers combined microfluidics, reactor cultivations, transcriptomics, and offline analytics to understand why single cells of *C. glutamicum* are growing 50 percent faster in microfluidics systems as in shake flask systems [46,47]. With their combinatory approach, they identified the iron-chelating medium compound protocatechuic acid (PCA) as the responsible factor for the observed elevated growth rates (Figure 3D – right). In population-scale cultivations, the minute amounts of PCA are metabolized within minutes, while in microfluidic perfusion PCA is continuously resupplied as an additional carbon source. Here, microfluidics revealed the phenomenon of higher growth rates in single-cell cultivations and conventional population tools for cultivation and analysis identified the responsible compound and the underlying mechanism. These examples impressively demonstrate how integrative and combinatory approaches advance microfluidics from an observation-based experiment towards a quantitative understanding of cellular processes at the single-cell level.

The integration of tools within microfluidic systems, as well as the integration of these systems into the existing lab-scale workflows will lay the foundation for improved mechanistic understanding of cellular processes. In a short term, this lays the foundation of next-generation bioprocesses, especially through control and exploitation of cellular heterogeneity [6,48] and the metabolic engineering of robust production strains [49], but also through improved engineering strategies to control cellular behavior within large-scale bioprocesses. On long-term biotechnological principles such as mass balancing of single cells will become close to reaching within the next years, fulfilling the systems biology dream of quantifying cellular heterogeneity with a never available resolution.

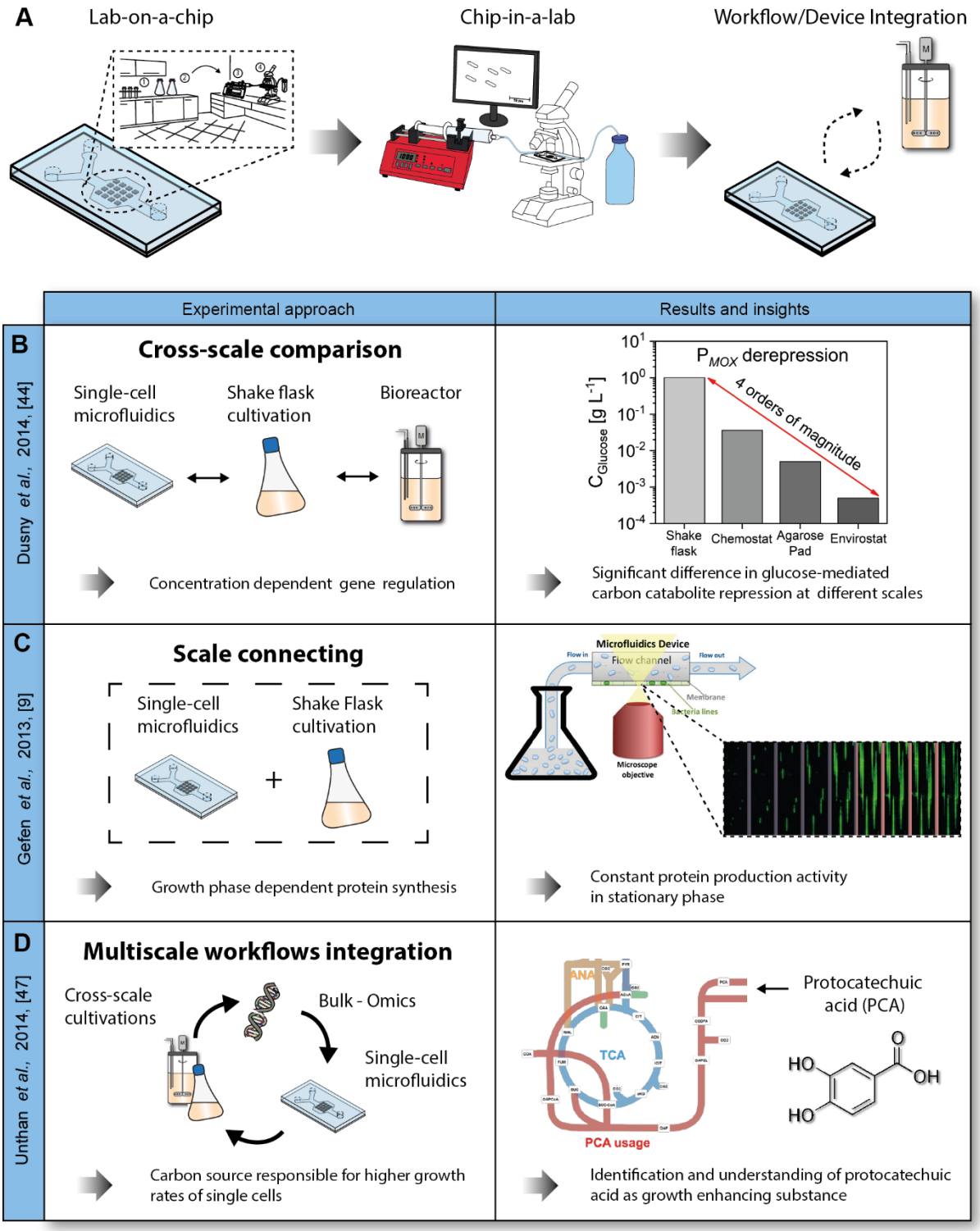

Figure 3: Integration of microfluidic systems into biotechnological workflows. (A) The dream of "Lab-on-a-chip" systems can currently be more seen as "chip-in-a-lab" systems, which in the future can be integrated into biotechnological workflows (B-.D). Example studies that integrated microfluidic systems into biotechnological workflows. (B) The cross-scale analysis up to several orders of magnitudes across cultivation scales (pl-ml scale) of carbon-catabolite repression in the yeast *Ogataea polymorpha.* (C) A study where a microfluidic system was coupled to a bioreactor to analyze cellular gene expression upon true batch conditions. (D) Multiscale workflow integration, where different "Omics" technologies and

cultivation in bioreactor, shake flask and microfluidic systems were used to identify the molecule PCA which is responsible for increased growth rates of *C. glutamicum* during MSCA.

Final remarks

This work intends to stimulate rethinking the role of microfluidic single-cell analysis for biotechnology. Microfluidics mature towards an important technology for quantitative physiology with single cells and can provide considerable additional value when properly integrated and combined with population-based workflows. As novel cultivation technologies and analytics enable to control cell input, quantitatively measure intracellular processing and cell output, mass and energy balancing of individual cells now comes into reach. Knowledge of mass and energy flows is invaluable for understanding the working principles of the target cellular physiology beyond observing heterogeneities. In the near future, microfluidic single-cell analysis will enable the improvement of microbial cell factories via the identification of novel targets for metabolic, reaction and process engineering strategies.

Acknowledgments

This project was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - 428038451.

References

1. Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B: **Industrial biocatalysis today and tomorrow**. *Nature* 2001, **409**:258-268.
2. Lin B, Tao Y: **Whole-cell biocatalysts by design**. *Microbial cell factories* 2017, **16**:106-106.
3. Dusny C, Schmid A: **Microfluidic single-cell analysis links boundary environments and individual microbial phenotypes**. *Environmental Microbiology* 2015, **17**:1839-1856.
4. Fritzsch FSO, Dusny C, Frick O, Schmid A: **Single-cell analysis in biotechnology, systems biology, and biocatalysis**. *Annual Review of Chemical and Biomolecular Engineering* 2012, **3**:129-155.
5. Grünberger A, Wiechert W, Kohlheyer D: **Single-cell microfluidics: Opportunity for bioprocess development**. *Current Opinion in Biotechnology* 2014, **29** 15-23.
6. Binder D, Drepper T, Jaeger K-E, Delvigne F, Wiechert W, Kohlheyer D, Grünberger A: **Homogenizing bacterial cell factories: Analysis and engineering of phenotypic heterogeneity**. *Metabolic Engineering* 2017, **42**:145-156.
7. Rosenthal K, Oehling V, Dusny C, Schmid A: **Beyond the bulk: disclosing the life of single microbial cells**. *FEMS Microbiol Rev* 2017, **41**:751-780.
8. Wang P, Robert L, Pelletier J, Dang WL, Taddei F, Wright A, Jun S: **Robust Growth of *Escherichia coli***. *Current Biology* 2010, **20**:1099-1103.
9. Gefen O, Fridman O, Ronin I, Balaban NQ: **Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity**. *Proceedings of the National Academy of Sciences* 2014, **111**:556-561.
10. Burmeister A, Hilgers F, Langner A, Westerwalbesloh C, Kerkhoff Y, Tenhaef N, Drepper T, Kohlheyer D, von Lieres E, Noack S, et al.: **A microfluidic co-cultivation platform to investigate microbial interactions at defined microenvironments**. *Lab on a Chip* 2019, **19**:98-110.

11. Liu J, Martinez-Corral R, Prindle A, Lee D-yD, Larkin J, Gabalda-Sagarra M, Garcia-Ojalvo J, Süel GM: **Coupling between distant biofilms and emergence of nutrient time-sharing**. *Science* 2017, **356**:638-642.
12. Osmekhina E, Jonkergouw C, Schmidt G, Jahangiri F, Jokinen V, Franssila S, Linder MB: **Controlled communication between physically separated bacterial populations in a microfluidic device**. *Communications Biology* 2018, **1**:97.
13. Mustafi N, Grünberger A, Mahr R, Helfrich S, Nöh K, Blombach B, Kohlheyer D, Frunzke J: **Application of a genetically encoded biosensor for live cell imaging of L-valine production in pyruvate dehydrogenase complex-deficient Corynebacterium glutamicum strains.** *PLOS ONE* 2014, **9**:e85731.
14. Kaiser M, Jug F, Julou T, Deshpande S, Pfohl T, Silander OK, Myers G, van Nimwegen E: **Monitoring single-cell gene regulation under dynamically controllable conditions with integrated microfluidics and software**. *Nature Communications* 2018, **9**:212.
15. Wang D, Bodovitz S: **Single cell analysis: the new frontier in 'omics'**. *Trends in Biotechnology* 2010, **28**:281-290.
16. Boulineau S, Tostevin F, Kiviet DJ, ten Wolde PR, Nghe P, Tans SJ: **Single-Cell Dynamics Reveals Sustained Growth during Diauxic Shifts**. *PLOS ONE* 2013, **8**:e61686.
17. Solopova A, van Gestel J, Weissing FJ, Bachmann H, Teusink B, Kok J, Kuipers OP: **Bet-hedging during bacterial diauxic shift**. *Proceedings of the National Academy of Sciences* 2014, **111**:7427-7432.
18. Lee SS, Vizcarra IA, Huberts DHEW, Lee LP, Heinemann M: **Whole lifespan microscopic observation of budding yeast aging through a microfluidic dissection platform**. *Proceedings of the National Academy of Sciences* 2012, **109**:4916-4920.
19. Ho P, Westerwalbesloh C, Kaganovitch E, Grünberger A, Neubauer P, Kohlheyer D, Lieres Ev: **Reproduction of Large-Scale Bioreactor Conditions on Microfluidic Chips**. *Microorganisms* 2019, **7**:105.
20. Kaganovitch E, Steurer X, Dogan D, Probst C, Wiechert W, Kohlheyer D: **Microbial single-cell analysis in picoliter-sized batch cultivation chambers**. *New Biotechnology* 2018, **47**:50-59.
21. Lindemann D, Westerwalbesloh C, Kohlheyer D, Grünberger A, von Lieres E: **Microbial single-cell growth response at defined carbon limiting conditions**. *RSC Advances* 2019, **9**:14040-14050.
22. Rojas E, Theriot JA, Huang KC: **Response of *Escherichia coli* growth rate to osmotic shock**. *Proceedings of the National Academy of Sciences* 2014, **111**:7807-7812.
23. Uhlendorf J, Miermont A, Delaveau T, Charvin G, Fages F, Bottani S, Batt G, Hersen P: **Long-term model predictive control of gene expression at the population and single-cell levels**. *Proceedings of the National Academy of Sciences* 2012, **109**:14271-14276.
24. Lugagne J-B, Dunlop MJ: **Cell-machine interfaces for characterizing gene regulatory network dynamics**. *Current Opinion in Systems Biology* 2019, **14**:1-8.
25. Elf J, Barkefors I: **Single-molecule kinetics in living cells**. *Annual Review of Biochemistry* 2019, **88**:635-659.
26. Si F, Le Treut G, Sauls JT, Vadia S, Levin PA, Jun S: **Mechanistic origin of cell-size control and homeostasis in bacteria**. *Current Biology* 2019, **29**:1760-1770.e1767.
27. van Vliet S, Dal Co A, Winkler AR, Spriewald S, Stecher B, Ackermann M: **Spatially correlated gene expression in bacterial groups: The role of lineage history, spatial gradients, and cell-cell interactions**. *Cell Systems* 2018, **6**:496-507.e496.
28. Dormeyer M, Lentes S, Ballin P, Wilkens M, Klumpp S, Kohlheyer D, Stannek L, Grünberger A, Commichau FM: **Visualization of tandem repeat mutagenesis in *Bacillus subtilis***. *DNA Repair* 2018, **63**:10-15.
29. Robert L, Ollion J, Robert J, Song X, Matic I, Elez M: **Mutation dynamics and fitness effects followed in single cells**. *Science* 2018, **359**:1283-1286.
30. Uphoff S: **Real-time dynamics of mutagenesis reveal the chronology of DNA repair and damage tolerance responses in single cells**. *Proceedings of the National Academy of Sciences* 2018, **115**:E6516-E6525.

31. Monteiro F, Hubmann G, Norder J, Hekelaar J, Saldida J, Litsios A, Wijma HJ, Schmidt A, Heinemann M: **Measuring glycolytic flux in single yeast cells with an orthogonal synthetic biosensor**. *bioRxiv* 2019:682302.
32. Steffen V, Otten J, Engelmann S, Radek A, Limberg M, Koenig BW, Noack S, Wiechert W, Pohl M: **A Toolbox of genetically encoded FRET-based biosensors for rapid l-Lysine analysis**. *Sensors* 2016, **16**:1604.
33. Okumoto S, Jones A, Frommer WB: **Quantitative imaging with fluorescent biosensors**. *Annual Review of Plant Biology* 2012, **63**:663-706.
34. Dusny C, Lohse M, Reemtsma T, Schmid A, Lechtenfeld OJ: **Quantifying a biocatalytic product from a few living microbial cells using microfluidic cultivation coupled to FT-ICR-MS**. *Analytical Chemistry* 2019, **91**:7012-7018.
35. Demling P, Westerwalbesloh C, Noack S, Wiechert W, Kohlheyer D: **Quantitative measurements in single-cell analysis: towards scalability in microbial bioprocess development**. *Current Opinion in Biotechnology* 2018, **54**:121-127.
36. Duncan KD, Fyrestam J, Lanekoff I: **Advances in mass spectrometry based single-cell metabolomics**. *Analyst* 2019, **144**:782-793.
37. Ali A, Abouleila Y, Shimizu Y, Hiyama E, Emara S, Mashaghi A, Hankemeier T: **Single-cell metabolomics by mass spectrometry: Advances, challenges, and future applications**. *TrAC Trends in Analytical Chemistry* 2019.
38. Haidas D, Bachler S, Köhler M, Blank LM, Zenobi R, Dittrich PS: **Microfluidic platform for multimodal analysis of enzyme secretion in nanoliter droplet arrays**. *Analytical Chemistry* 2019, **91**:2066-2073.
39. Stratz S, Verboket PE, Hasler K, Dittrich PS: **Cultivation and quantitative single-cell analysis of Saccharomyces cerevisiae on a multifunctional microfluidic device**. *Electrophoresis* 2018, **39**:540-547.
40. Hammar P, Angermayr SA, Sjostrom SL, van der Meer J, Hellingwerf KJ, Hudson EP, Joensson HN: **Single-cell screening of photosynthetic growth and lactate production by cyanobacteria**. *Biotechnology for Biofuels* 2015, **8**:193.
41. Krone KM, Warias R, Ritter C, Li A, Acevedo-Rocha CG, Reetz MT, Belder D: **Analysis of enantioselective biotransformations using a few hundred cells on an integrated microfluidic chip**. *Journal of the American Chemical Society* 2016, **138**:2102-2105.
42. Au AK, Lai H, Utela BR, Folch A: **Microvalves and micropumps for BioMEMS**. *Micromachines* 2011, **2**:179-220.
43. Streets AM, Huang Y: **Chip in a lab: Microfluidics for next generation life science research**. *Biomicrofluidics* 2013, **7**:011302.
44. Dusny C, Schmid A: **The MOX promoter in *Hansenula polymorpha* is ultrasensitive to glucose-mediated carbon catabolite repression**. *FEMS Yeast Research* 2016, **16**.
45. Dusny C, Fritzsch FSO, Frick O, Schmid A: **Isolated microbial single cells and resulting micropopulations grow faster in controlled environments**. *Applied and environmental microbiology* 2012, **78**:7132-7136.
46. Grünberger A, van Ooyen J, Paczia N, Rohe P, Schiendzielorz G, Eggeling L, Wiechert W, Kohlheyer D, Noack S: **Beyond growth rate 0.6: *Corynebacterium glutamicum* cultivated in highly diluted environments**. *Biotechnology and Bioengineering* 2013, **110**:220-228.
47. Unthan S, Grünberger A, van Ooyen J, Gätgens J, Heinrich J, Paczia N, Wiechert W, Kohlheyer D, Noack S: **Beyond growth rate 0.6: What drives Corynebacterium glutamicum to higher growth rates in defined medium**. *Biotechnology and Bioengineering* 2014, **111**:359-371.
48. Wang T, Dunlop MJ: **Controlling and exploiting cell-to-cell variation in metabolic engineering**. *Current Opinion in Biotechnology* 2019, **57**:10-16.
49. Wehrs M, Tanjore D, Eng T, Lievense J, Pray TR, Mukhopadhyay A: **Engineering robust production microbes for large-scale cultivation**. *Trends in Microbiology* 2019, **27**:524-537.

Outstanding Paper

* of special interest
** of outstanding interest

* Grünberger *et al.*: This review discusses the application of single-cell cultivation systems for application in applied biotechnology and bioprocess engineering.

* Elf *et al.*: A recent review that summarizes and discusses progress in the analysis of single-molecule kinetics in single cells

* Haidas *et al.*: Demonstration of multi-modal cell analysis by combining microfluidics-coupled mass spectrometry and fluorescence analysis.

* Ali *et al.*: A comprehensive overview of mass spectrometry approaches for single-cell analysis.

**Uhlendorf *et al.*: Microfluidics single-cell cultivation, image analysis, and modeling were used for model-based predictive feedback control of gene expression at the population and the single-cell level.

**Monteiro *et al.*: Development and application of a novel fluorescent biosensor for measuring glycolytic flux in single yeast cells. The sensor was applied to investigate flux heterogeneity among single cells.

** Dusny *et al.*: Demonstrates the quantification of L-Lysine produced by a few living microbial single cells via chip-coupled mass spectrometry.

** Unthan *et al.*: Combinatory approach that uses microfluidics, labs-scale cultivation and omics analyses for gaining a deeper understanding of elevated growth rates of *C. glutamicum* in microfluidics. An iron-chelating medium component was identified to enable extraordinary high growth rates.

**Gefen *et al.*: Coupling of a microfluidic single-cell cultivation device and batch cultivation in shake flask to analyze growth phase-dependent gene expression capabilities under true batch conditions.

** Dusny *et al.*: A cross-scale analysis that revealed a previously underestimated ultrasensitivity of a strong promoter system in the yeast *Ogataea polymorpha* to glucose-induced carbon-catabolite repression.