Quantifying a biocatalytic product from a few living microbial cells using microfluidic cultivation coupled to FT-ICR-MS

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ABSTRACT: The *in vivo* quantification of metabolic products from microbial single cells is one of the last grand challenges in (bio-)analytical chemistry. To date, no label-free analytical concept exists that is powerful enough to detect or even quantify the minute amounts of secreted low molecular weight compounds produced by living and isolated single bacteria or yeast cells. Coupling microfluidic cultivation systems with ultra-high resolution electrospray-ionization mass spectrometry with its exquisite sensitivity and specificity offers the prospect of single-cell product analysis and quantification, but has not been successfully implemented yet. We report an analytical framework that interfaces non-invasive microfluidic trapping and cultivation of a few bacterial single cells with the analysis of their catalytic products by Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). Cell trapping was performed with the microfluidic Envirostat platform for cultivating bacterial cells under continuous perfusion via negative dielectrophoresis (nDEP). 1.5 µL product-containing cell supernatant was sampled into microcapillaries using a dead volume-reduced world-to-chip interface. The samples were analyzed with a nanoESI ion source coupled to a FT-ICR-MS (limit of detection for lysine: 0.5 pg). As biocatalytic model system, we analysed few *Corynebacterium glutamicum* DM 1919 pSenLys cells that synthesized L-lysine from D-glucose. Secreted lysine was quantified from a few cells (down to 19). Single-cell specific lysine productivities were 2 and 10 fmol/cell/h. This demonstrates that coupling microfluidics and mass spectrometry (SIC-MS) now enables the quantification of catalytic products and extracellular metabolites from only a few living microbial cells.

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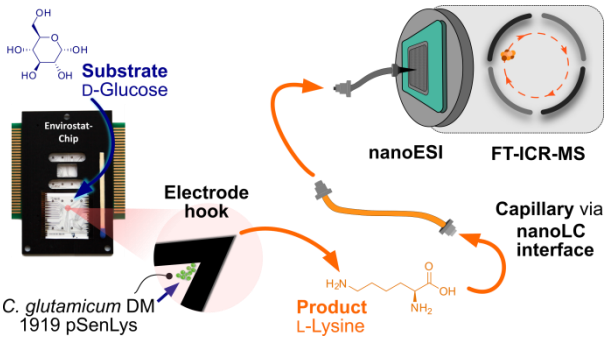

Introduction

Our understanding of the single cell as a catalytic unit of bio(techno)logy is built on bulk data from populations or tissues. [1] However, averages from bulk experiments do not contain information on individual cell states.[2] With the emergence of single-cell technologies, it became apparent that heterogeneity is a fundamental trait of cellular systems. The origins of heterogeneity are manifold, provoked by stochastic events in transcription and translation, unequal partitioning during cell division and changes in the cellular microenvironment.[3, 4] As a consequence, individual cells respond differentially to environmental cues despite clonality.[5] This aspect is of importance for biotechnological processes, where mostly microbial cells are used as living catalysts that perform complex chemical syntheses.[6] In such technical scenarios, cellular heterogeneity is tightly linked to the productivity and stability of the biotechnological process.[7]

Single-cell analytics for profiling cellular heterogeneity cover the wide spectrum of molecular hierarchy from genomes to metabolomes.[2] Targeted amplification-based methods such as PCR for DNA- or RNA-sequencing of single cells are well-advanced and can be used on a routine basis to assess cellular heterogeneity based on genome-scale molecular information.[8] Single-cell proteome or metabolome analyses are in contrast much less advanced than amplification-based methods.[9] However, untargeted analysis of metabolites and synthesis products in single cells is still a significant challenge, mainly due to the low abundance and high variety of the analytes and the resulting analytical requirements in terms of sensitivity, specificity, as well as lossless sample transfer and processing.[10] Among the wealth of technologies for the chemical analysis of metabolites, only mass spectrometry (MS) is capable to meet above requirements. MS can simultaneously detect, identify and quantify molecules in biological matrices with a high diversity over a large dynamic mass range at theoretical single-cell sensitivity.[11] However, major challenges for analyzing single cells are found in ion suppression due to matrix effects, analyte dilution in flow-through systems and the mostly small molecular weight of the target compounds. Studies reporting the successful analysis of metabolites from single cells via MS typically worked mainly with large cell types such as mammalian or plant cells.[12-14] Even non-invasive secretory measurements via mass spectrometry could be successfully demonstrated for several mammalian cell types.[15] However, cellular dimensions and hence analyte amounts pose vastly different challenges to the sensitivity of any single-cell metabolite analysis: a single rod-shaped *Escherichia coli* cell with a cell length of 3 µM contains approximately 3 x 10^8 metabolite molecules, whereas mammalian cells of 20 µM cell diameter can contain up to 1 x 10^{12} metabolite molecules.[16]

Only a handful of studies exist that demonstrate metabolite analysis from single microbial cells via MS. Noticeable are the pioneering studies of the Zenobi group using microarray-based single-cell MALDI (matrix-assisted laser desorption/ionization) coupled to a TOF-MS for obtaining insight into the ATP metabolism in *Saccharomyces cerevisiae* single cells.[17-20] Another recent study demonstrated the multimodal analysis of enzyme secretion from only 50-100 yeast cells via the microarray MALD-MS technology.[21] One key aspect that enabled such challenging secretome analysis is that produced compounds accumulate in batch environments to achieve concentration levels above the LOD of the MS analytics.

Next generation static SIMS (secondary ion mass spectrometry) coupled with an orbitrap mass analyzer allows higher spatial and mass resolutions.[22] An alternative approach used micro-droplet encapsulated cells directly infused into an electrospray source.[23] However, MS is generally difficult to apply for analyzing living cells and their dynamics as cells are typically disrupted for analyses. For many disciplines, such as biotechnology, it is precisely the dynamics of single cells and their catalytic activity that are of highest interest.

Microfluidic cell cultivation enables performing analyses with single-cell resolution under controlled environmental conditions. Microfluidic devices have been used to analyze the enantioselective biotransformation of a few hundred living *Escherichia coli* cells by means of deep UV time-resolved fluorescence.[24] In terms of untargeted product analysis from catalytically active, living microbial cells, this study represents a benchmark in single-cell analysis.

We hypothesize that the necessary technologies and methods to analyze catalytic products from single cells via MS, such as microfluidic chips for cultivation, interfacing solutions and suitable mass spectrometers, are available, but the synthesis towards a novel analytical concept for the functional integration of these technologies is still missing.

In this study, we explore the possibilities of coupling a microfluidic chip for cell cultivation to a Fourier-transform ion cyclotron resonance mass spectrometer for analyzing the catalytic products of only a few living microbial cells. As a biocatalytic model system, we investigated *Corynebacterium glutamicum* DM1919 pSenLys, which is capable to convert D-glucose to L-lysine via a multi-step reaction cascade.[25] A few single microbial cells of lysine-producing *C. glutamicum* DM 1919 pSenLys were cultivated under unperturbed physiological conditions in the chemically-inert Envirostat microfluidic single-cell cultivation system.[26] With the Envirostat, microbial cells can be specifically isolated and trapped via negative dielectrophoresis (nDEP) in a continuous flow of medium. Cellular phenomena such as regulation and physiological responses to environmental perturbations that are inaccessible via average values from cell populations can be analysed using the Envirostat concept.[27]

A simple, but reliable method was developed enabling direct infusion of the product-containing cell supernatants from microfluidics into a FT-ICR-MS.[28] Ion-suppression effects were minimized for the efficient sample collection from the microfluidic Envirostat and the lossless transfer of the sample by using a customized low-salt buffer system at nonetheless high cellular productivities during microfluidic cultivations (Figure 1).

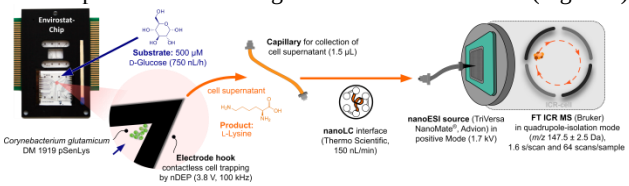

Figure 1: Conceptual approach for quantifying lysine produced by a few living microbial cells via single-cell microfluidics coupled to FT-ICR MS (SIC-MS).

Experimental section

Single-cell cultivation and sampling. Cell populations of *C. glutamicum* DM1919 were cultivated in batch mode using CGXII medium, harvested, washed and transferred to the Envirostat. For details on cell preparation and cultivation please see section S-1 in the supporting information. Microfluidic cultivations with the Envirostat were performed in glucose-containing ammonium bicarbonate (ABC) buffer. For experimental details on microfluidic cultivation, please see section S-2. The cell loading and sampling procedure is illustrated in Figure S-1. For sampling, cell supernatants from Envirostat cultivations were collected in polyether ether ketone (PEEK) capillaries (ID: 100 µm, OD: 1/16", l: 1910 mm, V = 1.5 µL). The open end of the capillary was exposed to a water saturated atmosphere in order to prevent sample evaporation and crystal formation at the capillary tip. A dead volume-reduced

microfluidic world-to-chip-interface was used to connect the capillary to the Envirostat chip.[29, 30] After 12 h of incubation, the capillary, which was filled with the supernatant produced during the preceding 2 h, was removed, sealed with PEEK-MicroTight adapters (P-882, IDEX Health & Science LLC, WA, USA) and immediately transferred to MS analysis.

FT-ICR-MS analysis. All MS measurements were performed with an FT-ICR mass spectrometer with a dynamically harmonized analyzer cell (solariX XR, Bruker Daltonics, Billerica, MA, USA) and a 12 T refrigerated actively shielded superconducting magnet (Bruker Biospin, Wissembourg, France). The mass spectrometer was controlled with ftmsControl 2.1.0 (Bruker Daltonics, MA, USA) and data was processed with Compass DataAnalysis 4.4 (Bruker Daltonics, MA, USA). Spectra were recorded with low mass range setting ($73 < m/z < 1000$; Time of flight = 0.6 ms) in broadband mode using magnitude mode processing (8 MWord; $m/\Delta m$ = 1,200,000 for m/z = 147). Mass calibration was done with Arginine cluster (Sigma-Aldrich). For Envirostat samples, Q-isolation spectra with a quadrupole-window of m/z 147.5 ± 2.5 Da were recorded. A TriVersa Nanomate® (Advion BioSciences, Ithaca, NY, USA) nanoESI ion source was used in positive ion mode. The distance of emitter chip to MS-inlet was 0.4 cm. Parameters for the NanoESI source were as follows: dry gas flow: 8 L/min, dry temperature: 150 °C, capillary voltage: 1.7 kV. For a lossless transfer of the Envirostat samples from the sampling capillary to the MS, LC-coupling mode was used. An LC-coupler (20 cm fused silica capillary, Advion BioSciences, Ithaca, NY, USA), was connected to a six-way autosampler valve of a NanoLC (Ultimate 3000 nanoRSLC, Thermo Fischer Scientific, Waltham, MA, USA) via a nanoViper-capillary (ID: 20 µm, OD: 1/16", length: 750 mm, Thermo Fischer Scientific, Waltham, MA, USA). LC-Coupler and nanoViper capillary were connected with a PEEK-MicroTight adapter (P-882, IDEX Health & Science LLC, WA, USA). The Envirostat sample was transferred into a 1 µL nanoViper sample loop and subsequently transported to the nanoESI source with a flow rate of 150 nL/min. A solution of 1:1 (V/V) ultrapure water and methanol, supplemented with 0.1% (V/V) formic acid was used as eluent. This setup ensured stable nanoESI flow rate and spray conditions as well as extensive rinsing of the capillaries before and after each injection. For method blank measurements and $^{15}N_2$-labelled L-lysine standards (Sigma-Aldrich), PEEK capillaries were either manually filled using a syringe or via automatic filling at the outlet of the Envirostat under real sample conditions (before cell transfer) and injected as described above. At standard measurement conditions, 64 scans were added with an accumulation time of 1.6 s per scan. Peaks were considered detected, if the signal-to-noise (S/N) ratio was greater than four and peak heights were used for quantification. Instrumental and method limit of detection and quantification were determined by taking average peak intensities plus 3 and 9 times the respective standard deviation of repeated blank measurements.

Results

Low sample volume detection of lysine with FT-ICR-MS. All instrumental parameters (nanoLC flow rate, nanoESI voltage, ion transfer optics) were optimized for the detection of lysine. Long transients with matching long ion pre-accumulation times were found to produce the highest absolute intensities per used amount of analyte (data not shown). Such long ion accumulation times required the use of a small Q-isolation window of 5 Da to reduce the overall number of ions present in the ICR cell. No substantial decrease in signal magnitude was observed as compared to larger Q-isolation windows. Fragmentation of ions within the Q-window during accumulation still occurred, especially loss of H_2O, due to the long accumulation times (Figure S-2A). H_2O loss from lysine was below 4%.

To demonstrate that the sensitivity of modern ultra-high resolution mass spectrometers is sufficient for detecting catalytic products of a single microbial cell, a lysine standard solution (Sigma-Aldrich) was diluted to the expected product concentration range of the model system *C. glutamicum* DM1919 pSenLys (0.8 – 5 ng/mL). Measurements of standards were performed from vials with the nanoLC-nanoESI-FT-ICR-MS setup as described in the methods section. Based on absolute intensities, 0.8 ng/mL lysine could be detected from 0.6 µl sample within 4.1 min measurement time (approx. 0.5 pg lysine). While the observed S/N of the lysine signal was well above the peak detection limit, a blank signal was also constantly detected at the same accurate mass (Figure S-2B). The calculated instrumental limit of detection (LOD) and limit of quantification (LOQ) correspond to lysine concentrations of 0.5 ng/mL and 1.1 ng/mL, respectively (Figure S-2C).

To correlate the estimated single-cell lysine productivity and the determined LOD and LOQ of the FT-ICR-MS for lysine, we estimated the resulting product concentrations in the sampled outflow of the microfluidic cultivation systems. Typical volumetric flow rates in microfluidic single-cell systems range from 0.1 to 1 µL/h. Based on this, final lysine concentrations of 0.2 – 2 ng/mL could be expected in the supernatant of a single cultivated cell, which is above the lower limit of detection for our FT-ICR-MS method.

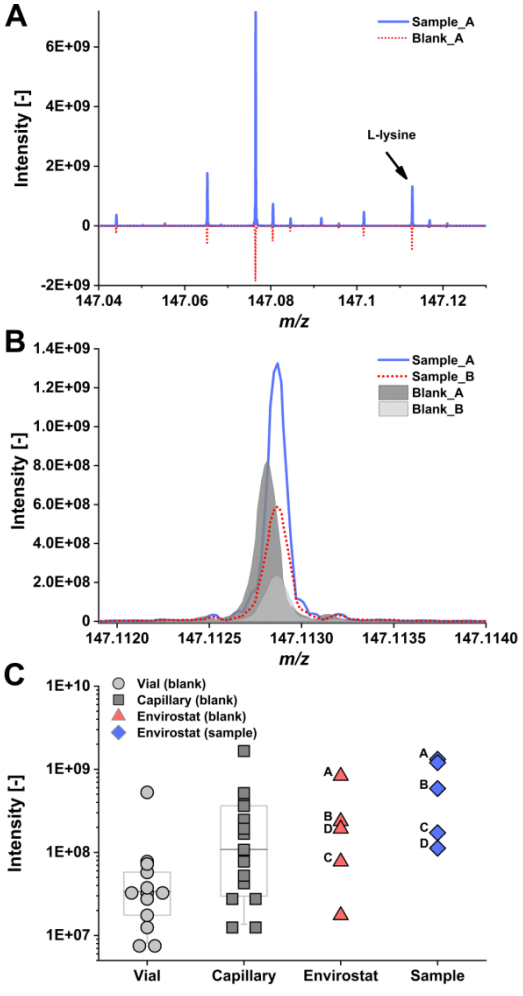

Figure 2: (A) Zoom into isolation spectrum of a cell supernatant from 24 cells (experiment A) and corresponding full method blank showing nominal mass 147. (B) Absolute intensities of lysine (m/z = 147.1128) for cell supernatants and

corresponding full method blanks for experiments A and B. At a mass resolution of 1.2 Mio, all interfering peaks at this nominal mass are resolved.(C) Variability of absolute lysine intensities among different blank conditions. For comparison, the absolute lysine intensities in the cell supernatant samples (Exp. A-D) from the Envirostat are shown. Y-axis is log10. For panels A-C, 64 spectra were co-added with 1.6 s ion accumulation time in the quadrupole.

Microfluidic cell trapping and sampling. As its core component, the developed approach used the Envirostat microfluidic single-cell cultivation and sampling system.[33, 39] Low ABC buffer concentrations of initial instrument sensitivity experiments (5 mM) hampered the trapping of cells via nDEP in the Envirostat due to the low electrical conductivity of the solution. Therefore a slightly higher buffer concentration of 20 mM was chosen for further experiments, which resulted in an optimal trapping performance of the cells via nDEP. For sampling experiments, a few cells (15 – 24 individual cells) were trapped in the hook region of the Envirostat and continuously perfused with glucose-containing buffer. After an equilibration phase of one hour under a constant flow rate for ensuring steady-state conditions, lysine-containing cell supernatants were sampled for 12 h into the sampling capillary. Microscopic observation of the cells confirmed cellular integrity of the cells during the whole experiment. Cell viability was verified via monitoring of the intracellular yellow fluorescent protein (YFP)-fluorescence. Cells did not grow or divide during the experiment duration due to the nutrient-limited conditions in the ABC reaction buffer.

Detection of lysine in cell supernatants. The current workflow to measure cell supernatant sampled by the Envirostat resulted in one set of sample plus blank per day. The blank was always obtained before the cell suspension was loaded into the Envirostat. A representative FT-ICR mass spectrum of the obtained cell supernatant (Experiment A) is shown in Figure 2. The section of the spectrum around the mass peak of lysine reveals > 10 peaks within a 0.1 Da window (Figure 2A, m/z 147). Most of them were also present in the corresponding method blank, although at lower absolute intensities. As expected, also at the m/z of lysine, a mass peaks was found in the method blanks (Figure 2A). These initial attempts to measure lysine in the supernatant of 15 and 24 cells yielded variable lysine peak intensities (Sample A+B, Figure 2B). In the corresponding method blanks from the same day the mass peaks at m/z of lysine had consistently lower absolute intensities (Blank A+B, Figure 2B). Although absolute intensities for the method blank samples for the Envirostat were higher than the respective medium blanks from vials (Figure 2C), the lysine signals in the samples were an order of magnitude larger than the highest artificial standard concentration (5 ng/mL) used for the sensitivity tests (see above). To distinguish between method blanks and daily instrumental variability, 10 ng/mL $^{15}N_2$-L-lysine was added to the medium and used in further cultivation experiments as internal standard (IS). This also allowed for a reliable quantification of lysine for extended experimental series.

Effect of ABC concentration and calibration. The influence of the ABC buffer concentration on lysine ion suppression was evaluated in a physiologically-relevant concentration range between 5 mM and 50 mM. Samples were measured from vials and the internal standard was added to compensate for instrument variability. The absolute lysine signal decreased by a factor of 9 with increasing buffer concentration, whereas the normalized intensity was less affected (Figure 3A). Using 5 ng/mL lysine, the S/N of the mass peak at 20 mM ABC concentration was still well above the peak detection limit. The presence of 50 µg/mL Kanamycin in the medium did not further influence the lysine signal intensity (data not shown). Due to the overall high absolute intensities of the lysine mass peak in the two initial experiments A and B, a matrix-matched calibration series was prepared between 5 and 40 ng/mL lysine (in 20 mM ABC buffer, 500 mM glucose and 10 ng/mL IS) and measured from vials with the nanoLC-nanoESI-DI-FT-ICR-MS method (Figure 3B). We then used the derived calibration function to quantify the lysine concentration in the cell supernatants samples from the Envirostat and in the corresponding method blank sample.

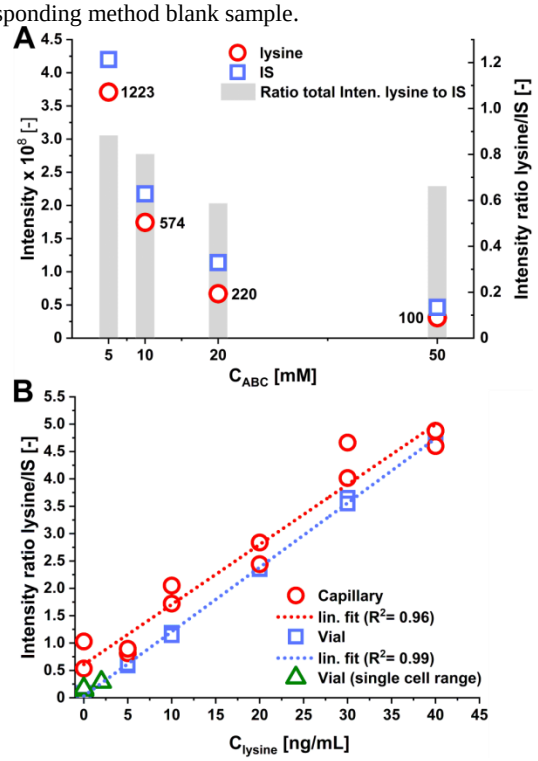

Figure 3: (A) Effect of ABC buffer concentration on lysine (5 ng/mL, red) and IS (10 ng/mL, blue) signal intensity and the corresponding lysine/IS ratio. The S/N ratio of the respective lysine peak is indicated. (B) Calibration of lysine using the nanoLC-nanoESI-FT-ICR-MS setup. Duplicated injection of standards from vials (squares and triangles) and manual filling of capillaries (circles) with respective linear fits are shown. Samples were prepared in 20 mM ABC buffer with 500 mM glucose. The IS concentration was 10 ng/mL and the lysine calibration range adjusted to the expected concentrations from the experiments C and D.

Quantification of lysine produced by a few living microbial cells. Experiments were conducted from individual cell batches in order to quantify cell-specific lysine productivity of *C. glutamicum* DM1919 pSenLys cells cultivated in the Envirostat system (Figure 4A+B). The absolute intensities of the lysine signal from the microfluidic cell supernatant varied substantially between experiments (between 1.1 x 10^8 and 1.3 x 10^9, n = 4, Figure 2C) whereas intensities normalized to the internal standard showed less variability (3.5 – 7, Figure 4C). Full method blanks were prepared for each experiment and the corresponding lysine concentrations were calculated accordingly (11 – 21 ng/mL). The lysine concentrations in the samples after subtractions of the corresponding method blanks were 38.8 and 8.5 ng/mL for 21 and 19 cells, respectively. These values correspond to a cell-specific supernatant concentration of 1.9 and 0.5 ng/mL/cell. The sampled volume of 1.5 µL represents a time-averaged productivity of 2 h and hence an average cell-specific productivity of 5.9 fmol/cell/h (range: 2.3 – 9.5 fmol/cell/h) and mass output of 0.9 ng/cell/h (range: 0.3 – 1.4 ng/cell/h). The

coefficient of variation of lysine standards close to the instrument limit of detection was 6% (2 ng/mL, N = 7).

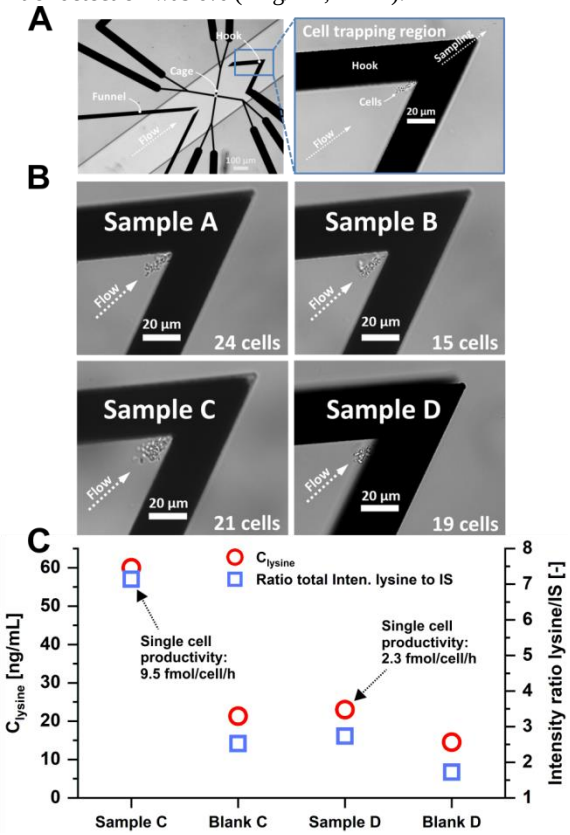

Figure 4: (A) Cell trapping and cultivation with the Envirostat single-cell analysis system. The micrograph of the cell manipulation and cultivation area shows functional electrode elements for cell focusing (Funnel), cell manipulation and presorting (Cage), and hook-shaped electrodes for cell retention (Hook). (B) Microscopic images of *C. glutamicum* cells that were trapped via nDEP at the hook electrodes. Corresponding numbers of trapped cells are given in the images. (C) Concentration of lysine (circles) and underlying intensity ratios (lysine/IS, squares) in experiments and corresponding method blanks. Blank concentrations were subtracted from the samples.

Discussion

The determination of metabolites from single cells based on mass spectrometry poses significant challenges in terms of sample transfer and matrix effects. Hence, matrix effects introduced by the applied buffer system become especially important for analyzing the minute product amounts from single cells. 5 mM to 20 mM ABC was found to be the optimal concentration range for production and nDEP cell trapping with negligible matrix effects for lysine quantification from microfluidic cultivations. Moreover, the application of volatile ABC buffer dispensed with the need of lossy preseparation of analyte and buffer compounds, while still supplying the necessary amounts of nitrogen for lysine synthesis to the cells.

Next to matrix effects, lossless sample transfer from microfluidics to the MS is a critical aspect in single-cell analysis. By using a dead volume-reduced world-to-chip interface that was directly coupled to sampling capillaries which served as a sample loop, sufficient amounts of cell supernatant could be transferred for quantification. By sealing the capillaries, sample storage at 4°C for up to 12 hours was possible without any loss in signal intensity.

Microfluidic probing of single cells for metabolite quantification results in a combination of low sample volumes (nL - µL range) and low analyte concentrations (low ng/mL range). Modern mass spectrometers are capable of detecting and quantifying fg to pg amounts of analytes, if they ionize sufficiently. With the nanoLC-nanoESI-FT-ICR-MS workflow developed in this study, limit of detection for the amino acid lysine in the range of 0.5 ng/ml (30 nM) was achieved. The absolute amount of lysine still being detected (0.5 pg or 30 fmol) is thus in the same range as achievable via LC-MS/MS with MRM or LC-FLD detection of derivatized amino acids.[40] It should be noted that background contribution of the analyte rather than instrumental limitations determined the LOD/LOQ for lysine. The source of the lysine background could not be fully eliminated despite rigorous cleaning and rinsing protocols.

The comparison of the blanks between vials and capillaries showed that most of the background lysine was introduced by the handling of the samples likely due to the ubiquitous presence of lysine. The lysine background also resulted in a lower fit of the calibration curve from capillaries as compared to the vials (Figure 3B). The measurement of the full method blanks showed that the Envirostat itself contributed little additional lysine background, confirming that the chip cleaning protocol was effective. To achieve lower LOD/LOQ for this particular analyte for single-cell analysis, either an even more stringent instrument cleaning and blank control, or further purification of chemicals is needed. Due to the long equilibration time of 12 h prior sampling as well as the reverse flow in the side channel used for cell loading, a contamination from the initial cell suspension could be excluded.

The observation of mass peaks in the blanks at the same m/z values as those in the samples highlights the need for proper blank controls, also in subsequent data processing, as ionization efficiencies in nanoESI-DI-FT-ICR-MS experiments are highly matrix dependent and thus vary between blanks and real samples.[31] This is especially important for the quantification of ultra-low amounts of metabolites from few cells (as demonstrated in this study), although here the matrix composition may be less variable and inherently determined by the medium due to the overall low concentration of metabolites.

The introduction of an internal standard to the glucose medium reduced the buffer composition-related variability as well as daily instrumental ionization variability. Based on the established calibration and an average of 19 – 21 cells, the cell-specific productivity ranged between 2 and 10 fmol/cell/h. This residual variability in productivity of after blank subtraction might be attributed to biological variability.

The averaged cell-specific productivities from shake flask experiments were lower by a factor of up to five compared to those obtained from the single-cell cultivations (see section S-3). A potential explanation for this interesting phenomenon can be found in the well-controlled and constant environmental conditions that prevail during the microfluidic cultivations. As demonstrated before, single cells can grow faster under continuous microfluidic perfusion with fresh medium than in populations.[32] This is due to the optimal supply of nutrients to the cells by the flow. This activation effect on metabolism might also be the explanation for the observed elevated productivities of single microbes under perfusion, although the cells were not able to proliferate in the MS buffer. Nevertheless, the calculated cell-specific lysine productivities from the quantification experiments are still well within the range of published productivities of *C. glutamicum*.[25]

Conclusions

Interfacing single-cell microfluidics and mass spectrometry, as demonstrated here, resulted in the possibility to quantify cellular productivity from living microbial cells in precisely controlled environments. The sensitivity of the method allowed detecting catalytic products from only a dozen of living microbial cells, which is now only one order of magnitude from a quantitative

product analysis of single isolated cells and thus closer to the isolated single-cell level than ever before. Adjusting microfluidic flow rates and further minimizing the blank levels from the buffer might be the key to reach single cell sensitivity. Additional clean-up or chromatographic steps to separate the analyte from medium compounds are considered to further minimize matrix effects.

For this study, we used the Envirostat in steady-state mode to obtain one sample for metabolite quantification. However, our microfluidic system allows fast manipulations of the chemical environment during incubation of a cell. We can now study the dynamic response of metabolite production of the same cell(s) under such rapid perturbations by sequential sampling of cell supernatant. This data complements high-throughput single-cell methods such as droplet microfluidics.

Our concept is basically adaptable to virtually any flow-through-based microfluidic cell cultivation system. The range of possible applications of the developed approach is very broad and spans from studying heterogeneity in production scenarios to mechanistic studies that enable linking environmental conditions and cellular performance. Label-free MS analysis thus holds the potential as a new standard in single-cell product quantification and opens the door towards quantifying microbial activity at the lowest possible biological level, the single cell.

References

1. Single-cell microbiology. In *Nat. Biotechnol.*, 2016; Vol. 34, p 1077.
2. Rosenthal, K.; Oehling, V.; Dusny, C.; Schmid, A., Beyond the bulk: disclosing the life of single microbial cells. *FEMS Microbiol. Rev.* **2017,** *41* (6), 751-780.
3. Nikel, P. I.; Silva-Rocha, R.; Benedetti, I.; de Lorenzo, V., The private life of environmental bacteria: pollutant biodegradation at the single cell level. *Environ. Microbiol.* **2014,** *16* (3), 628-42.
4. Balazsi, G.; van Oudenaarden, A.; Collins, J. J., Cellular decision making and biological noise: from microbes to mammals. *Cell* **2011,** *144* (6), 910-25.
5. Rego, E. H.; Audette, R. E.; Rubin, E. J., Deletion of a mycobacterial divisome factor collapses single-cell phenotypic heterogeneity. *Nature* **2017,** *546* (7656), 153-157.
6. Schrewe, M.; Julsing, M. K.; Buhler, B.; Schmid, A., Whole-cell biocatalysis for selective and productive C-O functional group introduction and modification. *Chem. Soc. Rev.* **2013,** *42* (15), 6346-77.
7. Delvigne, F.; Goffin, P., Microbial heterogeneity affects bioprocess robustness: Dynamic single-cell analysis contributes to understanding of microbial populations. *Biotechnol. J.* **2014,** *9* (1), 61-72.
8. Yuan, G. C.; Cai, L.; Elowitz, M.; Enver, T.; Fan, G. P.; Guo, G. J.; Irizarry, R.; Kharchenko, P.; Kim, J.; Orkin, S.; Quackenbush, J.; Saadatpour, A.; Schroeder, T.; Shivdasani, R.; Tirosh, I., Challenges and emerging directions in single-cell analysis. *Genome Biol.* **2017,** *18*.
9. Fritzsch, F. S.; Dusny, C.; Frick, O.; Schmid, A., Single-cell analysis in biotechnology, systems biology, and biocatalysis. *Annu. Rev. Chem. Biomol. Eng.* **2012,** *3* (1), 129-55.
10. Zenobi, R., Single-cell metabolomics: analytical and biological perspectives. *Science* **2013,** *342* (6163), 1243259.
11. Zhang, L.; Vertes, A., Single-cell mass spectrometry - Approaches to explore cellular heterogeneity. *Angew. Chem. Int. Ed. Engl.* **2018,** *57* (17), 4466-4477.
12. Guillaume-Gentil, O.; Rey, T.; Kiefer, P.; Ibanez, A. J.; Steinhoff, R.; Bronnimann, R.; Dorwling-Carter, L.; Zambelli, T.; Zenobi, R.; Vorholt, J. A., Single-cell mass spectrometry of metabolites extracted from live cells by fluidic force microscopy. *Anal. Chem.* **2017,** *89* (9), 5017-5023.
13. Comi, T. J.; Do, T. D.; Rubakhin, S. S.; Sweedler, J. V., Categorizing cells on the basis of their chemical profiles: Progress in single-cell mass spectrometry. *J. Am. Chem. Soc.* **2017,** *139* (11), 3920-3929.
14. Zhao, J.-B.; Zhang, F.; Guo, Y.-L., Quantitative analysis of metabolites at the single-cell level by hydrogen flame desorption ionization mass spectrometry. *Anal. Chem.* **2019**.
15. Fujita, H.; Esaki, T.; Masujima, T.; Hotta, A.; Kim, S. H.; Noji, H.; Watanabe, T. M., Comprehensive chemical secretory measurement of single cells trapped in a micro-droplet array with mass spectrometry. *Rsc Advances* **2015,** *5* (22), 16968-16971.
16. Schmid, A.; Kortmann, H.; Dittrich, P. S.; Blank, L. M., Chemical and biological single cell analysis. *Curr. Opin. Biotechnol.* **2010,** *21* (1), 12-20.
17. Zenobi, R.; Amantonico, A.; Urban, P. L.; Fagerer, S. R.; Balabin, R. M., Single-cell MALDI-MS as an analytical tool for studying intrapopulation metabolic heterogeneity of unicellular organisms. *Anal. Chem.* **2010,** *82* (17), 7394-7400.
18. Urban, P. L.; Schmidt, A. M.; Fagerer, S. R.; Amantonico, A.; Ibanez, A.; Jefimovs, K.; Heinemann, M.; Zenobi, R., Carbon^{13} labelling strategy for studying the ATP metabolism in individual yeast cells by micro-arrays for mass spectrometry. *Mol. Biosyst.* **2011,** *7* (10), 2837-40.
19. Amantonico, A.; Oh, J. Y.; Sobek, J.; Heinemann, M.; Zenobi, R., Mass spectrometric method for analyzing metabolites in yeast with single cell sensitivity. *Angew. Chem. Int. Ed. Engl.* **2008,** *47* (29), 5382-5.
20. Ibanez, A. J.; Fagerer, S. R.; Schmidt, A. M.; Urban, P. L.; Jefimovs, K.; Geiger, P.; Dechant, R.; Heinemann, M.; Zenobi, R., Mass spectrometry-based metabolomics of single yeast cells. *Proc Natl Acad Sci USA* **2013,** *110* (22), 8790-8794.
21. Haidas, D.; Bachler, S.; Kohler, M.; Blank, L. M.; Zenobi, R.; Dittrich, P. S., Microfluidic platform for multimodal analysis of enzyme secretion in nanoliter droplet arrays. *Anal. Chem.* **2019,** *91* (3), 2066-2073.
22. Passarelli, M. K.; Pirkl, A.; Moellers, R.; Grinfeld, D.; Kollmer, F.; Havelund, R.; Newman, C. F.; Marshall, P. S.; Arlinghaus, H.; Alexander, M. R., The 3D OrbiSIMS—label-free metabolic imaging with subcellular lateral resolution and high mass-resolving power. *Nat. Methods* **2017,** *14* (12), 1175.
23. Mahler, L.; Wink, K.; Beulig, R. J.; Scherlach, K.; Tovar, M.; Zang, E.; Martin, K.; Hertweck, C.; Belder, D.; Roth, M., Detection of antibiotics synthetized in microfluidic picolitre-droplets by various actinobacteria. *Sci. Rep.* **2018,** *8* (1), 13087.
24. Krone, K. M.; Warias, R.; Ritter, C.; Li, A.; Acevedo-Rocha, C. G.; Reetz, M. T.; Belder, D., Analysis of enantioselective biotransformations using a few hundred cells on an integrated microfluidic chip. *J. Am. Chem. Soc.* **2016,** *138* (7), 2102-5.
25. Becker, J.; Zelder, O.; Hafner, S.; Schroder, H.; Wittmann, C., From zero to hero--design-based systems metabolic engineering of *Corynebacterium glutamicum* for L-lysine production. *Metab. Eng.* **2011,** *13* (2), 159-68.
26. Rosenthal, K.; Falke, F.; Frick, O.; Dusny, C.; Schmid, A., An inert continuous microreactor for the isolation and analysis of a single microbial cell. *Micromachines* **2015,** *6* (12), 1836-1855.
27. Dusny, C.; Schmid, A., The MOX promoter in Hansenula polymorpha is ultrasensitive to glucose-mediated carbon catabolite repression. *FEMS Yeast Res.* **2016,** *16* (6).
28. Hofstadler, S. A.; Severs, J. C.; Smith, R. D.; Swanek, F. D.; Ewing, A. G., Analysis of single cells with capillary electrophoresis electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. *Rapid Commun. Mass Spectrom.* **1996,** *10* (8), 919-22.

29. Kortmann, H.; Chasanis, P.; Blank, L. M.; Franzke, J.; Kenig, E. Y.; Schmid, A., The Envirostat - a new bioreactor concept. *Lab Chip* **2009,** *9* (4), 576-585.
30. Fritzsch, F. S. O.; Kortmann, H.; Lonzynski, J.; Blank, L. M.; Schmid, A., Pressure-resistant and reversible on-tube-sealing for microfluidics. *Microfluid. Nanofluid.* **2011,** *10* (3), 679-684.
31. Giavalisco, P.; Hummel, J.; Lisec, J.; Inostroza, A. C.; Catchpole, G.; Willmitzer, L., High-resolution direct infusion-based mass spectrometry in combination with whole C-13 metabolome isotope labeling allows unambiguous assignment of chemical sum formulas. *Anal. Chem.* **2008,** *80* (24), 9417-9425.
32. Dusny, C.; Fritzsch, F. S.; Frick, O.; Schmid, A., Isolated microbial single cells and resulting micropopulations grow faster in controlled environments. *Appl. Environ. Microbiol.* **2012,** *78* (19), 7132-6.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Figure showing H_2O loss in CASI spectra, absolute intensities for blanks and standards and instrumental LOD/LOQ from vial-calibration (PDF)

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Notes

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