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Mass Spectrometric Method for Analyzing Metabolites in Yeast with Single Cell Sensitivity**

*Andrea Amantonico, Joo Yeon Oh, Jens Sobek, Matthias Heinemann, and Renato Zenobi**

Almost all currently employed analytical "-omics" methods provide data that are averaged over an entire cell population, but even genetically identical cells exposed to the same environmental conditions can show strong variations in molecular content and even in phenotypes.[1] Such heterogeneity is involved in many cellular and also disease-related processes, such as antibiotic resistance,[2] competence for DNA uptake,[3] and in viral life-cycle decisions.[4] The increasing number of such findings suggests that cellular heterogeneity is underestimated, which in turn calls for development of novel single-cell-based analytical methods.

Today, several techniques for chemical analysis of single cells exist, most of which focus on detection of peptides and proteins. In addition to the high sensitivity that is generally required for single cell analysis, the detection of intracellular metabolites (typically small molecules with MW < 1000 Da), poses additional challenges. In contrast to the protein and transcription levels, the metabolic level presents 1) turnover rates on the order of seconds, thus demanding fast cell processing, 2) a large chemical diversity, and 3) small molecular weights, which renders fluorescent tagging difficult without impacting the biological function of metabolites. Only very few studies have so far demonstrated the feasibility of small-molecule analysis in single cells. Capillary electrophoresis coupled with laser-induced fluorescence, a method named metabolic cytometry,[5] has been successfully used to monitor the oligosaccharide cascade of biodegradation products generated in single yeast cells,[6] in HT29 colon cancer cells,[5] and has recently been employed to characterize the metabolism of sphingolipids in single pituitary tumor cells.[7] Fluorescence resonance energy transfer (FRET) sensors have also been used to detect small molecules, such as glutamate,[8] in single cells. A variety of other techniques, for example, laser-induced native fluorescence[9] and capillary electrophoresis coupled with voltammetric detection[10] and UV adsorption[11] have been utilized to detect neurotransmitters and amino acids in single cells. What most of the currently existing approaches to small molecule analysis in single cells have in common, however, is that they are tailored to analyze a specific metabolite of interest, not allowing a broad measurement of many different metabolites at the same time, which is the goal of the emerging field of metabolomics.

In the field of classical (that is, population-averaging) metabolomics, mass spectrometry (MS) is increasingly used because, owing to its versatility, it can cope with the large chemical diversity of the metabolome. Although MS has been employed for single-cell analysis,[12] in particular for peptides and proteins, and in two studies detected very highly concentrated metabolites (histamine and serotonin in single mast cells[13]), MS has so far been severely limited for the detection of endogenous metabolites in single cells because of an inadequate sensitivity to detect the small, polar molecules constituting the primary metabolism. For these molecules, the sensitivity should be on the order of tens of attomoles; for example, a single yeast cell contains approximately 100 amol of ATP.[14] One MS-based technique that shows this promise is desorption/ionization on porous silicon (DIOS).[15] However, although DIOS is extremely sensitive for the detection of peptides,[16] we found the detection of metabolites from central metabolism to be impossible with the necessary sensitivity for single cell analysis, probably owing to their polarity (see the Supporting Information). Recently, a matrix-free method, nanostructure-initiator mass spectrometry (NIMS), was introduced.[17] NIMS has the capability to obtain single-cell mass spectra and to detect molecules with m/z lower than 1000, and may have the potential for single-cell analysis; yet it remains to be established which analytes are detectable by NIMS.

Herein, we present a mass-spectrometric method capable of detecting a wide set of endogenous metabolites with a sensitivity that allows the chemical analysis of a single yeast cell. Our strategy employs the combination of MALDI[18] with microscale sample preparation,[19] thin layer matrix deposition,[20] and negative-ion mode detection with the matrix 9-aminoacridine.[21] The sample is prepared by spotting picoliter amounts onto a target precoated with a thin, homogeneous

[*] A. Amantonico, Dr. J. Y. Oh, Prof. Dr. R. Zenobi
Department of Chemistry and Applied Biosciences
ETH Zurich
8093 Zurich (Switzerland)
Fax: (+41) 44-632-1292
E-mail: zenobi@org.chem.ethz.ch
Homepage: http://www.zenobi.ethz.ch
Dr. J. Sobek
Functional Genomics Center Zurich
UZH/ETH Zurich
8057 Zurich (Switzerland)
Dr. M. Heinemann
Institute of Molecular Systems Biology
ETH Zurich
8093 Zurich (Switzerland)

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layer of matrix. The spotting is performed using a commercially available noncontact piezoelectric dispensing system. The method is extremely fast and amenable to high throughput operation; the preparations of hundreds of spots can be done in a few minutes.

The approach presented herein allows the detection a few tens of attomoles of important endogenous metabolites (Figure 1). We calculate limits of detection (LODs) varying

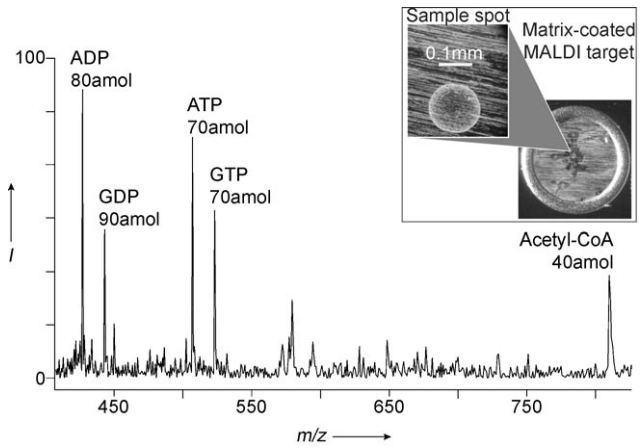

Figure 1. Single-cell sensitivity by MALDI microscale sample preparation. MALDI-TOF spectrum of an aqueous solution of five synthetic metabolites (100 ng mL^{-1}, 390 pL); metabolites and their amounts are indicated. Metabolite levels are all well above the detection limit. ATP content analyzed is lower than the content of a single cell. Inset: a photomicrograph of the sample spot resulting from the microscale sample preparation.

from 5 to 12 attomoles for important species such as ADP, GDP, ATP, GTP, and acetyl-CoA. The MALDI-based technique used herein is thus sufficiently sensitive for the analysis of single yeast cells, showing clear peaks for quantities of analyte significantly lower than the average content of a single cell. Previously reported LODs for these compounds were three orders of magnitude higher for an analogous MALDI method.[22] The excellent sensitivity for small molecules was achieved through a series of experiments and improvements (see the Supporting Information). A key aspect concerns the microscale layered sample preparation. Depositing matrix and analyte sequentially not only improves the sensitivity of MALDI,[20] but also facilitates the liquid handling with the piezo dispenser. Also, the piezo dispensing system creates a sample spot with a size comparable to that of the laser spot (Figure 1 inset). Conventional MALDI sample preparation using a micropipette and volumes on the order of microliters spread the analytes over a relatively large surface, and when analyzing small amounts of sample, the local concentration easily falls below the LOD. In our approach, the sample is spatially confined, giving detectable peaks for almost every laser shot.

The method was then tested on a biological sample. Previous studies have already demonstrated that negative ion mode MALDI with 9-AA as matrix is an effective tool for metabolome analysis.[22,23] We detected a variety of important components of the primary metabolism, such as phosphorylated nucleotides and coenzyme A derivatives, in a *Saccharomyces cerevisiae* cell extract (Figure 2). The chemical background, including peaks from the matrix itself, was surprisingly low. MS/MS experiments (data not shown) were

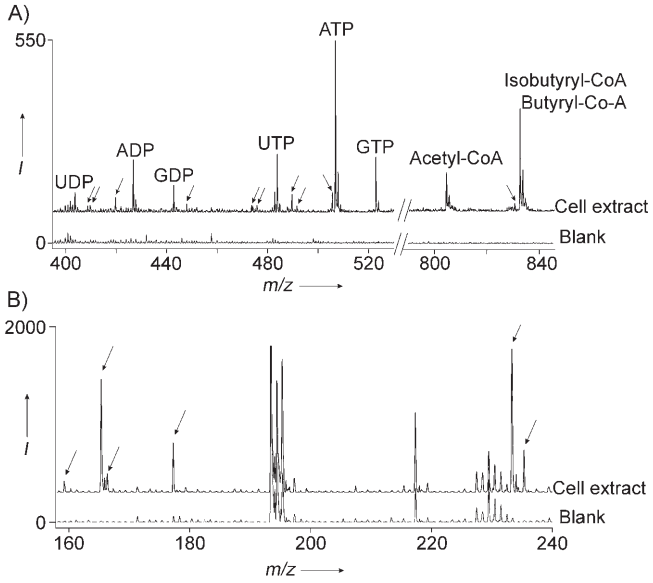

Figure 2. Use of 9-aminoacridine as matrix in negative-ion mode MALDI-MS, allowing the detection of different metabolites with a low chemical background. MALDI-TOF mass spectrum obtained from 400 nL of *Saccharomyces cerevisiae* cell extract in water (upper trace) and from 400 nL of pure water (blank; lower trace) dispensed onto a thin layer of matrix. The upper traces have been shifted vertically for clarity. A) Mass range showing signals of UDP, ADP, GDP, UTP, ATP, GTP, acetyl-CoA, and butyryl/isobutyryl-CoA. Identifications were made by MS/MS studies and by comparing accurate *m*/*z* values with the list of metabolite detected with 9AA in negative mode in a previous MS/MS study.[23] Numerous further peaks appear in the spectra (arrows), even in the low mass range (B), which also contains matrix signals.

conducted to identify some of the peaks in Figure 2, for example ATP at *m*/*z* 506.0 and acetyl-CoA at *m*/*z* 808.1, by comparison with spectra of metabolite standards and with available literature data. Accurate mass measurements, tandem-MS, and MS^n experiments could be used to identify the compounds corresponding to the numerous additional peaks observed in the spectra (arrows in Figure 2). The cell extract was analyzed directly, without previous chromatographic separation, a great advantage for single cell studies.

The single cell sensitivity was demonstrated using cell extract and the strategy illustrated in Figure 3. A certain number of *S. cerevisiae* cells (the exact number was estimated by flow cytometric analysis) was taken from a culture, and processed (see the Experimental Section).

This number of cells per volume allowed us to estimate the cell extract aliquot that corresponded to one single cell. Mass spectra of 4 nL and 390 pL of the cell extract corresponding to 6 and 0.6 cells are shown (Figure 4). These spectra show that this method is capable of direct detection of important endogenous metabolites in a complex matrix,

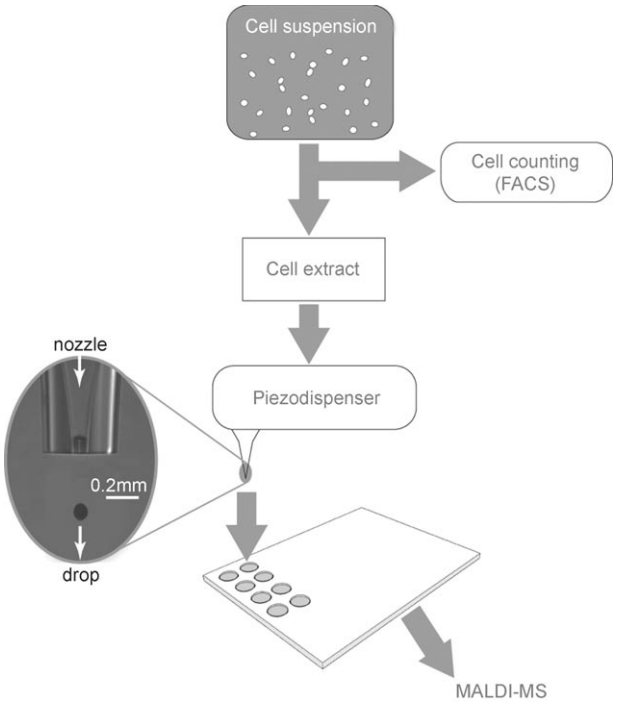

Figure 3. Strategy to analyze the metabolites contained in a single yeast cell. Fluorescence-activated cell sorting (FACS) was used to determine the number of cells from which the metabolites were extracted.

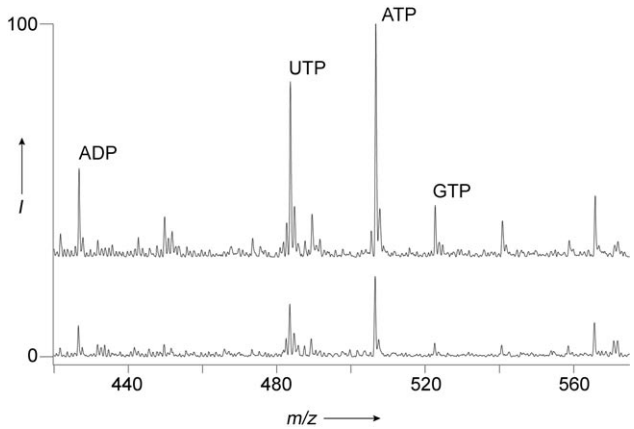

Figure 4. Analysis of the cell extract from less than a single cell. MALDI-TOF spectra of *Saccharomyces cerevisiae* cell extract, 4 nL upper spectrum) and 390 pL (lower spectrum), corresponding to approximately 6 and 0.6 cells, respectively. The upper trace has been shifted vertically for clarity.

such as the cell extract, even for a sample amount that corresponds to less than a single yeast cell.

The key innovation in this work is an unprecedented sensitivity for analyzing metabolites of the primary metabolism in a complex biological sample by mass spectrometry. The combination of a layered sample preparation with an optimized matrix leads to a large increase of the overall MALDI-MS sensitivity, opening the door for MS-based single-cell studies of the metabolism. As cell extract is analyzed directly, without any labeling or tedious sample work-up (i.e. without chromatographic separation), we now have a versatile detection method at hand that can, for example, be coupled to a microfluidics-based cell handling/lysis to handle the minute metabolite quantities originating from a single cell. We envision that such a combined platform will be key to tomorrow's systems biology as it will allow for new discoveries on metabolic variations in single cells.

Experimental Section

All metabolites (ADP, GDP, ATP, GTP, and acetyl-CoA) used in our standard mixture sample are commercially available and used without further purification. A piezo dispenser (sciFLEXARRAYER, Scienion AG, Berlin, Germany) was utilized for sample deposition. The volume of drops spotted by the dispenser depends on the solvent and on the nozzle orifice size (90 μm). In our case, each drop consisted of a volume of (390 ± 10) pL as determined by weighing. The sample was deposited onto a target precoated with matrix by spotting between 1 and 1000 drops. The spots were allowed to dry at room temperature; the evaporation took place within a few seconds. The thin matrix layer was produced by electrospraying or casting 0.7 μL of a 8 mg mL^{-1} solution of 9-aminoacridine in acetone onto a conventional MALDI plate. For more information on the matrix preparation, see the Supporting Information. All mass spectra shown were measured with a commercial MALDI time-of-flight mass spectrometry (Axima-CFR, Shimadzu/Kratos Analytical, Manchester, UK). Samples were ionized using a nitrogen laser (337 nm, 4 ns pulse width) and ions were accelerated with 20 kV. MS/MS spectra were performed on a 4800 MALDI-TOF/TOF (Applied Biosystems). All mass spectra were measured in negative ion mode. The yeast *Saccharomyces cerevisiae* CEN.PK 113-7D was cultivated in a bioreactor under fully aerobic conditions on a 5 g L^{-1} glucose minimal medium[24] at pH 5. The cell concentration in the culture broth was determined with a flow cytometer (BD Bioscience, Franklin Lakes, USA). At an optical density (600 nm) of 0.82, a sample (1.45 mL) was taken from the culture, and to stop any metabolic activity, cells were quenched with methanol at −40 °C according to a procedure slightly modified from that in reference [25]. Metabolites were then extracted with boiling ethanol according to a modified procedure.[26] The resulting solution of metabolites was stored at −80 °C. For the MS analysis, the sample was completely dried in a vacuum centrifuge and then water (10 mL) was added. The number of cells in the sample taken from the culture broth was determined to be 1.03×10^7 cells mL^{-1} × 1.45 mL = 1.49×10^7 cells. As we used 3.9 nL (or 0.39 nL) of the 10 mL metabolite solution for our analysis, our sample contained the metabolites from 6 (or 0.6) yeast cells.

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