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Single-Cell MALDI-MS as an Analytical Tool for Studying Intrapopulation Metabolic Heterogeneity of Unicellular Organisms

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Heterogeneity is a characteristic feature of all populations of living organisms. Here we make an attempt to validate a single-cell mass spectrometric method for detection of changes in metabolite levels occurring in populations of unicellular organisms. Selected metabolites involved in central metabolism (ADP, ATP, GTP, and UDP-Glucose) could readily be detected in single cells of *Closterium acerosum* by means of negative-mode matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS). The analytical capabilities of this approach were characterized using standard compounds. The method was then used to study populations of individual cells with different levels of the chosen metabolites. With principal component analysis and support vector machine algorithms, it was possible to achieve a clear separation of individual *C. acerosum* cells in different metabolic states. This study demonstrates the suitability of mass spectrometric analysis of metabolites in single cells to measure cell-population heterogeneity.

Heterogeneity is a characteristic feature of all populations of living organisms, and in fact it is observed even in monoclonal populations of unicellular microorganisms. Cell-to-cell variability can be due to a number of reasons; for example, nonuniformity of the cellular environment, position of a given cell within the cell cycle, genetic differences resulting from mutations,[1,2] or stochastic events in gene expression.[3] However, examination of the underpinnings of intrapopulation variability of cells can only be achieved with single-cell analysis. In fact, single-cell analysis is an emerging field that relies on modern analytical tools to study the biological processes responsible for the development of cell individuality.[4] Several microanalytical techniques are already available for the studies of genome, transcriptome and proteome on the single-cell level.[4]

Nonetheless, analysis of minute quantities of small molecules in complex matrices is challenging. Fluorometric assays generally require the introduction of fluorescent tags; thus, their applicability to studies of low-molecular-weight compounds in cells and cell extracts is limited to very few analytes (e.g., refs 5−10). Mass spectrometry (MS) is a label-free analytical technique which permits identification of virtually any analyte as well as structural analysis. During the past few years, the use of mass spectrometry in the analysis of small molecules has increased dramatically, and MS has become the key enabling tool in the field of metabolomics.[11,12]

Single-cell mass spectrometry (SCMS) is a rapidly growing branch of analytical chemistry, and a number of examples of its applicability to different cells can be found (e.g., refs 13−17). A few exciting studies demonstrate mass spectrometric analysis of several metabolites (usually small molecules, <1000 Da) present in single cells.[18−29] A recent example of a SCMS study using electrospray ionization (ESI) is the work by Mizuno et al.,[25] in

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which mass spectra corresponding to individual cells obtained from different cell lines were classified. Applications of SCMS based on matrix-assisted laser desorption/ionization (MALDI) to study biomolecules in single neurons have been presented by Sweedler and co-workers (e.g., refs 15, 16, 29). In general, MALDI-MS is widely used in proteomics, however its potential in metabolomics has recently been demonstrated.[30−32] MALDI-MS can provide efficient utilization of the limited amounts of analytes, and its sensitivity toward primary metabolites such as nucleotides is sufficient to detect the amounts present in single cells.[33] Moreover, MALDI-MS is less dependent on sample matrix composition than other techniques such as ESI-MS.[34] In this study, we show that MALDI-MS is capable of observing differences among individuals within a cell population on the metabolome level.

Up to date, research in cellular metabolomics could mainly be carried out on bulk samples composed of many cells. The main objective of this study is to demonstrate that MALDI-based SCMS can provide quantitative information on metabolic heterogeneity of cell populations and can distinguish subpopulations of cells with different metabolic states. This is achieved by depositing a single unicellular organism on every sample post on the MALDI target plate. By measuring metabolite levels in such organisms, we can learn about the primary, short-term response of populations of individual cells to environmental perturbations.

MATERIALS AND METHODS

Cell Culture. In order to study metabolic phenomena in the populations of single cells, we have chosen a unicellular eukaryotic alga, *Closterium acerosum* Ehrenberg (*Chlorophyta*). Being an autotroph, this species can easily be cultured in a dilute growth medium, compatible with MALDI-MS, making it a good biological model for this early application of the SCMS methodology in the analysis of small molecules in cell populations. *C. acerosum* cells were obtained from Carolina Biological Supply Company (Burlington, NC) and cultured in "Alga-Gro" medium. The starting culture of *C. acerosum* was initially chilled and etiolated for 4 days. On day 0 of the experiment, the culture was split into two aliquots. One of the aliquots was subsequently kept at a temperature of ~25 °C (photoperiod 16/8 h, day/night), further denoted as "warm". The other aliquot was kept in the dark at a temperature of ~4 °C, further denoted as "cold". The cultures were aerated on a regular basis by pumping air with a 100 mL syringe. The "warm" culture was illuminated with an electric bulb (13 W, 900 lm; Osram Dulux, Munich, Germany) placed at a distance of 30 cm. Before analysis, the cells were transferred into 10% "Alga-Gro" medium mixed with water (cf. Figure 5A).

Sample Preparation. Figure 1 presents our approach to single-cell mass spectrometry (SCMS) based on MALDI-MS, applicable to studies of metabolic individuality in cell populations.

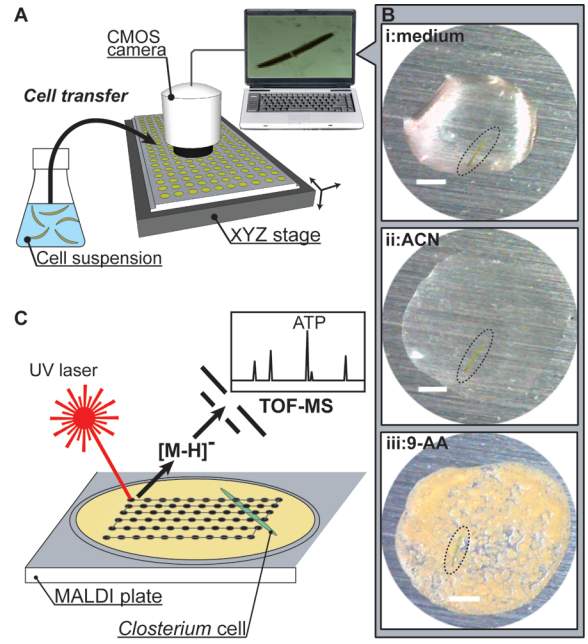

Figure 1. Single-cell mass spectrometry. (A) Concept of the experimental setup used for cell selection, transfer, deposition, counting and visual documentation. (B) Single-cell sample preparation on a MALDI plate (i. A single cell inside a droplet of a dilute growth medium. ii. Quenching of metabolism and initial extraction of metabolites by addition of acetonitrile. iii. Single cell embedded in MALDI matrix). The scale bars correspond to 200 μm. (C) MALDI-MS measurement of the metabolites contained within the matrix deposit.

For sample preparation, a small aliquot of the cell suspension (300 nL) is aspirated with a pipet and transferred onto a sample post on a stainless steel MALDI plate. A digital CMOS-based microscope mounted right above the MALDI plate (Figure 1A) enables counting the cells transferred, estimation of their size, visual documentation of cell appearance and quality control of the deposition process (Figure 1Bi). This additional information contributes to gathering basic information on the phenotype of the individual cells, which are subsequently analyzed by mass spectrometry. Following successful cell deposition, 300 nL of acetonitrile (spiked with uridine 5′-monophosphate (UMP) used as an internal standard; m/z 324) was pipetted into the droplet of medium containing a single cell (Figure 1Bii) in order to quench cellular metabolism and extract the metabolites. The natural content of UMP in the cells is very low compared with the amount spiked. The release of metabolites from the cell continues following the addition of the MALDI matrix solution (600 nL of 9 mg mL^{-1} 9-aminoacridine in acetone, Figure 1Biii), until all solvent has evaporated and cocrystallization of the matrix with the released metabolites has occurred. In this final step of sample preparation, a uniform distribution of metabolites with the crystalline layer of the MALDI matrix across the whole area of the sample spot is achieved. In order to ascertain a uniform distribution of analyte, it is important that all the three steps are performed in rapid sequence to avoid evaporation of solvents (assay medium and acetonitrile) between the steps. As the last step, the MALDI-MS analysis of the metabolites contained within the matrix deposit

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is performed (Figure 1C). 9-aminoacridine was used as a MALDI matrix[35] because it enables detection of nucleotides and other phosphate-containing metabolites with high sensitivity.[30,33]

Mass Spectrometry. A MALDI-TOF-MS instrument (Axima; Shimadzu/Kratos Analytical, Manchester, UK) equipped with a 337 nm nitrogen laser and controlled with Launchpad/Kompact software (ver. 2.4.1; Kratos Analytical) was used. The diameter of the laser beam focus on the target plate was ~100 μm. A rectangular serpentine raster (size: 1.3 × 1.3 mm; spacing: 100 μm; number of sampling spots: 196), covering ~80% of the sample spot area, was applied during data acquisition. Only sample spots with one microorganism were analyzed.

Sample Classification with Support Vector Machines. Support vector machines (SVMs) constitute a chemometric classification method introduced by Vapnik[36] that can be used for data evaluation in various fields.[37−39] Detailed information about SVM classifier can be found in refs 36, 40, 41. In the present study, four data subsets (130 samples in total) were used to build and test the SVM classification model: day 0 (30 samples), day 10 "warm" (34 samples), day 10 "cold" (30 samples), and day 10 "warm" + "cold" (36 samples). The classification task involved building a model that separates the day 10 "warm"/day 10 "cold" samples. This let us to define the region in the PCA score plot, in which the data points corresponding to the cells with low metabolic activity are localized. The Gaussian Radial Basis Function (RBF) kernel was used with a scaling factor (σ) of 0.25. Other SVM kernels (linear, quadratic, multilayer perceptron (MLP), etc.) were also tested. The quadratic programming method was used to find the separating hyperplane. All these calculations were performed using the Support Vector Machine toolbox (MATLAB software, ver. 7.6.0.324 (R2008a); MathWorks, Natick, MA).

RESULTS AND DISCUSSION

Targeted Metabolite Analysis of Single Cells. Previously it was demonstrated that by reducing the sample presentation area on the MALDI plate it is possible to achieve extremely sensitive detection of metabolites in a cell extract.[33] In the sample preparation protocol depicted in Figure 1, the confinement of the single-cell extract to a relatively small and homogeneous spot is therefore an important feature. In the case of relatively large cells such as *C. acerosum*, the confinement was achieved by adding only very small amounts of the cell medium, quencher and matrix solution. The extraction procedure with acetonitrile and matrix/acetone solution leads to the release of metabolites from the cell and a relatively even distribution of the extracted metabolites within the spot, with negligible dependence of the signal on the distance from the cell residue. While this is evident when spotting relatively large cells (*C. acerosum*), adaptation of this protocol for analysis of smaller cells would need to involve further minimization of the sample spot on the MALDI plate.

Figure 2 shows MALDI mass spectra corresponding to the single cells, deposited on the MALDI plate and documented in the pictures taken with the miniature CMOS camera. Peaks of several metabolites can be recorded from the samples of individual cells. We focus on four metabolites that give rise to a relatively high MS signal, ADP, ATP, GTP and UDP-Glucose (Figure 2A and B). The identities of all these peaks were confirmed in separate MS/MS analyses (Supporting Information (SI) Figure S1). Except for GTP, the metabolites were detected in all three cells whose spectra are shown in Figure 2A−C. Figure 2D shows a spectrum corresponding to the medium without cells, which has only few features characteristic of the MALDI matrix used. Cells (A) and (B) were cultured at a temperature of ~25 °C (photoperiod 16/8 h, day/night; further denoted as "warm"), wheres (C) was obtained from a culture kept at a temperature of ~4 °C for 10 days in the dark (further denoted as "cold").

By inspecting these spectra it is easy to observe differences in the signals corresponding to the metabolites studied. In order to study cell-to-cell variability of the metabolite content, the analytical method presented in Figure 1 needs to be characterized in terms of uncertainty related to the single measurement. Since each measurement is conducted on a single cell, it is not possible to study repeatability and other figures of merit in a classical way.

Instrumental vs Biological Variability. Figure 3 compares instrumental and biological variances observed in the MALDI-MS results. The instrumental variance was evaluated based on the ATP/ADP ratio obtained for multiple spots containing the same amounts of ATP and ADP standards. Analogically, the biological variance was evaluated based on the corresponding peaks in MALDI-MS spectra obtained for multiple spots containing single cells. An F-test clearly showed that the variance of the ATP/ADP ratio measured for the standard mixture is significantly lower than that observed in the analysis of individual cells. While the absolute individual amounts of ATP, ADP, and AMP may vary widely, the ATP/ADP ratio is a more reliable indicator of metabolism among cells; detection of ATP/ADP ratio is particularly valuable because this ratio determines (along with the free inorganic phosphate concentration) the actual free energy available for cellular reactions via ATP hydrolysis.[5]

A calibration plot obtained for metabolite standards (SI Figure S2) shows that within the anticipated limits of quantitative analysis by MALDI-MS (60−500 fmol), there is a linear dependence of the MALDI signal and the amount of the spotted analyte. UMP also proved to be a good internal standard: the correlation coefficients for 5-point calibration plots made by using ATP/UMP and ATP/^{13}C-ATP peak area ratios were exactly the same (R^2 = 0.999). Moreover, UMP was demonstraed to correlate with the ATP signal irrespective of the content of biological matrix (SI Figure S3). Thus, UMP can be considered a reliable internal standard which can be used for correction of the MALDI-MS signal in cases when ion suppression or heterogeneous crystallization of the matrix affect the absolute intensities of the MS peaks.

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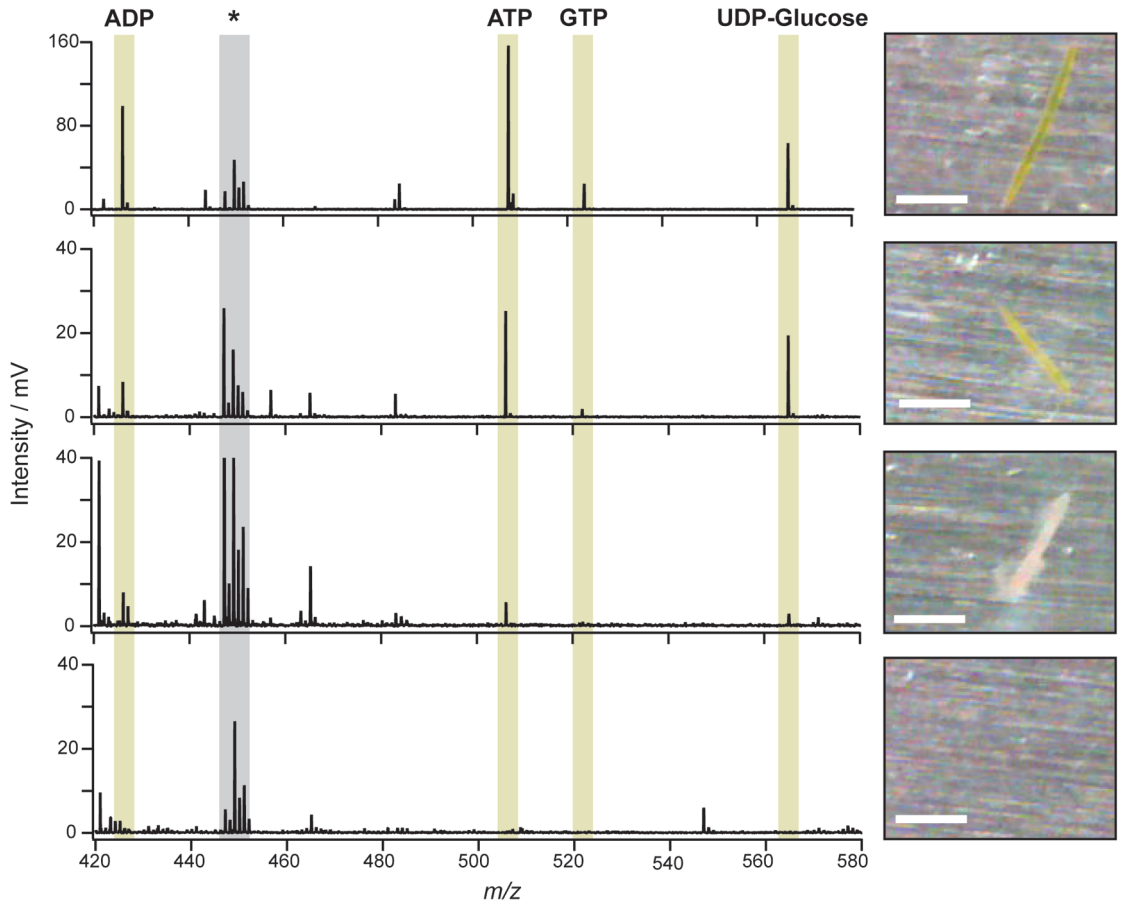

Figure 2. Metabolic individuality of single cells. (A−C) The three individuals of *Closterium acerosum* shown in the microscope pictures present different metabolic characteristics, as observed in the corresponding MALDI-MS spectra. Cells (A) and (B) were obtained from the "warm" culture, whereas (C) was obtained from the "cold" culture. (D) The spectrum corresponding to the blank (no cell deposited on the MALDI plate) contains several peaks characteristic of the MALDI matrix used (9-aminoacridine). All spectra were normalized by dividing the signal intensities by the intensity of the internal standard peak (UMP, not visible in the *m*/*z* range shown). The asterisk (*) denotes a spectral feature which is not associated with the biological sample. The scale bars correspond to 200 μm.

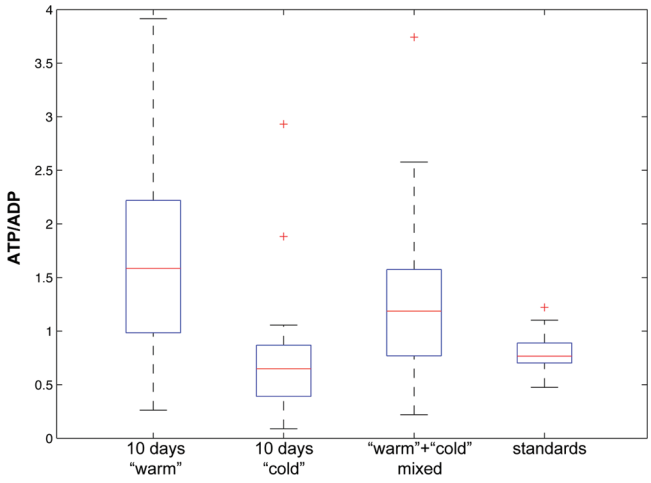

Figure 3. Box-and-whisker plot showing instrumental and biological variance observed in the MALDI-MS results. F-test showed that variance of the ATP/ADP ratio measured for the standard mixture is significantly lower than that observed in the analysis of individual cells on day 10.

Single-cell MS of Cell Populations. In order to verify the suitability of this SCMS method to studies on cell populations, we applied it to a simple biological system. By changing culture conditions ("warm" and "cold") we aimed to induce physiological changes in the cells that would enable testing the method depicted in Figure 1. Figure 4 is divided into three parts, in which the SCMS results corresponding to the "warm" cell culture (red-shaded), the "cold" cell culture (blue-shaded) and a mixture of cells obtained from these two cultures (gray-shaded) are displayed. Box-and-whisker plots for ATP/ADP and ATP/UDP-Glucose ratios are shown in Figure 4A and C. It was observed that the median values as well as the variances of the populations increased from day 0 to day 10 in the case of ATP/ADP (Figure 4A). On the other hand, the median values and variances obtained for the "warm" cell culture on day 0 and the "cold" cell culture on day 10 are comparable. In the case of the ATP/UDP-Glucose result, the variance increased already after day 1 and it ceased to increase further (Figure 4C). Similar trends as that observed in Figure 4A are also evident in the case of the triphosphates, ATP and GTP (SI Figure S4). Unlike in the case of ATP/UDP-Glucose (Figure 4C), the median values and apparent variances of ATP/ADP are higher in the mixture of cells ("warm" + "cold") than in the "cold" cell culture (Figures 3 and 4A). This is due to contribution of the high cell-to-cell ATP/ADP variability in the "warm" cell culture, observed on day 10 (cf. SI Figure S5). The observed changes in the variances of metabolite content can be explained with an increased cell-to-cell variability with respect to the energy charge

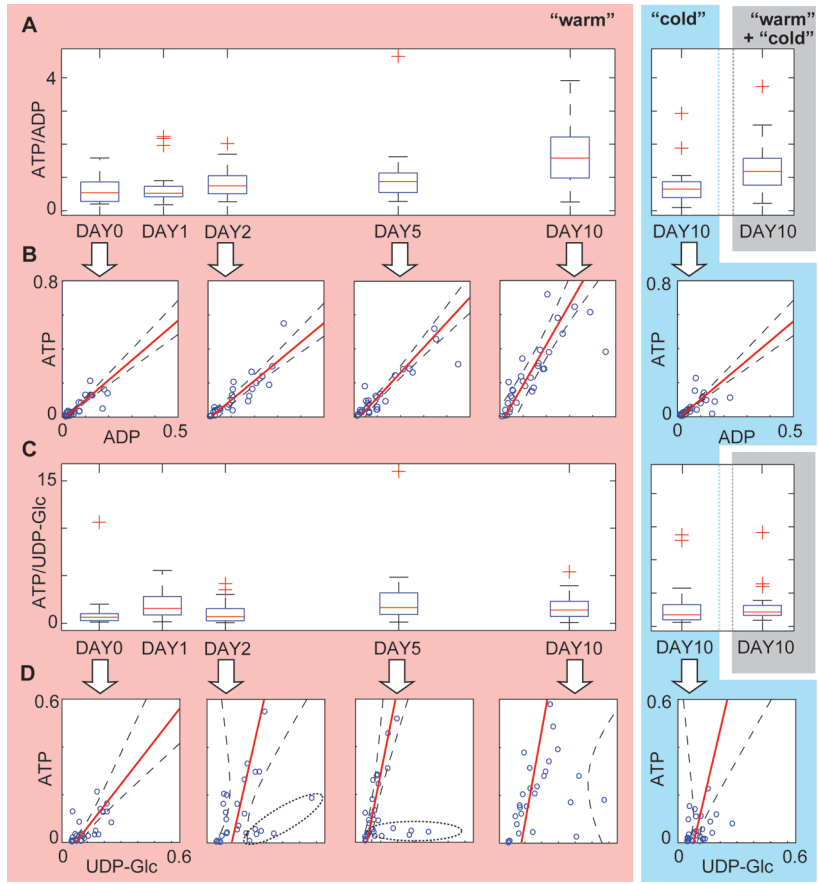

Figure 4. Metabolic heterogenity of cell populations studied by SCMS. Box-and-whisker plots for (A) ATP/ADP and (C) ATP/UDP-Glucose ratios calculated for all the cells analyzed in the experiment. (B) Peak intensities of ATP plotted against those of ADP obtained for a population of single cells. (D) Peak intensities of ATP plotted against those of UDP-Glucose, obtained for a population of single cells. In (A) and (C), red crosses (+) represent outliers. In (B) and (D), each data point (○) corresponds to a single cell; red lines are obtained by linear regression using robust fitting and the black dashed lines are the relative prediction bands (95% confidence). The red-shaded section presents results obtained for the "warm" cell culture. The blue-shaded section presents results for the "cold" cell culture. The gray-shaded section indicates the results obtained on day 10 for a mixture of the two above-mentioned cell cultures.

in the studied population of algal cells. Probability distribution plots enable putative deconvolution of the composite population ("warm" + "cold") into the constituent subpopulations (SI Figure S5). All these suggest that the SCMS approach presented here could enable monitoring metabolic changes in cell populations in time.

Inter- and Intrapopulation Metabolic Variability. In order to confirm the ability of this approach to differentiate populations of unicellular organisms based on their metabolic state, we performed a proof-of-concept classification of the obtained MS data to compare the different cell populations (Figure 5). The intensities of the four peaks identified in Figure 2, that is, ADP, ATP, GTP, and UDP-Glucose, were normalized to the peak of the internal standard. These were used to create a 2D principal component analysis (PCA) score plot for the cells analyzed on day 10, obtained from the "warm" (●) and the "cold" (○) cell cultures, Figure 5B.

By performing PCA on the relative signal strengths of primary metabolites, it is possible to differentiate single-cell specimens obtained from different cell cultures based on their individual metabolic fingerprints, with the presence of illumination and the temperature being the only discriminating factors (Figure 5B). The support vector machine (SVM) algorithm is a universal chemometric method for classification of data points by defining boundaries,[42] and it could readily be applied to the results presented in Figure 5B. The data obtained for cells harvested on day 10 were used as training sets: the resulting boundary between the two classes is represented in Figure 5B with a black line. Although only the data points for the "cold" cell culture showed a clustering tendency, a clear separation of the data points pertaining to these two cohorts of cells could be observed.

The data points for cells obtained on day 0 (*) are displayed in a separate score plot (Figure 5C), which incorporates the previously calculated SVM boundary (Figure 5B). Importantly, all of them were found to lie in the area delimited by the previous SVM classification (Figure 5C, black line): as expected, the cells from the starting culture (analyzed on day 0) and the cells from the "cold" culture (analyzed on day 10) possess similar metabolic states. In the final experiment, cells harvested from both cell cultures on day 10 were mixed and analyzed ("warm" + "cold"). It was observed that the data points obtained for this combined sample (+) are distributed between the area delimited by the SVM boundary and the area outside this boundary (Figure 5C, black line). Since the cells obtained from the two cultures were not

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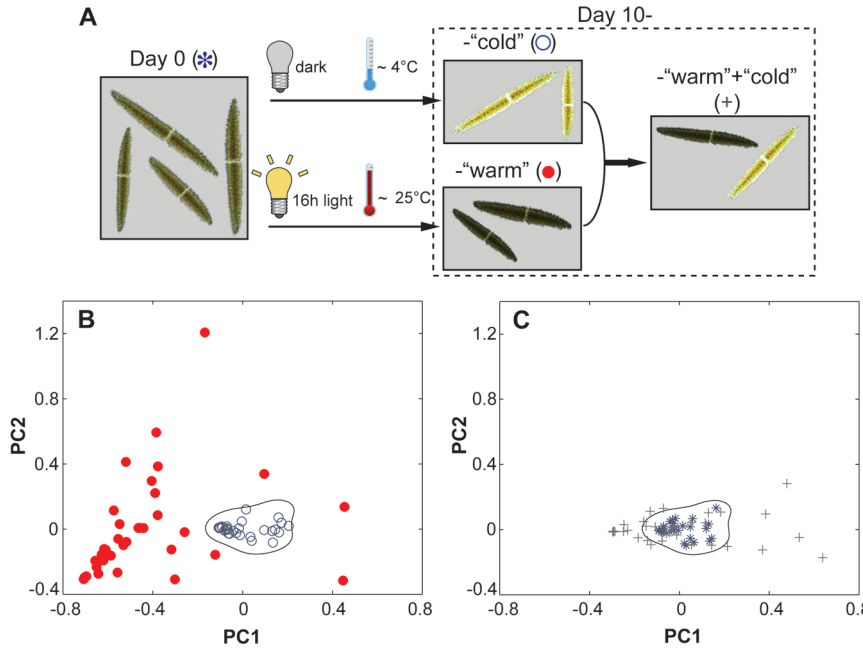

Figure 5. Assessment of metabolic state of individuals within cell populations. (A) Cell culture conditions used to produce metabolically different cell populations. Colors of the cells in cartoons are chosen arbitrarily to illustrate the differential treatment of cell cultures. (B) and (C) Principal component analysis (PCA) of the single-cell results. The PCA was conducted using sets of data composed of intensities of the four peaks identified in Figure 2, that is, ADP, ATP, GTP, and UDP-Glucose, normalized against the peak of an internal standard. Each cohort is composed of 30−36 individual cells. Classification of the data points in (B) was conducted using the support vector machine algorithm (data points inside the black curve). The cells obtained on day 10 of the experiment ("warm" (●) and "cold" (○)) were used for the training of this classification method (B). The data points obtained on day 0 (*) were subsequently plotted in an equivalent PCA plot (C) and found to fall inside the curve previously generated by the SVMs (B). The data points obtained for the mixed cell cultures on day 10 ("warm" + "cold", +) were also plotted and found to be distributed between the area delimited by the SVM boundary and the area outside this boundary (C).

labeled, this can be considered a "blind study" demonstrating single-cell classification capabilities of the combination of single-cell MALDI-MS with PCA and SVM.

Based on all these findings, we conclude that, using this simple SCMS approach that incorporates on-plate quenching of the metabolism and extraction of the metabolites with organic solvents, in conjunction with the SVM classification method, one could study phenotypic differences within cell populations. Overall, due to the fact that using the MALDI-based SCMS in conjunction with SVM classification we were able to observe the increased metabolic variability of the artificially constructed cohort of single cells (+ points outside the SVM boundary in Figure 5C), we can conclude that this approach is applicable to studies on intrapopulation metabolic variability.

Metabolic Divergence within Cell Populations. While the PCA and the SVM algorithm enabled discrimination between whole populations of cells using a limited data set (only four metabolites), analysis of metabolic variability can even be carried out by correlating contents of two metabolites within individual cells. For example, increase of dispersion of the data points can already be observed in the graphs correlating ATP and ADP signals obtained for the sets of single cells studied (Figure 4B). The ATP and ADP values, especially those obtained on day 2 and 5, are seen to be well correlated (Figure 4B). On the other hand, the corresponding correlations obtained for ATP and UDP-Glucose in all the cells studied on day 2 and 5 are much lower (Figure 4D). Closer examination of this data suggests a possibility of the cell population diverging into discrete groups of cells, which differ with respect to the ATP/UDP-Glucose ratios, supporting much more pronounced effects observed for the whole populations of cells, including four different metabolites (Figure 5B). In fact, it was found that a group of cells with relatively low ATP content and high content of UDP-Glucose falls outside the band of confidence obtained with the robust fitting algorithm (Figure 4D, data points surrounded by a black dotted oval). Based on these observations one could speculate that by correlating contents of two metabolites it should be possible to observe incidence of metabolically diverging individuals giving rise to new subpopulations.

CONCLUSIONS

While changes in the mean content of metabolites can be studied using conventional analytical methods, studying the variance of the metabolite content within a cell population is a task that requires a single-cell approach, such as the one demonstrated in this paper. Indeed, the MALDI-based SCMS method presented here enabled observation of the increase of the population median as well as the sample variance measured on the single-cell level.

The protocol presented here is characterized by the following features: facile selection of single cells, fast and reliable quenching of metabolism with nanoliter volumes of organic solvents, the

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possibility of visual inspection of the cells, a simple and direct analysis without prior separation, potential for high-throughput operation, and low cost. While in the present study we investigated populations of a relatively large unicellular organism, it is anticipated that single-cell MALDI-MS can readily be adapted to smaller microbiological specimens, following appropriate adjustments of the cell transfer and sample preparation protocol (for example using capillaries, microfluidics or piezoelectric spotting[33]), and use of state-of-the-art MALDI-MS instrumentation. In further work, it is appealing to adapt the SCMS also to less abundant metabolites, some of which are indicative of important physiological processes taking place in microbial cells.[43] To further understanding of the responses of biological organisms to environmental perturbations, it is also appealing to integrate both transcriptome and metabolome data,[44] and we envisage achievement of this goal following further extension of the SCMS methodology.

Using 9-aminoacridine as a MALDI matrix, we can detect mainly the species containing phosphate groups, such as nucleotides. By choosing an appropriate MALDI matrix, it is possible to focus on a specific class of metabolites, for example, neuropeptides, sugars, lipids, or amino acids. Since the number of MALDI and LDI methods available for analysis of a wide range of molecules is rapidly increasing,[26,31,45−47] one can readily extend the range of detectable metabolites.

The method presented here enabled observation of single cells with distinct metabolic states, and it could potentially be applied to classify individuals within cell populations with unknown "history", for example those obtained from the natural environment. This emphasizes the potential of SCMS in the areas such as bioindication.

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SUPPORTING INFORMATION AVAILABLE

Additional information including Table S1 and Figures S1−S5. This material is available free of charge via the Internet at http://pubs.acs.org.

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