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COMMUNICATION

Carbon-13 labelling strategy for studying the ATP metabolism in individual yeast cells by micro-arrays for mass spectrometry†

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Isotopic labelling of cellular metabolites, used in conjunction with high-density micro-arrays for mass spectrometry enables observation of ATP metabolism in single yeast cells.

Heterogeneity even in clonal populations of microbial cells seems to be a common phenomenon.[1] In order to unravel physiological aspects of cell population heterogeneity, each cell has to be considered as an individual entity, and analysed separately.[2] Studying single cells necessitates analytical methods[3] that allow qualitative and quantitative characterization of the cell's genome, proteome, and metabolome. Significant progress has been made towards single-cell genomic[4,5] and proteomic[6,7] protocols; however, analysis of metabolites in single cells still remains a challenge (for related reviews see, for example, ref. 8–14).

It has been realized that matrix-assisted laser desorption/ionization (MALDI)-MS[15] can be a useful tool for analysis of numerous metabolites in single cells (*e.g.* ref. 16–19). MALDI-MS has high sensitivity for various metabolites,[20] which permits biochemical analysis of relatively small microbial cells, including the standard model organism—baker's yeast (*Saccharomyces cerevisiae*).[21] This powerful analytical technique also exhibits high salt tolerance,[22] has potential for integration of sample preparation steps into MALDI target plates,[23] accommodates use of small sample volumes (down to picolitres),[20,21] and enables high-throughput analysis (in some cases, >2 samples s^{-1}).[21] However, compared to other mass spectrometric techniques, MALDI-MS has the reputation of having poorer quantitative capabilities. This issue can, at least in part, be solved by the use of isotope-labelled internal standards.[24] The present study verifies the possibility of using isotopic labels in conjunction with MALDI-MS to reveal biosynthetic activity in single yeast cells.

In the method developed here, biosynthetic activity of a cell is assessed based on the appearance of ^{13}C-labelled forms of adenosine triphosphate (ATP)—the major energy carrier molecule—which can readily be detected by MALDI-MS in the samples composed of single or few cells, following very little sample preparation.[21] In order to detect metabolites in yeast cells, we implemented high-density micro-arrays for mass spectrometry (MAMS), which integrate single-cell aliquoting with sample confinement to enable highly sensitive analysis with high throughput.[21]

In the first experiment, *S. cerevisiae* cells (YSBN6 (mat a ho::HphMX4)) growing exponentially on a $^{12}C_6$-glucose minimal medium were washed by resuspension in minimal medium without a carbon source, and immediately transferred into minimal medium containing $^{13}C_2$-ethanol. From this point onwards, the labelled ethanol was the only carbon source available. MALDI-MS analysis of a pooled sample composed of several cells cultured for 24 h with $^{13}C_2$-ethanol revealed labelling of ATP molecules (from m/z 506—no label, to m/z 516—fully labelled), Fig. S1 (ESI†). However, the "average spectrum" obtained is not representative of any of the cells present in the sample. Therefore, we further used MAMS to study individual ^{13}C-labelled cells. The results revealed quasi-random labelling patterns of ATP that are characteristic for the studied yeast cells, and which do not exactly match the labelling pattern obtained for the sample composed of several cells (Fig. S1, ESI†): strikingly, the intensity ratios of highly labelled ($^{13}C_9$) and non-labelled ($^{12}C_{10}$) ATP peaks recorded for these two samples (4 cells and 1 cell) are different. This highlights the necessity for recording mass spectra of single cells.

In subsequent experiments, samples of the cell suspension were collected periodically, washed, and individual cells were analyzed by MAMS-MALDI-MS (*cf.* ESI†). We observed that the isotopic pattern of ATP, recorded for the cells sampled at different times, shifts towards higher mass (from m/z 506 to m/z 516), Fig. 1. It is striking that for a given time point (*e.g.* 10 h incubation with $^{13}C_2$-ethanol) the observed isotope distributions of ATP molecules vary strongly from cell to cell (Fig. 2 and Fig. S2, ESI†). A low level of labelling may be indicative of either a large pool of unlabelled ATP in the cell at "time zero" (*i.e.* when the cells were transferred to

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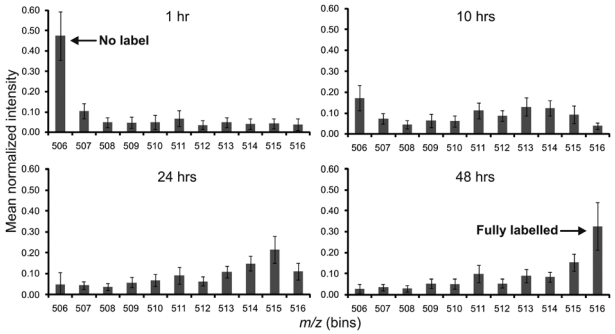

Fig. 1 Normalized mean intensities of ATP peaks showing isotopic distribution in single yeast cells (strain YSBN6 (mat a ho::HphMX4)) analyzed after 1 h (n = 61 cells), 10 h (n = 19 cells), 24 h (n = 100 cells), and 48 h (n = 73 cells) culture with minimal medium containing 0.5% $^{13}C_2$-ethanol (see ESI† for experimental details). The error bars represent standard deviations.

the ^{13}C-ethanol medium), or a low rate of ATP *de-novo* biosynthesis in a particular cell (see also Fig. S3, ESI†). The incorporation of carbon-13 into the structure of ATP relies on multiple reactions and enzymes responsible for individual biosynthetic steps. Since this process is relatively slow—compared with phosphorylation of adenosine diphosphate (ADP; not measured) and dephosphorylation of ATP—the result provided by the assay is believed not to be directly related to the so-called ''energy charge'' of a cell.

The mass spectra obtained for >200 individual cells analyzed by MAMS-MALDI-MS at different time points (n = 61, 19, 100, and 73 cells sampled following 1, 10, 24, and 48 h incubation with minimal medium containing 0.5% $^{13}C_2$-ethanol, respectively) were subjected to principal component analysis (PCA; see ESI† for details). In the PCA plot (Fig. 3) each point corresponds to a single yeast cell, and is described by 11 variables (binned and normalized intensities of the isotopic forms of ATP, containing from 0 to 10 ^{13}C atoms). A clustering behaviour, underlining the progress of ^{13}C-labelling in the cellular ATP within the studied population of single cells, can be seen. Clearly, the data points obtained for the samples collected after 1 and 24 h are separated due to a significant difference in relative abundances of the non-labelled (m/z 506) and highly labelled (m/z 514, 515) forms of ATP (Fig. 3). The whole experiment was conducted three times with minor modifications.

The ability to separate populations of cells based on their MS fingerprints (Fig. 3) suggests that, irrespective of the contribution of non-biological (*e.g.* method or instrument-related) variability, the proposed combination of MAMS and MALDI-MS with carbon-13 labelling can indeed point out possible systematic differences in metabolic rates of individual yeast cells. The methodological variability, which likely contributes to the scatter in each cluster of Fig. 3, may originate from (i) inability to preserve the native metabolite composition throughout the whole analytical procedure, and (ii) possible differences in rates of degradation of metabolites in various cells during the sample preparation and storage. However, we have taken precautions to avoid influencing the content of the metabolites present within the cells substantially (*cf.* ESI†). It should also be pointed out that although the absolute concentrations of metabolites are likely affected in the course of sample preparation, it is less likely that the relative abundances of various isotopic forms of one chosen metabolite will rapidly be affected (*cf.* Fig. S4, ESI†).

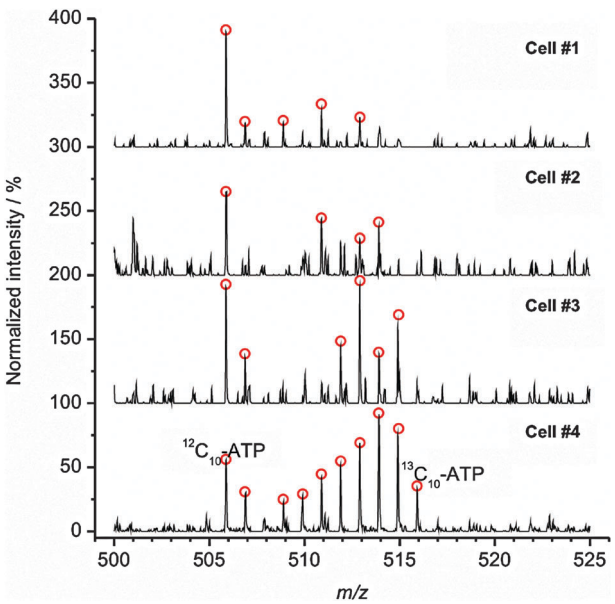

Fig. 2 Raw MALDI mass spectra showing different isotopic distributions of ATP in various single cells of yeast analyzed after 10 h culture with $^{13}C_2$-ethanol (examples of four cells with unequal levels of incorporation of ^{13}C), ordered according to the relative quantity of heavier forms of ATP (cell #1—low level, cell #4—high level of ^{13}C incorporation). Red circles mark various isotopic variants of ATP. Spectra are normalized to base peaks, and displaced in the y direction for clarity. The corresponding binned and normalized mass spectra are shown in Fig. S2 (ESI†). MALDI matrix: 9-aminoacridine; negative-ion mode.

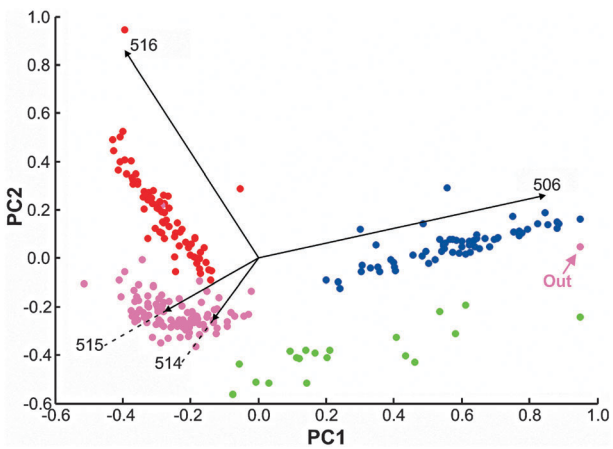

Fig. 3 Principal component analysis scores plot based on the normalized intensities of the signals pertaining to the isotopic pattern of ATP. Each point corresponds to one yeast cell. Samples collected 1 (blue), 10 (green), 24 (pink), and 48 h (red) after transferring the cells to the medium containing $^{13}C_2$-ethanol. Some of the variables (intensities of the isotopic forms of ATP measured at the m/z 506, 514, 515, and 516) are represented by vectors; direction and length of these vectors indicate how those variables contribute to the two principal components included in the plot. The ''Out'' label marks an outlying data point which corresponds to the population of cells incubated with $^{13}C_2$-ethanol for 10 h.

Following successful analysis of tens of single yeast cells incubated with $^{13}C_2$-ethanol, in order to verify the presence or absence of sequential labelling of ATP, we attempted localization of the ^{13}C-label within ATP molecules by tandem MS. Although it was feasible to obtain satisfactory MS/MS spectra of non-labelled ATP in single yeast cells (strain with larger cell phenotype, BY4741 (mat a his3Δ1/leu2Δ0/met15Δ0/ura3Δ0/rox3Δ::kanMX4)) grown on a non-labelled substrate (Fig. S5, ESI†), it was not possible to record MS/MS spectra of the labelled ATP forms from single yeast cells incubated with $^{13}C_2$-ethanol. This is due to the fact that the main ATP peak ($^{12}C_{10}$) "splits" into several peaks with different numbers of the carbon-13 label ($^{13}C_n{}^{12}C_{10-n}$), which have lower signal-to-noise ratios. Therefore, to elucidate possible partitioning of ^{13}C between adenine and ribose groups, we conducted MS/MS analysis using samples composed of multiple cells, which had been incubated with minimal medium containing 0.5% $^{13}C_2$-ethanol. As shown in Fig. S6 (ESI†), carbon-13 is incorporated into the adenine as well as ribose moieties of ATP. The ^{13}C label seems to be distributed between the two C_5 moieties. This is explained as follows: after the cells are transferred to the medium containing $^{13}C_2$-ethanol, carbon-13 is initially passed onto C_2 carriers such as acetyl-CoA. Subsequently, the label is carried to pentose and purine biosynthesis pathways, and built into the structure of the ATP molecule. Due to the large number of intermediate steps, quasi-random labeling of ATP carbons is observed (Fig. 1 and 2 and Fig. S1 and S2, ESI†). It is expected that following the incubation of the yeast cells with $^{13}C_2$-ethanol, the carbon-13 label should eventually find its way to all metabolic pathways. On the other hand, after a short ($\sim$30 min) incubation of yeast cells with $^{13}C_6$-glucose, batch incorporation of 5 ^{13}C atoms into ATP molecules present in individual yeast cells could be observed (Fig. S3, ESI†); this is unlike the quasi-random labelling observed following longer incubation ($\geq$ 1 h) with $^{13}C_2$-ethanol (Fig. 2). In the future, if the sensitivity of our method can be improved further, one should conduct MS/MS analysis on single rather than multiple ^{13}C-labelled cells; this will allow one to verify the occurrence of any preferential partitioning of ^{13}C atoms between adenine and ribose moieties of ATP in a cell when the label is delivered either *via* ethanol or glucose.

Interestingly, the method can also reveal the occurrence of yeast cells with negligible levels of ATP labelling (Fig. 3, "Out"). Thus, the single-cell metabolic activity assay described here might also be used to identify multimodal distribution of metabolic activities in populations of microbial cells, for example, in response to an external stimulus. It would be especially interesting to couple the ^{13}C/MAMS method with fluorescent labels of gene expression products since one could cross-reference the metabolomic and proteomic data obtained for every cell.

Since the use of a ^{13}C-labelled substrate allows one to probe various parts of the metabolic network (*e.g.* glycolysis, pentose and purine pathways), which are involved in biosynthesis of ATP, the single-cell ^{13}C/MAMS analysis could become a convenient alternative to the conventional tests of microbial viability. One particular advantage of the proposed single-cell protocol is that the result is based on the efficiency of ATP *de-novo* biosynthesis, which involves numerous reactions and enzymes. Compared to other tools for single-cell analysis, which also include various forms of optical detection, microspectroscopy and electrochemistry (for a review see, for example, ref. 9), the present mass spectrometric method is characterized by high selectivity, as it provides information on the relative abundances of various isotopic forms of the target intracellular metabolite. The weaknesses of the method include its destructive nature, and the inability to perform absolute quantification; these drawbacks should, however, be acceptable in a number of biological applications of this single-cell analysis method.

Moving prototype single-cell analytical technologies forward to enable their use in biology laboratories is considered an important short-term goal in chemical biology research.[1] Along these lines, the present study demonstrates that isotopic labelling, used in conjunction with MAMS, enables observation of metabolic activity in individual yeast cells, and thus opens new routes of inquiry in the area of systems biology. Employing the ^{13}C/MAMS protocol, we could observe progressive incorporation of the carbon-13 label into ATP in a population of yeast cells. Only limited sample preparation is required, and separation of the sample components prior to MS is not necessary, which facilitates studies on numerous cells. In the future, detection of isotopically labelled molecules should be extended to various groups of metabolites, including the less abundant ones, which could possibly be detected using various matrices and other modes of the laser desorption/ionization mass spectrometry. For example, the ability to detect labelled forms of amino acids would allow one to conduct classical ^{13}C-flux analysis[25] on single yeast cells.

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Notes and references

1 M. E. Lidstrom and M. C. Konopka, *Nat. Chem. Biol.*, 2010, **6**, 705–712.
2 S. V. Avery, *Nat. Rev. Microbiol.*, 2006, **4**, 577–587.
3 M. Leslie, *Science*, 2011, **331**, 24–26.
4 K. Zhang, A. C. Martiny, N. B. Reppas, K. W. Barry, J. Malek, S. W. Chisholm and G. M. Church, *Nat. Biotechnol.*, 2006, **24**, 680–686.
5 T. Kalisky and S. R. Quake, *Nat. Methods*, 2011, **8**, 311–314.
6 J. R. S. Newman, S. Ghaemmaghami, J. Ihmels, D. K. Breslow, M. Noble, J. L. DeRisi and J. S. Weissman, *Nature*, 2006, **441**, 840–846.
7 H. B. Gutstein, J. S. Morris, S. P. Annangudi and J. V. Sweedler, *Mass Spectrom. Rev.*, 2008, **27**, 316–330.
8 E. V. Romanova, S. S. Rubakhin, E. B. Monroe and J. V. Sweedler, in *Single Cell Analysis: Technologies and Applications*, ed. D. Anselmetti, Wiley-VCH, Weinheim, 2009.
9 A. Amantonico, P. L. Urban and R. Zenobi, *Anal. Bioanal. Chem.*, 2010, **398**, 2493–2504.
10 M. Heinemann and R. Zenobi, *Curr. Opin. Biotechnol.*, 2011, **22**, 26–31.

11 Y. Lin, R. Trouillon, G. Safina and A. G. Ewing, *Anal. Chem.*, 2011, **83**, 4369–4392.
12 S. S. Rubakhin, E. V. Romanova, P. Nemes and J. V. Sweedler, *Nat. Methods*, 2011, **8**, S20–S29.
13 A. Svatoš, *Anal. Chem.*, 2011, **83**, 5037–5044.
14 N. Tsuyama, H. Mizuno and T. Masujima, *Anal. Sci.*, 2011, **27**, 163–170.
15 *MALDI MS: A Practical Guide to Instrumentation, Methods and Applications*, ed. F. Hillenkamp and J. Peter-Katalinić, Wiley-VCH, Weinheim, 2007.
16 S. S. Rubakhin, W. T. Greenough and J. V. Sweedler, *Anal. Chem.*, 2003, **75**, 5374–5380.
17 M. Shimizu, N. Ojima, H. Ohnishi, T. Shingaki, Y. Hirakawa and T. Masujima, *Anal. Sci.*, 2003, **19**, 49–53.
18 A. Amantonico, P. L. Urban, S. R. Fagerer, R. Balabin and R. Zenobi, *Anal. Chem.*, 2010, **82**, 7394–7400.
19 D. Miura, Y. Fujimura, M. Yamato, F. Hyodo, H. Utsumi, H. Tachibana and H. Wariishi, *Anal. Chem.*, 2010, **82**, 9789–9796.
20 A. Amantonico, J. Y. Oh, J. Sobek, M. Heinemann and R. Zenobi, *Angew. Chem., Int. Ed.*, 2008, **47**, 5382–5385.
21 P. L. Urban, K. Jefimovs, A. Amantonico, S. R. Fagerer, T. Schmid, S. Mädler, J. Puigmarti-Luis, N. Goedecke and R. Zenobi, *Lab Chip*, 2010, **10**, 3206–3209.
22 K. D. Greis, S. Zhou, T. M. Burt, A. N. Carr, E. Dolan, V. Easwaran, A. Evdokimov, R. Kawamoto, J. Roesgen and G. F. Davis, *J. Am. Soc. Mass Spectrom.*, 2006, **17**, 815–822.
23 P. L. Urban, A. Amantonico and R. Zenobi, *Mass Spectrom. Rev.*, 2011, **30**, 435–478.
24 M. W. Duncan, G. Matanovic and A. Cerpa-Poljak, *Rapid Commun. Mass Spectrom.*, 1993, **7**, 1090–1094.
25 U. Sauer, *Mol. Syst. Biol.*, 2006, **2**.

