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 that allow qualitative and quantitative characterization of the cell's genome, proteome, and metabolome. Significant progress has been made towards single-cell genomic4,5 and proteomic6,7 capabilities. This issue can, at least in part, be solved by the use of isotope-labelled internal standards.24 The present study highlights the necessity for recording mass spectra of single cells. 

In the method developed here, biosynthetic activity of a cell is assessed based on the appearance of 13C-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 12C6-glucose minimal medium were washed by resuspension in minimal medium without a carbon source, and immediately transferred into minimal medium containing 13C2-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 13C2-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 13C-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 (13C9) and non-labelled (12C10) ATP peaks recorded with MALDI-MS to reveal biosynthetic activity in single yeast 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 13C2-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...
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†).
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 his3A1/leu2A0/met15A0/ura3A/roc3A::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 is initially passed onto C$_2$ carriers such as acetyl-CoA. Subsequently, the yeast cells with $^{13}$C$_2$-ethanol, the carbon-13 label should and S2, ESI† labeling of ATP carbons is observed (Fig. 1 and 2 and Fig. S1). 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 (~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 (≥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. 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 on single yeast cells.

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Notes and references