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Fluorescence Probing Live Single-cell Mass Spectrometry for Direct Analysis of Organelle Metabolism

Tsuyoshi ESAKI and Tsutomu MASUJIMA†

Quantitative Biology Center (QBiC), RIKEN, 6-2-3 Furuedai, Suita, Osaka 565-0874, Japan

Mitochondria in a live HepG2 cell were visualized with a fluorescent probe to specify their location and state in a living cell. Then, mitochondria were selectively captured with a nanospray tip under fluorescence microscope, and thousands of small molecular peaks were revealed and unique steroids specific to mitochondria were also found. This fluorescence imaging combined with live single-cell mass spectrometry opens the door to the analysis of site- and state-specific molecular detection to elucidate precise molecular mechanisms at the single-cell and organelle level.

Keywords Single-cell analysis, live single-cell mass spectrometry, fluorescence imaging, mitochondria, organelle

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We have developed live single-cell mass spectrometry in combination with bright field microscopy to detect variations in many small molecules in relation to cell morphology, as the dynamics of individual cells in the same culture dish show heterogeneity.[1-3] A general protocol for plant cells has been published recently.[4] However, in addition to morphological information, fluorescence live imaging techniques are also useful in revealing functional, site-specific morphological, and state heterogeneity even at the organelle level in a single cell, which are not observable by simply looking at the cell shape.[5-8] For apoptosis stage specific recognition, for instance, FITC-probed Annexin V can detect early stage apoptosis.[9] In addition, measurements of variations in fluorescence intensity enable us to estimate the concentration of molecules in living cells. For example, a fluorescent probe, Fluo-4, can be used to analyze the pattern of Ca^{2+} flux in living cells.[10] However, the disadvantage of fluorescence probing is the limited number of markers available. Since fluorescent protein probes, such as GFP, can not be detected by mass spectrometry for small molecular detection range of metabolites and a small molecule fluorescent probe will make only one peak in the mass spectrum, fluorescence probing is very comprehensive with mass spectrometry which performs an exhaustive analysis of metabolomics. Therefore, it is quite advantageous to ideally combine live single-cell mass spectrometry with fluorescence probing.

For exhaustive detection of metabolites, the live single-cell mass spectrometry method has been developed.[1-4,11] However, since organelles are extremely small and difficult to capture within cells, until now, targets that could be captured were limited. We have thus developed methods to capture single vacuoles and single granules.[1-3,11] When we combined live fluorescence imaging techniques with live single-cell and organelle mass spectrometry, we found we can trap even mitochondria. Mitochondria are a very important target in biology because of the important roles they play. For example, they produce the energy molecule ATP and control apoptosis. However, metabolites of mitochondria in a single living cell have not been disclosed yet. In this study, we successfully analyzed the metabolites of mitochondria in a single living cell under a fluorescence microscopic visualization. We detected not only the usual well-known metabolites of mitochondria, but also some other specific molecules.

Human hepatocellular carcinoma cell line (HepG2) was cultured in Dulbecco's modified Eagle medium high glucose with 10% fetal calf serum (FBS), 100 mg/mL penicillin, and 100 mg/mL streptomycin G in 35 mm glass bottom dishes at 37°C and 5% CO_2. Thirty minutes before sampling, HepG2 cells were treated with mitochondria specific fluorescent marker MitoRed (Dojindo)[12] to a 100 nM final concentration. After washing the medium twice with PBS (–), HepG2 cells were set under the confocal laser scanning microscope (FV-1000, Olympus), and observed with 559 nm excitation wavelength and 580 nm filter. Stained mitochondria of target cells were directly sucked into a nanospray tip (CT-1, HUMANIX) with a micro-manipulator operation (Fig. 1(a)). In order to compare mitochondria spectra, the area where fluorescent probes were not introduced in the cell was also sucked into the tip as cytosol blank samples (Fig. 1(b)). After trapping the mitochondria aggregates from the target cell, 2 μL of ionization solvent (80% methanol) was added into the nanospray tip from the back of the tip. The sample was fed into a high resolution mass spectrometer LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific) equipped with a nano-electro spray ionization source. The spray voltage was applied around –0.8 kV to the tip. The measuring m/z range was set from 100 to 1000.

Raw data were analyzed by MarkerView (Sciex) software in order to perform the peak alignment of the obtained spectra, the paired two-side t-test analysis, and the principal component analysis (PCA). Detected peaks were estimated by the exact m/z value of ±3 ppm tolerance, and annotated by KEGG database (http://www.genome.jp/kegg/kegg_ja.html) and LIPID MAPS (http://www.lipidmaps.org/). Specific peaks of interest

† To whom correspondence should be addressed.
E-mail: masujima@riken.jp

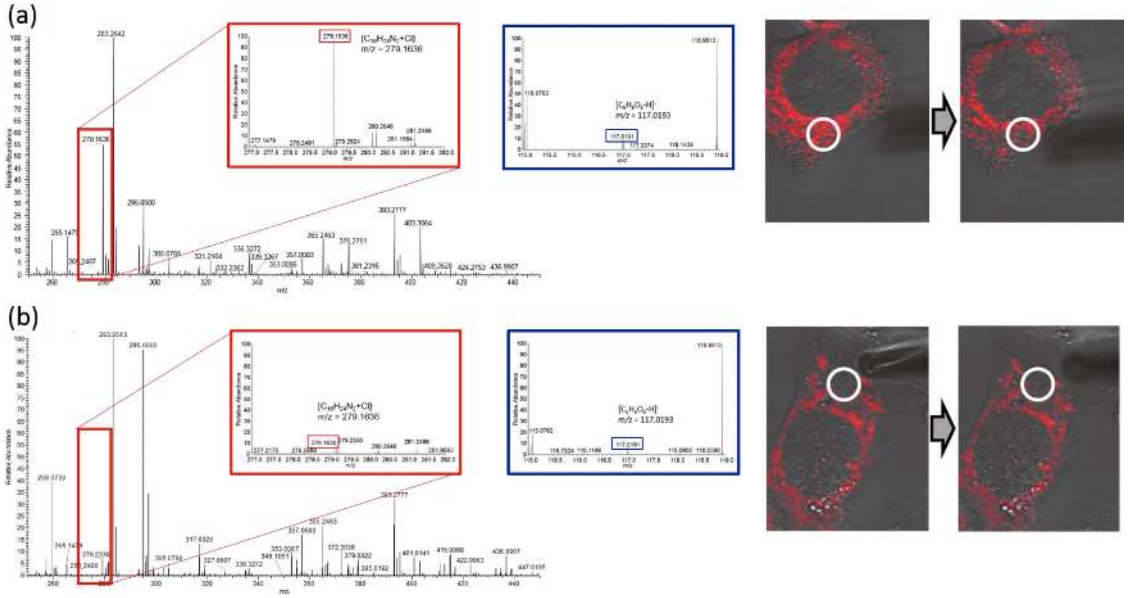

Fig. 1 Capturing of (a) a mitochondria rich spot, (b) a cytosol rich spot, and spectra peaks detected from a HepG2 cell. Red frames show peaks of xylometazoline, a mitochondrial-specific metabolite. Blue frames show succinic acid.

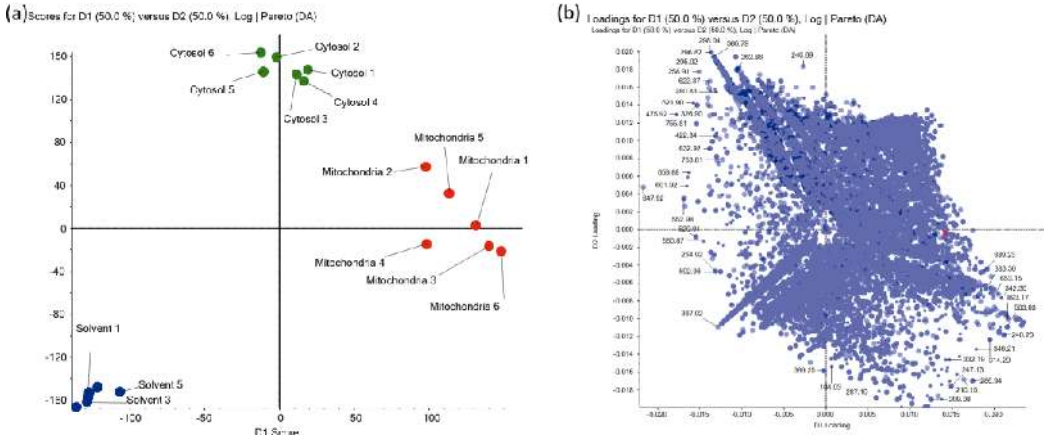

Fig. 2 Principal component analysis (PCA) for detected spectra. (a) The score plot shows that the spectra of mitochondria (red), cytosol (green), and solvent (blue) groups were separated from each other in two-dimensional space by two principal components. (b) A loading plot shows that the scattered peaks which are far from the center are specific ones and those gathered at the center are common peaks. The red point (b) is also shown in Fig. 3.

were analyzed by MS/MS for molecular identification. Obtained fragment peaks were identified by referring to MetFrag web tool (http://msbi.ipb-halle.de/MetFrag/) and MassBank database (http://www.massbank.jp/). Furthermore, in order to estimate the concentration of captured metabolites in mitochondria, *m/z* peak intensities were normalized with fluorescence intensity of MitoRed, using ImageJ software (http://imagej.nih.gov/ij/).

From a change in fluorescence intensity in the target cell, it was confirmed that mitochondria had been captured into the nanospray tip successfully. The amount of mitochondria was estimated. From obtained spectra, over 5000 peaks were detected. Among them, more than 1700 metabolites, such as amino acids, metabolites of the tricarboxylic acid (TCA) cycle, fatty acids, and sterol lipids were successfully annotated. While the TCA cycle-specific metabolite succinic acid peaks (blue framed inserts) were detected in both samples in Figs. 1(a) and 1(b) with similar intensity, the mitochondria specific amount could not be identified at this stage. Peaks at *m/z* 279.16 of xylometazoline (red framed inserts) confirm that detected peaks in Fig. 1(a) are specific to the mitochondria and that it is possible to discriminate mitochondria specific metabolite peaks.

All these peaks were analyzed by PCA, as shown in Fig. 2. The score plot (Fig. 2(a)) shows sample distribution based on peak pattern. The mitochondria cluster is clearly separated from the cytosol and the ionization solvent groups. Furthermore, the cytosol group is separated from the ionization solvent group. This result means that our method allowed a clear distinction between mitochondria, cytosol, and ionization solvent groups, each presenting different spectrum patterns. Additionally, this result indicates that the targeted organelles can be successfully

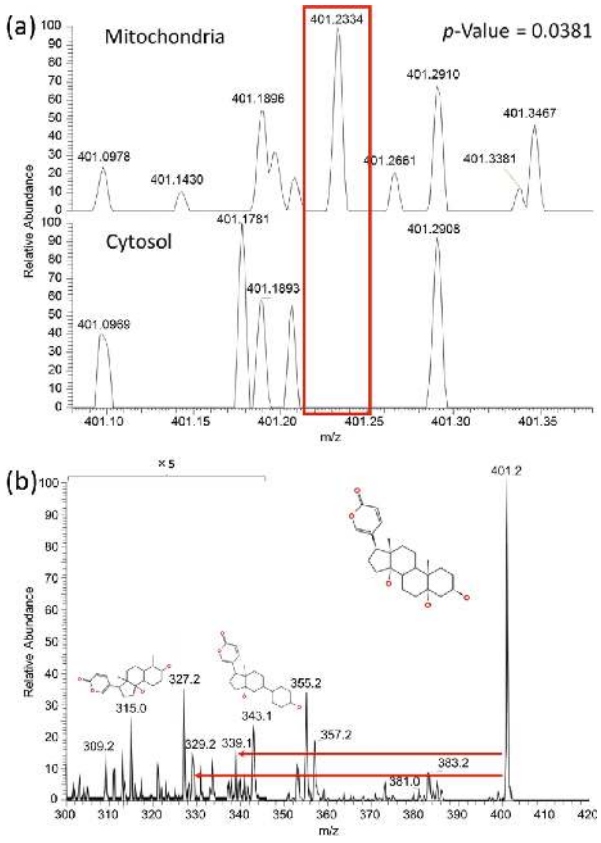

Fig. 3 (a) Mitochondrial specific peak of mitochondria at *m/z* 401.23. (b) The MS/MS spectra of $[M–H]^-$ at *m/z* 401.23 was identified as telocinobufagin by MetFrag.

captured in a nanospray tip and measured. The PCA loading plot shows intensity of peaks after normalization (Fig. 2(b)). Peak dots in the central area of the loading plot represent common peaks detected in all samples, while peaks in the scattered peripheral area represent sample specific peaks. One peak at *m/z* 401.23 was selected for MS/MS analysis. This peak was only detected from the mitochondrial spectrum (Fig. 3(a)) [*t*-test, $p < 0.05$].

The MS/MS analysis of the peak at *m/z* 401.23 showed fragments at both *m/z* 329.2 and *m/z* 339.1 to be annotated as telocinobufagin, a kind of steroid (Fig. 3(b)).

Through the described method, we have detected mitochondrial metabolites from a single living cell exhaustively, which opens the possibility to detect new specific metabolites of clearly visualized mitochondria. This can be used to clarify mechanisms of mitochondrial diseases and also to develop new pharmaceutical products.

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