Current Topics

Challenge of Mass Spectrometry toward the Elucidation of Life Phenomena

Molecular and Functional Analysis of Cellular Phenomena Using Single-Cell Mass Spectrometry

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Single-cell analysis has attracted attention in many fields of biological studies as a tool to survey the precise mechanisms of cellular and molecular behavior. The development of sensitive mass spectrometry allows the study of molecules in single cells or small regions. Matrix-assisted laser desorption/ionization imaging mass spectrometry and secondary-ion mass spectrometry use *in situ* ionization of specimens on sample plates to visualize molecular distributions as images from mass spectra. Several single-cell mass spectrometry technologies that initially recover a single cell followed by ionization have been developed. Among them, only nanospray-mediated sampling and ionization named Live Single-cell Mass Spectrometry can be used for real-time analysis. This paper explains that method in detail.

Key words single-cell mass spectrometry; cell heterogeneity; metabolome; drug metabolism

1. INTRODUCTION

Biological specimens consist of numerous heterogeneous cells even though their genetic information is identical. General studies of biomolecules such as DNA, RNA, proteins, and small metabolites use relatively large sample amounts, although recent analytical methods allow highly sensitive studies. From this viewpoint, it is plausible that studies using bulk samples of many cells yield averaged results and information on cellular individuality is thus lost. Phenomena with high heterogeneity or short periods with asynchronicity cannot be captured in bulk-sample analysis.

Only methods combining optical approaches such as fluorescence detectors enable single-cell and/or single-molecule analysis.^{1,2)} In fluorescent microscopy for single molecules or cells, molecules with strong fluorescence can be observed in combination with optical devices that have photon-multiplying or photon-accumulating functions.³⁾ Most molecules lacking fluorescence cannot be analyzed or require labeling with a fluorescent tag, although tag-labeling sometimes alters the nature of the target molecule, especially of small metabolites. Direct analysis of biomolecules is necessary to avoid the tag-labeling problem.

Mass spectrometry (MS) has the greatest potential to detect many types of molecule without the need for a label.^{4,5)} The development of MS, especially of ionization methods, enables sensitive analysis of biomolecules.⁶⁾ However, conventional MS approaches use large amounts of cells, similar to general biological approaches. Sampled cells are extracted with organic solvent, and extracts are pretreated before liquid chromatographic separation. Thus, information on cellular origin or heterogeneity is lost.

Therefore, one of the approaches to develop MS is focused

on molecular analysis of limited regions of biological specimens such as tissue, single cells, or organelles in a cell. For single-cell analysis, two approaches are used: *in situ* ionization of the region containing the target; and direct recovery of targeted cells followed by ionization (Fig. 1). In the former approach, matrix-assisted laser desorption/ionization-imaging mass spectrometry (MALDI-IMS)⁷⁾ and secondary-ion MS (SIMS)⁸⁾ are widely used. In the latter approach, single-cell MALDI in which an isolated single cell is mixed with matrix and analyzed using routine MALDI-MS is used.⁹⁾ We developed Live Single-cell MS in which a nanospray needle is used both to isolate single cellular content and to ionize solubilized molecules.^{10,11)} These methods are discussed in detail below.

2. *IN SITU* IONIZATION METHOD

2.1. MALDI-IMS MALDI-IMS developed by Caprioli's group is now widely used by medical biologists.^{12–15)} The method involves spraying or multiple spotting of a chemical matrix solution on the target tissue specimen attached to a MALDI sample plate. Molecules contained in an area (50 μm or less) overlaid by matrix droplets are extracted and co-crystallized after the organic solvent has dried. Then crystallized samples are ionized by laser irradiation. Ionized molecules are introduced into the mass spectrometer to obtain the spectrum of the irradiated region. The spatial resolution of this method depends on several factors such as the matrix overlaying technique, microdroplet size of the matrix solution, or laser beam diameter. In a conventional MALDI apparatus, the resolution is 50 μm , which is usually larger than the size of a single cell. Thus, cell separation should be conducted to obtain a single-cell mass spectrum, as discussed below.

2.2. SIMS The SIMS procedure includes focused

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Fig. 1. Approaches in Single-Cell MS

Fig. 2. Scheme of Live Single-Cell MS

ion-beam irradiation from a primary ion source to the sample surface, release of secondary ions from the bombarded surface due to the collapse of primary ions, and analysis using a mass spectrometer, usually a time-of-flight (TOF) analyzer. Low-intensity primary ions are usually used for biomolecular analysis called static SIMS, which allows the recovery of secondary ions of high molecular weight. Although the resolution is as small as 100 nm, the entire procedure should be carried out under a vacuum, which is inappropriate for real-time analysis.¹⁶⁾

3. SINGLE-CELL RECOVERY AND IONIZATION

3.1. Single-Cell MALDI Single-cell MALDI MS analysis was initially developed to detect neuropeptides in mollusk neurons.¹⁷⁾ This method is applicable to several types of cell

such as those of mammalian origin.¹⁸⁾ The technology consists of several steps. A single cell is collected with a glass capillary under a microscope and placed on a MALDI plate, followed by washing with buffer, extraction with matrix solution in organic solvent, drying *in situ* to form a co-crystal of the matrix and extracted molecule, and acquisition of the mass spectrum by laser irradiation of the crystalized sample-matrix complex. Our group also reported that small molecules such as histamine can be detected in a mast cell using a similar method.^{19,20)} Zenobi's group performed bacterial single-cell MALDI-MS showing cellular heterogeneity.²¹⁾

3.2. Microarrays for MS For the separation of individual cells, high-density microarrays for mass spectrometry (MAMS) were developed. Urban *et al.* prepared conductive MALDI plates with a high-density array of microorganism-compatible spots using laser ablation.²²⁾ Cells are

Fig. 3. PCA Analysis of the Mass Spectra of Seven Cell Lines

Extracted peaks of each single-cell mass spectrum were aligned with m/z and subjected to PCA. Clusters of cell spectra are circled with different patterns attached to their images.

Fig. 4. Microregional MS Analysis

(a) Granule or cytoplasm of RBL-2H3 cells were collected with nanospray tips. (b) Single-cell mass spectrum of a granule (left) and cytoplasm (right). (c) Histamine and serotonin signal intensity in single-cell granules and cytoplasm ($n=4$).

captured and attached to the spots (*ca.* 100- μ m interval grid) to form a single-cell array, followed by MALDI-MS analysis. They showed that several types of single cell such as a ciliate, bacterium, and yeast could be used to detect endogenous nucleotides at the attomole level. They also combined MAMS with fluorescent microscopy and Raman spectroscopy for synergistic multimodal analysis.

3.3. Single-Cell Laser Desorption/Ionization MS As an alternative to achieve single-cell analysis, matrix-free laser desorption/ionization mass MS (LDI-MS) was also attempted for single-cell MS by Urban *et al.*²³ The lack of a matrix sometimes results in less sensitivity than conventional MALDI, although it affords higher resolution because molecules in the sample do not disperse over a droplet-soaked area in combination with a narrowly focused laser beam.

Fig. 5. Detection of Tamoxifen and Its Metabolites in HepG2 Cytoplasm and Vacuoles

The cytoplasm and vacuoles of tamoxifen-treated HepG2 cells were recovered with nanospray tips. Tamoxifen and target N-DM-T SIM spectra are indicated.

They obtained single unicellular algae cell-by-cell spectra to show phospholipid signal variations in combination with the green fluorescence of proplastids and the characteristic peak of β -carotene with the Raman spectra of each individual cell.

3.4. Live Single-Cell MS To achieve real-time microregional analysis of single cells, we developed the Live Single-cell MS technique.^{10,11,24} The procedure for this method (Fig. 2) consists of the selection of a cell with the desired phenotype, recovering it with a metal-coated nanospray needle attached to a micromanipulator on the microscope stage, and introducing a very small amount of cell content (<1 fL) into the tip by applying negative pressure with a syringe. An ionizing solvent appropriate to the nature of the target molecule is added to the back end of the tip, and the tip is set to the nano electrospray ionization ion source of an LTQ-Orbitrap mass

Fig. 6. Tafluprost and 7-EC Metabolism in Primary Human Hepatocytes

(a) SIM spectra of tafluprost, 7-EC, and their metabolites are presented sequentially with metabolic turns. (b) Cell-by-cell intensity of tafluprost and 7-EC metabolites. The metabolite intensity in each cell is indicated by different symbols.

spectrometer, followed by charging at about 1000 V. Spraying continues for more than 5 min. The spectrum is obtained following the experimental objective: if the target molecule is clear, the selected ion-monitoring mode at the mass range of m/z 10 is applied; or the full-scan mode is used when the target is not specified. After obtaining the spectrum, peaks are extracted manually, followed by peak alignment and statistical analysis with MarkerView software (AB SCIEX, Framingham, MA, U.S.A.). The use of the nanospray with a very small pore at the forefront (around 3 μ m) gives efficient ionization because of the small electrospray droplet, less ion suppression, and very high sensitivity as a result. Quick recovery of the targeted region also enables real-time spatiotemporal analyses of biological samples.

This method is applicable to various types of cells. Among

mammalian cells, adherent cells as well as nonadherent cells can be used, and among plant cells, cells from any position such as the leaf and stem can be used. To clarify whether single-cell spectra contain cell-specific characteristics, we cultured seven cell lines, human fetal lung fibroblasts TIG-1 and TIG-3, human hepatocellular carcinoma HepG2, human renal cell carcinoma ACHN and CAK11, human EB-transformed B lymphoblastic cell C007, and mouse teratocarcinoma P19, and performed Live Single-cell MS. Peaks extracted from each spectrum were analyzed using principal component analysis (PCA) with MarkerView (AB SCIEX). Score plots showing cell line-specific clusters of spectra indicated that each single-cell spectrum contained a unique class of peaks (Fig. 3). In addition, cellular heterogeneity appearing in the mass spectra was smaller than cellular differences. It is noteworthy

Fig. 7. Plant Single-Cell MS

(a) A geranium leaf, stem, and petal were observed under a stereomicroscope and single cells from each tissue were recovered with a nanospray tip. Directions of nanospray insertion are indicated by arrows. (b) Single-cell mass spectra of a leaf (left), stem (center), and petal (right). (c) Peak intensity of m/z 169.122 in each single-cell spectrum. (d) MS/MS spectrum of m/z 169.122 identified as geranic acid.

that cell clusters with same tissue or cancer origin, *i.e.*, TIG-1 and TIG-3, and CAK11 and ACHN, were located near each other.

We could also detect subcellular region-specific molecules by targeted sampling of a specific region inside a cell. Rat basophilic leukemia cells, RBL-2H3, are conveniently used as mast cell model.²⁵⁾ They contain allergy-response molecules inside their granules. We isolated granules and cytoplasm with the nanospray tip to detect microregion-specific molecules (Fig. 4). A few dozen peaks out of several hundred were found to be specific to granules and cytoplasm. Among these peaks, m/z 112.09 and m/z 177.08 were identified as histamine $[M+H]^+$ and serotonin $[M+H]^+$, respectively. Histamine was granule specific ($p < 0.03$), whereas serotonin was detected in both granules and cytoplasm. Metabolites of these molecules could be monitored for information on subcellular localization.

The biological activity of single cells was also monitored at the single-cell level. We showed that drug metabolism could be measured in human hepatocytes using tamoxifen and tafluprost.^{26,27)} The human hepatocellular carcinoma cell line HepG2 was treated with tamoxifen $5 \mu M$ for 12 h, and the

tamoxifen metabolites were traced using single-cell cytoplasm recovery with a Cellomix tip (Fig. 5). Selected-ion monitoring (SIM) analysis showed that tamoxifen (m/z 372.232) and its metabolites, *N*-desmethyl tamoxifen (*N*-DM-T, m/z 358.216), 4-hydroxytamoxifen, tamoxifen-*N*-oxide (m/z 388.227), 3,4-dihydroxy tamoxifen, 4-hydroxy tamoxifen *N*-oxide (m/z 404.222), were detected. Among these metabolites, *N*-DM-T was the most abundant, reflecting high expression of cytochrome P450 (CYP)3A4, an isozyme of cytochrome P-450. In addition, intracellular localization of tamoxifen metabolites was analyzed. A vacuole or nucleus in a single cell was recovered and analyzed similarly. Contrary to our expectation, no tamoxifen metabolites were detected in vacuoles, although tamoxifen itself was detected. Neither tamoxifen nor its metabolites was detected in nuclei. These results indicate that this method allows not only the detection of drug metabolites in a cell but also the clarification of their cellular distribution.

Next, we changed from the use of HepG2 cells to human primary hepatocytes because metabolic enzyme activity is lowered by oncogenic transformation. We treated primary hepatocytes with $5 \mu M$ of tafluprost, a prostaglandin derivative,

and 10 μM of 7-ethoxycoumarin (7-EC) (Fig. 6a). Hepatocytes with various shapes were selected, and their cytoplasm with granules was collected with nanospray tips. Peaks extracted from single-cell spectra were aligned, and peak intensity corresponding to these ions was evaluated. SIM in negative-ion mode was used to measure tafluprost, 7-EC, and their metabolites [tafluprost acid (TA), 1,2-dinor-tafluprost acid (dinor-TA), 1,2,3,4-tetranor-tafluprost acid (tetranor-TA), hydroxylated-1,2-dinor-tafluprost acid (dinor-TA-OH), hydroxylated-1,2,3,4-tetranor-tafluprost acid (tetranor-TA-OH), and 7-hydroxycoumarin (7-HC)]. Each compound was detected as $[\text{M}-\text{H}]^-$ ion at m/z 451.228, 161.024, 409.183, 397.148, 381.152, 371.132, 355.138, and 189.056, respectively. Quantitative levels of tafluprost and its metabolites varied significantly, whereas 7-HC levels were relatively similar among cells (Fig. 6b). When cell spectra were categorized according to TA level (high and low), the dinor-TA level was similar to the TA level. On the other hand, the 7-HC level was similar irrespective of the TA and dinor-TA levels. It is possible that tafluprost metabolic activities varied among hepatocytes, whereas the activity of 7-EC was constant. Because both tafluprost and 7-EC are metabolized to TA by esterase and further metabolism is performed by cytochrome P-450s, it is also possible that esterase activity varies among cells.

In plant tissue, cells of any target position can be recovered in combination with a stereomicroscope^{28,29)} (Fig. 7a). As the size of a single plant cell is larger than that of ordinary mammalian cells, manipulation can be performed at lower magnification. Mass spectra are obtained easily in the full-scan mode because of the positive inner pressure of cells, as shown in Fig. 7b. The peak intensities are compared similarly, and differential peaks among cells from different tissue can be obtained. A representative peak of m/z 169.122 is shown in Fig. 7c, which is from a geranium petal. MS/MS analysis of m/z 169.122 showed that this peak is the $[\text{M}+\text{H}]^+$ ion of geranic acid.

4. FUTURE PERSPECTIVES

The development of MS for single cells/small regions is currently directed toward qualitative rather than quantitative analysis. The sensitivity of mass spectrometry is still too low to detect small numbers of molecules, which decreases its quantitative sensitivity. Improvements in ionization methods, ion transfer efficiency, and detector sensitivity are important to achieve higher sensitivity. In addition, optimization of every experimental procedure is important. On the other hand, clarification of the characteristics of single cells with MS profiles is necessary to understand the phenomena related to their molecular profiles. For precise cellular characterization in combination with mass spectra, the use of other markers such as the Raman spectrum as reported by Zenobi's group supports a better understanding of cellular phenomena at the single-cell level.^{22,23)} We plan to combine Live Single-cell MS with such methods to clarify phenomena-related molecular profiles.

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