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Review

In situ metabolomic mass spectrometry imaging: Recent advances and difficulties☆

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ABSTRACT

MS imaging (MSI) is a remarkable new technology that enables us to determine the distribution of biological molecules present in tissue sections by direct ionization and detection. This technique is now widely used for *in situ* imaging of endogenous or exogenous molecules such as proteins, lipids, drugs and their metabolites, and it is a potential tool for pathological analysis and the investigation of disease mechanisms. MSI is also thought to be a technique that could be used for biomarker discovery with spatial information. The application of MSI to the study of endogenous metabolites has received considerable attention because metabolites are the result of the interactions of a system's genome with its environment and a total set of these metabolites more closely represents the phenotype of an organism under a given set of conditions. Recent studies have suggested the importance of *in situ* metabolite imaging in biological discovery and biomedical applications, but several issues regarding the technical application limits of MSI still remained to be resolved. In this review, we describe the capabilities of the latest MSI techniques for the imaging of endogenous metabolites in biological samples, and also discuss the technical problems and new challenges that need to be addressed for effective and widespread application of MSI in both preclinical and clinical settings. This article is part of a Special Issue entitled: Mass spectrometric interrogation and imaging of tissues Mass spectrometric interrogation and imaging of tissues.

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1. Introduction

Understanding the complex biochemical processes that occur within living organisms requires not only the elucidation of the molecular entities involved in these processes, but also their spatial distribution within the organism. Analytical technologies for elucidating multiple molecular dynamics in the micro-region that retain the spatial information of the target tissue are thought to be important for understanding biological complexity of disease progress. Chemical stains, immunohistochemical tags and radiolabels are common methods for visualizing and identifying molecular targets. However, there are limits to the sensitivity and specificity of these methods and to the number of target compounds that can be monitored simultaneously. Thus, the simultaneous multiple molecular imaging with high sensitivity will be a technical breakthrough for pathophysiological research.

Metabolites are the result of the interactions of a system's genome with its environment, and are the end products of gene expression. The metabolome is defined as the total quantitative collection of small-molecular-weight metabolites present in a cell, tissue, or organism, that participate in the metabolic reactions required for growth, maintenance, and normal function [1–3]. Unlike the transcriptome and proteome that represent the processing of information during the expression of genomic information, the metabolome more closely represents the phenotype of an organism under a given set of conditions and can be defined as the "compound-level phenotype" of the genomic information. Metabolomics, the measurement of the global endogenous metabolite profile from a biological sample under different conditions, can lead us to an enhanced understanding of disease mechanisms, the discovery of diagnostic biomarkers, the elucidation of mechanisms for drug action, and an increased ability to predict individual variation in drug response phenotypes [4,5]. Thus, this rapidly developing discipline has important potential implications in the field of biomedical research.

To date, MS coupled with pre-separation techniques such as LC-MS or GC-MS has been known to be a conventionally used strategy for metabolomics [6–8]. However, these methods have a drawback in the analysis of tissue samples because of the requirement of metabolite extraction, which causes the loss of information on the spatial localization of the metabolites. In contrast, imaging techniques capable of determining the spatial localization of molecules have revolutionized our approach to diseases by allowing us to directly examine the pathological process, thereby giving us a better understanding of the pathophysiology. In most cases, however, there is a tradeoff among sensitivity, molecular coverage, spatial resolution, and temporal resolution. For example, magnetic resonance imaging (MRI), positron emission tomography (PET), and fluorescence microscopy can visualize the spatial localization of targeted molecules with high sensitivity, but these techniques have low molecular coverage (only a few molecules at a time) [9]. The simultaneous and spatially resolved detection with high sensitivity of a broad range of molecules is still a challenging issue.

MS imaging (MSI) is an emerging technology that makes it possible to determine the distribution of biological molecules present in tissue sections by direct ionization and detection. MSI has received considerable attention as a potential imaging technique for a molecular *ex vivo* review of tissue sections from an animal or plant based on label-free tracking of endogenous molecules with spatial resolution and molecular specificity [10–12]. The MSI technique was initially developed as a tool for intact protein imaging from the tissue surface using MALDI-MS [12–16]. In current research, proteins or peptides are still the main targets. However, the analysis of a wide variety of low-molecular weight compounds, including endogenous metabolites, using MSI combined with several soft ionization methods is emerging as a research target. In this review, we describe recent advances and difficulties in developing an analytical platform for MSI of endogenous small metabolites.

2. Ionization platform for MSI of endogenous metabolites

2.1. *MALDI*

MALDI is one of the laser desorption ionization (LDI) methods that can softly ionize several biological molecules. This ionization technique is usually used combined with TOF MS. A conventional MALDI source is equipped with a UV laser such as a nitrogen laser (337 nm) or Nd-YAG (355 nm). Spatial resolution is dependent mostly on the diameter of the laser; the diameter is usually more than 5 μm [17]. However, because MALDI-MSI requires a matrix application step, potential limitations in spatial resolution can be introduced by the matrix. The main limitations are the diffusion of metabolites within the tissue during matrix application and the heterogeneous size of crystal formation.

At the first decade of development, MALDI-MS has been used for synthetic polymer or protein (peptide) analysis. In the post-genomic era, the dramatic progress of bioinformatics research accelerated the use of MALDI-MS in proteomics research for identifying vast numbers of proteins [18]. MALDI-MS is known as a highly sensitive analytical method that can be used to analyze low concentrations (~fmol) of tryptic peptide. Sensitivity is extremely important characteristics for MSI because numerous biological molecules exist in a very small amount on the thin tissue section. However, MALDI-MS has rarely been used for low-molecular-weight metabolite analysis because many kinds of matrix and/or matrix-analyte cluster ion peaks are observed in the low-mass range ($m/z<700$), and the strong peaks that they

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generate interfere with the detection of the target low-molecular-weight compounds.

Based on these early observations, lipid molecules became the first targets for MSI studies of endogenous metabolites because the *m/z* range of most lipid molecules was more than 700. Lipids are also abundant in tissues (e.g., more than 60% dry weight of brain tissue) and are easily ionized because of the presence of a polar head [17,19]. For example, glycerophospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (PS), which have positively charged polar heads, were detected in the positive ion mode. On the other hand, glycerophospholipids such as phosphatidylinositol (PI) and phosphatidylglycerol, which have negatively charged polar heads, were detected in the negative ion mode. In addition, glycosphingolipids such as gangliosides, sulfatides (ST), and galactosyl-ceramide were visualized by MALDI-MSI. These lipids can easily ionize, and their region-specific distribution on tissue sections has been observed using traditional matrices such as 2,5-dihydroxy benzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA). In response to extracellular stimuli, several of the fatty acids in the phospholipids are released and converted into bioactive lipids that mediate important biological processes [20]. Thus, information on the unique distributions of the various phospholipids has contributed to a better understanding of the molecular basis of diverse biological phenomena [21].

On the other hand, a minority of researchers has tried to apply MALDI-MSI to investigate smaller endogenous metabolites. Metabolites in the low-mass range (*m/z*<700) with distinct distributions in various tissues were searched from a massive forest of background peaks that were generated as a result of using conventional matrices such as DHB and CHCA. Heme B (*m/z*=616) [22], GABA (*m/z*=104) [23], and α-tocopherol (*m/z*=431) [24] have been successfully detected, and their unique distributions on the surface of the plant and animal tissue sections have been visualized. However, the unfavorable features of conventional matrices, because of both the interference of matrix peaks and the low ionization efficiency, have made it difficult to detect other metabolites. Recently, 9-aminoacridine (9-AA) was reported as a suitable matrix for low-molecular-weight metabolite analysis [25]. When 9-AA was used in negative ion mode, only a few peaks derived from the matrix were observed in the low-mass range (m/z ~500). In addition, the excellent ionization efficiency of 9-AA against important cellular metabolites (in the order of attomoles) was demonstrated [26,27]. Using the same 9-AA matrix, several endogenous metabolites were detected from the extract of *Escherichia coli* and yeast [28–30]. Shroff *et al.* succeeded in visualizing the distribution of anti-herbivore glucosinolates in *Arabidopsis thaliana* leaves using 9-AA [31]. These researchers discovered that there were differences in the proportions of the three major glucosinolates in different leaf regions, and that their distributions appeared to control the feeding preference of the *Helicoverpa armigera* larvae. Benabdellah *et al.* reported that the location of 13 metabolites in the normal rat brain, although almost all were nucleotide derivatives, was observed using MSI [32]. We recently showed, for the first time, the applicability of MALDI-MS for obtaining chemically diverse metabolite profiles on a single-mammalian cell [33]. Human HeLa cells mounted on indium tin oxide glass were imaged using 9-AA. Negative mode MALDI-MS spectra corresponding to 50 individual signals were collected, and ATP, fructose-1,6-bisphosphate, and citrate were successfully identified as the representative metabolites [33]. This result indicated that the 9-AA-based MALDI-MSI system allowed single-cell metabolomic analysis to be successfully performed. Furthermore, the ultra-sensitive MALDI-MS technique enabled the spatially resolved detection of a broad range of metabolites with their unique distributions, and helped in the identification of more than 30 metabolites that included nucleotides, cofactors, phosphorylated sugars, amino acids, lipids, and carboxylic acids in normal mouse brain tissue. The application of this technique and metabolic pathway analysis to a rat transient middle cerebral artery occlusion (MCAO) model visualized a spatiotemporal behavior of metabolites in the central metabolic pathway regulated by an ischemia-reperfusion (Fig. 1) [33]. Hattori *et al.* have also reported spatiotemporal changes in energy charge, adenylates, and NADH during focal ischemia in a mouse MCAO model [34]. These findings highlight the potential applications of the *in situ* metabolomic imaging technique to visualize spatiotemporal dynamics of the tissue metabolome, which will facilitate biological discovery in both preclinical and clinical settings.

Together, these findings show the great potential for the realization of "metabolomic imaging" using MALDI-MSI. Although the present MALDI method is highly sensitive and well-established on the MSI platform, some limitations remain to be overcome before the broad range of endogenous metabolite imaging can be achieved. It is generally known that, in MALDI, the detection of molecules is completely dependent on the matrix. In addition, the crystal size of the deposited matrix strongly affects both experimental reproducibility and spatial resolution in MALDI-MSI. To accelerate the use of the MALDI-based metabolite imaging platform, substantial progress in matrix development and its application is required. In MALDI-MSI, because damage of the biomedical tissue section induced by laser irradiation is relatively modest, histological and biochemical evaluations can be performed on the same tissue slice after the MSI experiment is complete. The additional information that can be obtained using this approach allows high-precision and reliable molecular pathological evaluation of the results based on the combination of the different imaging modalities, MSI and the other pathological and biochemical imaging.

2.2. NIMS

Direct ionization on silicon (DIOS), one of the LDI methods without matrix, was first reported by Siuzdak *et al.* [35]. This method uses porous silicon to trap analytes deposited on its surface and laser irradiation to vaporize and ionize them. Because DIOS does not require a matrix, it has a great advantage over the MALDI method in which the matrix interferes with the detection of low-molecular-weight metabolites. Recently, the same researchers have developed nanostructure-initiator MS (NIMS) by improving the DIOS method [36,37]. NIMS is a surface-based MS technique that uses a nanostructured silicon surface to trap liquid ('initiator') compounds. Analyte materials adsorbed onto this 'clathrate' surface are subsequently released by laser irradiation for mass analysis. This method can change

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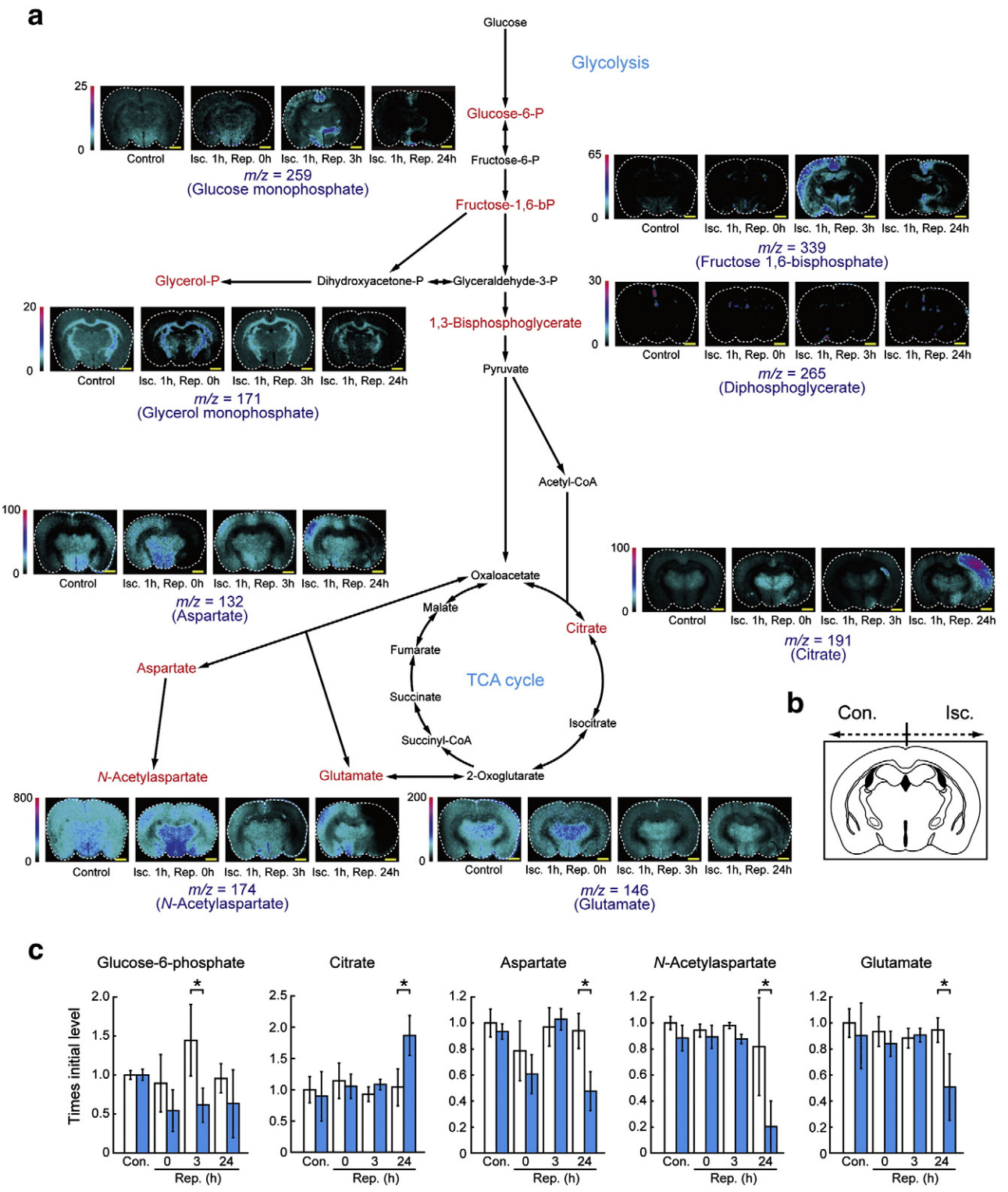

Fig. 1 – *In situ* metabolic pathway imaging visualizes drastic changes of spatiotemporal metabolite distribution in MCAO rat brain. Wistar rat brains of control (no operation) or from rats after various periods of reperfusion following 1 h of MCAO were extirpated and immediately frozen under −80 °C. Coronally sectioned brain slices (10 μm thickness) were then used for in situ metabolite imaging. Mass imaging data were acquired in negative ionization mode with 50 μm spatial resolution (14,000 μm × 11,000 μm, 10 shots/data point). All imaging data were normalized with the average mass spectrum for quantitative comparison of the concentration of each metabolite at different times. (a) These data were put on the central metabolic pathway map. (b) A schematic illustration represents the structure of coronally sectioned brain. (c) Relative changes in concentrations of metabolites extracted from whole cortex (CTX). Data is shown as the mean ± SD (n = 5), and represents the relative concentration of each condition to the concentration of contralateral CTX in control (Con.). Asterisk mark indicates significant differences (P < 0.05) between contralateral (open bar) and ischemic (closed bar) CTXs. Adapted with permission from ref. [33].

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the target molecule through the modification of the NIMS surface by choosing the 'initiator' compounds that are used. The analytical technique showed a higher sensitivity (~attomole) for biological molecules such as peptides and low-molecular-weight metabolites. NIMS can also detect several peaks from a single cell in the low-mass range (<1000). Interestingly, 650 yoctomoles (about 400 molecules) of the antiarrhythmic drug propafenone was detected as a significant peak. Furthermore, NIMS analysis can potentially be applied to the imaging of endogenous metabolite such as sterols or carbohydrates that have low ionization efficiency on other ionization platforms such as MALDI. For example of cation-enhanced NIMS imaging, Na+–enhanced NIMS was used to image sucrose in a flower stem and Ag+–enhanced NIMS was applied to visualize cholesterol in a mouse brain slice (Fig. 2) [38]. Compared with MALDI-MSI, the spatial resolution of NIMS has few limitations because NIMS does not require sample preparation, and therefore it allows for the direct analysis of tissue immediately after it is applied onto the NIMS surface [38–40]. That spatial resolution is completely dependent on the diameter of the laser irradiation is thought to be an advantage; however, compared with other MSI method, this is also a disadvantage because a tissue slice can easily be destroyed during imaging process, making it difficult to perform histological and biochemical evaluations on the same tissue slice after the MSI experiment is complete.

2.3. *DESI*

Desorption electrospray ionization (DESI), developed by Cooks *et al.*, is a soft ionization method under ambient conditions [41]. In this method, a fine spray of charged droplets hits a surface from which the target molecules are picked up. The target molecules are then ionized and analyzed using a mass spectrometer. The ionization mechanism of DESI is thought to be similar to ESI, and this can produce a wider range of ionizable target molecules than other imaging platforms such as MALDI and NIMS. Indeed, various kinds of chemicals and metabolites were analyzed using DESI [42,43]. Recently, DESI-MSI has been frequently used for lipid and free fatty acid imaging of animal tissue sections [44,45]. This imaging technique has also been applied to lipid-soluble vitamins, sugars, and cholesterol by derivatizing them with selective reagents added to the spray [46]. Cooks *et al.* have also succeeded in reconstructing a three-dimensional visualization of lipid distribution in mouse whole brain using DESI-MSI (Fig. 3) [47]. The distribution of several lipid molecules was reconstructed from the data of 36 representative sequential sections of mouse brain (200–500 μm) after imaging experiment. DESI-MSI was also used to analyze the lipid profiles of thin tissue sections of 68 samples of human prostate cancer and normal prostate tissue; cholesterol sulfate was identified as a possible candidate diagnostic marker for prostate cancer [48].

However, DESI-MSI has two major limitations for high-precision and reliable MSI. While results for relatively abundant molecules in tissue sections have been reported, there is little information available for the lower molecular weight and minor tissue components. This observation may indicate a potential difficulty in using DESI-MSI to visualize a broad range of the molecules found in tissues. Another drawback of DESI-MSI is that the spatial resolution that it can produce is approximately 200 μm, a value that is lower than that obtained using MALDI or NIMS, 20 μm [49]. However, unlike other LDI methods, neither the high vacuum condition nor sample preparation is required for DESI [42]. In addition to this advantage, DESI is one of the chemical ionization MS methods, and its molecular coverage can be expected to improve by more effectively and appropriately choosing chemical reagents that are suitable for inducing the ionization of diverse metabolites [50].

3. Strategy for metabolite identification

When MSI experiments are performed using any of the ionization methods described, tens or hundreds of peaks are

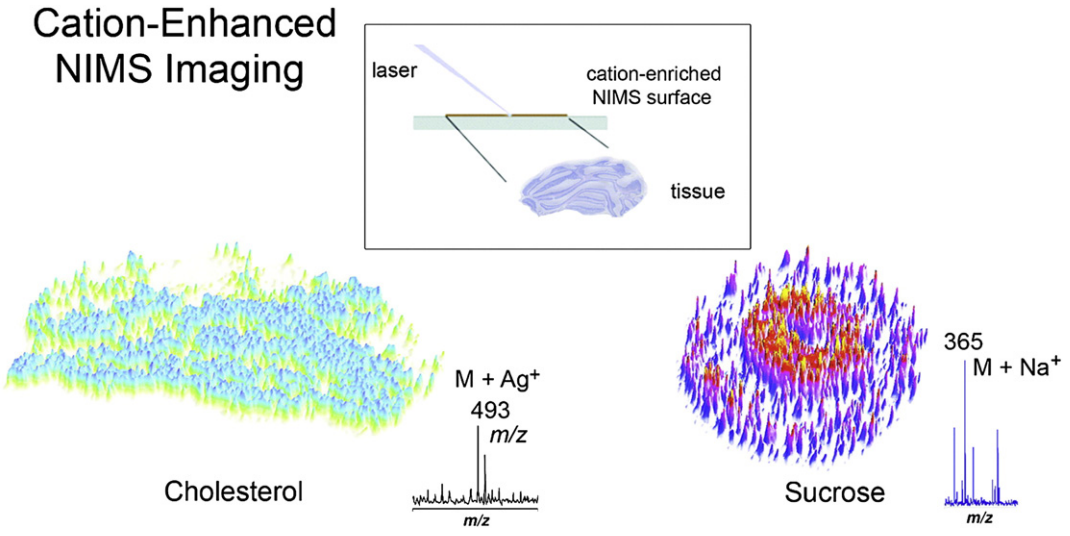

Fig. 2 – Cation-enhanced NIMS tissue imaging. Mass-based images of analytes that are traditionally difficult to detect, such as steroids and carbohydrates, can be generated with cation-enhanced NIMS. For example, $AgNO_3$ was deposited onto a NIMS surface to detect cholesterol in a mouse brain (bottom, left). NaCl was deposited onto a NIMS surface to detect sucrose in a flower stem (bottom, right). Adapted with permission from ref. [39].

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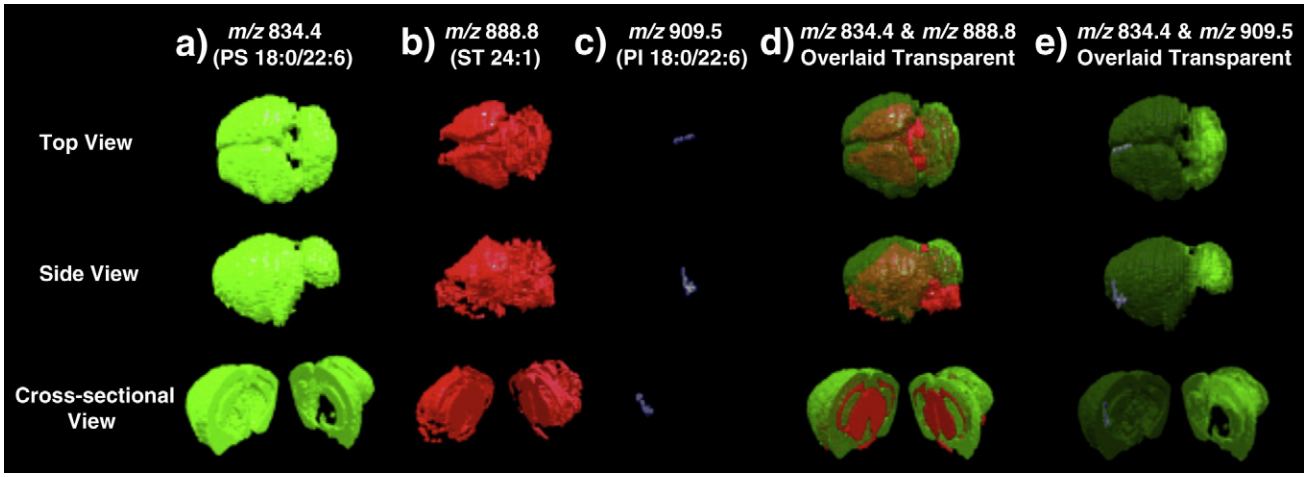

Fig. 3 – Three-dimensional (3D) models of the mouse brain by DESI-MS. Top, side, and cross-sectional views are shown for the 3D construction of the distribution of a) PS 18:0/22:6 in green, b) ST 24:1 in red, and c) PI 18:0/22:6 in blue. The same views are shown for the transparent overlaid distributions of the lipids d) PS 18:0/22:6 and ST 24:1, and e) PS 18:0/22:6 and PI 18:0/22:6. Adapted with permission from Ref. [47].

simultaneously detected in a single run. Understanding their unique distributions in the tissue section should provide important information. However, the crude analytical data without peak identification is difficult to interpret, and discovery of biomarkers or elucidation of biological and disease mechanisms is hindered. In the metabolomics research field, the comprehensive measurement of the metabolites extracted from whole cells and tissues is usually carried out by conventional analytical methods such as LC-MS or GC-MS. Each metabolite is usually identified by comparing two-dimensional data of the observed peak, its retention time and either the exact *m/z* or its MS/MS spectrum, with that of an authentic standard peak. In contrast, MSI experiments directly analyze a series of metabolites on the surface of a tissue without the extraction and separation processes that are required for the conventional methods. Thus, the identification of metabolites must be performed by only one-dimensional data, the exact *m/z* or the MS/MS spectrum of the observed peak.

Generally, an MS/MS approach is applied to indentify metabolites in MSI experiments [32,51]. In-house library or public database (for example, Mass Bank [52], the METLIN database [53] and/or the Human Metabolome Database [54]) search strategies using the available MS and MS/MS mass spectra patterns of known and available compounds are usually used as the well-established chemical annotation of the experimental MS data. This strategy is not data-driven; rather, it is completely dependent on the spectral databases and on comparisons with reference authentic standard spectra. There are some problems in using these methods. First, the commercially available compounds cannot cover all biological metabolites because there are thought to exist large numbers of unknown metabolites [55]. Second, poor mass resolution can cause the peaks of several metabolites to overlap; in such cases, the MS/MS spectrum becomes a mixed spectrum of the overlapping metabolites. TOF-MS (resolution ~50,000) and Orbitrap-MS (resolution ~100,000) are widely used as the MS instruments for MSI experiments; however, their mass resolution power is low. These problems mean that it is difficult to identify individual peaks in MSI spectra using an MS/MS strategy. Indeed, only 20–30% of all peaks detected from mouse brain tissue were successfully identified by an MS/MS approach [33]. Clearly, compared with the ability of an MS/MS approach to identify proteins, which is usually performed by Mascot algorithm [56], this approach alone is not effective enough to identify metabolites in target tissues. The identification of various biological metabolites on the basis of MS data still remains a challenging issue.

The elemental composition of an unknown metabolite is one of the most important pieces of information for reliable structure determination. Several researches have focused on the direct determination of elemental composition directly from mass spectra. In principle, one elemental composition has one molecular weight and one molecular weight determines one elemental composition; however, this relationship strongly depends on the accuracy of the molecular weight measurement. In fact, many hundreds of candidates based on the elemental composition of metabolites are found at lower accuracy. Mass spectra with high accuracy (<1 ppm) effectively narrow down the number of candidates based on elemental composition [57]. Furthermore, elemental composition can be determined effectively by combining accurate *m/z* value and MS^n spectra [58]. Recently, it was reported that ultra-high-resolution (resolution >500,000) MS analysis enabled the elemental composition to be determined directly using isotopic fine structure (Fig. 4) [59]. High-resolution MS provides another advantage for MSI because low mass resolution causes the critical problem that the observed imaging map for single *m/z* value is at high risk of containing overlapped images of proximate multiple metabolite peaks. In low mass resolution MSI, individual metabolites can be detected by MS/MS fragment imaging [60]; however, because this is a completely targeted analysis, a preliminary MS/MS experiment of the standard of target molecule is required beforehand. Very close mass peaks that are observed as a single overlapped peak by low mass resolution MS can be separated clearly and visualized as independent multiple images by ultra-high resolution MSI [11]. However, digital

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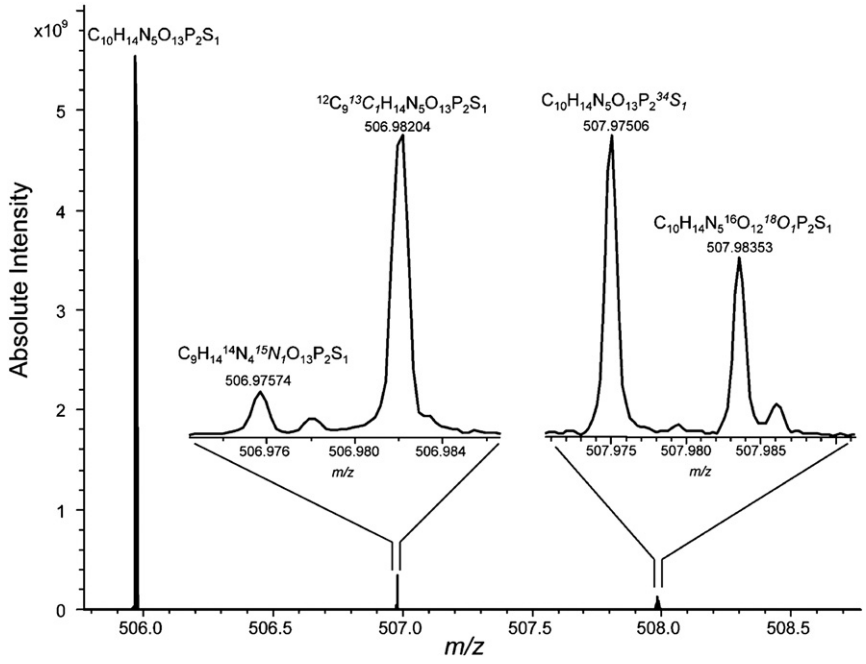

Fig. 4 – Mass spectral observation of PAPS ($C_{10}H_{15}O_{13}N_5P_2S$) in broadband mode by using high magnetic field fourier transform ion cyclotron resonance (FT-ICR)-MS. Ionization source of FT-ICR-MS was operated in an ESI negative ion mode. Multiplet isotopic peaks were observed at $(M-H^+ + 1)^-$ and $(M-H^+ + 2)^-$ region, and these peaks were successfully assigned to the substitution of stable isotope for each element. Adapted with permission from Ref. [59].

data from a single ultra-high resolution MSI experiment will be over a terabyte in size. Therefore, researchers must choose the MS platform for MSI that best matches the experimental circumstance and purpose.

4. Conclusion and future perspectives

In this review, we discussed the recent advances and difficulties of endogenous metabolite MSI. Each MSI platform, MALDI, NIMS, and DESI, has advantages and disadvantages, and improvement of the methods and development of instruments are still in progress in the attempt to overcoming diverse technical disadvantages, especially molecular coverage and metabolite identification.

In the future, a combination of an *in situ* endogenous metabolite MSI technique with other analytical platforms such as multivariate statistical analysis and *in vivo* non-invasive imaging techniques (such as anatomic imaging including MRI and computed tomography, or functional imaging including functional MRI and PET) may become the compulsory technology for unraveling and understanding the molecular complexities of local tissues or for *in situ* pharmacometabolomics, biomarker discovery, early diagnosis, and cytodiagnosis [61].

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