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Novel Techniques in Histologic Research: Morphometry and Mass Spectrometry Imaging

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Abstract

Application of mass spectrometry (MS) has recently been developed for the identification of biomarkers using pathologic samples, whereas its application using formaldehyde-fixed paraffin-embedded (FFPE) tissues remains limited. In this review, we introduce MS imaging (MSI) using FFPE tissue. This method is still challenging for peptide ionization, and various pretreatment techniques have been conducted for enhancing the ionization signal of peptides. A simple chemical pretreatment method involving heating in acetonitrile-containing buffer under pressurized conditions is introduced. Further, two-dimensional MSI data are summarized in a DM for region of interest (ROI) and hierarchical cluster analyses. These techniques enable MALDI-MSI analysis of archived pathological FFPE samples to identify new biomarkers.

Keywords: mass spectrometry imaging, formalin-fixed paraffin-embedded (FFPE) tissues, acute myocardial infarction

1. Introduction

Over the past several decades, MS-based techniques are used in blood and urine analyses; however, their application to tissue analysis requires further development. Recently, matrix-assisted laser desorption/ionization (MALDI) has been applied for MS analysis for the identification of diagnostic markers [1–4]. As one of the applications of MALDI-MS (mass spectrometry), MALDI-MS imaging (MALDI-MSI) has been recently developed for the identification of phospholipids [5, 6] and peptides [7–10] and pharmacological monitoring for drug delivery system in various tissues using frozen samples [11–13]. Further, the MSI technique has been further developed in the use of formalin-fixed paraffin-embedded (FFPE) tissues and cells [7–10].

Thus, these new strategies and analytical methods are useful for the study of drug pharmacokinetics, metabolism, discovery of biomarkers, and treatment-specific effects on the proteome.

1.1. Tissue pretreatment of FFPE for MS imaging

Most of human tissues are preserved as FFPE samples in bioresource repositories. Formaldehyde reacts amino acid residues such as arginine containing amino group by methylene bridging for prevention of decomposition. There are a number of reports discussing procedures for pretreatment of FFPE sections for MALDI-MS [14, 15]. Researchers performed microdissection of pathologically diagnosed disease regions, followed by protein extraction and digestion of frozen samples for proteomic study using MS technique, but this technique is costly and time-consuming for storage of frozen samples. For broad application with the use of FFPE, there is a critical issue for study using MS technique, because the methylene bridge between amino acid residues makes it difficult to ionize peptides in samples. In fact, for immunohistochemical analysis of pathological samples, FFPE tissues are subjected to a retrieval protocol, including protein digestion by proteinase, microwave, or boiling treatment. Although the treatment using detergents is available for the retrieval of FFPE, this procedure may cause noise in the MS spectrum. There are some reports that surfactants improved MS sensitivity, but there is considerable limits regarding the subjects and the amounts to use for stable protocol [16]. In addition, enzymatic digestion is generally used to promote ionization by fragmentation; however, this enzymatic digestion is not sufficient to ionization for gaining intense MS signals. In general, collagens and elastin are commonly rich in most of all tissues, and these caused background noise of MS imaging and LC-MS. Angel et al. described availability of a matrix metalloproteinase (MMP) enzymes to access [17]. PAXgene, a type of alcohol-based non-cross-linking tissue fixative, can be used as an alternative fixative for multiomic tissue analysis [18].

A simple in situ histologic pretreatment technique for FFPE sections was developed for MALDI/TOF-MS imaging. Application of immunohistochemical method such as heating sample slides in a solution of ethylenediaminetetraacetic acid (EDTA) or citric acid may be available for enhancement of signal intensity [7]. Ronci et al. reported the imaging of human lens using this method. MALDI-MSI revealed a concentric distribution pattern of proteins of apolipoprotein E (ApoE) and collagen IV alpha-1 on the anterior surface of surgically removed lens capsule [19, 20].

1.2. MALDI-MSI of acute myocardial infarction (AMI)

Next, let us review a method for the identification of molecular markers to precisely identify mitochondrial and sarcomeric proteins in cardiac tissues. Ischemic heart failure remains a major cause of sudden cardiac death. MSI is a promising technique for understanding the pathogenesis of lethal acute myocardial infarction (AMI). MSI of cardiac tissue was recently reported by several groups including these authors [21, 22].

Histological analysis is certainly one of the key methods employed in cardiac muscle damage studies. Chronic cardiac infarcted lesions demonstrate gross fibrosis, and scarring; however, acute cardiac infarcted lesions rarely include such distinct histologic and macroscopic changes. However, the microscopic findings observed in AMI are not sufficiently informative of the phase immediately following the onset of the infarction episode [23]. Of course, wavy fibers

and contraction bands are histological hallmarks of myocardial injury [24], but these indicators are localized and not necessarily obvious. Depleted H-FABP staining could be seen in areas that normally show hematoxylin-eosin (H&E) staining [25].

Therefore, it is often difficult to diagnose AMI on autopsy for pathologist. In macroscopic findings in the autopsied heart, it is known that dark mottling is detectable within 4 h after the onset but variable by cases. A yellow tan may be detectable on damaged tissue within a few days. Transient thrombus in acute coronary syndrome (ACS) with coronary spasm is frequently lost. Thus, macroscopic findings are limited and not sufficiently informative at the phase immediately after the onset of the infarction episode. Therefore, evaluating the range of the infarction histologically remains difficult. Rather than these pathologic findings, a guideline for ECG has been recently developed for diagnosis of ACS [26, 27], such as ST-elevation and/or Q-wave, which is diagnostic data as well as chest pain or other pains on the onset of the infarction or other symptoms. Further, several studies have used ischemic models that facilitate a deeper understanding of cardiac muscular remodeling after hypoxic stress [28, 29]. Biochemical data of creatine kinase MB (CK-MB) is an alternative important diagnostic indicator. In clinical diagnosis, troponins [30] have been utilized for blood test. Serum markers are sensitive to time-dependent myocardial damage that is mainly caused by disruption of coronary artery blood flow. Heart-type fatty acid-binding protein (H-FABP) [31] is in clinical use, but their sustainability and sensitivity are insufficient (**Table 1**). Several studies have used ischemic models that facilitate a deeper understanding of cardiac muscular remodeling after hypoxic stress for identification of alternative markers [28, 29].

1.3. Laser-captured microdissection and proteomics

Liquid chromatography (LC)-MS techniques using laser microdissection technique were useful for identification of a promising biomarker for myocardial infarction [32].

In this process, contraction band and wavy fibers are hallmarks of AMI for dissection, which are localized in the infarcted area. In collection of cardiac tissues, fibrotic area was excluded to prevent contaminating other types of injury. To extract proteins followed by liquid chromatography-mass spectrometry (LC-MS), each microdissected sample was suspended in NH_4HCO_3 containing CH_3CN . Tubes were heated at 95°C and cooling trypsin digestion was performed. Tubes were incubated at 37°C overnight and then heated at 95°C . After drying, samples were resuspended in trifluoroacetic acid CH_3CN . Following this process, 1.0×10^4 peptides and 10^{2-3} proteins are identified. The list of identified proteins is shown below (**Table 2**). An example of spectrum in LC-MS is shown in **Figure 1**.

	<2 h	2–4 h	4–6 h	6–12 h	12–24 h	24–72 h	72 h<
Myoglobin	○	○	○	○	○	△	×
H-FABP	○	○	○	○	○	△	×
Cardiac troponin	×	△	○	○	○	○	○
CK-MB	×	△	○	○	○	△	×
Myosin light chain	×	△	○	○	○	○	○

Table 1. Representative biomarkers of AMI. CK, creatine kinase.

1.4. MALDI-MSI and ROI analysis

The permeability of reaction buffer into FFPE tissues for hydrophilization has recently been improved such usability without tissue damage. The procedure enhances the crystallization of the peptide and matrix and organic low weight molecule, such as DHB, for MALDI/TOF-MS analysis. Here, let us review a method for the identification of molecular markers using MSI and tandem MS [6]. In detail, the tissue was incubated for 1 h at 37°C in the buffer containing NH_4HCO_3 and CH_3CN . After removal of the buffer, the tissue was incubated again with a

Collagen α -1 (I) chain
 Collagen α -1 (III) chain
 Filamin-C
 Serum albumin
 Hemoglobin subunit- β
 Collagen α -2 (I) chain
 Hemoglobin subunit- α
 Keratin, type II cytoskeletal

Table 2. Nonspecific proteins in analysis of cardiac myocyte proteins in LC-MS using FFPE. These are commonly detected proteins of various organs that should be removed from protein filing.

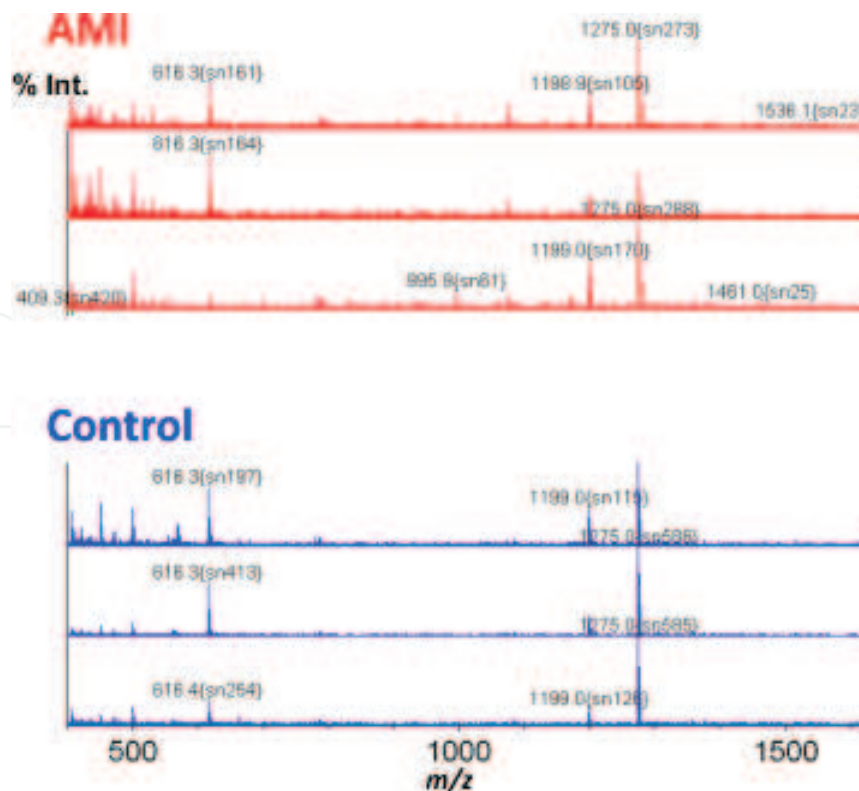


Figure 1. An example of LC-MS spectrum representing hemoglobin beta in AMI and control.

volume of buffer to cover the sample. The slide is subsequently covered with a cover glass and heated at 94°C on an aluminum hot plate. Next, trypsin solution is added on the tissue for protein digestion, and the slide is incubated at 37°C overnight (Figure 1). Long-term incubation in a buffer containing CH₃CN may increase in spaces between tissue fibers in cardiac tissue. The matrix (2,5-dihydroxybenzoic acid, DHB) solution (Figure 2), which is known to be suitable for analysis of peptides, is deposited in droplets at a spatial interval using several devices that were developed for matrix deposition (Figure 2). After drying, mass spectra were gained using a MALDI TOF/TOF mass spectrometer equipped with a nitrogen laser. Using this method, histone H2A was reported as a marker of colon cancer [6].

MALDI-MSI of hemoglobin beta demonstrates the two-dimensional distribution of blood flow in the cardiac tissue (Figure 3). Imaging MS Solution is a novel tool for ROI that was developed (Shimadzu Co. Ltd).

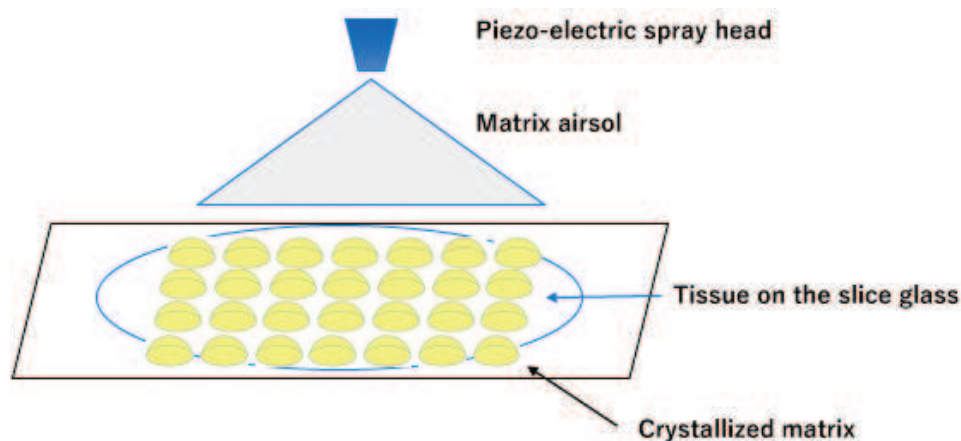


Figure 2. DHB in a solution of 50% methanol was deposited onto the sections using a piezoelectric head.

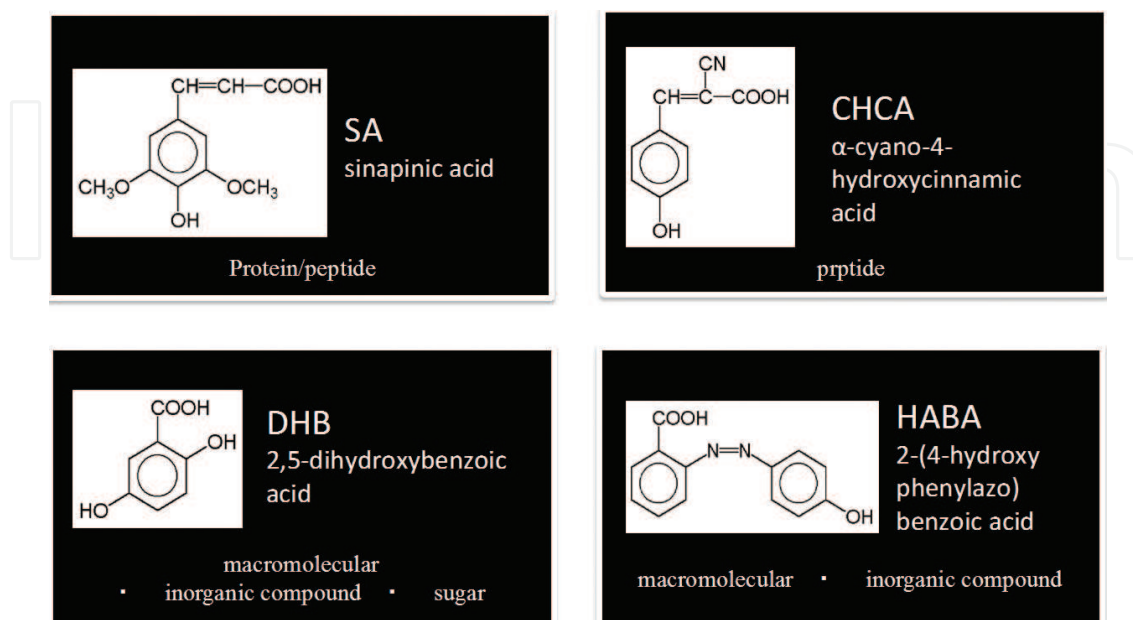


Figure 3. Available matrix for ionization of peptide.

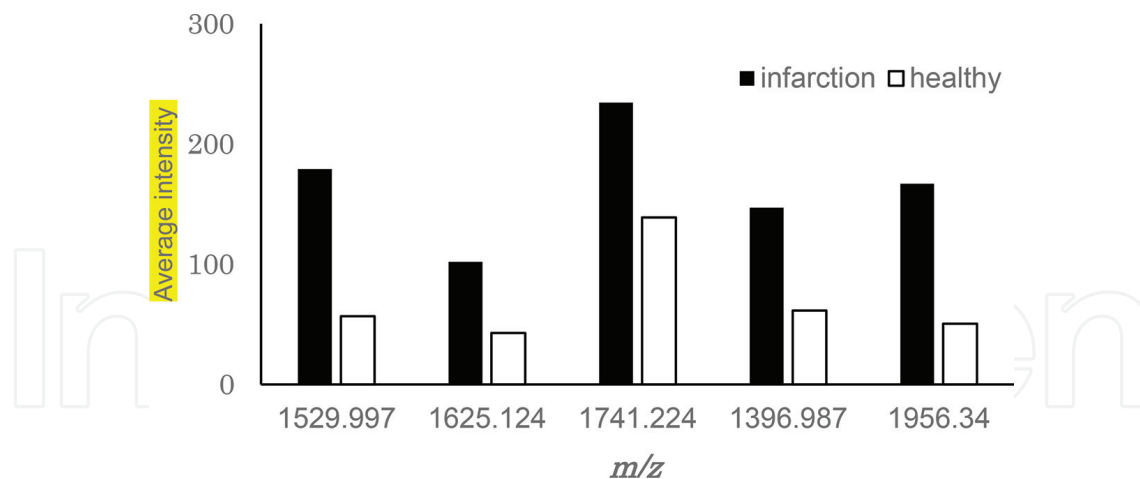


Figure 4. Examples of comparative ROI analysis. Black bar and white bar represent the average intensity of individual m/z values. The value 1530 corresponds to hemoglobin subunit alpha. HBA signals in the infarcted ROI of samples were measured for all pixels in the respective regions ($N = 4126$; healthy area, $N = 7554$).

Using this program, the intensity in individual measured point pixels is summarized in a DM that is used for computation (**Figure 4**). For this analysis, the observations are based upon PTAH and H&E staining; the endocardial area is selected as ROI, in which contraction bands, dilated arterioles, and endothelial damage are observed. In this way, ROI analysis is performed to test for intensity differences between infarcted ROI and healthy area in each batch of imaging data over all pixels. ROI analyses distinguished molecules that are specific to ROI (**Figures 5 and 6**).

Note: In analysis of big imaging data, t -test is not necessarily sufficient because p -value depends on a number of data. In particular, the pixel point in ROI analyses is greater than 1000. For ROI analysis, Cohen's d -value, which is independent of the numbers of data, supports t -test analytic data. The d -value is defined by

$$d = \frac{s_i - s_h}{\sqrt{\frac{(n_i-1)\sigma_i^2 + (n_h-1)\sigma_h^2}{n_i + n_h - 2}}} \quad (1)$$

s_i and s_h represent the average signal intensity of the pixels in ROI and the control area, respectively. n_i and n_h represent the pixel numbers in ROI and the control, respectively. σ_i and σ_h represent the standard deviation of the intensity in the pixels ROI and the control, respectively [33]. Cohen's criteria are as follows: $d < 0.2$, not significant; $0.2 < d < 0.5$, small; $0.5 < d < 0.8$, medium; and $d > 0.8$, great (significant).

1.4.1. Hierarchical cluster analysis of MSI

Imaging MS solution® (Shimadzu, Kyoto, Japan) is applied for hierarchical clustering analysis (HCA) based on the IMS data. According to the algorithm, DM clustering is performed using intensity value information for each m/z peak. When performing hierarchical cluster analysis,

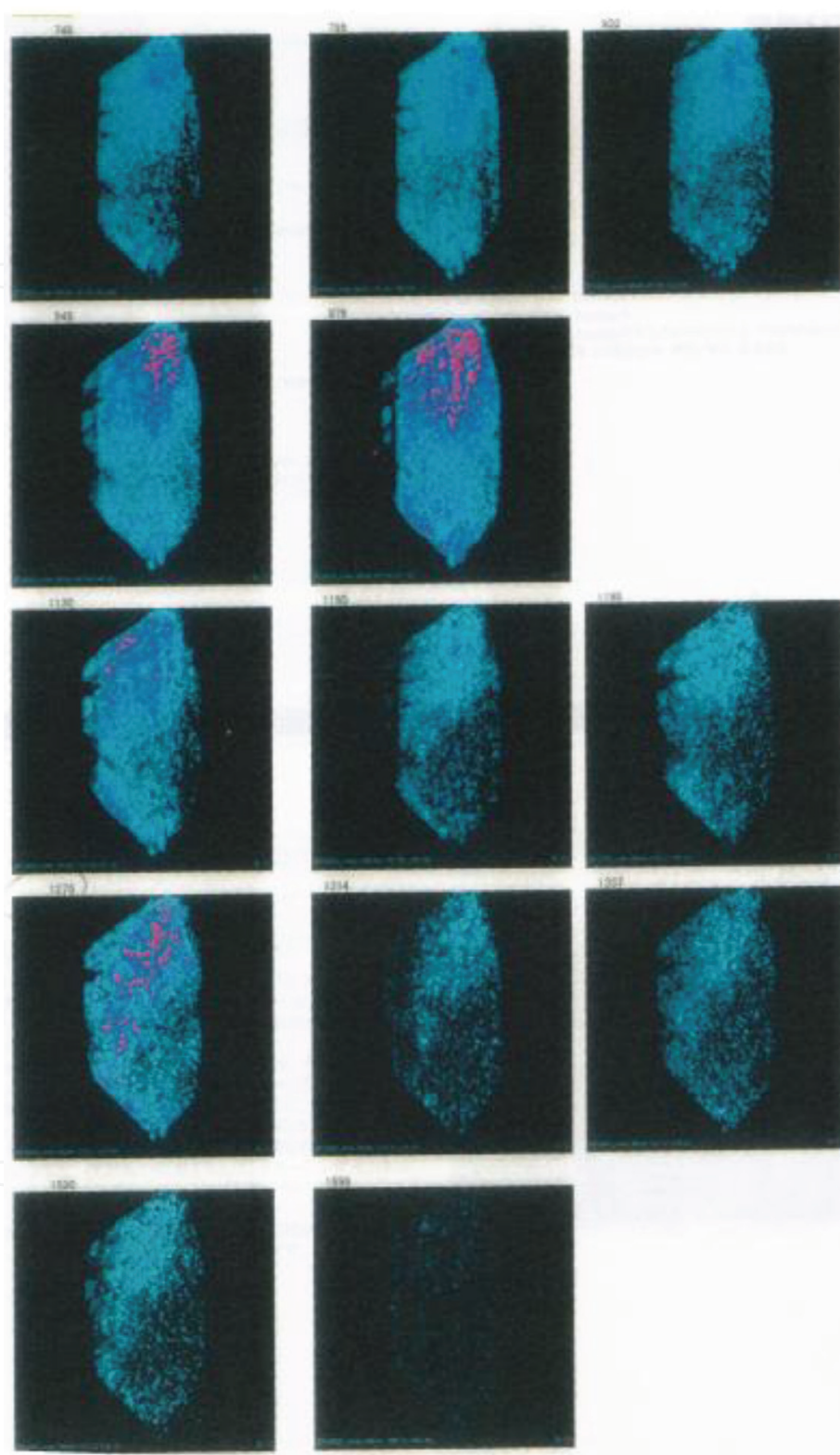


Figure 5. MALDI-MSI: m/z values are 748, 795, 932, 945, 976, 1130, 1165, 1194, 1275, 1314, 1337, 1530, and 1696, respectively. The intensity is illustrated on the cardiac tissue in a heat map manner. For visualization, raw MALDI-MS data were converted into the Imaging MS Solution ver. 1.20 (Shimadzu).

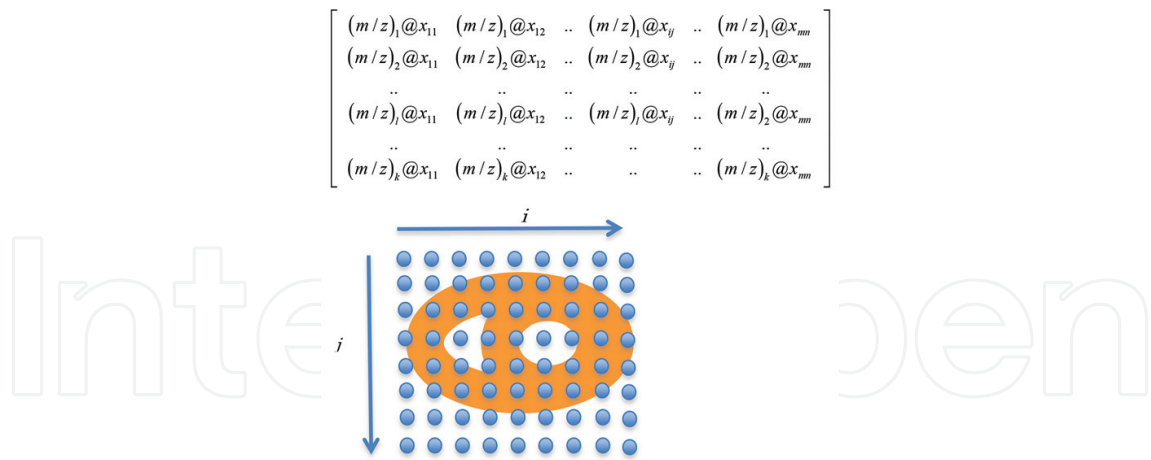


Figure 6. Examples of DM. The component $(m/z)_l @ x_{ij}$ in the matrix signifies the l th m/z values ($1 \leq l \leq k$) for the pixels at the coordinate of x_{ij} ($1 \leq i \leq m, 1 \leq j \leq n$) on the sample issue. This DM is the platform of the ROI analysis and other static analyses. The bottom illustration represents the coordinate on the cardiac tissue (indicated by orange circles). First, we measured the intensity values for $m/z = (m/z)_1$ of each pixel in the image and arranged the value as a column vector. Similarly, another value $m/z = (m/z)_2$ was measured, and the value was arranged as the next column vector. Further measurements of all detected intensity values of m/z ($1, 2, \dots, l, \dots$) are arranged in the row direction. Row and column vectors yielded a DM for a single whole dataset of a tissue.

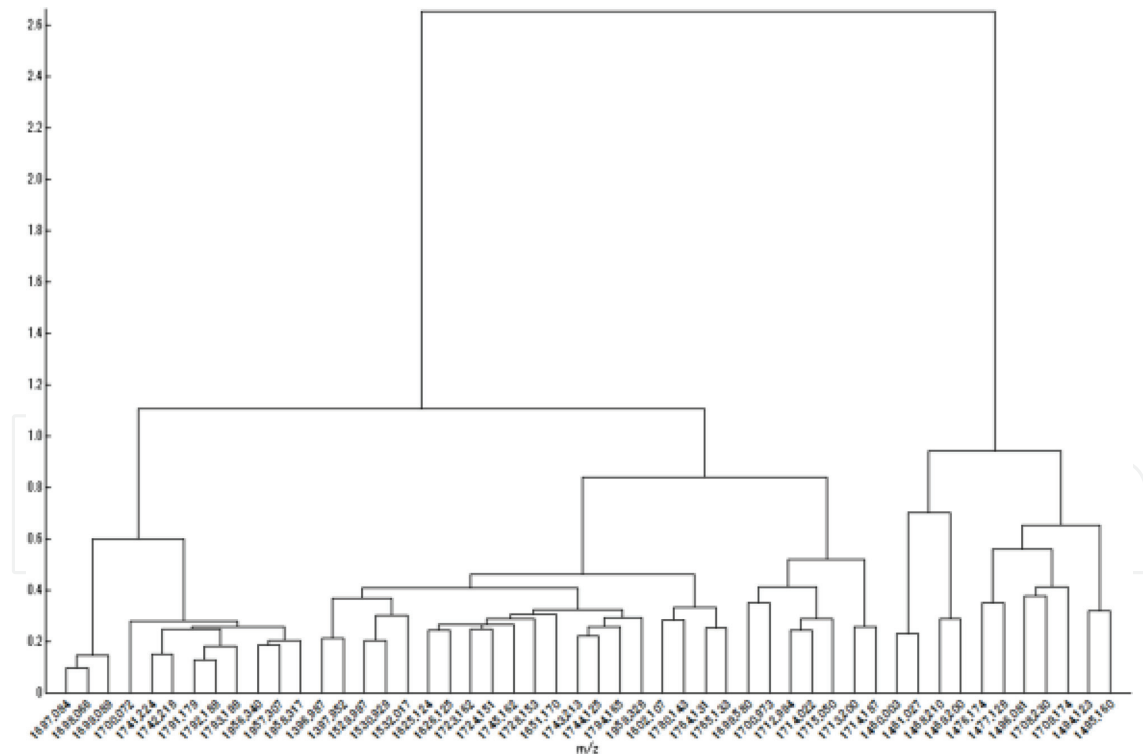


Figure 7. An example of HCA analysis. Distribution of hemoglobin subunit alpha peptide signal ($m/z = 1529$) is shown.

the Euclidean distance between data matrices was defined as performed (scalar values). We summarized the images close in distance as one larger cluster, and we then calculated the distance between the newly grouped individual clusters. By repeating this process, clustering

was carried out hierarchically in the dendrogram. For HCA, we analyzed data using Ward's method and the group average method. The vertical axis represents distance, and m/z values are represented on the horizontal axis. Comparing the distribution between hemoglobins is anticipated to provide an important data of relevance of blood flow carried by hemoglobin and mycotic injury due to infarction (**Figure 7**).

2. Conclusion

In this article, we reviewed analytical method in MSI analyses of FFPE tissues. This method for protein extraction from FFPE enabled proteomic analysis. With the combination of label-free LC-MS accompanied by precise laser microdissection enabled in situ proteomic analysis, new pathological findings are obtained.

Proteomic studies by MSI have recently been developed extensively for the identification of peptides in tissues [5, 34]. In this study, the application of MSI method leads to a significant improvement in the signal intensity FFPE tissues. The treatment improved the quality of signals obtained from FFPE specimens by swelling of the deparaffinized sample and increasing tissue permeability. For precise diagnosis, FFPE specimens provide more informative histologic findings than frozen samples. Therefore, establishment of technique for proteomic study using FFPE contributes to the identification of novel diagnostic markers. To obtain specific markers, it is necessary to exclude nonspecific abundant proteins, including collagens, keratins, beta tubulin, and vimentin. Proteomic studies represent a promising approach to the discovery of novel diagnostic markers and understanding of the pathogenesis of cardiac remodeling. MSI may provide information about the histologic diagnosis of AMI, and several sarcomeric proteins may be a promising marker for the diagnosis of AMI. ROI and HCA analyses proved to be useful tools for the analysis of signal distribution patterns of infarcted tissues. When supplemented by these analyses, IMS may be a promising technique for the identification of biomarkers for pathological studies that involve the comparison of diseased and control areas.

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Conflicts of interest

The authors have declared no conflicts of interest.

Appendices and nomenclature

MALDI	Matrix-assisted laser desorption ionization
MALDI-MS	MALDI mass spectrometry
MALDI-MSI	MALDI-MS imaging
FFPE	Formalin-fixed paraffin embedded
EDTA	Ethylenediaminetetraacetic acid
AMI	Acute myocardial infarction
DM	Dataset matrix
ROI	Region of interest
HCA	Hierarchical clustering analysis

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