Chapter 4

Imaging Mass Spectrometry: Sample Preparation, Instrumentation, and Applications

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Contents

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1. INTRODUCTION

The ability to visualize the molecular distribution in biological material such as tissue samples has helped scientists to provide a better understanding of the principles of life. The study of biomolecule distribution in organs and its alterations with disease remains one of the most challenging and intriguing scientific issues of recent times. Various techniques are used in laboratories around the world to visualize molecular systems—techniques such as magnetic resonance imaging (MRI) technology (Hurd and Freeman, 1989) and positron electron tomography (PET) (Ametamey *et al.*, 2008). The Nobel Prize–winning MRI and PET technologies are known as noninvasive techniques for medical diagnosis. Nuclear magnetic resonance spectroscopy (NMRS) is also helpful for imaging and identification of biomolcules in tissue sample (Hiltunen *et al.*, 2002). The limitations of these techniques are the relatively poor resolution, sensitivity, and requirement of labeling of molecules for detection (in the case of the PET method).

Imaging mass spectrometry (IMS) was introduced for spatial distribution analysis of biomolecules without the need for extraction, purification, separation, or labeling of biological samples. Recent developments in molecular imaging have created new opportunities to perform molecular diagnostic and therapeutic procedures. The technique can be exploited to visualize cellular and molecular processes that occur in two-dimensional (2D) or three-dimensional (3D) fashion without perturbing the structure of the system (Caprioli *et al.*, 1997; Setou *et al.*, 2010).

Mass spectrometry (MS) is a technique based on the measurement of the charged ions in an electric or magnetic field. Generally, a mass spectrometer contains three distinct parts: (1) an ion source producing ions from sample molecules; (2) a mass analyzer separating the different molecules with respect to their mass-to-charge ratios (m/z), and (3) a detector, registering the ion m/z and the intensity at which the ions were detected. Data are collected and visualized in a mass spectrum where the different m/z ratios are displayed as a function of their signal intensity (Gross, 2004). MS is a great scientific tool because of the wide range of molecules that can be accurately detected and identified: large organic compounds and biomolecules of low molecular weight.

In the beginning, mass spectrometric analysis was limited to samples that had undergone excessive preparation procedures, such as purification, separation, and concentration steps. These procedures not only jeopardize sample integrity, but also lead to the complete loss of any spatial distribution information. MS instruments are equipped with different ionization methods, including electron ionization and chemical ionization (Fales *et al.*, 1972), fast atomic bombardment (Morris *et al.*, 1981), electrospray ionization (ESI) (Fenn *et al.*, 1989), and matrix-assisted laser desorption/ionization (MALDI) (Karas *et al.*, 1985) for the analysis of a wide range of organic and bio-organic molecules. The introduction of the "soft" ionization sources such as ESI and MALDI transfigured MS, as it offered the capability to analyze large intact biomolecules.

At present, IMS is a well-recognized technique for profiling the distribution of biomolecules in tissue sample at micrometer to nanometer resolution (Caprioli *et al.*, 1997; Goodwin *et al.*, 2008; McDonnell and Heeren, 2007; Pol *et al.*, 2010; Shimma *et al.*, 2008). Data acquisition is performed through scanning a tissue section with a laser, thereby obtaining one mass spectrum for every pixel. The main principle of IMS is based on desorption and ionization of biomolecules from the surface of the tissue sample. There are currently four important desorption/ionization methods: desorption electrospray ionization (DESI) (Takats *et al.*, 2004), secondary ion mass spectrometry (SIMS) (Benninghoven, 1973), MALDI (Tanaka *et al.*, 1988) and laser ablation electrospray ionization (LAESI) (Nemes and Vertes, 2007).

2. IONIZATION METHODS FOR IMAGING MASS SPECTROMETRY

2.1. Desorption Electrospray Ionization

DESI was introduced by R.G. Cooks in 2004. In DESI, the molecules are ionized at atmospheric pressure without the use of any organic matrix (Dill *et al.*, 2009) in a combination of ESI and desorption ionization (DI). The charged droplets of solvent generated during the electrospray stage are used to ionize molecules from the surface of the sample and the ions produced thereby are directed into an atmospheric inlet of the MS. The components and use of DESI in IMS are presented in Figure 1a. The spatial resolution obtained by this method is 0.3–0.5 mm, which is a low resolution of tissue sample in IMS studies. DESI has been successfully applied to IMS for the identification of lipids, drug metabolites, and antifungal molecules in seaweeds (Dill *et al.*, 2009; Lane *et al.*, 2009; Wiseman *et al.*, 2008).

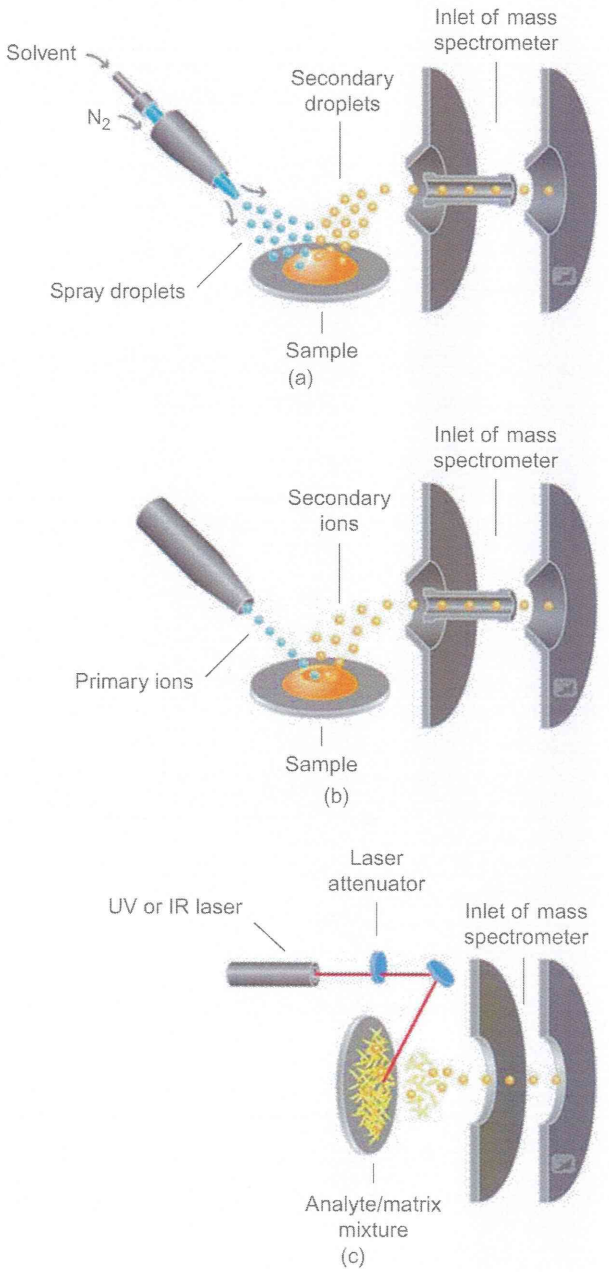

FIGURE 1 Desorption-ionization techniques used in mass spectrometry imaging. (a) Desorption electrospray ionization (DESI). (b) Secondary ion mass spectrometry (SIMS). (c) Matrix-assisted laser desorption ionization (MALDI). UV, ultraviolet; IR, infrared. Reprinted from Pol *et al.* (2010) with permission from Springer.

2.2. Secondary Imaging Mass Spectrometry

SIMS is a sophisticated technique that uses ion beams from metal ions such as Ar^+, Ga^+, and In^+ (here denoted *primary ion beams*) to produce secondary ions from molecules on the surface of a sample. Ionization is performed in high vacuum to avoid a collision with surrounding gas molecules, and the primary ion beams can be focused down to 50 nm on the sample surface, with the resolution depending on the current and charge state of the ions. SIMS coupled with time-of-flight (TOF-SIMS) is a superior tool for high-spatial, submicron resolution (< 10 nm). Thus SIMS can be applied for the differentiation of biomolecules that are present all the way down to the cellular level. However, fragmentation of larger molecules on the sample surface is observed when strong laser energy is applied for the primary ion beam. Hence, SIMS is primarily applicable for the analysis of small molecules (< 1000 Da) (Heeren *et al.*, 2006; Slaveykova *et al.*, 2009). Figure 1b shows the process of SIMS ionization of molecules from the sample surface. SIMS has been applied for imaging of samples such as single cells, embryos, brain, cocaine, and cinnamoylcocaine in coca (Colliver *et al.*, 1997; Jones *et al.*, 2007; Wu *et al.*, 2007). The fragmentation of molecules in SIMS can be overcome through the treatment of an organic MALDI matrix; this approach is known as matrix-enhanced (ME)-SIMS (Altelaar *et al.*, 2007).

2.3. Laser Ablation Electrospray Ionization

LAESI was developed by Nemes and Vertes (2007) and is a method for MS analysis of tissue samples without sample preparation under atmospheric pressure (Nemes and Vertes, 2007). Laser ablation from a mid-infrared (mid-IR) laser is combined with a secondary ESI process. The spatial resolution for tissue samples using LAESI technique is better than DESI and can be used for imaging of biomolecules from the surface of tissue sample at a lateral resolution of < 200 μm. The technique has been applied for imaging and identification of plants, tissues, cell pellets, and even single cells (Nemes *et al.*, 2010; Shrestha *et al.*, 2010; Sripadi *et al.*, 2010). Recently it has also been used in 3D imaging of molecules from the sample (Nemes *et al.*, 2009).

2.4. Matrix-Assisted Laser Desorption/Ionization

MALDI was introduced as a soft ionization technique that causes little or no fragmentation of the target molecules, allowing for the analysis of molecules at several hundred kilodaltons (i.e., high m/z values). This allows for mass spectrometric analysis of a wide range of molecules such as amino acids, peptides and proteins, carbohydrates, and nucleic acids and drugs and has proven to be one of the most powerful MS technologies

to date. In traditional MALDI, an organic matrix is mixed with the sample on the target plate and irradiated by a ultraviolet or IR light generated by a pulsed and focused laser. The matrix absorbs the light at the wavelength of the laser, leading to a soft desorption/ionization of the intact compounds of interest (Gross, 2004; Karas *et al.*, 1985; Tanaka *et al.*, 1988). Figure 1c illustrates the MALDI mechanism.

3. MALDI IMAGING

By scanning a sample surface with the MALDI matrix/laser setup and registering individual mass spectra for each pixel, a 2D ion density map can be reconstructed using appropriate software. Direct MALDI-IMS analysis of clinical samples offers a unique approach to reveal the spatial expression of biomolecules linked with pathological disease and other clinical information. MALDI imaging is also suitable as a biomarker discovery tool by comparing the relative quantities and/or spatial distribution patterns of molecules in pathological and normal samples. The localization and abundance of biomarkers identified in tissue sections are used to understand disease progression at a molecular level. The main advantages of a direct biomarker analysis using MALDI imaging are that it provides spatial distribution patterns and is free from extraction, purification, or separation steps, hence avoiding procedures that are both time-consuming and jeopardize sample integrity (Chaurand *et al.*, 2006; Hayasaka *et al.*, 2010; Herring *et al.*, 2007; Schwartz *et al.*, 2003; Sugiura *et al.*, 2009). With the currently available imaging software packages, we can construct multiplexed imaging maps of selected biomolecules within tissue sections. The laser energy is used in a raster scan pattern to ionize the molecules, which are present as discrete spots or pixel. For each pixel the full mass spectrum is represented. The data acquisition time for IMS was shortened by the introduction of N_2 (337-nm) or neodymium-doped yttrium aluminum garnet (Nd:YAG) (355-nm) lasers with repetition rates of 200–1000 Hz with pulse lengths of 3 ns. The laser spot size of MALDI-MS is decreased from 100–150 to 20 μm, rendering higher spatial resolution of biomolecules on the tissue surface. Further, a higher spatial resolution can be attained with a MALDI instrument equipped with a highly focused laser. Chaurand *et al.* (2007) used a laser beam at 7 μm, which is in the order of the diameter of a single cell to detect protein ions. However, a decrease in sensitivity is observed while increasing the resolution in this manner. Figure 2 shows an example of MALDI-IMS analysis of protein from tissue section.

In addition to increased sample integrity, the great advantage of IMS is that it allows the construction of numerous ion images of molecules detected in a single run. This technique does not require previous

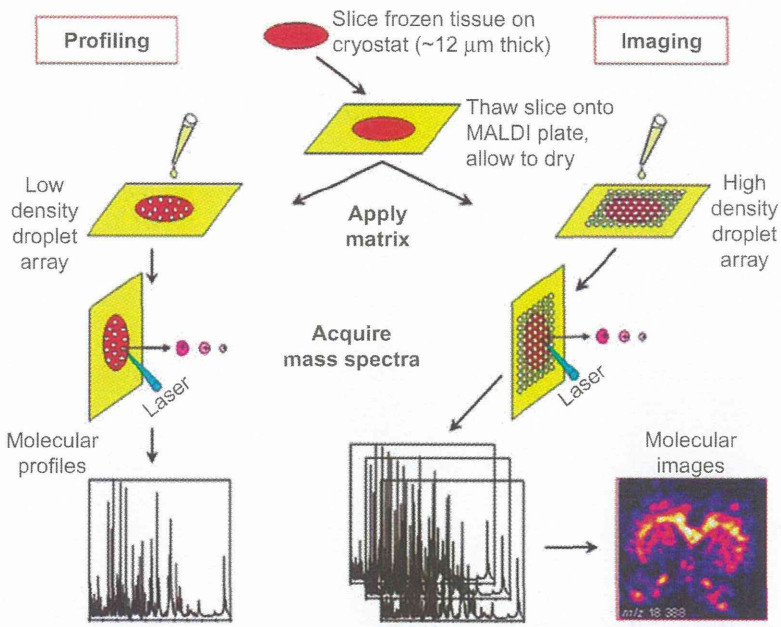

FIGURE 2 Scheme presenting the protein profiling and imaging analytical strategies from thin tissue sections. Reprinted from Chaurand (2006) with permission from American Chemical Society.

labeling with fluorescent probes or radioactive isotopes. MS analyses may be performed for imaging of biomolecules at low concentrations; the detection of 500 attomol has been reported in a single cell (Northen *et al.*, 2007). Another advantage when using MS is the specific identification of molecules; tandem MS is used to identify compounds for which no previous knowledge is required. For this, two MS analyzers are used: one for the selection of the ion of interest before fragmentation, and the second is used for the analysis of fragmented masses. Thus the use of MS is rapid, sensitive, and free from complicated sample procedures for the analysis of unknown biological tissue samples.

3.1. Sample Handling

Sampling handling is a very important concern for imaging and identification of biomolecules in tissue samples. Consideration must be given to the storage of the tissue sample after surgical removal from the human or animal body to prevent *ex vivo* degradation and alteration processes. The sectioning, washing, and staining of tissue, the choice of matrix, and its application on the tissue section are other parameters to optimize in order to obtain better-quality data.

3.1.1. Storage of Samples

Tissue storage is the most important part of the protocol for IMS studies to maintain the integrity of both the molecular composition and the spatial localization of analytes. When sampling is performed through surgical removal of tissue, molecular processes such as protein degradation continue in the *ex vivo* state. These processes should be halted immediately, either through freezing in liquid nitrogen or heat stabilization (Schwartz *et al.*, 2003). Chaurand *et al.* (2008) reported a long preservation method of tissue samples with ethanol for generating high-quality histological sections that enable high-quality images of biomolecules in tissue sample. Previously archived samples, on the other hand, are often fixed with paraformaldehyde and embedded in paraffin. Due to the cross-linking between molecules caused by this preservation method, special methods for specific tissue digestion have been developed (Wisztorski *et al.*, 2010).

3.1.2. Sectioning of Tissue

The next important part of imaging experiments is the sectioning of tissue sample into thin slices and the subsequent mounting of these tissue slices onto an appropriate target. Before tissue sectioning, the frozen tissue samples are transferred from the −80°C freezer to the cryostat chamber at −20°C for 30 minutes to thermally equilibrate the tissue. The tissue is usually embedded on an optimal cutting temperature (OCT) polymer, which supports easy handling and precise microtoming of sections. However, the use of OCT compounds causes a suppression of MALDI analyte signals in MS and should, if possible, be avoided (Schwartz *et al.*, 2003). Figure 3 shows the mass spectra of rat liver with suppression of MALDI-MS signals when OCT is used as a supporting material.

The use of gelatin is an alternative method for embedding the tissue sample where the mass spectrum is free from background signals compared with the use of OCT (Chen *et al.*, 2009). The embedded tissue is fixed on a sample stage and the temperature is maintained between 5°C and −25°C. The optimal temperature is set depending on the type of tissue to be analyzed and is followed by slicing of tissue with a steel microtome blade. For MALDI-IMS, the tissue sections are usually 5–20-μm thick (Chaurand *et al.*, 2006; Schwartz *et al.*, 2003).

The next step is the proper transfer of the sliced tissue section onto an electrically conductive steel plate or a glass slide. Thicker sections of tissue are more suitable when transferring them to the target plate because thinner sections break more easily. The first method of tissue transfer is performed by simply placing the plate in the cryostat chamber kept at −15°C while sectioning. An artist's brush is used to pick up the tissue section and gently place it on the cold plate, followed by gentle warming

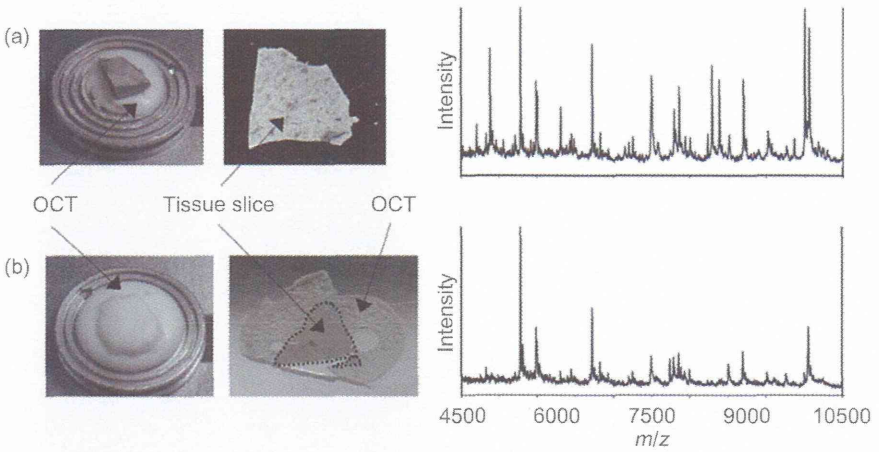

FIGURE 3 Effect of optimal cutting temperature (OCT) on MALDI signal from rat liver. (a) Optimal procedure where OCT is used to adhere the tissue to the sample stage but does not come into contact with the sliced tissue. The resulting spectrum shows many intense signals between m/z 4500 and 10500. (b) The tissue was embedded in OCT and attached to the sample stage. The resulting tissue slice is surrounded by OCT on the MALDI plate, and the resulting spectrum contains of only about half of the signals as in (a). Reprinted from Schwartz *et al.* (2003) with permission from John Wiley and Sons.

of the plate by touching the backside of the plate with a fingertip. The tissue is thereby thaw-mounted on the target plate. In the second method, the plate is kept at room temperature and placed over the sliced frozen section, and the tissue is thereby simply thawed on the target plate. Great care should be taken with both methods to retain the shape of the tissue. Obviously, folding or stretching caused during the sectioning of tissue section may affect the molecule distribution analysis and prevents detection of some of the molecules from the tissue surface.

3.1.3. Washing Tissue Sections

A tissue sample is generally washed to remove contaminants such as tissue-embedding media as well as lipids or biological salts that may affect the profiling and identification of peptides and proteins in MALDI-MS analysis. Washing a tissue section with 70% ethanol can remove salts, followed by a 90%–100% ethanol wash to dehydrate and fixate the tissue (Lemaire *et al.* 2006b; Schwartz *et al.*, 2003). Lemaire *et al.* demonstrated a procedure for washing a tissue section with five different organic solvents (chloroform, xylene, toluene, hexane, and acetone) for the identification of proteins in tissue samples and repeated the procedure with fresh solvents. The detection of protein signals is increased when the tissue

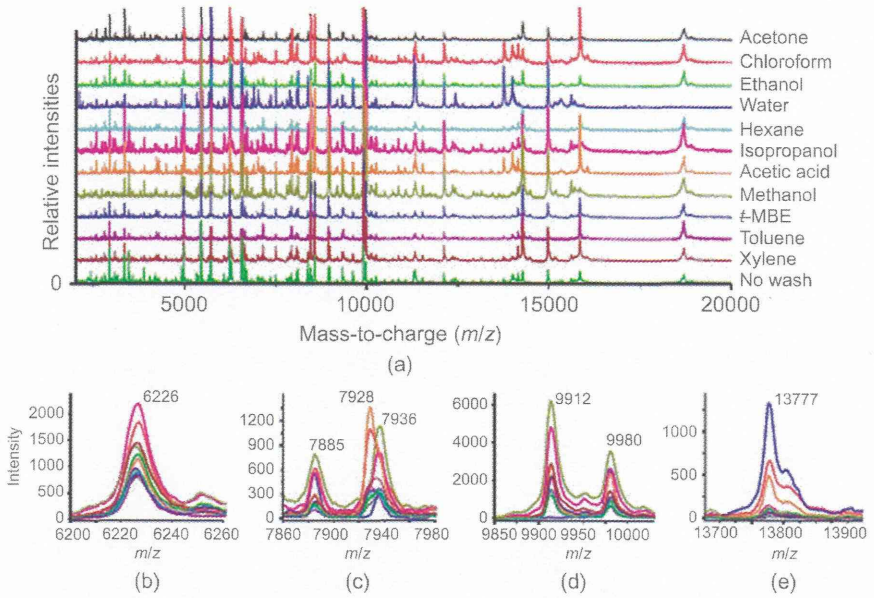

FIGURE 4 Average MALDI-IMS protein profiles directly acquired from serial mouse liver tissue sections not washed or washed with different solvent systems. (a) Full mass range; (b)–(e) selected mass signals showing specific behaviors for the different washes. Reprinted from Seeley *et al.* (2008) with permission from Springer.

sections are washed with organic solvent compared with untreated samples (Lemaire *et al.*, 2006b). Seeley *et al.* (2008) reported a new washing procedure to enhance protein detection in terms of both the number of observed peaks and the signal intensity. They demonstrated that the use of 12 different washing solvents established the most effective condition for direct protein analysis from the surface of tissue section. They also obtained a high detection sensitivity of protein signals, matrix crystal formations, and histological integrity of the tissues by washing with 70% isopropanol for 30 seconds followed by a 90% isopropanol wash for 30 seconds. Figure 4 shows the MALDI-IMS results for protein detection in mouse liver tissue sections after washing with different organic solvents.

3.1.4. Histological Staining of the Section

Histological staining of the tissue section is necessary to interpret the ion images obtained from the IMS results with the tissue section used in the experiments. The optical image obtained by the microscope is also used to superimpose the images acquired by IMS analysis to see the localization of molecules in tissue section. Hematoxylin-eosin (H&E) staining is a very popular histological method for MALDI-IMS results (Walch *et al.*, 2008). In IMS, two serial sections are sliced from tissue; one is used for imaging