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High Spatial Resolution Imaging Mass Spectrometry and Classical Histology on a Single Tissue Section

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Abstract

The work presented in this report describes and demonstrates a protocol for protein imaging analysis of biological tissue using MALDI IMS where histological staining and MS analysis are performed on the same tissue section. Spatial image resolution is shown at 35 μm for sagittal sections of the cerebellum from rat brain.

Introduction

MALDI imaging mass spectrometry (IMS) has emerged as a powerful tool for the molecular imaging of thin tissue sections, providing information about the spatial distribution and relative abundance of compounds within these sections[1–2]. The technology is advantageous for discovery because it does not require target specific markers, can map proteins, peptides, drugs and metabolites, has high sensitivity (detection limits in the low femtomole range for small molecules [3–5]) and high throughput using high repetition rate lasers (>1 kHz). Thus, many hundreds of molecular images are generated in a single raster of the tissue by laser.

Significant innovations especially in sample preparation protocols have allowed analysis of both fresh frozen and formalin fixed paraffin embedded (FFPE) tissue [6–8]. In many cases, staining protocols can be used to correlate mass spectrometry data with histological features in the sections. Previous reports have described several different protocols for staining and subsequent correlation of the MS data with histology using serial sections [9], where one section is used for the MALDI analysis and adjacent section for staining to allow optimization of the preparation steps for each procedure. However, the disadvantage is that this requires the registration and correlation of two distinct non-identical sections. Another approach employs histological staining of a section prior to the MALDI IMS [10]. This minimizes the registration/correlation issue since both procedures use the same section, but is limited in practice because the stain can interfere with the subsequent MALDI MS analysis. For example the H&E stain commonly used in histology significantly interferes with the MS ionization process. A third sample preparation approach uses a single section to perform MALDI IMS analysis, and then is followed by histological staining. An initial report demonstrated excellent correlation between the histological and MALDI images [11], although it did not provide histological evaluation of the tissue prior to the MALDI measurement.

We report here further development of a protocol that performs MALDI IMS on a tissue section followed by elimination of the matrix by washing and subsequent normal histology staining and microscopic examination of the tissue. The major advance in this protocol is that it accomplishes MALDI IMS and HC on the same section at high spatial resolution (35 μm) for proteins. This protocol separates the processes of matrix coating from analyte

extraction through a recrystallization process. A key result of this protocol is that it limits delocalization of proteins to enable imaging at high spatial resolution.

This protocol employs a robotic spray apparatus for matrix application, providing a standardized spraying system that gives high reproducibility and a thin and homogeneous matrix coating. The device uses a combination of a solvent-matrix solution and heated inert gas, which are constantly flowing through a nozzle, resulting in a very fine matrix spray. This protocol is essentially a dry process where most of the solvent evaporates before the remaining matrix reaches the sample. In a second step, the extraction of proteins is then maximized by rehydrating/recrystallizing the matrix on the slides, similar to a method previously described [12]. The process is robust and reproducible and is amenable to high spatial resolution imaging.

The total sample preparation time is less than one hour following sectioning and the process can be multiplexed for high throughput operation. H&E staining after the MALDI measurement makes this protocol adaptive for many applications in histology/pathology, where a comparison of the MALDI images and histology is desirable. We have successfully used this protocol for the image analysis of tissue sections from brain, heart, kidney and lymphoid tissue.

Materials and Methods

Chemicals

70% and 100% ethyl alcohol HPLC grade (EtOH), acetic acid, acetonitrile HPLC grade and chloroform were obtained from Fisher Scientific (Fair Lawn, NJ, USA); trifluoroacetic acid 99.5% (TFA) from Acros Organics (Morris Plains, NJ, USA); sinapinic acid (SA) from Sigma-Aldrich Co. (St. Louis, MO, USA).

Experimental animals

Unstripped rat brains were purchased from Pel-Freez Biological (Rogers, AK, USA). The tissue was obtained from mixed gender Sprague-Dawley rats, 7–8 weeks old. The brains were collected fresh, meninges intact, frozen on dry ice within minutes of sacrifice and stored at -20°C ; individual tissue weight was approximately 1.5 grams.

Instrumentation

Tissue sections were cut on a Cryomicrotome (Leica CM3050S, Wetzlar, Germany) and frozen on Iridium Tin Oxide (ITO) coated glass slides ($25 \times 75 \times 1.1\text{mm}$; $30\text{--}60\Omega$) (Delta Technologies, Stillwater, MN, USA). The matrix was applied using the TM-Sprayer (HTX Technologies, Carrboro, NC, USA). For the rehydrating/recrystallizing, a stainless steel-polished blank MALDI sample plate (Applied Biosystems, Foster City, CA, USA), copper tape ($3''$ Core, $\frac{1}{2}''$ (W) $\times$ 54 ft (L)) (Electron Microscopy Sciences, Hatfield, PA, USA) and an oven (Thermolyne Oven Series 1297, Dubuque, IA, USA) were used. The mass spectrometry analysis was performed on a Bruker Autoflex Speed MALDI TOF MS (Bruker Daltonics, Billerica, MA, USA) in the linear positive ion mode. Images of the H&E stained sections were acquired with the Mirax slide scanner (Carl Zeiss MicroImaging GmbH, Gottingen, Germany).

Tissue sections

Tissue was stored at -80°C . The rat cerebellum was removed from the brain with a razor blade and attached to the sample holder with a small amount of NEG50 (Richard Allan Scientific, Kalamazoo, MI, USA) so that the cutting blade does not contact the NEG50. The tissue samples were sectioned at $10\text{ }\mu\text{m}$ at -22°C and transferred to chilled ITO glass slides

where they were thaw mounted onto the slides and allowed to dry for 30 minutes in a dessicator. Washing procedure: The tissue on the glass slides was washed in different solvents in Petri dishes following the protocol described below and allowed to dry in a desiccator for 15 minutes afterwards. The washing process involved sequential washing using the following protocol: 70% ethanol for 30 seconds, 100% ethanol for 30 seconds, Carnoy's fluid (ethanol: chloroform: acetic acid 6: 3: 1) for 2 minutes, 100% ethanol for 30 seconds, H₂O with 0.2% TFA acid for 30 seconds, 100% ethanol for 30 seconds.

Matrix deposition and rehydration

The matrix solution containing 5mg/ml SA in 90% ACN, 9.7% purified water, 0.3% TFA was sonicated for 3 minutes. The complete loop of the sprayer(5 ml) was flushed with the matrix solution and then applied to the sample plate. For a relatively dry spray a block temperature of 75 C was chosen. The stage of the spraying device was moved at a speed of 400 mm/min. The spray pattern was adjusted with a line spacing of 3 mm and 8 passes were performed applying a homogenous thin film of approximately $0.156 \text{ mg/cm}^2 \pm 0.0046 \text{ mg/cm}^2$ (n=8) matrix on the tissue. Between each pass, the spray pattern was changed by a 90° angle. The spray pattern of the passes 3, 4, 7 and 8 was shifted in whole due to a 1.5 mm to provide an offset pattern to achieve a more homogeneous matrix coverage. The matrix flow rate of the pump was 100 µl/min and the air pressure was set to 10 psi. The nozzle to glass slide distance of the sprayer was set to 46 mm.

After matrix application, the section was fixed on the inside of the top of a Petri dish and preheated to 85°C in the oven for 2 minutes (for better heat conduction the steel plate was put between the top of the Petri dish and the glass slide and fixed with a magnet). One ml H₂O containing 50 µl of TFA was pipetted onto filter paper located in the bottom part of the Petri dish. The preheated top and the bottom part of the Petri dish were put together. This "rehydration chamber" was heated in an oven to 85°C for 4 minutes. Subsequently the top was removed from the Petri dish and the slide allowed to dry for another minute in the oven.

MALDI Imaging Mass Spectrometry

The matrix coated sections were loaded onto the MALDI MS instrument equipped with a Nd:YAG operating at 1 kHz repetition rate. Calibration was performed collecting 5,000 lasershots of spots containing standard (bovine insulin [*m/z* 5,734.6], equine cytochrome C [*m/z* 12,361.2], equine ampomyoglobin [*m/z* 16,952.5]) mixed with the solvent-matrix solution. Images were acquired using the FlexControl 3.3 software (Bruker Daltonics, Billerica, MA, USA). A raster with 35 µm resolution was used and aligned to an optical image of the analyzed section using the FlexImaging 2.1 software (Bruker Daltonics, Billerica, MA, USA). One hundred shots were collected per pixel, at an accelerating voltage of 19kV, a grid voltage of 80%, and a delay time of 340 ns. The acquired data were processed (baseline subtracted, normalized) using the Bruker FlexImaging 2.1 software.

H&E staining of the tissue

After the MALDI measurement the remaining matrix was washed of the tissue by gently shaking it in a Petri dish in 95% Ethanol for 20 s. A standard H&E protocol was used for staining afterwards (95% EtOH, 70% EtOH, H₂O for 30 s each, hematoxylin for 3 minutes, H₂O, 70% EtOH, 95% EtOH for 30 s, eosin for 1 minute, 95% and 100% EtOH for 30 s each, xylene for 2 minutes). The sections were covered with cover slips using Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI, USA) and allowed to dry. The histological preparations were scanned using the MIRAX SCAN instrument.

MS Images

Figure 1 shows an assortment of ion density maps of the cerebellum at different m/z values between 5.000 and 14.500. These proteins show a distribution in the cerebellum, which correlates with one or two of the three main parts of the cerebellum (white matter, gray matter, granule cells). Several hundred peaks were obtained in the average spectrum between 5 and 20 kDa.

Figure 2 details the distribution of three different proteins. Each signal is specific for one main structure of the cerebellum: grey matter [m/z 10,812], granule cells [m/z 11,349] and white matter [m/z 18,397]. An overlay of these images was created and compared to the image achieved from the histological staining. As the MS analysis and the histological staining were performed on the same tissue section the anticipated correlation and a high degree of spatial resolution in the MS image is demonstrated.

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Figure 1. IMS analyses of a rat cerebellum section 10 μm thick. **a:** Ion density maps obtained at different m/z values with a spatial resolution of 35 μm . **b:** Average mass spectrum from this tissue section.

Figure 2.

Comparison of the MALDI IMS analysis with the histological H&E stain. **a–c:** Protein markers for different regions of the cerebellum. **d:** Overlay of these proteins in a single image. **e:** Overlay of the MALDI IMS image and the H&E stain (WM: white matter, GM: gray matter, GC: granule cells) showing the registration correlation of these sections.