Essential tactics of tissue preparation and matrix nano-spotting for successful compound imaging mass spectrometry

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ARTICLE INFO

Keywords:
Tissue-imaging MALDI-MS
Microdispenser
Matrix spotting
Rat lung
Tiotropium
MALDI Orbitrap

ABSTRACT

The ultimate goal of MALDI-Imaging Mass Spectrometry (MALDI-IMS) is to achieve spatial localization of analytes in tissue sections down to individual tissue compartments or even at the level of a few cells. With compound tissue imaging, it is possible to track the transportation of an unlabelled, inhaled reference compound within lung tissue, through the application of MALDI-IMS. The procedure for isolation and preparation of lung tissues is found to be crucial in order to preserve the anatomy and structure of the pulmonary compartments.

To avoid delocalization of analytes within lung tissue compartments we have applied an in-house designed nano-spotter, based on a microdispenser mounted on an XY table, of which movement and spotting functionality were fully computer controlled. We demonstrate the usefulness of this platform in lung tissue sections isolated from rodent in vivo model, applied to compound tissue imaging as exemplified with the determination of the spatial distribution of \(\text{(1,2\beta,4\beta,7\beta)-7-[(hydroxidi-2-thienylacetyl)oxy]-9,9-dimethyl-3-oxa-9-azoniatriacyclo[3.3.1.0^{2,4}]nonane, also known as tiotropium. We provide details on tissue preparation protocols and sample spotting technology for successful identification of drug in mouse lung tissue by using MALDI-Orbitrap instrumentation.}

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1. Introduction

Matrix assisted laser desorption/ionization tissue-imaging mass spectrometry (MALDI-IMS) is an emerging technology among the numerous applications of MALDI-MS, which possesses the ability to show spatial and temporal distribution of endogenous and administrated compounds in a tissue section [1]. To obtain high resolution images of analytes the tissue sections are sampled along a predetermined two-dimensional array collecting full mass spectrum at each measuring point [2]. Selective representation of an m/z value over the tissue surface in the form of two-dimensional intensity maps could provide details of compound localizations and their metabolic processing [3-5] or analytes with

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doi:10.1016/j.jprot.2010.02.021
clinical diagnostic value [6,7]. This approach has been demonstrated first for peptides and proteins applying the matrix directly on a C18-coated membrane holding the blotted molecular image of the tissue section [1], which was changed to place the tissue sections directly on the target plate and covered with matrix [8].

Apart from the applications of MALDI-IMS on peptide and protein analyses, the direct mapping of low-molecular-weight compounds has gained much attention [3,4,9–12], holding the promise to become a standard technology in the pharmaceutical industry. The knowledge about whether the drug can reach the site of action in the target organ has an outmost value at an early stage, during drug development. Accordingly, some reports have been focused on localization of drug molecules in rat brain [12] or human ovarian tumor tissues [13], using both MALDI-IMS and MALDI-MS, respectively. However, present methods for compound imaging in tissue, including autoradiography (e.g., whole body autoradiography) and fluorescence spectroscopy, require laborious and costly synthesis and labeling of a radioactive isotope or fluorophore tag, respectively. Such chemical modifications may not only severely alter the pharmacological properties of drug molecules but these approaches are limited to report on the labeling tag, making it difficult to differentiate between the intact compounds and their metabolites [12]. However, detecting the fragment ions of these compounds can lead to their unambiguous identification, which is especially powerful in combination with cross-validation by other molecular imaging techniques [10].

A lot of effort is focused on how to achieve homogenous matrix coating on tissue surfaces, since this is essential to obtain highly localized information of the analytes. In principle, the matrix solution can be applied on tissue sections by spraying or spotting. To maximize solubilization of analytes in the tissue section, facilitating their co-crystallization with matrix compounds while preserving their localization, many research groups have used spray nebulizers [14,15] depositing the matrix directly onto the tissue sections. Consequently, several sprayer systems have been reported as commercial products [16]. Alternatively, the spotting technologies offer the possibility to deposit matrix onto tissue sections in minuscule volumes (80–150 pL), resulting in crystallization on circular areas in the size of 100–200 μm. The accurate deposition of minute volumes of matrix solutions can be achieved by piezo-based dispensers, similar to the ones used in inject printers [17], or utilizing acoustic energy to eject matrix droplets directly from a fluid interface in a reservoir [18].

We have designed an in-house microdispenser platform that is built on a piezo-actuated microchip that allows the use of a wide range of organic solvents. The dispenser deposits approximately 100 pl droplets and the built-in software allows elaborate matrix deposition protocols to be performed. The importance of an accurate matrix deposition strategy and the influence of the substrate conditions for good MS signal generation are outlined. Demonstration of this compound tissue-imaging workflow is presented by employing MALDI LTQ XL Orbitrap instrumenta-

2. Material and methods

2.1. Chemicals

The matrix compounds, 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma-Aldrich (Steinheim, Germany) and Bruker Daltonics (Bremen, Germany), respectively. Methanol (MeOH) at HPLC grade (99.8+%) and trifluoroacetic acid (TFA) at Reagent-Plus® (99%) were from Sigma-Aldrich (Steinheim, Germany); whereas acetonitrile (AcN) at hypergrade for LC-MS and isopropanol (IP) at spectrophotometric grade (99+%) were from Merck (Darmstadt, Germany) and Acros Organics (Geel, Belgium), respectively.

2.2. Tissue preparation

The study was conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and was approved by the local animal ethics committee. Rats (n=5, male, Wistar, Taconic, Denmark), were 12 weeks old and weighed about 350 g at the start of dosing. Standard procedures and conditions were applied for animal care, feeding and maintenance of the room, caging and environment. The animals were administered tiotropium [(1α,2β,4α,7β)-7-[(hydroxidi-2-thienylacetyl)oxy]-9,9-dimethyl-3-oxa-9-azoniatriocyclo[3.3.1.0^2,4]nonane] via inhalation of an aerosol. The animals were sacrificed after 15 min after the administration of tiotropium. The lungs were rapidly dissected and slowly frozen by placement for 2 min on a plastic boat floating in a bath of isopentane that was supercooled with dry ice (−70 °C). The lungs were stored at −20 °C until sectioning. Cryostat thin slices of 10-μm thickness were prepared without embedding in OCT to reduce background contamination. A small amount of OCT was used to attach the lung to the cryostat holder. The matrix solution, either 20 mg/mL 2,5-dihydroxybenzoic acid (DHB) or 4–5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in 50% MeOH/0.1% TFA or 50% ACN/0.1% TFA, was deposited in several layers (cycles) by the ImagePrep instrument (Bruker Daltonics, Bremen, Germany), applying a spraying/drying cycle of 2 s spraying, 10 s incubation time followed by a drying period of 90 s; or the in-house designed microdispenser-based nano-spotter [19]. A Portrait® 630 (Labcyte Inc., Sunnyvale, CA) acoustic spotter instrument [18] was also employed to prepare matrix arrays on lung tissue sections, depositing 10 mg/mL CHCA in 50% ACN/0.1% TFA.

2.3. Matrix spotting platform

A microchip-based piezo-actuated dispenser was previously developed by our group [19], utilizing piezo-controlled liquid delivery that is principally similar to the commercial inkjet...
printers. The microdispenser is designed with a flow-through channel, which enables on-line filling of the matrix solution. To entirely cover the surface of lung tissue sections, a total volume of ca. 11 µL is needed (calculating with 1 cm² area = 1100 matrix positions in case of 300 µm pitch, 5 droplets/position and 20 repeats of the total area spotted). In the current application of MALDI matrix spotting, the dispenser was modified by affixing a 200 µL pipette tip as a matrix reservoir to one end of the microchip channel, enabling continuous operation during several hours of matrix spotting.

The matrix-spotting dispenser was then mounted on a fixed position holder 1 mm above the target plate, which was positioned on an XY table operated by high precision stepping motors. The microdispenser, the XY table and the signal generators were synchronously manipulated by an in-house developed software. The microdispenser was set to deposit matrix droplets at a rate of 50 Hz. The signal generators operated with a piezo actuation signal of 100 µs pulse duration, amplitude of 16–17 V, around 5 µs rise time and 150 µs fall time, depending on the composition of matrix solutions.

2.4. Fluorescence microscopy

Images of tissue and matrix deposition on tissue were acquired using a Leica DMX/RA microscope equipped with Nomarski differential interference contrast (DIC) optics. A variety of fluorescent excitation/filter combinations were employed between the range of 340–570 nm.

2.5. Mass spectrometry

A MALDI LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA) was used, operating the FT analyzer at 60,000 resolution (determined at m/z 400) in the 100–1,000 Da mass range for full mass scans in positive mode. For MS/MS data collection, the ion trap was utilized to fragmentize the parent ion of tiotropium (m/z 392.097) at normal scan rate, isolating the ions in m/z 3.0 width. Normalized collision energy was 50% during an activation time of 30 ms; and activation Q of 0.250 was applied with wideband activation. The minimal signal required for MS/MS was 500 counts. The laser energy was 10.0 µJ for 2 sweep laser shots (where no spectra collection was made) followed by 4 additional microscans for the collection of spectra. The visualization of parent and fragment ions of tiotropium was performed with the ImageQuest™ software (Thermo Scientific, Waltham, MA).

3. Results

3.1. Tissue preparation

Alteration in the tissue homogeneity is a challenge that is inherent in several organs when analyzed by compound tissue imaging. We have experienced that it is of particular importance in the pulmonary tissue compartments conducting respiration, since a sizable proportion of the tissue area is parenchymal alveolar bed and thus prone to be more easily disrupted during sectioning than stronger structural elements, such as vessels and bronchioles. As the resected lung tissue held a large surface area of air sacs, the preparation protocols developed for the lung tissue were found to be crucial. In order to preserve the three-dimensional structure of the organ upon surgical isolation, the following steps were developed to ensure high quality tissue sectioning: i) sufficient of the airways before organ removal; ii) freezing of the resected organ in a bath of super-cooled isopentane in dry ice to −80 °C in, over a 2-min period to allow preservation without water crystal formation; and iii) cryostat sectioning onto glass slides. In particular, it is important to develop protocols that allow sectioning without embedding in media support, such as the OCT polymer.

Upon drug compound quantification, regional distributions of cells and histological pulmonary compartments at different levels of the organ need to be accommodated by sampling throughout the 3-D volume of this heterogeneous organ. The multiple sections/slides from the same organ allow statistical comparisons within and between individual test animals to determine population variance.

Serial sectioning of the organ offers various opportunities for comparing and differentially staining adjacent tissue structures. We routinely include standard histopathology analyses on slides sampled for mass identification. These stained slides provide the pathology/histology context needed for interpreting possible biological connotation to the mass distributions achieved in a scan.

Fig. 1 illustrates the critical steps of the tissue preparation, whereby the cryostat sectioning is optimized to give correctly oriented lung tissue sections. We have cut along the frontal plane of the left lobe. It is also essential to cut from side to side to get full sections of the central airways as shown in Fig 1A. In addition, the current preparation protocol was found to be fully compatible with histology staining following the matrix spotting and compound tissue-imaging analysis. This latter development makes the histology-compound compartment localization investigation extremely powerful.

3.2. Matrix deposition

One of the key factors for success in tissue imaging is the strategy of matrix deposition, resulting in qualitative crystal formation on the surface of tissue sections. Owing to its simplicity, the most commonly used method for matrix deposition is spraying the matrix solutions by various tools [15,20,21], including TLC spray, airbrush or dedicated instruments (e.g., ImagePrep from Bruker Daltonics, Bremen, Germany). Although, the crystallization of matrices can be controlled by step-wise deposition interrupted with drying phases and the final thickness of the matrix coverage can be determined by measuring optical density through the glass plate at off-tissue position, the matrix crystal formation is far from homogenous, as it is often considered [15,22]. Investigating lung tissue sections fixed onto conductive ITO plates and covered with 5 mg/mL CHCA matrix by using the ImagePrep instrument programmed to achieve sufficient matrix thickness, we observed rather heterogeneous crystalization (Fig. 2). Most likely, the solvent droplets were large enough to flow down into “valleys” and accumulate in these lower parts of the tissue, resulting in “sweet spots” for MALDI-
MS measurements. Whereas the highest parts of tissue were seldom covered with matrix crystals thus these “tops” gave no or very low signal intensities (data not shown). Solvent droplets could only be randomly sprayed over the tissue section, accumulating at some positions but missing others as it is shown in Fig. 2C. An optional interpretation to the regional matrix accumulation is that when the solvent is simultaneously sprayed over the entire tissue section the fluid is accumulated in the regions where the wetting conditions are optimal with respect to hydrophobic or hydrophilic conditions. It should also be noted that micro- and nano-topography could also vastly influence the wetting conditions.

3.3. Nano-spotting platform

To improve crystallization of matrix, we employed our microchip piezo-actuated spotting technology, which was originally developed for transferring liquid chromatographic separations onto MALDI target plates [19,23]. However, in the present study, we modified the microdispenser platform for high-density spotting onto tissue sections in rectangular array formats. Due to the size of the nozzle of the microdispenser, the deposited volumes were kept at 100 pL/droplet, which in turn was one of the determinants that control the size of the matrix spot on the surface, along with the solvent/surface compatibility. Since the amount of matrix in a single droplet is insufficient for obtaining MS signals of analytes from tissue, the matrix solutions were applied as multiple droplets at a given position and also with multiple cycle-runs over the same array.

Additionally, the highest applicable concentration of a matrix compound was chosen to achieve crystallization within the shortest preparation time. Since DHB is highly water-soluble it could be used at high concentration (20 mg/mL in 50% MeOH). CHCA, on the other hand was found to be limited to saturation levels around 5 mg/mL. In order to
overcome clogging in the nozzle of the microdispenser, an addition of isopropanol (IP) to the matrix solution was used to circumvent crystal formation of CHCA. The inclusion of an additional organic modifier allowed continuous deposition over a 1 cm² (tissue) surface for at least 40 cycles, which corresponds to about 2 h of operational matrix deposition.

We optimized the experimental conditions such that our microdispenser could operate with 4–5 mg/mL CHCA dissolved in 50% AcN/5–10% IP depositing 5 droplets/position at 50 Hz stable droplet frequency. The resulting crystal spots on both stainless steel and liver tissue surfaces were about 200 µm (see Fig. 3). It was noted that the crystal formation on both liver and lung tissue appeared significantly different compared to that on stainless steel surfaces, providing larger matrix crystals, with somewhat poorer crystal coverage in general, as shown in Fig. 3B and C.

Obviously, the consecutive application of solvent droplets on predominantly hydrophilic tissue surfaces gives rise to compound extraction (followed by their co-crystallization with matrix) as postulated in general for MALDI-IMS [13,20]. However, the auto-fluorescence read outs of the matrix compounds revealed that free diffusion into the tissue in a distance, which is relatively far away from its original application area (shown by the arrow in Fig. 4B), is possible. We have also observed that the wetting conditions are substantially changed as the matrix array transits from the glass surface to the tissue sample. The tissue displays better wetting properties to the matrix solution and hence a larger crystal spot is seen. This is also reflected in the “doughnut” shaped crystals being formed on the tissue, whereas a smaller and more confined crystal spot is obtained on the glass surface (see Fig. 4). The “doughnut” shape is a well-known analyte concentration effect for droplet samples that dry on a surface with a low contact angle (good wetting) [24]. It should be commented that spraying of matrix might give rise to a larger extent of lateral diffusion of the compound within the tissue. These observations may consequently be the reason of impaired crystallization resulting in alteration of the local concentrations of compounds of interest at a given position.

In most cases, CHCA provides higher mass signals compared to DHB, both matrices were tested with our matrix array platform. Reproducible spot formation (about 200 µm in size) and homogeneous crystallization was achieved with CHCA, resulting in elevated tiotropium signals from lung tissue. Due to the fact that the matrix solution was spotted onto tissue sections in an array format, confined matrix crystallization within small areas was achieved. We were thereby able to successfully localize tiotropium in microenvironments within the lung tissue. The tiotropium signal was found to be widely distributed throughout the alveolar bed.

A simple comparison between the nano-spotter and an acoustic spotter (Portrait 630) and a sprayer (ImagePrep) was conducted in order to estimate the performance of our matrix deposition instrument (see Table 1 for some parameters). It was found that the signal intensities of the test analyte
(tiotropium at m/z 392.1) were moderate, achieved by our nano-spotter. In accordance, the noise was also measured to be somewhat higher; whereas the mass spectra appeared to be less busy compared with the results obtained by the other matrix deposition instruments (Fig. 5). Although, spraying the matrix solution provided higher absolute signals of tiotropium, it was clearly due to the presence of some "sweet spots", reflected in the very high variation values (see RSD values in Table 1). The resolution was about the same in all cases.

A MALDI LTQ Orbitrap XL mass spectrometer was used to collect information on the spatial distribution of tiotropium in rat lung tissue sections. The optimized lung tissue preparation protocol and process, along with the microchip piezo-actuated matrix dispensing system were the mandatory developments that made it possible to confirm the qualitative identity of tiotropium with corresponding signal responses, presented in Fig. 6. The tiotropium occurrence and localization found within the peripheral tracts of the lung were also clearly identified within the tissue compartment (see Fig. 6).

4. Discussion

Although, tissue sample preparation is regarded as the most critical part of the tissue-imaging mass spectrometry workflow, this has only recently been addressed more thoroughly [25,26] and summarized in a review [27]. Yet, most of the efforts have been centered on the preservation of peptide and protein localization within the brain, and to a less extent applied to other tissues. The limited reports on lung tissue for MALDI-IMS have been aimed at the determination of peptides/proteins in formalin-fixed and paraffin-embedded samples [28]. An important aspect of tissue preparation is to employ histology-image compatible methods [29] to make it possible to compare the MALDI-IMS readouts with high resolution image morphology of the same tissue slice. We hereby report on an optimal practice that allows high preservation of tissue morphology in pulmonary compartments, known for its cellular heterogeneity, when prepared for compound tissue imaging.

Generally, the choice of matrix depends on the chemical nature and characteristics of the analyte of interest and should be experimentally outlined and optimized. Our results indicate that the crystallization properties are also extremely important and may be significantly different off- and on-tissue, yielding signal intensities that are often 10-fold lower in the latter case. The reason for these findings are still not completely clear from our investigations, as ion suppression, may only partly be an explanation. Probably, insufficient extraction of analytes from the 3D structure of the tissue makes the drug compounds less available for matrix co-crystallization. Another reason could be that the physical/chemical properties of a tissue section will vary within the lung morphology in very short distances.

Since spraying of matrix solution was proved to cover tissue surfaces heterogeneously, resulting in uneven crystal distribution (Fig. 2), the nano-spotter techniques are recommended to deposit matrix in discrete spots. Even within these spots matrix crystals could not homogeneously cover the tissue using matrix compounds like DHB (Fig. 4). The crystals of DHB covered the glass surface completely and formed a thick layer, providing smaller spot sizes compared to the spots on lung tissue, where crystals built up usually around the spot’s edge, along the interface between the dry and wet areas. This phenomenon always occurred as soon as the matrix droplet is in contact with the tissue (see the spot in the middle of Fig. 4D) that absorbed an aliquot of solvent.

Long-term operation of continuous spotting could be achieved with our microdispenser platform using concentration levels and addition of isopropanol to the matrix solution, which would prevent crystal formation in the nozzle. Additionally, we have observed that clogging occurred more often when the microdispenser was not depositing continuously, i.e., in a single droplet/position mode when moving from a position to the next one. These limitations in spotting operations occur with longer periods of “dwell time” while the robot is moving in between positions. After careful investigation we found that a rapid deposition of a droplet sequence (4–5 droplets) is more advantageous than a single-droplet deposition, yet resulting in very similar spot areas. Moreover, the number of cycles required to obtain sufficient matrix thickness could be decreased, as well as the overall preparation time accordingly.

Direct comparison of three matrix deposition instruments proved that our nano-spotter performed similarly to the commercial Portrait® 630, and outperformed the ImagePrep sprayer in terms of more homogeneous signals and less chemical noise (Table 1). It is worth mentioning that the lower absolute signal intensities of tiotropium may only be due to the lower matrix concentration applied by our nano-spotter (and thus total amount of CHCA/spot).

Furthermore, ionic matrices may also be utilized for dispensing in tissue imaging [30] as they offer several

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<th>Table 1 - Comparison of the performance of two principally different spotters and a sprayer used for CHCA deposition in imaging mass spectrometry.</th>
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a) Calculated by the Xcalibur QualBrowser.
Fig. 5 – Optical and spectral comparison of three different lung tissue sections covered with matrix by (A) our nano-spotter (4.5 mg/mL CHCA in 50% AcN/10% IP/0.1% TFA), (B) Labcyte’s acoustic spotter (10 mg/mL CHCA in 50% AcN/0.1% TFA) or (C) ImagePrep sprayer (5 mg/mL CHCA in 50% AcN/0.1% TFA). The full mass spectra were collected from different parts of each tissue section and represent four averaged measurements at adjacent positions. The arrows indicate the tiotropium signals.
advantages over the conventional matrices in terms of homogeneity and high stability in vacuum [31]. It has also been reported that signal intensities were higher, whereas chemical noise was lower [30]. In a forthcoming work we will investigate some of the DHB- and CHCA-based ionic matrices (e.g., CHCA/aniline) in combination with our microdispenser platform on tissue sections.

5. Conclusions

Lung organ isolation with anatomic preservation, and an absolute three-dimensional, morphological structure are pre-requisites in order to be able to make correct microenvironmental localization of drug molecules by IMS. The protocols herein, are of particular importance and value, due to the fact that the pulmonary structure is highly porous, heterogeneous, and mostly comprises air in its natural state.

Spotting platforms in comparison with sprayers may be more advantageously utilized for matrix deposition in MALDI-IMS experiments as they allow accurate deposition of minute volumes repeatedly, building up the matrix layers required. Our experimental data indicates that the crystal structure and formations on tissue are linked to the final signal generated. In order to obtain optimal signals of a low-molecular-weight drug, a set of experiments has to be performed. Our in-house designed microdispenser allows the deposition of matrices in replicate runs according to the protocols that we found to be optimal for the drug compound investigated. To our knowledge, these features are first described in this successfully integrated MALDI-IMS workflow, providing proof-of-concept data from mouse lung tissue dosed with the cholinergic drug, tiotropium.

We believe that this approach will be the standard in the future and offers never before achieved understandings of the molecular distribution of specific ions in true biological context.

Acknowledgements

The authors are grateful for Martin Hornshaw and Egon Rosén at Thermo Fisher Scientific for mass spectrometry support and for the Swedish Research Council for funding.
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