MALDI Mass Spectrometry Imaging Reveals Distinct Spatio-Molecular Phospholipid Distributions in Mouse Lungs

While phospholipids play key roles in lung physiology and pathology, the complex composition and function of the lipidome in the lungs is poorly understood. We applied MALDI mass spectrometry imaging to characterize the spatial distribution of phospholipid molecules in mouse lungs.

Using a combination of MALDI Imaging and on-tissue MS/MS in negative ion mode, we imaged and identified phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidic acid (PA) species that were distinctly distributed in spatial subregions of normal mouse lungs. The obtained results will be useful for future studies that seek to map lipid alterations in pulmonary disease, including tuberculosis, asthma, pulmonary fibrosis, lung cancer, and metastatic spread to the lungs, among others.

Introduction

Phospholipids play a critical role in structure and function of lung tissue; however, the spatial distributions of these lipids are not well characterized. Lungs are a common site of disease,

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including cystic fibrosis, cancer, cancer metastasis, asthma, and infectious diseases. To understand how diseases affect phospholipids within lung tissue, normal lung phospholipid distributions must first be determined. To this end, we set out to develop a method to remove mouse lungs while maintaining the three-dimensional structure of the lungs in a way that is compatible with MALDI mass spectrometry imaging (MSI), which can be used in future studies of lung disease. Furthermore, we explored a range of MALDI Imaging applications including positive and negative ionization as well as tandem MS imaging to measure the spatial distributions of phospholipids in lung tissue in a well established mouse model.

Materials and Methods

All animal experiments were approved by the Johns Hopkins Institutional Animal Care and Use Committee and in compliance with the Animal Welfare Act regulations and Public Health Service (PHS) Policy. Normal female athymic nude mice were sacrificed by CO_2 inhalation. During necropsy, lungs were inflated by cutting a small hole in the trachea as recently published [1]. Polyethylene tubing (inner diameter of 0.75 mm) was inserted into the trachea and attached to a 5 mL syringe. The lungs were inflated with 2% gelatin in water (w/v) through the syringe. The trachea was tied off and the lungs were removed and placed in a 20x20x25 cm^3 mold (Sigma) with 2% gelatin. The gelatin was allowed to solidify at -20˚C for approximately 15 to 20 minutes. The mold was then flash frozen in liquid nitrogen and stored at -80˚C until sectioning. Indium tin oxide (ITO) coated slides were washed and sonicated with hexanes (2 x 10 mins) and ethanol (2 x 10 mins). Slides were dried overnight in a desiccator. Frozen lungs were equilibrated to -20˚C prior to sectioning. Lungs inflated and embedded in 2% gelatin were sectioned at 10 micron thickness at -10˚C onto cleaned ITO slides. Lung sections for positive ion mode imaging were sprayed with 2,5-dihydroxybenzoic acid (DHB). Lung sections for negative ion mode imaging were sprayed with 1,5-diaminonaphtalene (1,5-DAN). All matrices were sprayed using an HTX TM sprayer. MALDI Imaging was performed on a Bruker rapifleX MALDI TOF/TOF instrument in either positive or negative reflectron mode. Positive ion images were acquired with 20 micron raster size and 500 laser shots per pixel. Negative ion images were acquired with 50 micron raster size and 200 laser shots per pixel. MS/MS images were acquired in negative reflectron mode. MALDI images were generated using flexImaging and statistical analysis was performed with SCiLS Lab software. Following MALDI Imaging, all lung tissues were washed in 70% and 100% ethanol for matrix removal, followed by hematoxylin and eosin (H&E) staining and slide scanning to visualize morphological tissue features.

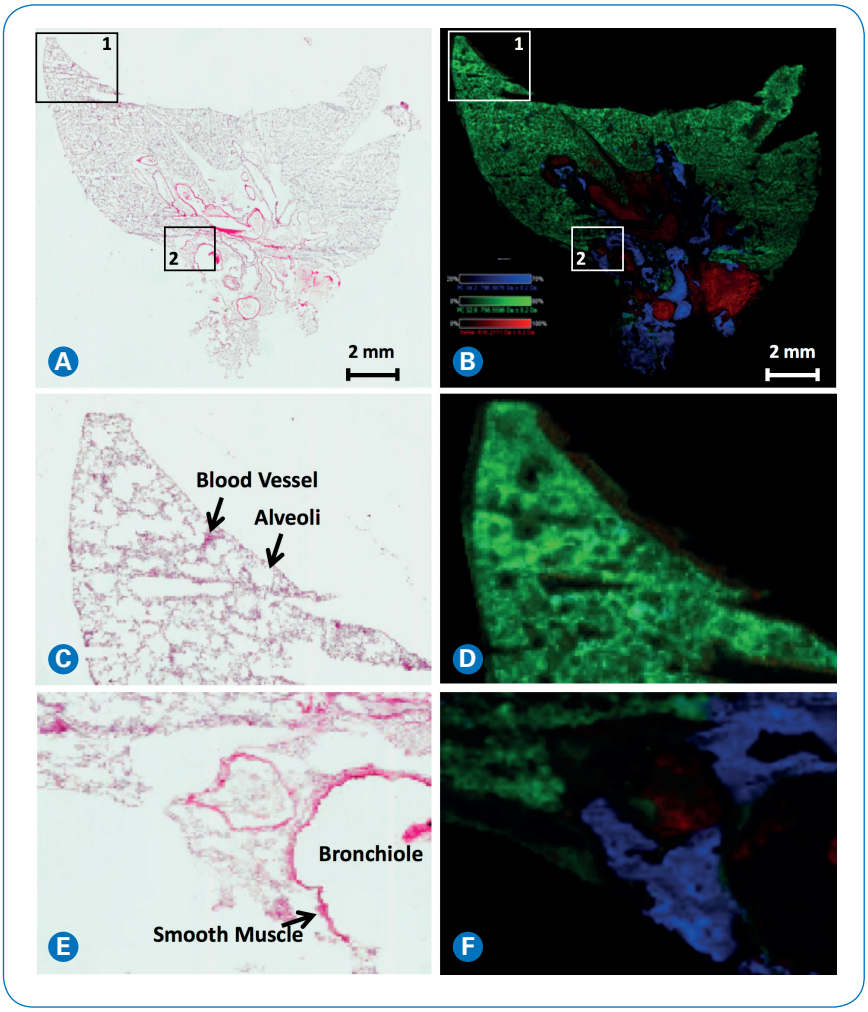

Figure 1: Lipid imaging of inflated mouse lungs in positive ion mode. Images show (A, C, E) hematoxylin-eosin stain following MALDI Imaging for morphologic referencing, (B, D, F) MALDI Imaging obtained on Bruker rapifleX instrument. (A, B) show the full lung image, (C, D) show the upper inset 1, and (E, F) show the lower inset 2. Color-coding in all MALDI images is as follows. Green: m/z 756.6, phosphatidylcholine (PC)(32:0), $[M+Na]^+$. Blue: m/z 796.5, PC(34:2), $[M+K]^+$. Red: m/z 616.2, heme, $[M+H]^+$. Pixel size of 20 µm, acquisition time of 3 hours 41 minutes, 239,434 pixels.

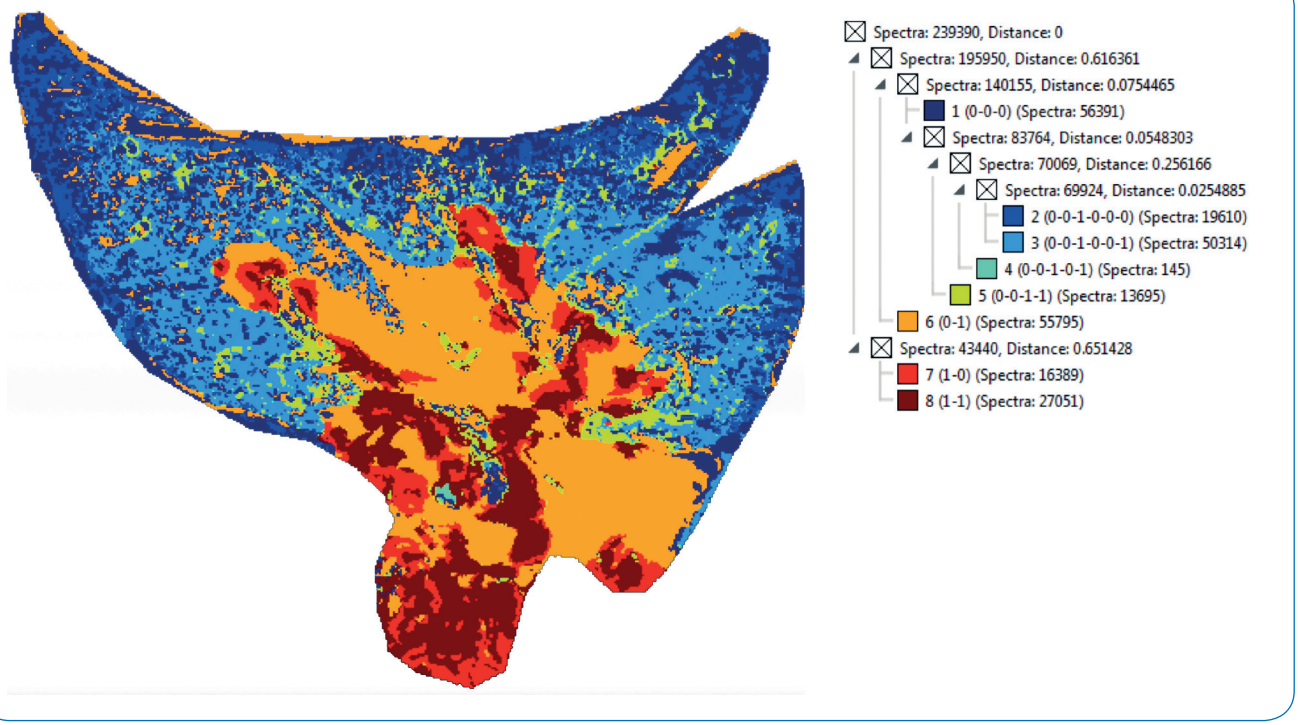

Figure 2: Tissue segmentation analysis of mouse lungs imaged in positive ion mode. Using the automated spatial segmentation feature of the SCiLS Lab software, prominent features were annotated in the obtained MALDI Imaging data. The same MALDI Imaging data from gelatin-inflated mouse lungs as in Figure 1 is shown here following automated tissue segmentation analysis, highlighting several distinct spatial features, which are extracted from characteristic spectral features.

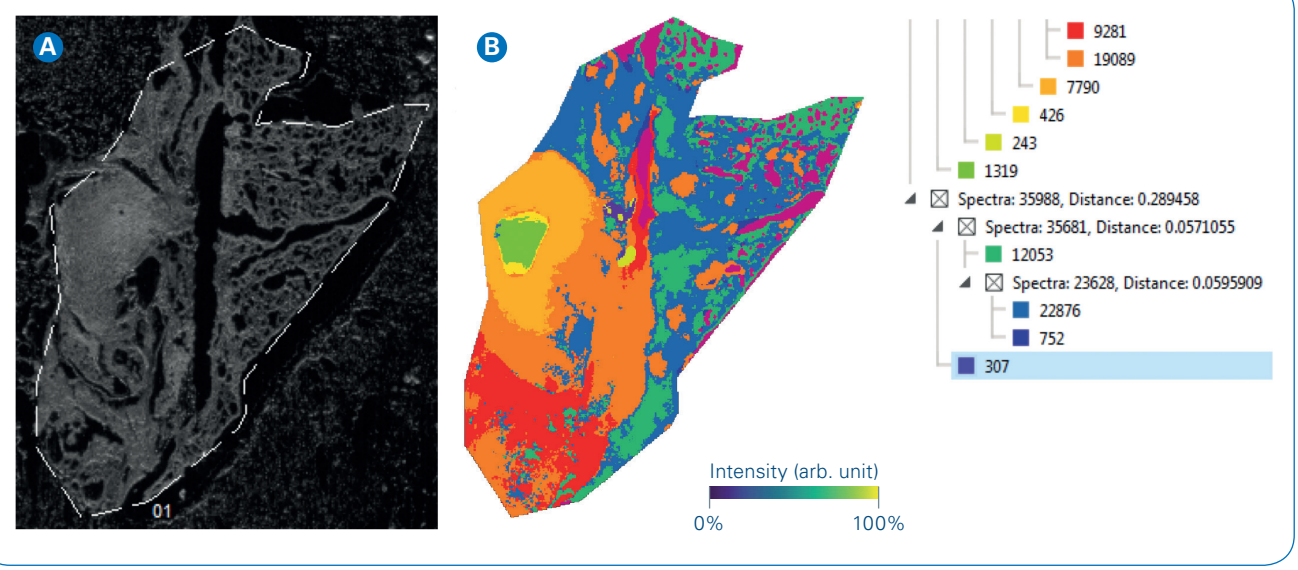

Figure 3: Lipid imaging of inflated mouse lungs in negative ion mode. Images show (A) optical image of inflated lung tissue and (B) segmentation map and hierarchical clustering obtained in SCiLS Lab. Unsupervised segmentation analysis revealed several distinct lung regions with specific lipid compositions, which included muscle tissue, trachea, bronchioles of various sizes, alveoli, connective tissue, and small blood vessels. Pixel size of 50 µm, acquisition time of 37 minutes, 79,425 pixels.

Results and Discussion

Positive Ion Imaging

Positive ion lung images revealed unique distributions of phospholipids, based on different structures within the lung. The MALDI images in Figure 1 show that *m/z* 756.6, identified as sodiated phosphatidylcholine (PC)(32:0), surrounds alveoli in the lobes of the lungs (Figure 1C, D, shown in green). In the more central part of the lungs, *m/z* 796.5, assigned to potassiated PC(34:2) was more abundant, surrounding bronchioles and the trachea (Figure 1E, F, shown in blue). Heme at *m/z* 616.2 was visible in the central part of the lungs (Figure 1E, F, shown in red). These data demonstrate that different phosphatidylcholines and heme outline unique morphological structures in the lungs. These phophatidylcholine distributions co-localize with structures visible in the H&E images showing little to no delocalization.

The positive ion lung image was imported into SCiLS Lab, and segmentation analysis was performed (Figure 2). The segmentation was able to differentiate prominent anatomical lung structures, which were also observed in the H&E image. The central area of the lungs contained relatively uniformly segmented lipid components, while the lobes of the lungs were segmented into finely differentiated anatomical structures composed of different phospholipids. The bronchioles and trachea were the first structures to be segmented. The lobes of the lungs were segmented into areas containing alveoli and blood vessels, which were composed of different phospholipid distributions.

Negative Ion Imaging and MS/MS Experiments

Depending on their molecular composition, specific classes of phospholipids ionize only in negative reflectron mode. Negative ion images were also

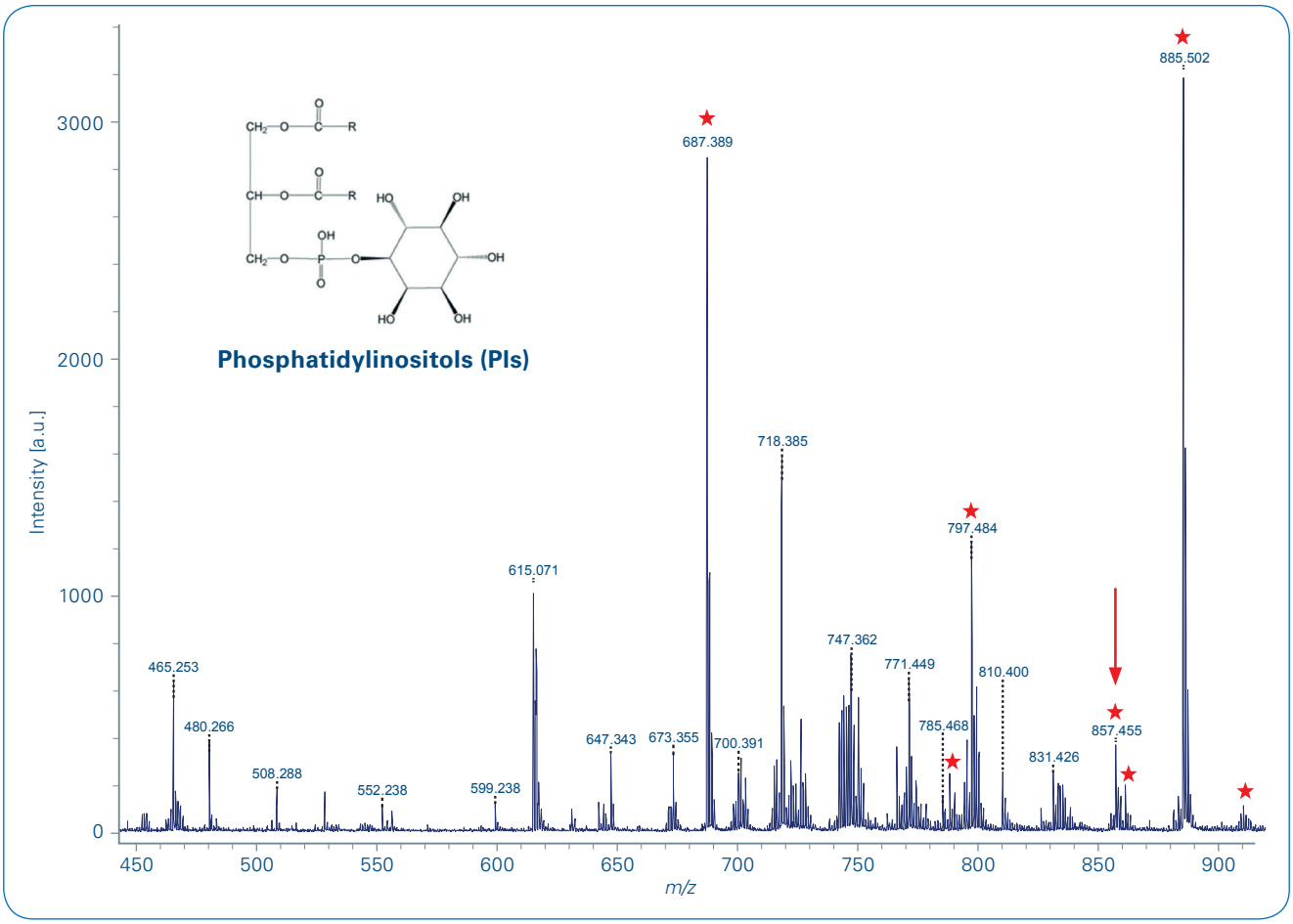

Figure 4: Overall negative ion lipid spectrum from lung tissue. Several high (m/z 885.5, m/z 687.5), intermediate (m/z 797.5, m/z 857.5), and low (m/z 909.5, m/z 861.5, m/z 797.5) abundance m/z signals, as indicated by red asterisks, were analyzed in MS/MS mode. Inset shows the overall structure of phosphatidylinositols (PIs). The red arrow points out m/z 857.5, which was analyzed by MS/MS imaging.

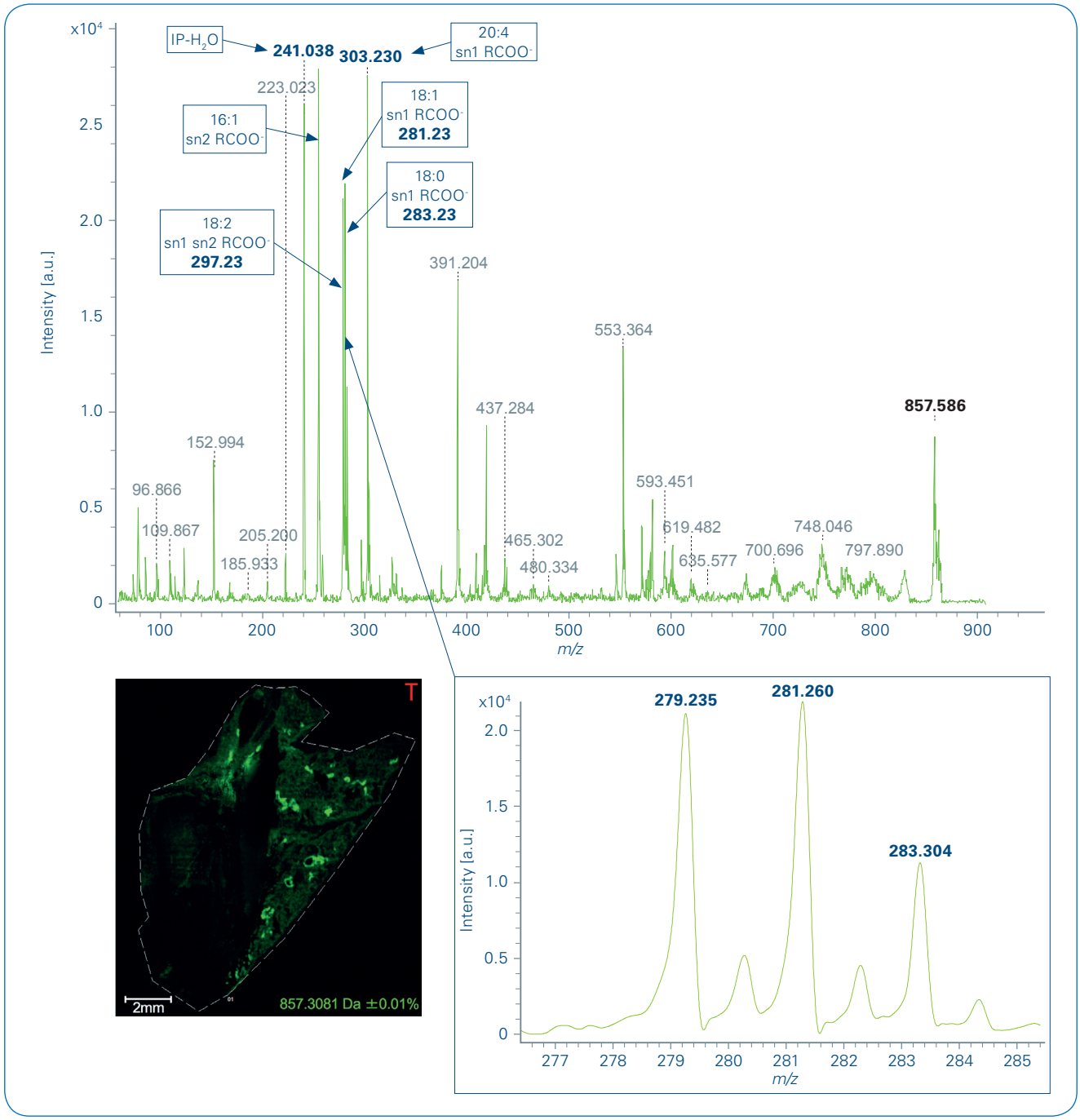

Figure 5: The parent ion m/z *857.6 was selected for MS/MS imaging on an adjacent section to determine identity and spatial distribution of all four phosphatidylinositol (PI) species. The inset shows the image of* m/z *857.6.*

acquired, and the data was used for unsupervised segmentation in SCiLs Lab (Figure 3). Different distributions of phospholipids were observed in specific anatomical structures, including muscle tissue from the heart or diaphragm, bronchiole, and alveoli. Notably, the muscle tissue was one of the first tissues to be revealed in the segmentation analysis, followed by the lobes of the lungs, showing unique phospholipid distributions in different types of tissue (i.e. muscle vs. lung), as well as specific localization of different phospholipids to different parts of the lung.

Several *m/z* signals were selected from the overall negative ion spectra for MS/MS experiments (Figure 4, red asterisks). MS/MS images revealed a number of peaks, which were analyzed in both flexImaging and using the glycerophospholipid precursor/product

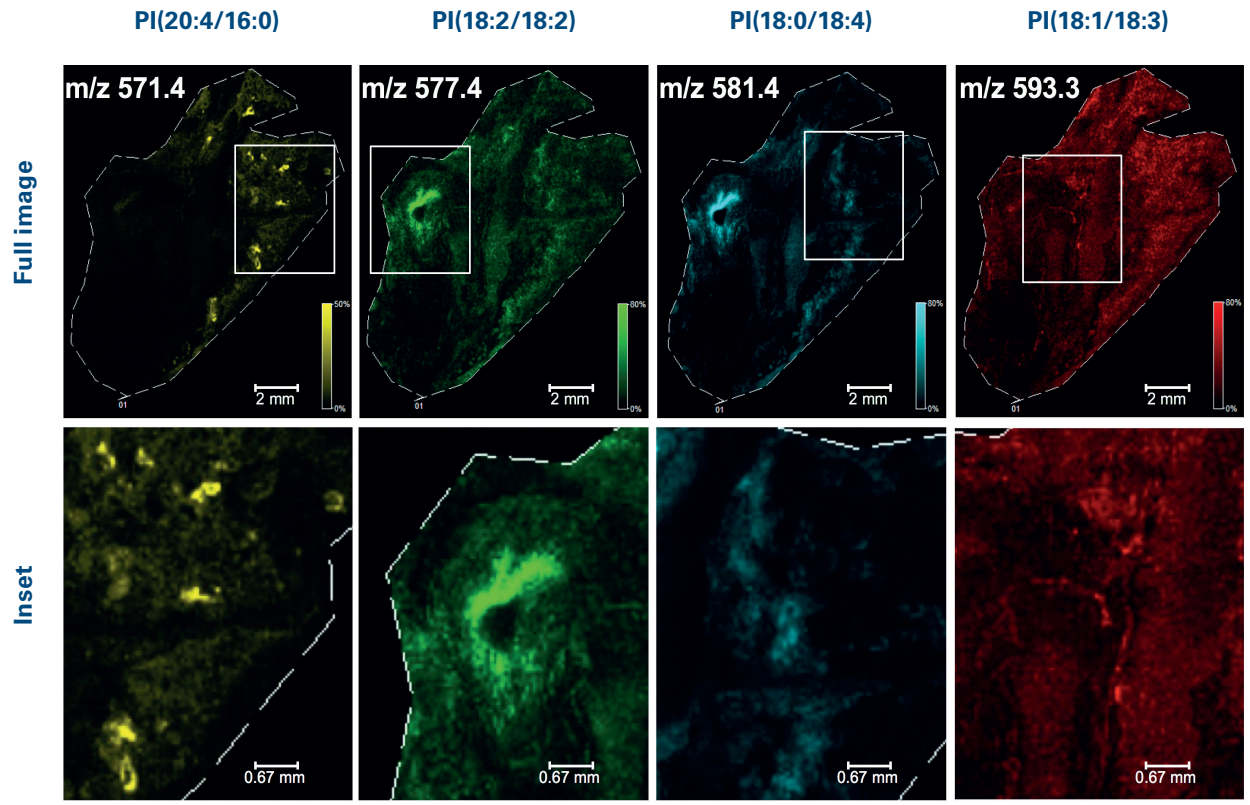

Figure 6: Selected fragment ion images obtained by MS/MS imaging of the parent ion m/z *857.6, highlighting unique fragmentation patterns of different lipid species that are constituting the parent ion of* m/z *857.6. Insets are enlarged by a factor of 3.* M/z *571.4, shown in yellow, corresponds to the loss of the sn1 acyl chain as (RCH=C=O) from [M-H]⁻ of PI(20:4/16:0), localizing to the periphery of the lung.* M/z *577.4, shown in green, corresponds to the neutral loss of the sn2/sn1 RCOOH group from [M-H]⁻ of PI(18:2/18:2), localized to muscle tissue.* M/z *581.4, shown in magenta, corresponds to the neutral loss of the sn2 RCOOH group from [M-H]⁻ of PI(18:0/18:4), localizing to connective tissues throughout the lungs, and with highest intensity in muscle tissue.* M/z *593.3, shown in red, corresponds to the loss of the sn1 acyl chain as (RCH=C=O) from [M-H]⁻ of PI(18:1/18:3), which is spatially distributed throughout the lungs.*

ion database in Lipid Maps [2]. We identified *m/z* 909.5 to be composed of a mixture of PI(22:6/18:0) and PI(20:4/20:2), localized to muscle tissue, connective tissue, and aveoli. *M/z* 885.5 was identified to be PI(20:4/18:0) and was nonspecifically distributed throughout the lung. *M/z* 861.5 was localized to the muscle tissue and was identified as PI(18:2/18:0). *M/z* 797.5 was identified as PG(18:0/20:4) and *m/z* 687.5 as PA(O-18:1/18:1), both of which localized to connective tissue in the lungs.

Negative Ion MS/MS Imaging of *m/z* 857.6

On-tissue MALDI ionization generates complex spectra, often containing multiple isobaric lipid species. Specific tissue distributions of these isobaric lipids can be identified by acquiring images in MS/MS mode. The fragment ion spectrum of *m/z* 857.6 (Figure 5) identified four different phosphatidylinositol isomers, all of which displayed different distributions throughout the lung (Figure 6). Selected fragment ion images obtained by MS/MS imaging of the parent ion *m/z* 857.6, highlight unique fragmentation patterns of different lipid species constituting the parent ion of *m/z* 857.6 (Figure 6). Insets are enlarged by a factor of 3. Fragment ion *m/z* 571.4, shown in yellow, corresponds to the loss of the sn1 acyl chain as (RCH=C=O) from $[M-H]^-$. Neutral loss of the sn2 RCOOH group and inositol from $[M-H]^-$ (*m/z* 391.2) and the sn1 $RCOO^-$ ion (*m/z* 303.2) were observed with the same spatial distribution as *m/z* 571.4. This fragmentation pattern identified

PI(20:4/16:0), which is localized to the periphery of the lung.

Fragment ion *m/z* 577.4, shown in green, corresponds to the neutral loss of the sn2/sn1 RCOOH group from $[M-H]^-$. The sn2/sn1 $RCOO^-$ ion (*m/z* 279.2) was observed with the same spatial distribution as *m/z* 577.4, which was identified as PI(18:2/18:2) and localized to muscle tissue as well as the periphery of the lung. Fragment ion *m/z* 581.4, shown in magenta, corresponds to the neutral loss of the sn2 RCOOH group from $[M-H]^-$. The loss of the sn2 acyl chain as (RCH=C=O) from $[M-H]^-$ (*m/z* 599.3), neutral loss of the sn1 RCOOH group and inositol from $[M-H]^-$ (*m/z* 411.2), and the sn1 $RCOO^-$ ion (*m/z* 283.3) were observed with the same spatial distribution as *m/z* 581.4. This fragmentation pattern was identified as PI(18:2/18:2) and localized to connective tissues throughout the lungs, and with highest intensity in muscle tissue. Fragment ion *m/z* 593.3, shown in red, corresponds to the loss of the sn1 acyl chain as (RCH=C=O) from $[M-H]^-$.

The neutral loss of the sn1 RCOOH group from $[M-H]^-$ (m/z 579.3), neutral loss of the sn2 RCOOH group and inositol from $[M-H]^-$ (m/z 417.2), and the sn1 $RCOO^-$ ion (m/z 281.3) were observed with the same spatial distribution as m/z 593.3. This fragmentation pattern identified PI(18:1/18:3) and was spatially distributed throughout the lungs. Morphological structures were visualized by H&E staining and slide scanning of the same tissue sections following MS/MS imaging. These MS/MS images clearly demonstrate the power of image acquisition in MS/MS mode to identify and differentiate isobaric phospholipids based on their fragmentation patterns and the tissue distributions of each fragment ion. The ability to visualize the tissue distribution of each fragment ion gave us additional confidence in their identification, as fragment ions generated from the same PI species showed the same tissue distribution patterns.

Conclusion

- MALDI Imaging of inflated mouse lungs in positive or negative reflectron mode results in highly multiplexed, high-spatial-resolution lipid images. The majority of positive ions are phosphatidylcholines, and negative ions detected are mostly phosphatidylinositols.
- Segmentation in SCiLs Lab can be applied to the data analysis of both positive and negative ion images to visualize prominent anatomical structures from phospholipid images.
- On-tissue MS/MS profiling or MS/MS imaging of lung tissue can be utilized to differentiate phospholipids with overlapping or isobaric m/z's, revealing unique phospholipid distributions within mouse lungs.
- MALDI Imaging and on-tissue MS/MS imaging of the lung lipidome is starting to shed light on the lungs' complex lipid composition.

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