

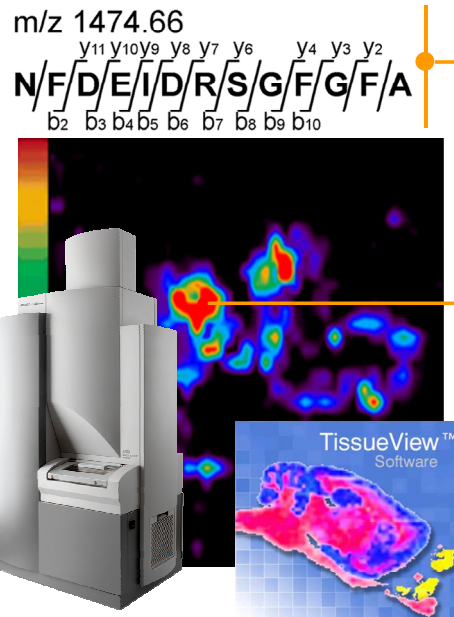
Technical Note

Three Dimensional Mapping and Imaging of Neuropeptides and Lipids in Crustacean Brain

4800 MALDI TOF/TOF™ Analyzer

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The functions of various biological compounds in complex tissues or organisms are often related to their locations in tissue structures. The emerging Mass Spectral Imaging (MSI) approach provides an attractive opportunity to detect and probe the complex biological molecules in an anatomical context, which reduces the time-consuming steps of sample extraction, purification and separation, while maintaining the topographical information about molecular distribution. The use of MSI to map the distribution of neuropeptides has gained increased attention^{1, 2}. MSI creates distribution maps of selected compounds in a similar manner to the more traditional biochemical techniques such as chemical staining, immunohistochemistry, and radiochemistry without



requiring a *priori* knowledge of the target analytes. It also enables the simultaneous differential localization of numerous peptides even when significant sequence homology exists. In addition, MS/MS fragmentation can be performed for unambiguous identification or even *de novo* sequencing of detected peptides. Furthermore, this technique has been extended to acquire images of serial sections from single specimen and provide a depth dimension to the data set, allowing three dimensional (3D) representations of multiple analytes in the target organs^{3, 4}. The in-depth profiling of 3D MSI provides more comprehensive spatial information for molecules of interest, such as localization of proteins and dynamic distribution and partition of pharmaceutical compounds into various target tissues.

This application note presents a simple scheme to obtain 3D mapping and imaging of multiple neuropeptides and lipids in a crustacean brain using a 4800 MALDI TOF/TOF™ Analyzer. The differential distribution of neuropeptides was determined using region specific direct tissue analysis, and collisional-induced dissociation (CID) fragmentation was performed directly from the brain tissue to confirm the identities of particular neuropeptides and lipids. For MSI sample preparation, both dry coating and regular coating with airbrush matrix application were investigated for the effect of matrix solvent on detection sensitivity and preferential extraction of lipids and peptides. 3D imaging was acquired by mapping seven layers of horizontal tissue slices followed by reconstructing ion images in three dimensions. The details of this study can be found in the article by Chen et al⁴.

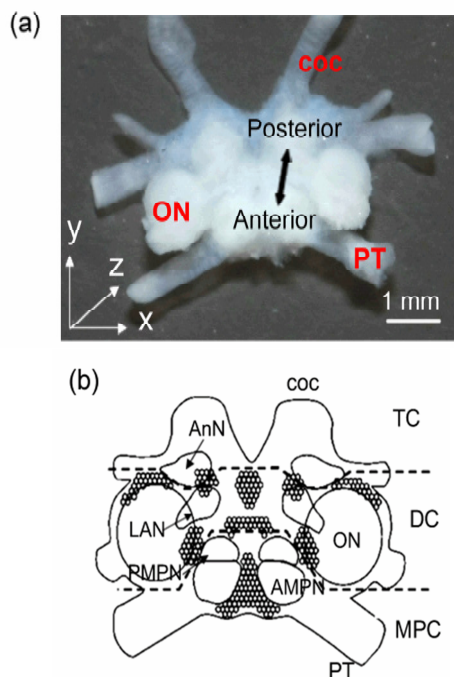


Figure 1. Morphology of *C. borealis* Brain. (a) Optical picture of isolated supraesophageal ganglion (brain) under microscope, with the major nerves and neuropils labeled. (b) Representation of the ventral surface of the isolated *C. borealis* brain with labeled neuropil regions showing differences between the fused ganglia.

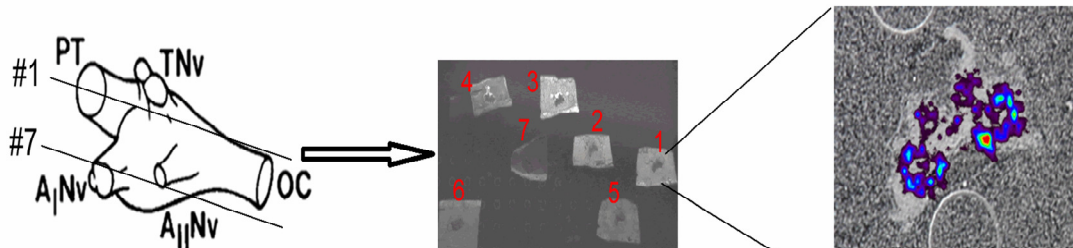


Figure 2. Three Dimensional Imaging Strategy. The schematic diagram of 3D imaging experiments showing serial sectioning along z-axis of the brain.

Experimental

Tissue Preparation: Brain tissues were harvested from Jonah crabs *Cancer borealis*, snap frozen in gelatin (100 mg/mL aqueous) and stored at -80°C freezer until further use. Sectioning into 12 μm slices at -25 °C was performed on a cryostat (Leica, Wetzlar, Germany), and the slices were thaw-mounted onto a MALDI plate that had been coated with a thin layer of 2, 5-dihydroxybenzoic acid (DHB) using an airbrush. As shown in Figure 2, a series of seven pieces of tissues was collected through the z axis of brain with 132 μm intervals in between. Tissues were dehydrated in a desiccator in -20°C freezer for at least 1 h prior to matrix application.

MALDI Matrix Application: 150 mg/mL DHB solution (50% methanol, v/v) was used for matrix application. Two types of coating conditions were tested. For normal spray, the airbrush was held 35 cm from the plate, and five coats were applied. Alternatively for dry spray, the airbrush was

held 55 cm from the plate and the flow rate was reduced to ensure most of the matrix solvent evaporated before reaching the plate.

Mass Spectrometry: A 4800 MALDI TOF/TOF™ Analyzer (Applied Biosystems) equipped with a Nd:YAG laser with a repetition rate of 200 Hz, λ = 355 nm (spot diameter of 75μm) was used for all mass spectral analyses. Acquisitions were performed in positive ion reflector mode. The tissue region to be imaged and the raster step size were controlled using the 4800 Imaging Tool Application (www.maldi-msi.org). To generate images, spectra were collected at 100 μm pixels in both the x and y dimensions across the surface of the sample. Each mass spectrum was generated by averaging 200 laser shots over the mass range *m/z* 600-1800. Mass spectra were externally calibrated using peptide standards applied on the MALDI target. Tandem mass spectra (MS/MS) were acquired with 2 kV collision induced dissociation (CID) using air as the collision gas at 2 X 10⁻⁸ Torr. 750 laser shots were averaged for each MS/MS spectrum, and sequence interpretation was performed manually.

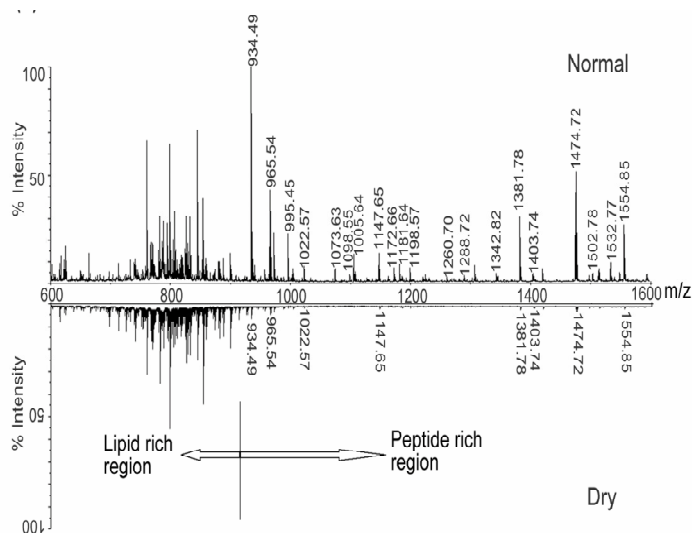


Figure 3. Comparison of Dry Matrix Spraying and Regular Matrix Spraying for Lipid and Neuropeptide Detection. Normal matrix spraying technique (top) provides for better detection sensitivity for higher mass lipids and neuropeptides.

Data Processing: Image files were processed, and extracted ion images were created using the TissueView™ Software package (Applied Biosystems). To compare images from different sections, the same intensity scales were used. 3D images were constructed and obtained using Image J (<http://rsbweb.nih.gov/ij/>, NIH). Gray scale images from seven layers of brain tissues constructed with TissueView™ Software were imported into Image J and integrated as image stack. Then slices were arranged and viewed using the stack function 3D projection. The orientation of each layer was adjusted slightly to match the proportions of original preparation.

Matrix Coating for MSI

To investigate the effect of solvent on lipid and peptide detection, two matrix application methods were compared, namely dry coating and regular coating using airbrush. As shown in Figure 3, these two matrix application methods yield similar signal intensities and peak patterns for lipids with abundant peaks below m/z 900. However for the dry coating, much fewer peptides with lower signal intensities are observed above m/z 900. This experiment suggests that a solvent free matrix application technique could be beneficial for MALDI imaging of lipids; however certain amount of solvent is crucial for extracting peptides from tissue samples to yield detectable signals.

Direct Tissue Analysis of Neuropeptides in *C. borealis* Brain

To study the neuropeptides present in *C. borealis* brain, direct tissue *in situ* analysis from different regions was performed. As shown in Figure 4, different neuropeptide profiles were observed from three different brain regions including both qualitative and relative quantitative differences. The peptide identities were further confirmed by CID fragmentation experiments performed directly on the tissue sample. Figure 5 shows two examples of MS/MS spectra, including *C. borealis* tachykinin-related peptide (CabTRP) 1a (m/z 934.5, Figure 5a) and an orcokinin peptide (m/z 1474.7, Figure 5b). As seen, almost complete series of fragment ions are observed, providing high confidence for peptide identification. Direct tissue analysis results demonstrate the complex structure of crustacean brain and extremely heterogeneous distributions of numerous neuropeptides.

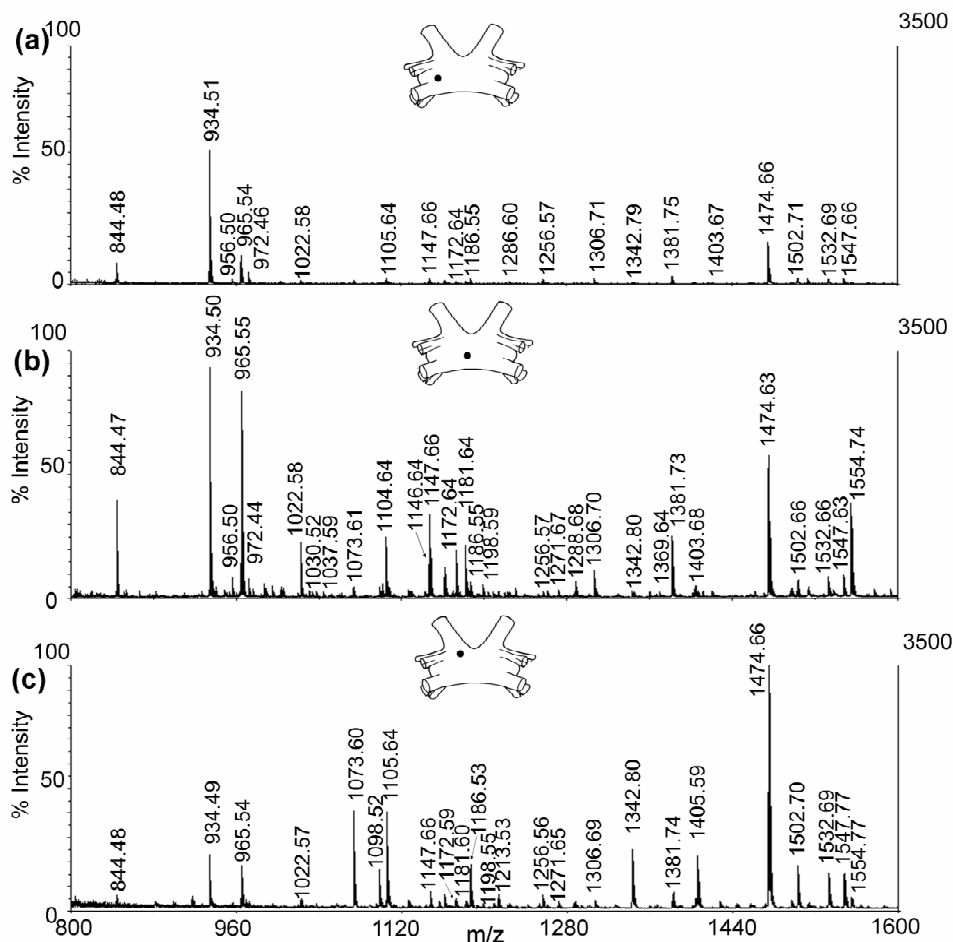


Figure 4. Comparison of Direct Tissue MS Spectra from Three Discrete Regions of *C. borealis* Brain. The solid dark circle indicates the position of tissue in the whole brain structure including (a) medium protocerebrum (MPC), (b) deutocerebrum (DC) and (c) tritocerebrum (TC) from which MS spectrum was acquired.

Three Dimensional Distribution of Neuropeptides

Figure 6 shows representative MALDI molecular ion images of six neuropeptides present in the brain across seven layers, including two orckinins, two RFamides, SIFamide and CabTRP 1a. Different neuropeptide families show differential distribution patterns in the crustacean brain. Interestingly, isoforms from individual families also exhibit distinct expression patterns in some cases. By acquiring and comparing images of serial sections, detailed information on neuropeptide distribution in 3D domains can be achieved.

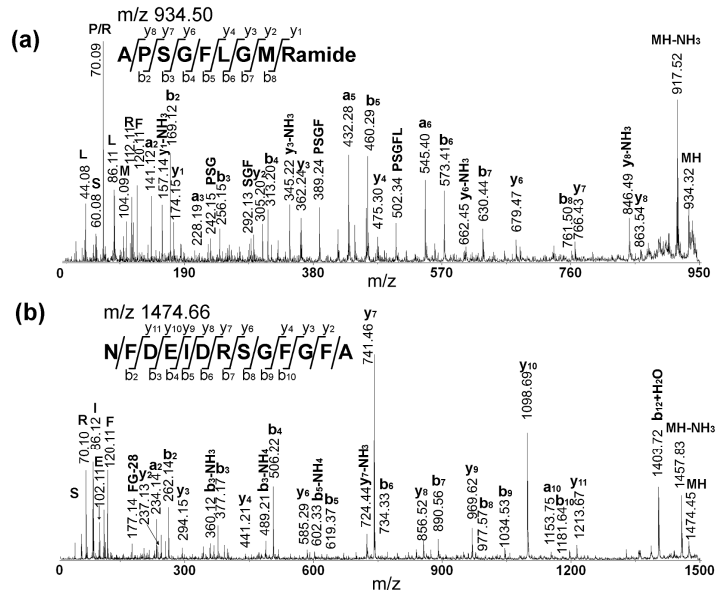


Figure 5. Representative MS/MS Sequencing of Two Neuropeptides Obtained Directly from the Brain Tissue Sample.

The amino acid sequence of each peptide is given above the spectrum. (a) MS/MS fragmentation of CabTRP 1a APSGFLGMRamide (m/z 934.49); (b) MS/MS fragmentation of orckinin NFDEIDRSGFGFA (m/z 1474.66). The presence of b and y ions is indicated by horizontal lines above (y ions) or below (b ions) the corresponding amino acid residues in the peptide sequence.

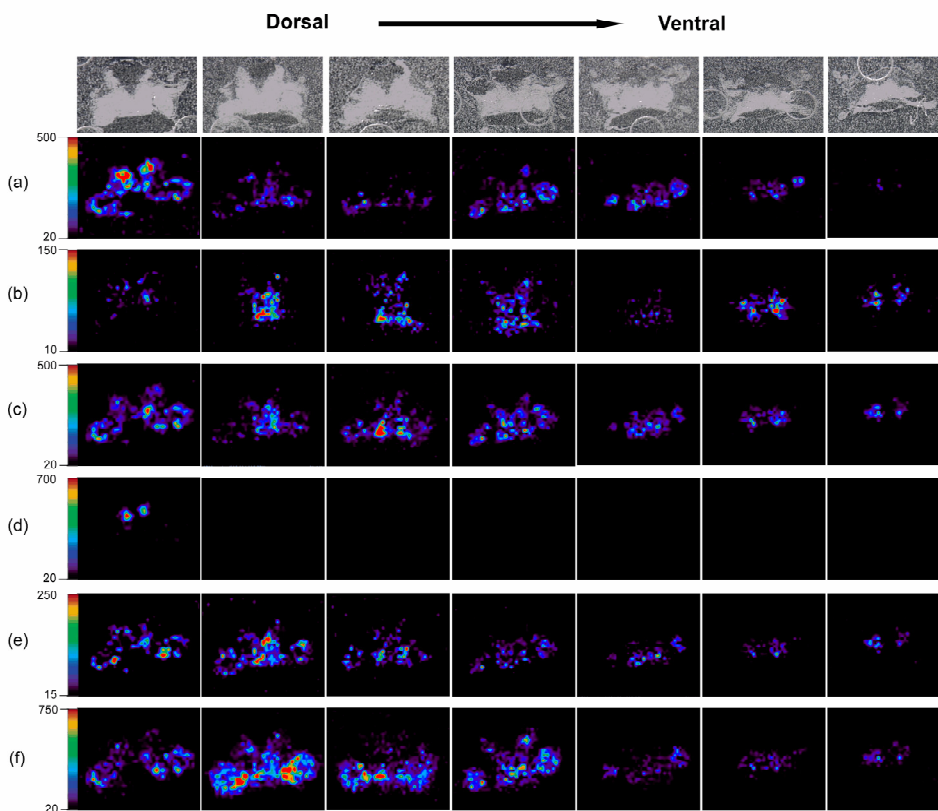


Figure 6. Three Dimensional Distributions of Neuropeptides in *C. borealis* Brain. From left to right, the slices were arranged from dorsal to ventral. The individual optical images of each layer of section are shown in the top panels. Six neuropeptides are shown, including two orckinins: (a) NFDEIDRSGFGFA (m/z 1474.7) and (b) NFDEIDRTGFGFH (m/z 1554.7); two RFamides: (c) NRNFLRFamide (m/z 965.5) and (d) SMPSLRLRFa (m/z 1105.6); (e) GYRKPPFNGSIFamide (m/z 1381.7); and (f) CabTRP 1a APSGFLGMRamide (m/z 934.5). Different distribution patterns are shown between different families as well as isoforms within individual families.

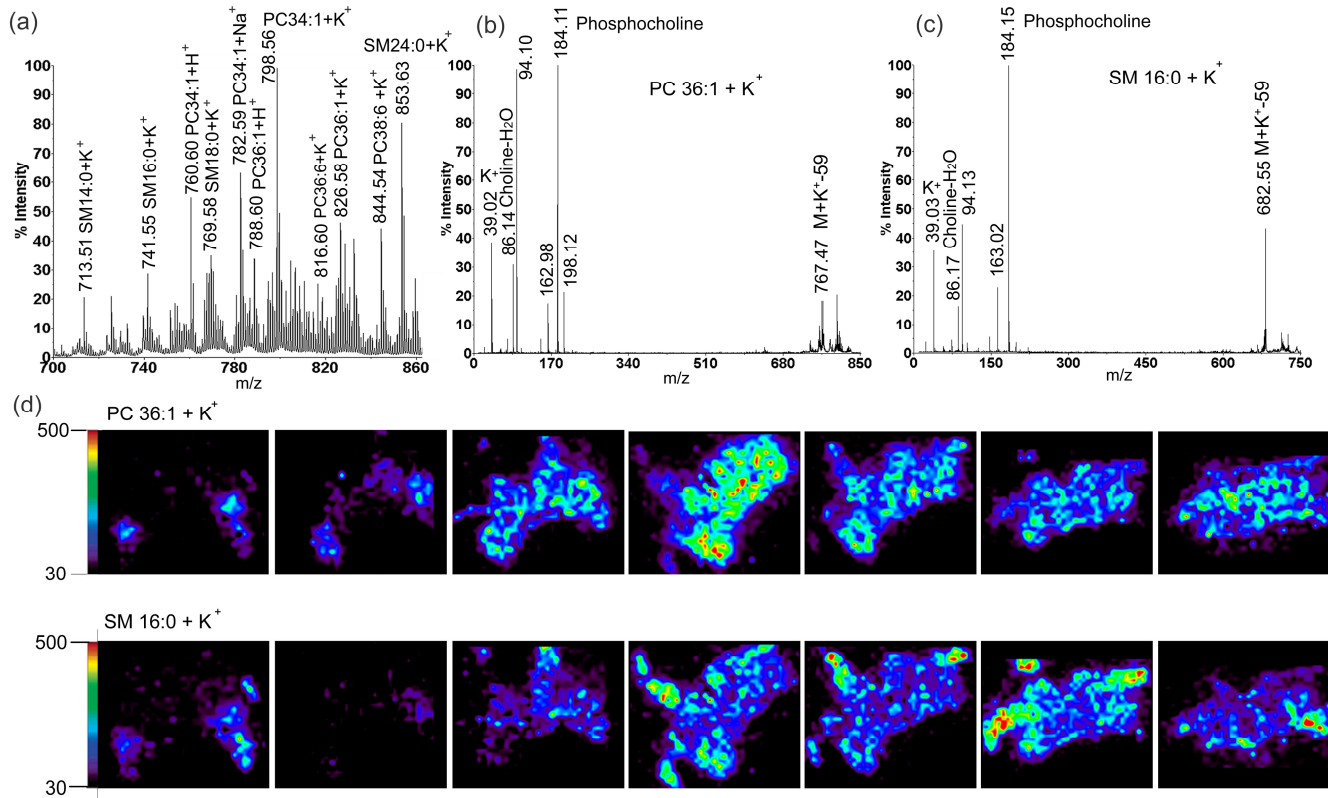


Figure 7. Identification and 3D Mapping of Lipids in the *C. borealis* Brain. (a) Representative MS spectrum of direct tissue analysis of lipids from brain slice with the masses and identities of each peak labeled; (b) MS/MS fragmentation of m/z 826.6 (PC 36:1+K⁺); (c) MS/MS fragmentation of m/z 741.6 (SM 16:0 + K⁺); Neutral loss of trimethylamine group ($\Delta 59$) and product ion m/z 184 demonstrate the presence of phosphocholine head chain in both molecules. (d) Comparison of 3D distributions of two different species of lipids PC 36:1 (upper) and SM 16:0 (lower).

Three Dimensional Distribution of Lipids

Figure 7 shows several abundant lipids present in the brain tissue slice. The identities of these lipids are assigned by accurate mass measurements in combination with MS/MS fragmentation. Figure 7b and 7c show representative spectra of two lipid species, including phosphatidylcholine (PC) and sphingomyelin (SM). Neutral losses of trimethylamine group $\Delta 59$ Da and product ion from the phosphocholine head chain (m/z 184.1) are observed as the characteristic fragmentation pattern. The detection of ion at m/z 39 suggests the formation of K⁺ adducts instead of protonated form. As shown in Figure 7d, two examples of 3D imaging of molecules from both PC and SM classes are presented. Different localization patterns of these two types of lipids are observed with PC localized primarily in the main body of brain, whereas SM has higher abundance in the fibers.

Figure 8 shows representative 3D images of a neuropeptide CabTRP 1a and a lipid generated using Image J by stacking seven images. The 3D images are visualized from z axis. Unlike neuropeptides, the lipids are distributed relatively evenly throughout the whole brain structure instead of being concentrated in a few neuronal clusters.

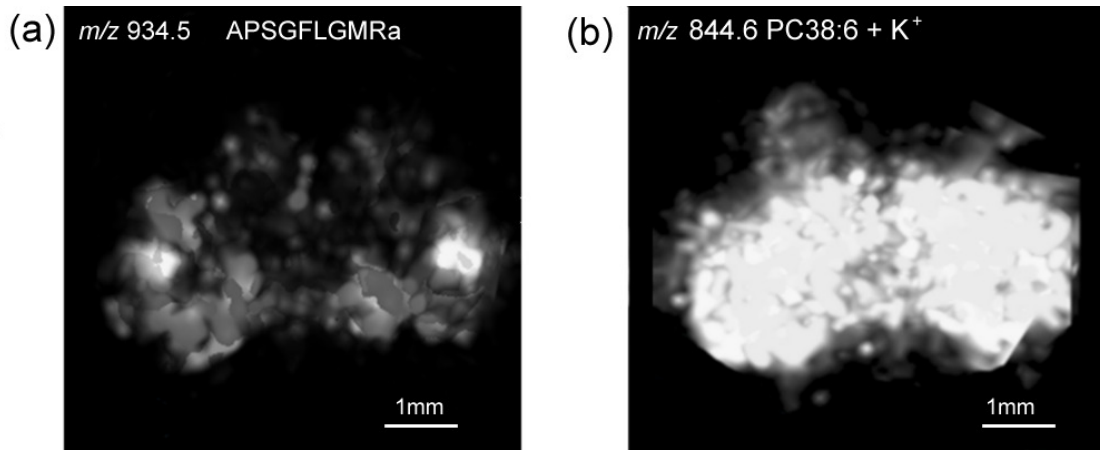


Figure 8. Representative Reconstructed Three-Dimensional Images of (a) CabTRP 1a and (b) Lipid PC 38:6. Lipids are distributed more evenly throughout the tissue than the neuropeptide.

Conclusions

Tissue imaging using MALDI MS and MS/MS is emerging as a powerful tool for identifying and localizing a variety of analytes in biological tissues. Simple sample preparation tools, robust acquisition and data analysis software and powerful visualization software are key to obtaining high quality localization information. Here, good MS resolution and high quality MS/MS as provided by the 4800 MALDI TOF/TOF™ Analyzer was key to confirm the identity of specific peptides and lipids in a crustacean brain and determine their localization.

References

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