APPLICATION NOTE

#56 Mass Spectrometry Imaging of Neuropeptides in Crustacean Brain Tissue

Application & Background

Neuropeptides are known to play important roles in almost every aspect of normal physiology, and we are increasingly understanding their varied roles in different disease pathologies.[1] An important consideration in studying neuropeptides is their ability to diffuse far from their secretory vesicle and influence cellular chemistry far from the original neuron in which they were produced.[1] Mass spectrometry imaging (MSI) is thus an excellent analytical technique with which to study neuropeptide distribution and quantification in both normal and diseased states, as it preserves the spatial location of each individual biomolecular signal, without need for prior knowledge of the analytes of interest. However, while many protocols exist for protein matrix-assisted laser desorption/ionization- (MALDI-) MSI, there are relatively few studies demonstrating the use of this analytical tool for imaging of neuropeptides.

In this study, a novel sample preparation protocol was developed for imaging neuropeptides in crustacean brain tissue via MALDI-MSI. Using an original washing method and optimized matrix spraying protocol developed using the HTX TM-Sprayer, changes in neuropeptide distribution in the crustacean brain were able to be analyzed in response to three different stress conditions compared to a control.

Experimental

Experimental Design

Female blue crabs (*Callinectes sapidus*) were obtained from Midway Asian Market (Madison, WI) or LA Crawfish Company (Natchitoches, LA). All crabs were originally housed in sea water at 35 parts per thousand salinity, 17-18°C, 80-100% oxygen (O_2) saturation, and a pH = 8.3 for over 5 days. Then, the crabs were divided into four experimental groups: (a) control (pH = 8.3, 100% O_2 water saturation), (b) severe hypoxia (pH = 8.3, 10% O_2 water saturation), (c) mild hypoxia (pH = 8.3, 50% O_2 water saturation), and (d) hypercapnia (pH = 7.6–7.8, 50% O_2 water saturation). Hypoxic experimental conditions were created by sparging tanks with nitrogen gas until the O_2 was at the desired level (approximately 30-40 minutes), and the hypercapnic experimental conditions were created by sparging a tank with CO_2 gas until the pH was lowered to the desired level (approximately 5 minutes). For each condition, after the experimental tank was prepared, one crab was placed in the tank for 2 hours. After anesthetization on ice, brains were removed from the crabs and embedded in gelatin. Tissues were sectioned at 12 µm using a Micro HM525 cryostat (Thermo Scientific) and mounted onto plain, glass microscope slides. Samples were stored at -80°C until needed.

Wash Protocol Optimization

Control brain samples were removed from the freezer and placed in a vacuum chamber for 10-20 minutes until the sample was dry. Samples were exposed to one of 14 different wash conditions using a Tissue-Tek slide staining system. These conditions were the 7 following EtOH:H_2O volume:volume (v:v) solutions for either 10 or 30 seconds: 100:0, 85:15, 70:30, 50:50, 30:70, 15:85, and 0:100. Three technical replicates were used to evaluate each washing condition. The osmolarity of the wash solution was analyzed pre- and post-wash using an Advanced Instruments Model 325 Single-Sample Osmometer in order to evaluate whether the wash was removing salts from the tissue. The post-wash solution was also then spotted in a 1:1 ratio with 150 mg/mL DHB in 50:50 MeOH:H_2O and 0.1% formic acid (FA) in order to evaluate whether the wash was removing any additional analytes, including some neuropeptides, from the tissue.

Sample Preparation

After washing, samples were placed under vacuum for 15-20 minutes to dry. DHB matrix was applied using the HTX TM-Sprayer with the parameters displayed in **Table 1**.

Matrix	DHB
Solvent	50:50 MeOH:H_2O + 0.1% FA
Matrix Concentration (mg/mL)	40
Flow Rate (mL/min, FR)	0.1
Spray Nozzle Velocity (mm/min, V)	1250
Spray Nozzle Temperature (°C)	80
Track Spacing (mm, TS)	3
Number of Passes (NP)	12
Nitrogen Pressure (psi)	10
Spray Pattern	CC
Drying Time (sec)	0
Nozzle Height (mm)	40

Table 1. Spraying parameters for DHB matrix on crustacean brain tissue.

Data Collection and Analysis

All experiments were performed on a MALDI-linear trap quadrupole (LTQ)-Orbitrap XL mass spectrometer (Thermo Fisher). Data were collected from a mass range of *m/z* 500-2000 at 75 µm spatial resolution and 30,000 mass resolution at *m/z* 400. Raw data files were exported in imZML format and imported into MSiReader (Version 1.00). Files were normalized in MSiReader to the total ion current (TIC), and images were generated. Accurate mass matching of ± 5 (condition comparisons) or 10 (method development) ppm to a home-build database was used to annotate neuropeptides, and accurate mass matching of ± 5 ppm to the LIPID MAPS online database was used to identify lipids within the images. All images were normalized to the same intensity scale. Using the Coloc2 FIJI plugin, images were overlaid in ImageJ to perform co-localization analysis based on a linear

Data Collection and Analysis cont'd

least-squares regression for the technical replicates. A minimum of five points were used as coordinates for each registration. A Pearson's correlation coefficient was calculated for each pixel between the intensities of different images to conduct intensity analyses. The Manders' split coefficient was then calculated for each analyte and used to evaluate how well the distribution of each analyte was conserved between the images of technical replicates. The raw data files were also imported into SCiLS software (Bruker Daltonics), and a student's *t* test was used to determine statistically significant changes between the four experimental conditions. A Bonferroni correction was applied in order to account for multiple tests.

Results

Wash Protocol Optimization

The different wash protocols were first evaluated based on the number of neuropeptide identifications. It was found that 50:50, 30:70, 15:85 EtOH:H_2O yielded the highest number of identifications (~30). Then, average normalized intensity was compared between the three wash solutions, and, while it was found that all the washes increased signal intensity compared to a control of no wash, the 50:50 EtOH:H_2O solution gave the highest overall signal intensity (approximately a 5-fold increase) at both the 10 and 30 second duration (**Figure 1**).

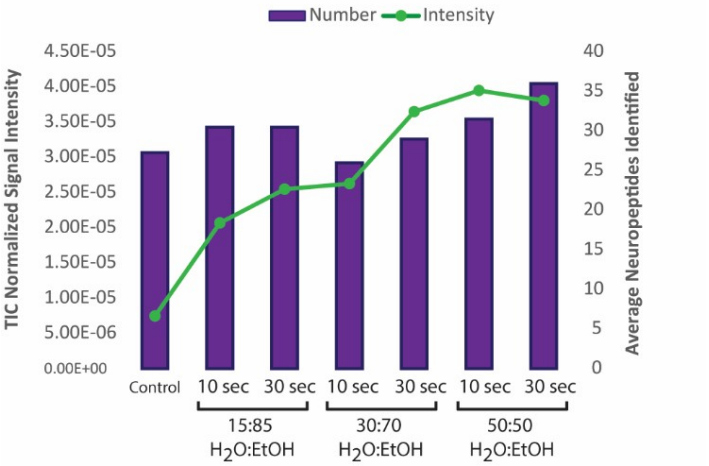


Figure 1. Comparison of the normalized signal intensity and average number of neuropeptides identified from the top three washing protocols compared with a control condition of no wash. The purple bars represent the number of neuropeptides identified, and the green dots represent normalized signal intensity across n = 3 technical replicates. It was found that the 50:50 H_2O:EtOH maximized both the intensity and number of neuropeptides among the three washes and was selected for further experiments and was selected for further experiments.

In order to identify the optimized time of washing, a colocalization analysis for five different known neuropeptides was performed. However, it was found that there was not significant difference between the 10- and 30-second washes, so the 10-second wash was used going forward in all experiments simply due to its brevity.

For the optimized wash system of 50:50 EtOH:H_2O for 10 seconds, the intensity of 34 neuropeptides were found to be enriched. In comparison, three neuropeptides showed no change in signal intensity, and six neuropeptides actually showed a decrease in signal intensity compared to the control (**Figure 2**).

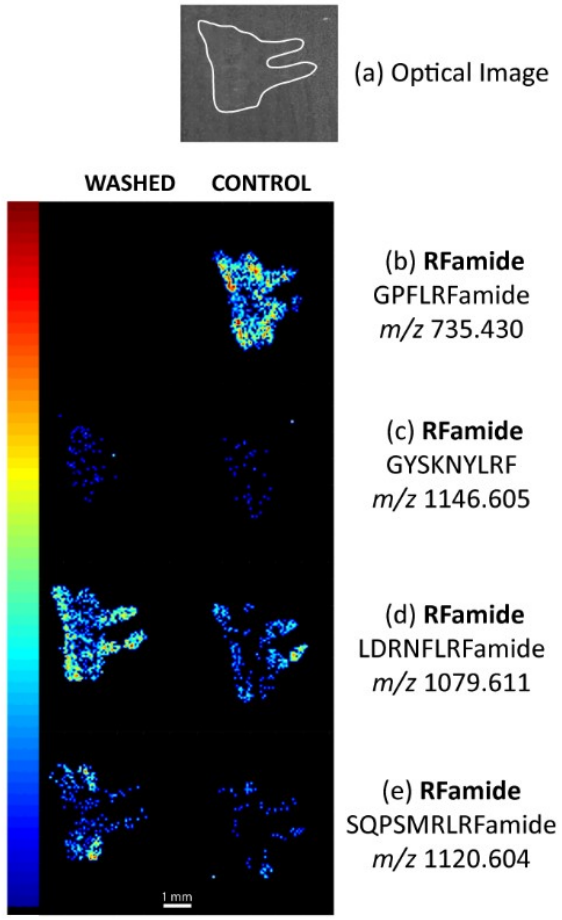


Figure 2. Examples of MALDI MS images of various neuropeptides in response to the optimized wash protocol. (a) An optical image of a crustacean brain, outlined in white. (b) A few neuropeptides displayed a decrease in signal intensity in the washed crustacean brain tissue versus the control (e.g. RFamide GPFLRFamide (*m/z* 735.430)). (c) Some neuropeptides displayed no change in signal intensity in the washed crustacean brain tissue versus the control (e.g. RFamide GYSKNYLRF (*m/z* 1146.605)). (d & e) Many neuropeptides displayed a clear increase in signal intensity in the washed crustacean brain tissue versus the control (e.g. RFamide LDRNFLRFamide (*m/z* 1079.611) and RFamide SQPSMRLRFamide (*m/z* 1120.604)). Scale bar represents 1 mm.

The effect of the optimized wash procedure on other analytes was also investigated. Overall, the signal intensity of lipids appeared to increase in the washed tissues compared to the control, which is likely due to the removal of some salts by the washing step. However, there was no significant change in the osmolarity of the washing fluid measured. The washing fluid was also analyzed for neuropeptides. A few were identified in the post-washing fluid, which was expected given the decrease in signal intensity noted for a few neuropeptides in the washed sample compared to the control.

Changes in Neuropeptide Distribution in Response to Stress

Differences in signal intensity of neuropeptides were noted between the control and each of three experimental conditions, with the most neuropeptides (16) changing significantly in signal intensity between the control and hypercapnic conditions (**Table 2, Figure 3**). Interestingly, there were also significant differences noted between the neuropeptide distribution in the moderate and severe hypoxia conditions, suggesting that the degree of hypoxia modulates the response of the distribution of neuropeptides. In addition, there were also several neuropeptides that displayed differences between moderately hypoxic and hypercapnic conditions, suggesting that each stress uniquely influences the response of neuropeptide distribution in crustacean brain tissue **(Table 2, Figure 3)**.

Experimental Conditions	Number of Neuropeptides with Significant Intensity Changes ($p < 0.0125$)
Control vs. 10% O_2	14
Control vs. 50% O_2	10
Control vs. pH	16
10% O_2 vs. 50% O_2	12
50% O_2 vs. pH	15

Table 2. The number of neuropeptides with significant intensity changes between each stress condition and the control condition and between each individual stress condition.

Experimental Summary

Sample Type	Crustacean brain (*Callinectes sapidus*)
Preservation	Gelatin-embedded, stored at -80°C after sectioning
Tissue Cut	12 µm

Instrumentation and Supplies

MALDI Plate	Plain glass slides
Matrix	DHB (Acros Organics, 40 mg/mL in 50%:50% MeOH:H_2O, 0.1% FA)
Matrix Sprayer	HTX TM-Sprayer™
MALDI MS	LTQ-Orbitrap XL (Thermo Fisher)
Acquisition Range	*m/z* 500-2000
Imaging Software	MSiReader (Version 1.00); SCiLS (Bruker Daltonics)

Conclusions

This novel sample preparation protocol for MALDI MSI of crustacean neuropeptides could be easily implemented in many other research labs due to its reproducibility and simplicity. As crustacean neuropeptides are homologous to human neuropeptides,[2] this research will be important as the role of neuropeptides in human health and disease continues to be investigated.

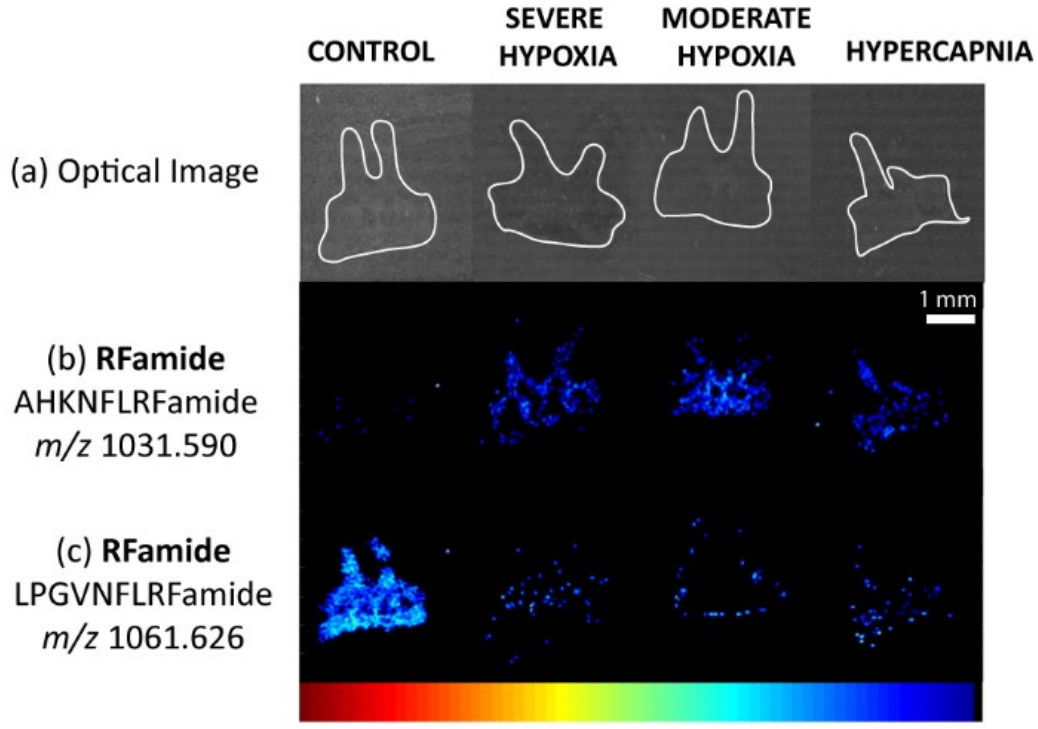


Figure 3. Examples of MALDI MS images of various neuropeptides in response to the three stress conditions tested. (a) An optical image of crustacean brains, outlined in white. (b) A few neuropeptides displayed a clear increase in signal intensity in the stressed crustacean brain tissue versus the control (e.g. RFamide AHKNFLRFamide (*m/z* 1031.590)). (c) Some neuropeptides displayed decreases in signal intensity iin the stressed crustacean brain tissue versus the control (e.g. RFamide LPGVNFLRFamide (*m/z* 1061.626)). Scale bar represents 1 mm.

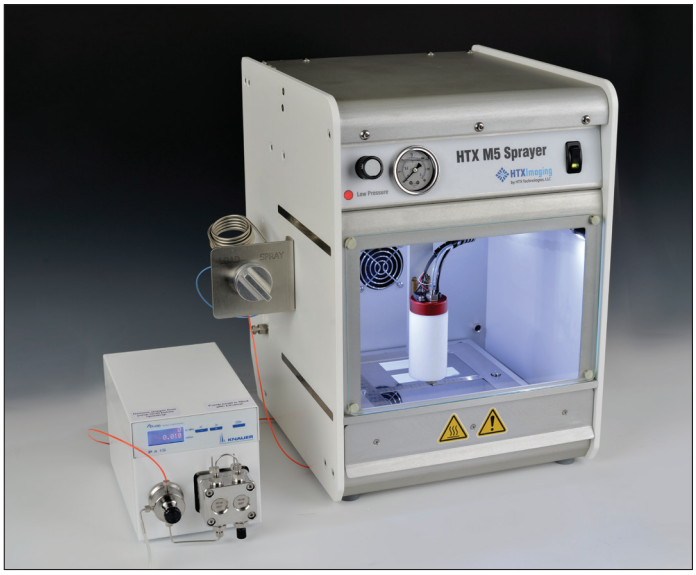
The tissue images and MS data presented here were provided by Dr. Amanda Buchberger, Nhu Q. Vu, Dr. Jillian Johnson, Kellen DeLaney, and Dr. Lingjun Li from the University of Wisconsin-Madison. This work was originally presented in: A Simple and Effective Sample Preparation Strategy for MALDI-MS Imaging of Neuropeptide Changes in the Crustacean Brain Due to Hypoxia and Hypercapnia Stress. J Am Soc Mass Spectrom. 2020 Mar 23. doi: 10.1021/jasms.9b00107.

References

[1]Russo AF. *Overview of Neuropeptides: Awakening the Senses?* Headache. 2017 May; 57 Suppl 2:37-46. doi: 10.1111/head.13084.

[2]Grimmelikhuijzen CJ, Leviev I, Carstensen K. *Peptides in the nervous systems of cnidarians: structure, function, and biosynthesis.* Int Rev Cytol.1996; 167:37-89.

HTX M5 Sprayer™ Tissue MALDI Sample Preparation System



HTX M5 Sprayer™ System is an Automated MALDI Matrix Deposition System Offering **High Reproducibility** and **Superior Data Quality** for Imaging Mass Spectrometry

The HTX M5 Sprayer™ is an easy-to-use, versatile spraying system that provides automated processes for sample preparation in imaging mass spectrometry.

The proprietary spray technology of the HTX M5 Sprayer™ guarantees a very fine, uniform and consistent matrix coating crucial for high-resolution imaging and relative quantification of analytes.

The unique ability to control liquid and propulsion gas temperature creates a fine solution mist that can be deposited in a precise and adjustable pattern over all or part of any MALDI plate.

Spray characteristics (wet or dry) are easily adjustable via the intuitive operator interface. Users can create and save methods for reproducible operation.

Key Characteristics

- Proprietary technology providing very small matrix droplets (<5 microns)
- High flow rate and fast sample prep (2 to 18 minutes per slide)
- Highly consistent matrix deposition across entire sample area (+/- 3% by weight)
- Unique use of temperature and nitrogen flow to control evaporation rate and matrix crystal formation
- More than 30 validated protocols covering trypsin and most matrices (e.g.: SA, CHCA, DHB, DAN, 9-AA, DHA, CMBT, THAP)
- Validated protocols for Trypsin digestion of FFPE
- Continuous matrix coverage as needed for high-resolution imaging
- Rugged operation and easy clean-up

Addressing the Matrix Deposition Challenge

The main challenge when preparing samples for MALDI Mass Spectrometry Imaging is to balance the positive effects of the matrix solution penetrating the tissue and co-crystallizing with the analyte, and the negative effects of analytes delocalization.

The all-new M5 chassis, high velocity stage and heated sample holder drawer contribute to a greater user experience and expanded process capabilities including:

- Faster and drier deposition capability
- On-tray trypsin digestion capability
- On-tray sample re-hydration

HTX M5 Sprayer™ is available worldwide exclusively from HTX Technologies, LLC.

To request further information contact:

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