Neuropeptide Mapping using MALDI-TIMS

Introducing a workflow that uncovers the hidden landscape of neuropeptides in bumble bees, shedding light on their role in insect behavior and neurobiology

Abstract

Neuropeptides play a vital role in regulating brain, endocrine, and exocrine functions, yet their detection directly from tissue remains a significant challenge in MALDI Imaging. Neuropeptide visualization is hindered by the high abundance of lipids and salts in biological samples. In this study, a workflow combining optimized washing steps with trapped ion mobility spectrometry (TIMS) is presented, to reduce chemical noise and enhance neuropeptide detection. Applied to the brain tissue of *Bombus impatiens* (the common eastern bumble bee), this approach enables high-resolution MALDI Imaging of numerous bioactive peptides. The integration of TIMS improves signal clarity and separation, offering new insights into the spatial distribution of neuropeptides in insect neurobiology.

Keywords:
Neuropeptides,
timsTOF fleX, Peptides,
Peptidomics,
MALDI Imaging

Introduction

Matrix-Assisted Laser Desorption/Ionization (MALDI) Imaging is a powerful technique for investigating the spatial distribution of biomolecules in tissue, offering high sensitivity and the ability to detect multiple analyte classes—including metabolites, lipids, peptides, and proteins—in an untargeted manner. While neuropeptides can be readily detected in tissue extracts, their direct detection from tissue sections remains difficult due to their relatively low abundance and the presence of more dominant species such as lipids and salts, which cause ion suppression.

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Various strategies have been explored to overcome these challenges, including chemical derivatization, enrichment protocols, and advanced mass spectrometry techniques. However, many of these approaches compromise spatial resolution or require extensive sample manipulation. Trapped Ion Mobility Spectrometry (TIMS), integrated into the timsTOF fleX platform, offers an advantage by separating ions based on their mobility, effectively reducing chemical noise and enhancing signal quality.

In this study, we combine a targeted washing protocol with TIMS to improve neuropeptide detection in the brains of *Bombus impatiens*. Bumble bees are an excellent model for studying neuropeptide function due to their complex social behavior and well-characterized neural circuits. Mapping neuropeptides in such organisms not only advances our understanding of insect neurobiology but also provides insights into conserved molecular pathways that govern behavior and decision-making across species, including humans [1]. By elucidating the spatial organization of neuropeptides, this work contributes to a deeper understanding of how sensory processing and neuroregulation shape behavior [2].

Methods

Heads of *Bombus impatiens* were carefully removed and embedded in a 2% carboxymethyl cellulose (CMC) solution, then frozen to preserve tissue integrity. The embedded samples were sectioned at a thickness of 10 µm using a cryostat and thaw-mounted onto IntelliSlides® (Bruker Daltonics GmbH & Co KG, Bremen, Germany).

To reduce ion suppression from lipids and salts, tissue sections underwent a sequential washing protocol: a 10-second wash in 70% ethanol, followed by two 10-second washes in 95% ethanol, with a 30-second drying period between each step. After washing, the slides were dried in a vacuum desiccator prior to matrix application.

The MALDI matrix consisted of 10 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) dissolved in 40% acetonitrile, 10% isopropanol, and 0.1% formic acid. Matrix deposition was performed using an HTX M3+ sprayer (HTX Technologies, Chapel Hill, NC, USA) with the following parameters: 80 µL/min flow rate, 70°C nozzle temperature, and 10 psi gas pressure.

MALDI Imaging data were acquired using a timsTOF fleX operated in TIMS ON mode. Imaging was performed at 20 µm spatial resolution from *m/z* 800–2500, with a 100 ms TIMS ramp time, $1/K_0$ range of 1.2–2.5, and 150 laser shots per pixel at a 10 kHz repetition rate.

Data visualization and analysis were conducted using SCiLS™ Lab, the SCiLS Lab API, and QuPath. Peptide annotations were based on predicted peptide sequences and confirmed via exact mass matching.

Results

Although MALDI Imaging of lipids is well-established, the complexity of lipid biosynthesis and functional diversity often complicates biological interpretation. Neuropeptides, in contrast, possess defined sequences and known bioactivities, enabling more direct correlations with physiological processes. However, their relatively low abundance compared to lipid species results in ion suppression during MALDI ionization, limiting their detectability.

To address this challenge, a targeted ethanol washing protocol was applied to tissue sections from *Bombus impatiens*, effectively reducing lipid and salt content and facilitating the detection of multiple neuropeptides. The brain of *Bombus impatiens* contains approximately one million neurons, with the mushroom bodies—responsible for integrating sensory input and guiding behavior—comprising over 300,000 neurons and approximately 20% of the total brain volume.

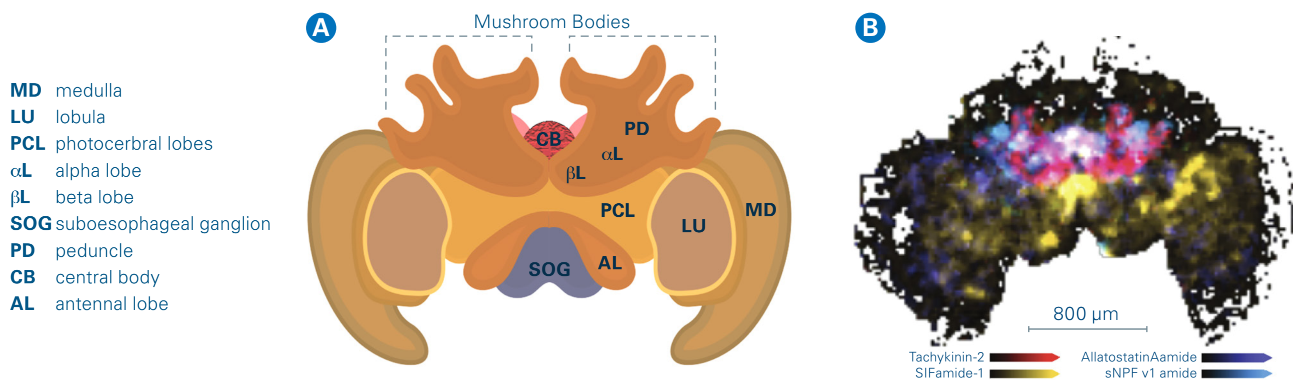

Figure 1. Ⓐ Schematic showing the frontal plane of *Bombus impatiens* with mushroom bodies labeled. Ⓑ Overlay of MALDI ion images showing the spatial distribution of tachykinin, allatostatin, SIFamide and sNPF. The overlay shows distinct spatial distribution of neuropeptides in the mushroom bodies and central body. Acquiring the data at 20 µm spatial resolution provides detailed ion images that show clear morphological detail while sampling in just a ~ 1 mm area.

Figure 1 presents a schematic of the bee brain and an overlay of MALDI ion images for tachykinin, allatostatin, SIFamide, and short neuropeptide F (sNPF). These neuropeptides exhibit distinct spatial distributions within the mushroom bodies and central body. Imaging at 20 µm spatial resolution enables detailed visualization of neuropeptide localization within a ~1 mm region, revealing clear morphological features. The overlay highlights the ability of MALDI Imaging to resolve neurochemical heterogeneity within anatomically defined regions, supporting functional interpretations of peptide localization.

The combination of ethanol washing and CCS-enabled MALDI Imaging using the timsTOF fleX platform enabled the putative detection of neuropeptides such as SIFamide, sNPF, orcokinin, allatostatin, apidaecin, and tachykinin. Note that identification was based on mass-matching from the known neuropeptides of *Apis mellifera*, as the *Bombus impatiens* proteome has not been mapped [3].

Table 1 summarizes the putative neuropeptides, including their sequences, measured and exact masses, and mass errors. The low ppm deviations support confident peptide assignments and demonstrate the effectiveness of the workflow. The presence of multiple isoforms of SIFamide and tachykinin suggests a complex neuropeptide signaling landscape in the bumble bee brain, potentially reflecting functional specialization across brain regions.

Table 1. Overview of detected neuropeptides in *Bombus impatiens*.
MALDI detected peptide sequences were obtained by taking known neuropeptides from *Apis mellifera* and aligning it to the *Bombus impatiens* proteome. Amide represents the C-terminus amide group.

Peptide name	Sequence	*m/z* measured	*m/z* exact mass	error [ppm]
Allatostatin4	RQYSFGL	913.454	913.453	1.09
Apidaecin7	PGPRPPHP	925.494	925.500	-7.02
LRNQLDIGDLQ-containing1	LRNQLDIGDLQ	1,284.693	1,284.691	1.79
Orcokinin2	NLDEIDRVGWSGFV	1,606.795	1,606.785	6.1
SIFamide1	AYRKPPFNGSIF	1,395.751	1,395.753	-1.86
SIFamide2	YRKPPFNGSIF	1,324.715	1,324.716	-0.83
SIFamide3	RKPPFNGSIF	1,161.651	1,161.653	-1.46
Tachykinin2	APMGFQGMR	993.479	993.476	3.02
Tachykinin11	ILDALEELD	1,030.527	1,030.530	-3.59
Short Neuropeptide F1amide	SPSLRLRF	974.590	974.589	0.21
sNPF v2amide	SQRSPSLRLRF	1,345.774	1,345.781	-5.65
Tachykinin3	APMGFQGMRG	1,051.477	1,051.481	-3.71
Allatostatin amide	RQYSFGL	869.465	869.463	2.07

Figure 2 illustrates the differential distribution of neuropeptides across serial coronal sections of the bee brain. Ion images of sNPF and tachykinin acquired from rostral to caudal regions reveal consistent and region-specific localization patterns. The bottom panels provide zoomed-in views of the mushroom bodies and central body, where integration of sensory inputs occurs. These data demonstrate the capacity of MALDI Imaging to capture dynamic changes in neuropeptide localization across adjacent tissue sections, offering insights into spatial regulation of neurochemical signaling.

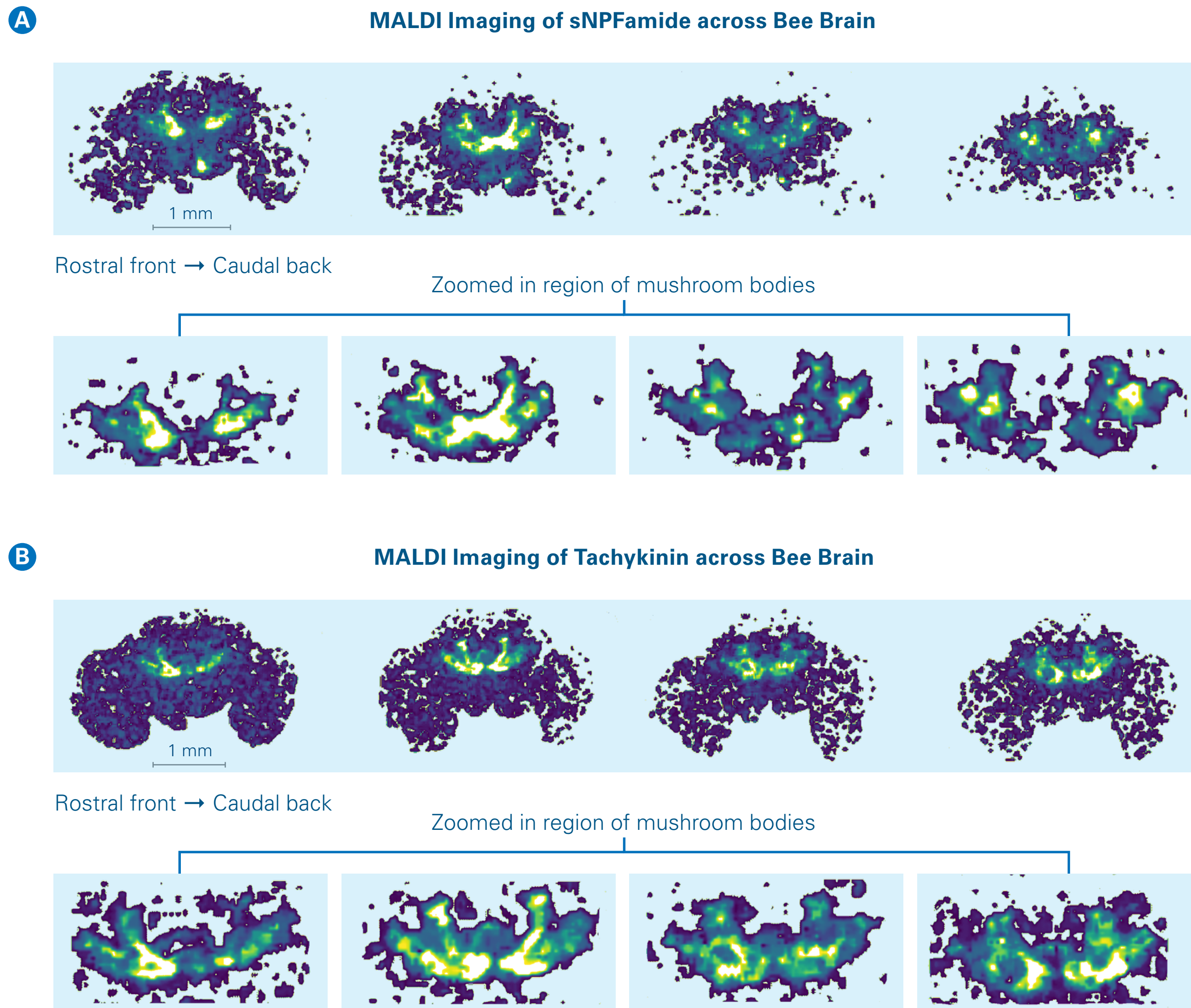

Figure 2. **Differential neuropeptide distribution across *Bombus impatiens* central body and mushroom bodies.**
(A),(B) top: MALDI ion images of sNPF and tachykinin of four serial coronal sections acquired coronal and moving from rostral to caudal. Acquiring data from adjacent tissues allows the study of how neuropeptide distributions change across the brain. (A),(B) bottom: Zoomed in region showing the central body and mushroom bodies where integration of all sensory inputs occurs.

To complement chemical imaging, hematoxylin and eosin (H&E) staining was performed post-MALDI acquisition. Figure 3A shows the stained tissue section, which was overlaid with ion images in SCiLS Lab to enable simultaneous visualization of tissue morphology and neuropeptide localization. Using QuPath, regions of interest were defined and annotated based on morphological features such as cell size, shape, and circularity (Figure 3B-C). These annotations were linked to ion intensity data, allowing statistical comparisons between regions. Figure 3D presents box and whisker plots for allatostatin and tachykinin intensities, comparing the central body to surrounding tissue. The plots reveal distinct signal distributions, supporting region-specific neuropeptide expression and enabling quantitative analysis of peptide localization.

Detection of low-abundance neuropeptides is often hindered by chemical noise and isobaric interferences. Without additional clean-up steps, such as fractionation, these signals may be obscured. TIMS on the timsTOF fleX provided a solution by separating neuropeptides from contaminants based on their collision cross section (CCS) values.

Figure 4 demonstrates the utility of ion mobility separation for resolving tachykinin signals. The average mass spectrum and corresponding mobilogram (Figure 4A) reveal the presence of tachykinin alongside an interfering contaminant. A zoomed-in view of the mobilogram (Figure 4B) distinguishes the tachykinin signal from the contaminant, enabling selective extraction. The non-CCS filtered image (Figure 4C) shows signal spread beyond the mushroom bodies, suggesting interference. In contrast, the CCS-filtered image (Figure 4D) accurately localizes tachykinin to the mushroom bodies, eliminating background signal. An image of the isobaric contaminant (Figure 4E) further illustrates how overlapping signals can distort interpretation in the absence of ion mobility separation.

These findings highlight the value of combining optimized sample preparation with CCS-enabled MALDI Imaging. The workflow enables high-resolution, chemically specific mapping of neuropeptides in insect brains, providing a robust platform for investigating conserved neuroregulatory mechanisms across species.

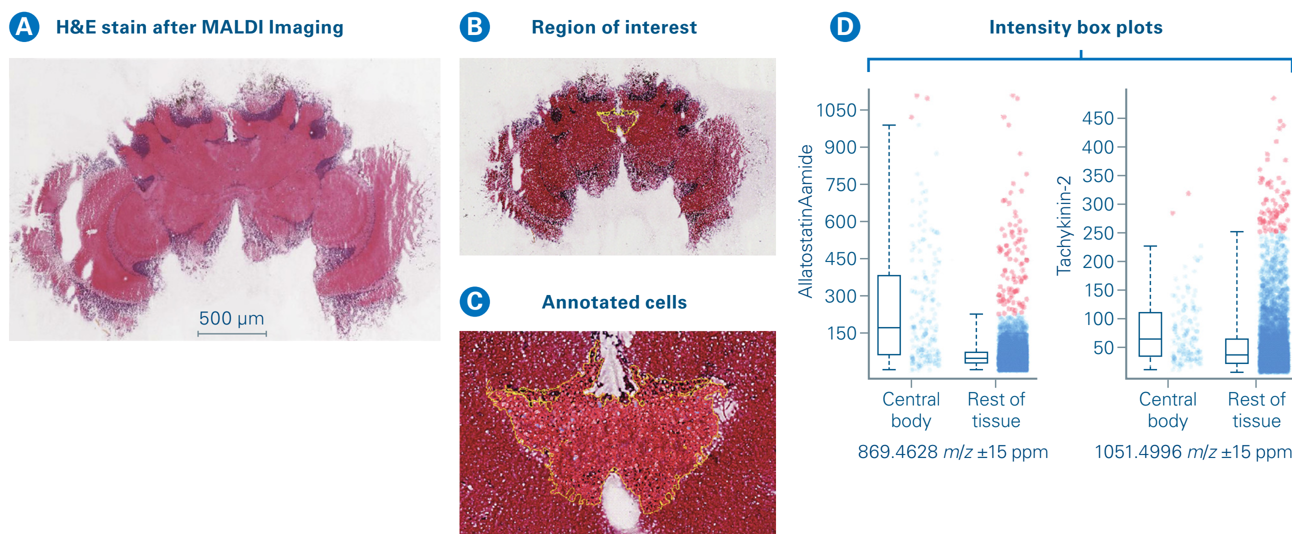

Figure 3. Histological images and cell type annotation in QuPath.
(A) Hematoxylin and eosin stain. (B) Region of interest defined in QuPath for cell annotation. (C) Zoomed-in image showing the annotated cells within the drawn region in yellow. Additional morphological information such as cell size, shape, and circularity can be extracted in a high-throughput fashion. The region information can then be imported into SCiLS Lab for further statistical analyses. (D) Two intensity box plots showing the signal intensity of allatostatinAamide and tachykinin 2 for the central body region versus the remaining tissue area.

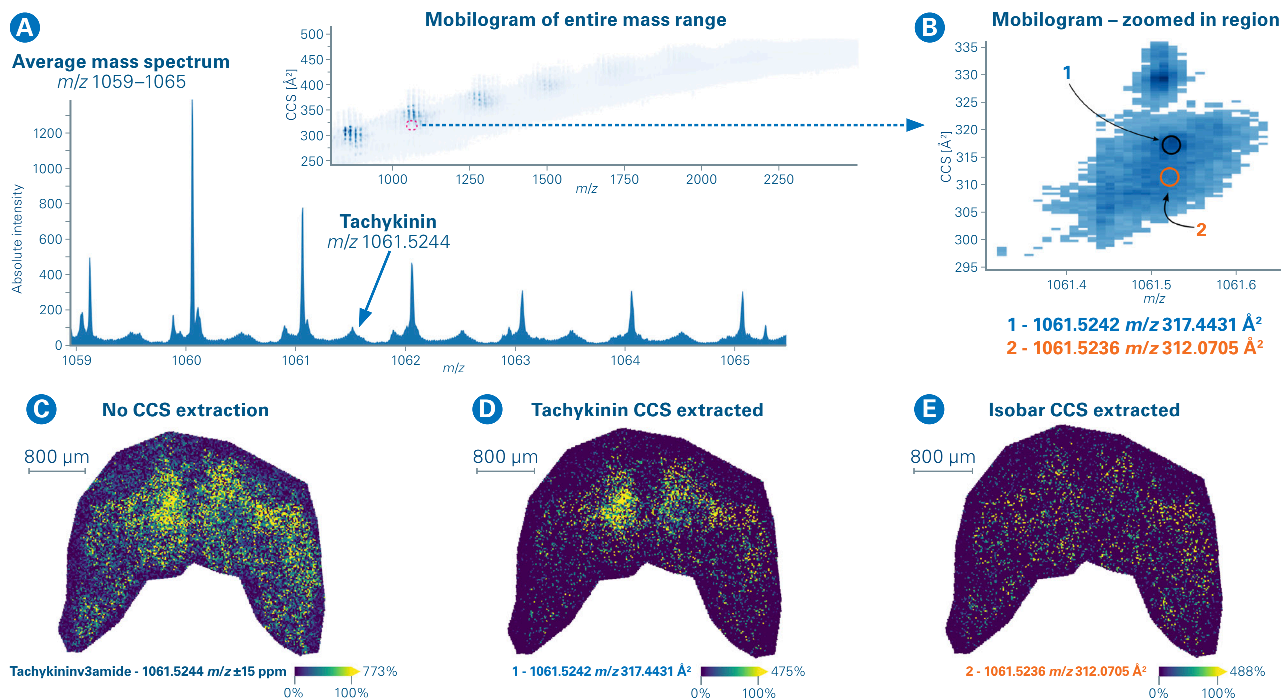

Figure 4. Ion mobility separation of tachykinin.
(A) Averaged mass spectrum and corresponding mobilogram depicting the location of Tachykinin peptide. (B) Zoomed in mobilogram region showing an interfering contaminant (1) and tachykinin peptide (2). (C) MALDI images showing non-CCS extracted tachykinin *m/z* 1061.524 where additional signal can be observed outside of the mushroom bodies. (D) CCS extracted tachykinin removes additional signal showing accurate tachykinin distribution throughout the mushroom bodies. (E) CCS extracted isobar *m/z* 1061.524 shows an additional signal that would have been incorporated into the MALDI image without ion mobility separation.

Conclusion

- High-resolution MALDI Imaging using the timsTOF fleX enables spatial mapping of neuropeptides in the brain of *Bombus impatiens*, providing insight into neurochemical organization in a model organism with complex behavior.
- TIMS enhances image quality by separating neuropeptides from isobaric interferences and chemical noise, resulting in more accurate and interpretable ion images.
- Serial section imaging supports three-dimensional interpretation of neuropeptide distribution across anatomical regions, revealing dynamic spatial patterns along the rostral–caudal axis of the brain.
- Post-MALDI histological staining with H&E, combined with QuPath-based cell annotation, enables correlation of neuropeptide localization with tissue morphology, facilitating region-specific and cell-type-specific analyses.

References

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