Supplemental Information

Visualizing neurotransmitters and metabolites in the central nervous system by high resolution and high accuracy mass spectrometric imaging

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Animal dissection

Animal experiments were performed according to institutional guidelines (UW-Madison IACUC). Blue crabs, C. *sapidus*, were purchased from the local market and maintained in recirculating artificial seawater aquaria at approximately 12-13 °C before use. Crabs were anesthetized by packing them on ice for 15 min, after which the dorsal carapace was removed. The crab's brain was dissected free from the connective stomach in chilled physiological saline. Wistar rats were terminally anesthetized with 5% isoflurane, transcardially perfused with saline solution and decapitated on P10 according to the approved experiment protocol. The details of dissection were described elsewhere.[32]

Sample Preparation

The crab brain was rinsed briefly in deionized water immediately following dissection to reduce the salt content, after which the excessive water was removed by KimWipes™. The brain was placed in a 100 mg/mL gelatin aqueous solution contained in a tissue block box. The rat brain was also embedded in the gelatin aqueous solution immediately after dissection. The tissue blocks were then flash-frozen in dry ice. The completely frozen tissues were sectioned to 12 µm sections that were mounted onto indium tin oxide (ITO) coated glass slides (Delta, Loveland, CO, USA). Optical images of the crab or rat brain sections were taken by scanning the glass slide with an office scanner (Epson V300 PHOTO) in professional mode with 2400 dpi. The crab or rat brain sections were then dehydrated in a desiccator at -20 °C for 30 min prior to matrix application.

For matrix application, 150 mg/ml DHB (50:50 methanol: water, v/v) was used and the airbrush (Paasche airbrush company, Chicago, IL, USA) was held 35 cm from the plate for regular spray. Five coats were applied, and the spray duration for each coat was 30 s with 1 min dry time between each cycle.

MALDI-TOF/TOF

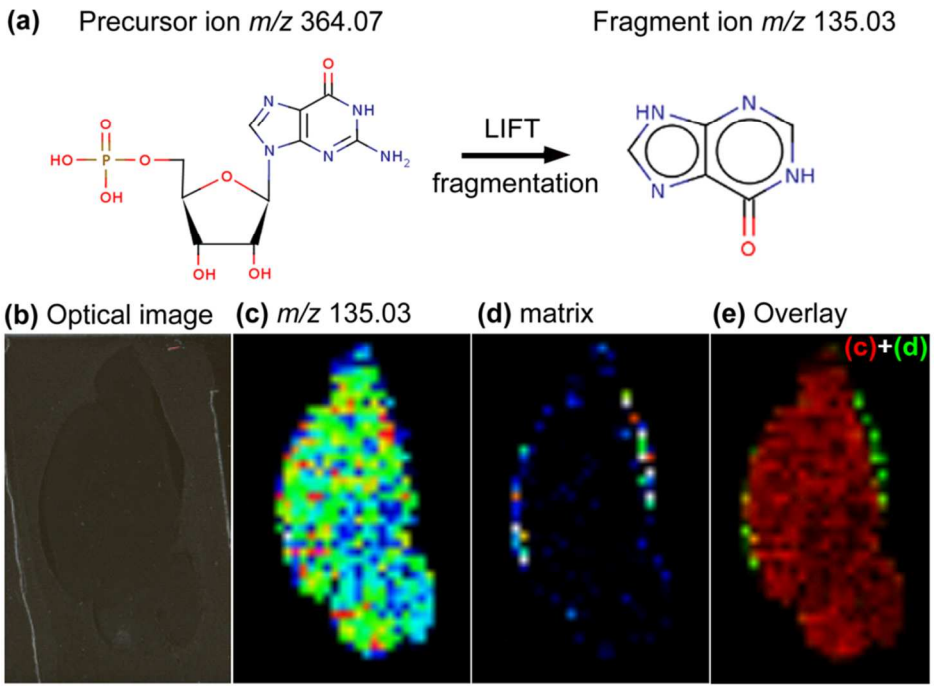
An Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with a 200 Hz smartbeam™ was employed for MALDI-MSI and tandem MSI. The following parameters were input in the positive reflectron mode for imaging acquisition: ion source 1 voltage 19.00 kV, ion source 2 voltage 16.62 kV, reflector 1 voltage 20.90 kV, reflector 2 voltage 9.64 kV and lens voltage 8.70 kV. The mass spectra data were acquired over a mass range of *m/z* 40-1000. Each spectrum consists of 200 laser shots, and the array of spectra was collected at 500 µm intervals in both x and y dimensions across the surface of the rat brain section. The following parameters were adopted in the positive LIFT™ mode for tandem MSI acquisition: precursor ion at *m/z* 364.07, ion isolation window ± 2Da, ion source 1 voltage 6.00 kV, ion source 2 voltage 5.30 kV, lens voltage 2.85 kV, reflector voltage 27.00 kV, Reflector 2 voltage 11.50 kV, Lift 1 voltage 19.00 kV and Lift 2 voltage 4.40 kV. The array of spectra was collected at a step-size of 500 µm, and each spectrum consists of 200 laser shots and was acquired over the *m/z* 40-400 mass range.

MALDI LTQ Orbitrap XL

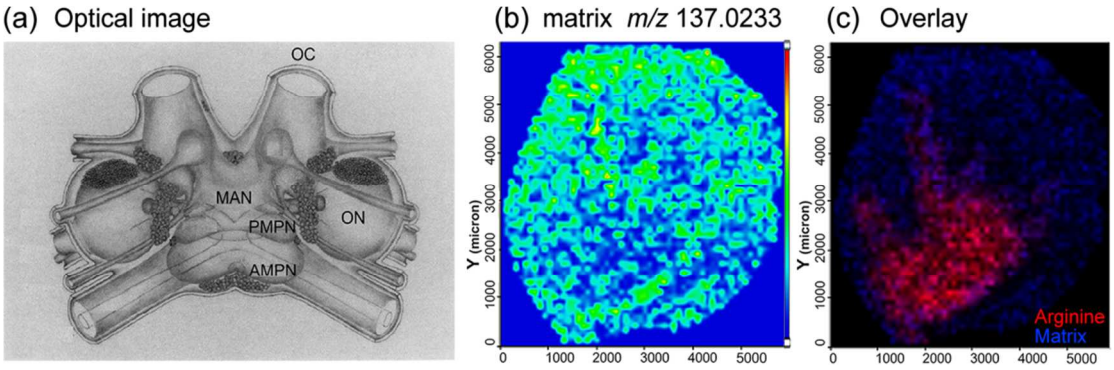
The MALDI LTQ Orbitrap XL instrument (Thermo Scientific, Bremen, Germany) equipped with a commercial 60 Hz N_2 laser at 337 nm (LTB Lasertechnik Berlin GmbH, Berlin, Germany) was used for MSI.[31] All experiments were performed with automatic gain control (AGC) turned on. MSI experiments were acquired using a step-size of 50 µm for crab brain and 500 µm for rat brain. Qualitative data were obtained using Thermo Scientific Tune 2.5.5 SP 2 instrument control software and Thermo Scientific Xcalibur™ software. The Orbitrap analyzer was fully tuned and calibrated with the aid of two calibration peptide mixtures (MSCal4, Sigma Aldrich, St. Louis, MO) for optimization in two mass ranges: *m/z* 50-1100 for small molecules and *m/z* 900-3000 for peptides. Data has been internally calibrated using a matrix peak $[DHB+H-H_2O]^+$ at *m/z* 137.0233 to improve mass accuracy using RecalOffline (Thermo Scientific) in a post-processing manner.

Supplemental Figures

Supplemental Figure 1. Tandem MSI of the *m/z* 364.07 ion on a LRMS MALDI-TOF/TOF. (a) Fragmentation pathway of the GMP precursor ion at *m/z* 364.07 to the monitored fragment ion at *m/z* 135.03. (b) Optical image of the rat brain section used for pseudo SRM imaging. (c) Tandem MS image of the major fragment at *m/z* 135.03. (d) Tandem MS image of an interfering ion resulted from fragmentation of matrix introduced simultaneously with the targeted precursor ion. (e) Overlay of the major AMP fragment and the matrix fragment. The presence of the interfering fragment ion demonstrated the interference of matrix in single-stage MSI when employing LRMS.



Supplemental Figure 2. (a) Illustration of the dorsal view of a crab brain, which reveals the main structure, including AMPN and PMPN from protocrebrum, MAN and ON from deutocrebrum and tracts that link neuropils like OC.[30] (b) MS image of a matrix-derived ion at *m/z* 137.0233. (c) An overlaid image of arginine in red and the *m/z* 137.0233 ion in blue. Their origins are revealed by their distinct distribution maps provided by HRMSI.



Supplemental Table 1. Metabolites identified from the crab brain specimen based on HRMSI.

Name	Formula	Theoretical Monoisotopic m/z	Orbitrap XL Measurement m/z	Δm (ppm)
Phenylalanine	$C_9H_{12}NO_2$	166.0863	166.0859	-2.4
Tyrosine	$C_9H_{12}NO_3$	182.0812	182.0814	1.1
NAD	$C_{21}H_{28}N_7O_{14}P_2$	664.1164	664.1177	2.0
Aminobenzoic acid+K	$C_7H_7NO_2K$	176.0108	176.0107	-0.6
Aminopentanoic acid	$C_5H_{12}NO_2$	118.0863	118.0863	0
Aminopentanoic acid+K	$C_5H_{11}NO_2K$	156.0421	156.0418	-1.9
GDP	$C_{10}H_{16}N_5O_{11}P_2$	444.0316	444.0317	0.2
ADP-ribose	$C_{15}H_{24}N_5O_{14}P_2$	560.0790	560.0797	1.3