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High throughput *in situ* DDA analysis of neuropeptides by coupling novel multiplex mass spectrometric imaging (MSI) with gas phase fractionation

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Abstract

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometric imaging (MSI) is a powerful tool to map the spatial distribution of biomolecules on tissue sections. Recent developments of hybrid MS instruments allow combination of different types of data acquisition by various mass analyzers into a single MSI analysis, which reduces experimental time and sample consumptions. Here, using the well-characterized crustacean nervous system as a test-bed, we explore the utility of high resolution and accurate mass (HRAM) MALDI Orbitrap platform for enhanced *in situ* characterization of the neuropeptidome with improved chemical information. Specifically, we report on a multiplex-MSI method, which combines HRAM MSI with data dependent acquisition (DDA) tandem MS analysis in a single experiment. This method enables simultaneous mapping of neuropeptide distribution, sequence validation and novel neuropeptide discovery in crustacean neuronal tissues. To enhance the dynamic range and efficiency of *in situ* DDA, we introduced a novel approach of fractionating full m/z range into several sub-mass ranges and embedding the setup using the multiplex-DDA-MSI scan events to generate pseudo fractionation before MS/MS scans. The division of entire m/z into multiple segments of m/z subranges for MS interrogation greatly decreased the complexity of molecular species from tissue samples and the heterogeneity of the distribution and variation of intensities of m/z peaks. By carefully optimizing the experimental conditions such as the dynamic exclusion, the multiplex-DDA-MSI approach demonstrates better performance with broader precursor coverage, less biased MS/MS scans towards high abundance molecules and improved quality of tandem mass spectra for low intensity molecular species.

Graphical Abstract

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Keywords

MALDI MS imaging; multiplex MS imaging; HRAM; neuropeptide; peptidomics; crustacean nervous system

Introduction

Since its introduction in 1997, MALDI MSI has become one of the most powerful tools for mapping the spatial distributions of *in situ* biomolecules in tissue samples [1]. MALDI MSI experiment generates ion density maps of thousands of biomolecules by acquiring mass spectra based on a predefined Cartesian grid. It has been increasingly utilized to study proteins, peptides, lipids, and small molecules for neuroscience studies [2–5], drug development and characterization [6–10], biomarker discoveries [11, 12], clinical diagnostics [13] and many other research areas. Moreover, novel ionization techniques have been developed for MSI to improved MSI performances, such as desorption electrospray ionization (DESI) [14–16], nanostructure initiator mass spectrometry (NIMS) [17, 18], matrix-assisted laser desorption electrospray ionization (MALDESI) [19], silver-assisted laser desorption ionization (LDI) [20] and laserspray ionization (LSI) [21, 22].

While MALDI MSI has undergone rapid development for nearly two decades, *in situ* biomolecule identification remains to be a major challenge in MALDI MSI studies. While putative identifications can be made by accurate mass matching, more confident identification relies on MS/MS fragmentation. *In situ* MS/MS experiments usually suffer from poor fragmentation efficiency caused by the low analyte abundance and complex biological context on the matrix coated tissue sections. Moreover, the limited fragmentation capability of most MALDI-TOF instruments prevents *in situ* MS/MS from achieving high efficiency and complete sequence coverage. In many tissue MSI studies, parallel LC-MS/MS experiments were performed using tissue homogenates for biomolecule identification [11, 23].

The development of MALDI-LTQ-Orbitrap XL hybrid mass spectrometer has revolutionized MALDI-MS analysis by combining an HRAM orbitrap with a fast scanning linear ion trap. This instrument can perform both collisional induced dissociation (CID) in the linear ion trap and high-energy collision dissociation (HCD) in the HCD cell, which provides flexibility to the MS/MS experiments [24, 25]. Furthermore, MALDI-LTQ-Orbitrap XL is capable of performing data-dependent acquisition (DDA) experiments to fragment top N ions after a full MS scan, which enables simultaneous high throughput distribution mapping and biomolecule identity verifying in complex samples. With the newly developed LSI and matrix assisted ionization vacuum (MAIV), MALDI-LTQ-Orbitrap XL can also be used in protein characterization and imaging [21, 22, 26].

The MALDI-LTQ-Orbitrap XL is an ideal instrument for multiplex MSI, a concept first introduced by the Lee lab to reduce data acquisition time, increase throughput and improve chemical information in MSI experiments [27]. Depending on the goal of each experiment, different scan combinations can be used in a multiplex experiment. For example, orbitrap and ion trap scans can be combined to reduce instrument time and improve spatial resolution [27]; full MS and MS/MS scans can be combined to map biomolecule distribution while elucidating structures of targeted biomolecules [28]; positive and negative ion mode scans can be combined to provide more chemical information [29]. It has been proven that multiplex MSI is a powerful tool in small molecule and lipid studies.

Decapod crustaceans have been utilized as model organisms to elucidate the function of neuropeptides in various physiological processes [30–34]. Their central nervous system (CNS) and stomatogastric nervous system (STNS) have been extensively studied as expedient models for investigating the generation [32], and modulation of rhythmic behavior [33] as well as regulatory roles of neuropeptides in food intake [34]. The neural circuits in their nervous system capable of producing motor patterns are extensively modulated by a collection of neuropeptides. The STNS is composed of several major neuronal ganglia, including the stomatogastric ganglion (STG), the paired commissural ganglia (CoG), the esophageal ganglion (OG) and other connecting nerves. The crustacean brain connects with the STNS via inferior ventricular nerve while each of the paired circumesophageal commissures connects to a CoG.

Herein, we adapted the idea of multiplex MSI to study the neuropeptides in the crustacean nervous system by multiplex-DDA-MSI approach. The combination of full MS scan with DDA scans in one run allows high-throughput MSI analysis, which shortens the acquisition time by half in comparison to performing full MS and DDA analysis in two separate acquisitions. Moreover, a novel strategy of fractionating m/z range coupled with DDA method was developed to analyze complex tissue samples with pseudo mass fractionation on the MALDI-LTQ-Orbitrap XL platform.

Experimental

Materials

All reagents were used without additional purification. Methanol, acetic acid and formic acid (FA) were purchased from Fisher Scientific (Pittsburgh, PA). 2, 5-dihydroxybenzoic acid

(DHB) was purchased from Acros Organics (Morris Plains, NJ). Microscope glass slides were purchased from VWR international, LLC (Radnor, PA). Physiological saline was composed of 440 mM NaCl, 26 mM MgCl₂, 13 mM CaCl₂, 11 mM KCl, 10 mM HEPES acid with pH value adjusted to 7.4 – 7.5. Distilled water mentioned in this work was Milli-Q water from a Millipore filtration system (Bedford, MA, USA).

Animal Experiment

Animal experiments were operated following institutional guidelines (University of Wisconsin-Madison IACUC). Rock crabs, *Cancer irroratus*, of similar size were purchased from Ocean Resources Inc. (Sedgwick, ME, USA). Blue crabs, *Callinectes sapidus*, were purchased from local seafood market. Animals were maintained for at least a week in a flow-through artificial seawater aquarium at ambient seawater temperature (12–13 °C) before use. Prior to dissection, animals were cold anesthetized by packing on ice for 20 minutes. Micro dissection was performed in chilled physiological saline. Supraesophageal ganglia (brain) and CoGs of crabs were harvested according to previously described dissection procedure [35].

MSI Sample Preparation

Tissue was embedded into gelatin solution (100 mg/mL in MilliQ water) and snap frozen on dry ice after dissection. The completely frozen tissue was sectioned into 12 µm slices on a cryostat (Thermo Scientific Microm HM 525) at –20 °C and thaw mounted onto a microscope glass slide (75×25×1 mm). The glass slide was dried in a desiccator at room temperature for 30 minutes before matrix application. DHB (50:50 methanol:water, vol:vol) was applied onto the tissue surface by a robotic TM sprayer (HTX Technologies, Carrboro, NC) for homogeneous matrix deposition. The nozzle temperature of the TM sprayer was set to be 80 °C with a moving velocity of 1000 mm/min. 10 passes of matrix was deposited with a flow rate of 0.25 mL/min and 30 seconds drying time between each pass. The slide was dried at room temperature after matrix application and stored in a desiccator in –80 °C until analysis.

MSI data acquisition

All MS experiments were performed on a MALDI-LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with 60 Hz 337 nm N₂ laser. Full scan mass resolution of 60,000 (at m/z 400), laser energy of 18 µJ and microscans of 4 were used for all analyses. MS/MS were performed in HCD mode with normalized collision energies of 45 and isolation window of 3 m/z (unless otherwise stated). Monoisotopic precursor selection was enabled. Different dynamic exclusion durations were tested and optimized. The multiplex MS imaging method was set up in Xcalibur software (Thermo Scientific, Bremen, Germany) and the imaging position file was defined in TunePlus software (Thermo Scientific, Bremen, Germany).

4-step linear-DDA-MSI and multiplex-DDA-MSI on crab brain tissue sections—

Two DDA-MSI experiments were performed to compare the influence of multiplexing on DDA-MSI of neuropeptide analysis using crustacean brain tissue sections. Four scan events were defined, with scan 1 as full MS scan and scans 2, 3 and 4 as data dependent MS/MS

scans. A raster step size of 50 μm was used for linear-DDA-MSI and a raster step size of 100 μm with spiral step size of 50 μm was used for multiplex-DDA-MSI.

9-step targeted multiplex-MSI on CoG tissue sections—A 9-step multiplex-MSI experiment was performed on CoG tissue sections. Nine scan events were defined: scan 1 was a full MS scan and scans 2–9 were targeted MS/MS scans of highly abundant neuropeptides observed in full MS scans. The precursor ions used for targeted MS/MS in steps 2–9 were listed in Table 1. The targeted MS/MS spectra were acquired in the linear ion trap with CID fragmentation at normalized collisional energy of 35. A raster step size of 150 μm and spiral step size of 50 μm were used.

9-step multiplex-DDA-MSI on brain tissue sections—Figure 1 illustrates the 9-step multiplex-DDA-MSI experimental set up on crab brain tissue section: each raster step of traditional MSI experiment is separated into 9 sub-steps or spiral steps. The number indicated the sequence of spiral plate movement. Steps 1, 4 and 7 were full MS scans at m/z ranges of 500–840, 840–1190 and 1190–1750, respectively. Steps 2/3, 5/6 and 8/9 were data dependent MS/MS scans of the top 2 most abundant ions detected in the previous full MS scans. The raster step size was 150 μm (i.e. step 1 to 1) and the spiral step size was 50 μm (i.e. step 1 to 2). The exact mass fractions for full MS scans and MS/MS scans could be varied for different tissue sections. The spatial distributions of biomolecules were assembled from each step 1, 4 or 7, while the identities of biomolecules were confirmed by MS/MS scans in steps 2/3, 5/6 or 8/9.

Data Analysis

Xcalibur software was used for spectrum processing. MSiReader (North Carolina State University, NC)[36] and ImageQuest (Thermo Scientific, Bremen, Germany) were used for MS image data processing. PEAKS DB (Bioinformatics Solution Inc, ON, Canada) was used for database searching.

Results and Discussion

MALDI MSI is a powerful tool to study the distribution of *in situ* biomolecules in various tissue samples. With the development of multiplex MSI by the Lee lab [27], more chemical information can be acquired with reduced instrument time and less amount of samples. DDA analysis on the LC-ESI-MS platform is often more powerful in peptide and protein identification than on the MALDI-MSI platform primarily due to the separation provided by LC before ESI-MS and the inherent more efficient fragmentation generated by multiply charged ions. However, MALDI-MSI grants the opportunity to investigate the chemical information directly in tissue with much less sample tampering in comparison to liquid phase sample preparation needed for LC. In this study, we adapted the concept of multiplex MSI on the MALDI-LTQ-Orbitrap XL platform with the goal to generate enhanced chemical information with limited sample amount. Utilizing the neuronal tissues from crustacean as a biological model system, a superior multiplex-DDA-MSI methodology was developed. By combining full MS with DDA in one analysis, the acquisition time was shortened by half compared to performing full MS and DDA in two separate acquisitions,

increasing the throughput of MSI analysis. In addition to traditional DDA experiments, we introduced an approach to fractionating the full m/z range into specific narrower sub-ranges and incorporating them into our multiplex-DDA-MSI setup. To achieve relatively even distribution of both peak number and peak intensity within each m/z sub-ranges, the original full MS scan was carefully tailored. Taking advantage of this pseudo fractionation strategy prior to DDA scans, we mimicked the separation process to make the precursor selection for MS/MS scans less biased and more efficient compared to the conventional DDA setup in MALDI-MSI.

Comparison between linear-DDA-MSI and multiplex-DDA-MSI

To compare the results from traditional DDA MSI (linear-DDA-MSI) with those from multiplex-DDA-MSI, two MSI experiments were performed with linear or multiplex-DDA-MSI on two consecutive crab brain tissue sections (Figure 2). Four scan events were set up with Step 1 as a full scan and Steps 2–4 as top 3 DDA scans for both experiments. A raster step size of 50 μm was used for linear-DDA-MSI (Figure 2a) and a raster step size of 100 μm with spiral step size of 50 μm was used for multiplex-DDA-MSI (Figure 2b). The distribution image of Total Ion Count (TIC) from MSI of linear-DDA-MSI (Figure 2c) appeared in a discontinued zigzag pattern, as only one out of four raster spots had the full MS scan information. In contrast, the TIC distribution image of multiplex-DDA-MSI (Figure 2d) displayed a continuous pattern with signals being distributed throughout the tissue, as the full MS scan information was available for every raster scan. In addition to the continuity of ion signals over the entire tissue, higher signal intensity on the olfactory and accessory lobes than the surrounding tissue also effectively demonstrated the variation of biomolecule abundances in different parts of the brain. The heterogeneous intensity distribution was not readily observed from the MSI image in the linear-DDA-MSI, as the isolated spots failed to produce signals representing the actual biomolecule concentrations in the remaining 3/4 of the tissue area where full scans were not acquired.

Furthermore, due to the heterogeneity of tissue surface, the neuropeptide species and abundance can be significantly different from spot to spot. For linear-DDA-MSI, the DDA scans were 50 μm (Step 2), 100 μm (Step 3), and 150 μm (Step 4) away from the full MS scan. The biochemical content in Step 3 or 4 may not be exactly the same as in Step 1 (full scan), which could lead to lower MS/MS quality of the DDA scans. In contrast, all DDA scans were 50 μm away from the full MS scan in multiplex-DDA-MSI mode, which more accurately represents the chemical information of the full MS scan.

To further demonstrate the advantages and unique features of multiplex-DDA-MSI, comparisons of peptides (HL/IGSL/IYRamide, m/z 844.4788 and VSHNNFLRFamide, m/z 1132.6010) for linear- and multiplex-DDA MSI conditions are shown in Figure 2. As shown in Figure 2e and 2g, only a few discrete spots were observed in the linear-DDA-MSI for both peptides as a result of the limited amount of full MS raster spots; while continuous distributions of both peptide ions were observed in the multiplex-DDA-MSI (Figure 2f and 2h). The identity of the first peptide (m/z 844.4788) was assigned by both accurate mass matching and DDA MS/MS results, while the identity of the second peptide (m/z 1132.6010) was assigned by accurate mass matching only. No DDA MS/MS scan was

acquired for the second peptide due to its low intensity in the full MS scans. Most of the precursor ions selected for MS/MS were from the lipid rich m/z range, where neuropeptides with lower intensity could not be selected. A fractionated mass range DDA method could significantly improve the precursor ion selection for lower intensity ions.

As shown, the traditional MSI is less compatible with DDA experiment because DDA scans sacrifice spatial resolutions for acquiring data dependent MS/MS scans. Nonetheless, multiplex-DDA-MSI acquires full MS scan in every raster position and simultaneously obtains data dependent MS/MS scans in subsequent spiral steps within the same raster step. This setup allows the image production of a more continuous distribution of neuropeptides on tissue surface while obtaining the MS/MS information to confirm the peptide sequences and identities.

The application of multiplex MSI for mapping neuropeptides in the CoG in blue crabs

To investigate the feasibility of applying the multiplex-MSI method to crustacean tissue, we performed experiments using the CoG isolated from the blue crab *C. sapidus*, which is a pair of neuronal ganglia that connect the CNS to the STNS in crustacean. Although previous study showed the presence of various neuropeptides in the CoG [37], the amount of neuropeptides in this minute size cellular cluster (typically ~ 500 μm in diameter) is much lower than in other bigger tissues such as the brain or the pericardial organ. In an MSI experiment, a 12 μm -thick section only contains about 1/40 of a single CoG ganglion. Using a multiplex-MSI setup containing 9 spiral steps, a full MS spectrum was acquired followed by eight MS/MS scans in every raster step. Due to the low abundance of analytes in the CoG and the instrument configuration, shorter travelling distance to the ion trap than to the HCD cell [24] is advantageous in preserving more precursor ions, which produces better quality MS/MS spectra when using CID fragmentation.

As a result of the HRAM measurement in full MS scans, 41 neuropeptides were putatively identified by accurate mass matching to our crustacean neuropeptide database [38], among which 18 were identified in the CoG for the first time. As shown in Figure 3a, 38 of the 41 matches were highlighted in color coding with corresponding neuropeptide families in the zoom-in m/z range of 800–1600. However, due to the complex tissue context, signals from neuropeptides were masked by higher intensity peaks (such as lipids, protein fragments and matrix etc.) when multiplex-DDA-MSI were adopted to confirm their identities. In order to obtain high quality MS/MS information to confidently identify the neuropeptides, a target list was generated and built in steps 2 to 8 in the 9-step-multiplex-MSI experiment. Figures 3b–3e are representative MS/MS spectra and distribution patterns of neuropeptides (overlaid with optical image) from 4 different neuropeptide families: tachykinin (Figure 3b), orcomyotropin (Figure 3c), SIFamide (Figure 3d) and orcokinin (Figure 3e). The sequence-specific b- and y-ions along with some internal fragment ions were produced with high abundance enabling good sequence coverage.

Although we demonstrated that simultaneous identification and distribution mapping were accomplished using targeted multiplex-MSI setup, this targeted method was not efficient enough for complex samples with more chemical information to be validated. Data

dependent acquisition in MSI is still of great importance. Therefore, we performed further method development with multiplex-DDA-MSI using more complex tissue samples.

Comparison between regular DDA and fractionated mass range DDA in multiplex-DDA-MSI

While the multiplex-DDA-MSI has improved the throughput of MSI experiment by acquiring distribution and identity of biomolecules in one analysis, it has some drawbacks. Most precursor ions selected for data dependent MS/MS scans were from the lipid rich mass ranges (m/z 500–840 and m/z 1400–1600). Very few neuropeptide ions were selected due to their relatively lower intensities compared to the abundant lipid ions. To circumvent this problem, a fraction mass DDA setup was developed to improve the multiplex-DDA-MSI method. The full mass range of m/z 500–1750 was divided into three fractions: m/z 500–840 (fraction 1), m/z 840–1190 (fraction 2) and m/z 1190–1750 (fraction 3). The m/z ranges of these fractions were determined by inspecting a profiling full MS scan and evenly dividing up the number of the analytes of interest into three fractions. Each fraction contained approximately 35–40 peaks for subsequent MS/MS analyses. In this more evenly fractionated mass-range DDA method, top 2 most abundant ions were selected for MS/MS within each mass range window. Since the dynamic exclusion for DDA is a global setting for all of the scan events, using similar number of target peaks to fractionate the full MS spectrum enabled an un-biased precursor selection across the entire m/z range. Fraction 1 was a complex mixture of matrix derived peaks, small neuropeptides and lipids while dominated by high abundance lipid species. The peak density and intensities in fraction 2 were much lower than in fraction 1. The rich neuropeptide information contained in fraction 2 was better separated from fraction 1 to achieve a less biased DDA setting for these low intensity species. Fraction 3 was a mixture of lipids and larger neuropeptides which had relatively higher signal intensities than the ions in fraction 2.

Interestingly, the number of peaks having MS/MS acquired in fraction 2 was lower than our expectation. It was noted that the peak intensities in m/z 840–920 were significantly higher than the rest of the analytes in fraction 2, which could lead to more biased MS/MS events for high abundant species, leaving these low intensity peptide peaks not selected for MS/MS scans in this m/z sub-range. To further optimize the performance of this fractionated mass DDA method, the profiling spectrum was re-evaluated and divided into the following segments: m/z 500–920 (fraction 1), m/z 920–1430 (fraction 2) and m/z 1430–1750 (fraction 3). Although the number of peaks differs from region to region, the signal intensities of the peaks within each fraction were more comparable. To accommodate these unevenly fractionated sub mass ranges, differential DDA setup was implemented. Top 3 most abundant ions were selected for MS/MS experiments from fraction 1, top 2 most abundant ions were selected from fraction 2, and top 1 most abundant ion was selected from fraction 3.

Figure 4 compares these three multiplex-DDA-MSI methods (regular multiplex-DDA-MSI, evenly fractionated mass multiplex-DDA-MSI and unevenly fractionated mass multiplex-DDA-MSI) from the brain tissue of blue crab *C. sapidus*. The number of precursor ions selected within each mass fraction window was compared in Figure 4a. As expected, in a regular DDA method without mass fractionation, most precursor ions selected for MS/MS

were from mass fraction 1. Only a few ions were selected for MS/MS in mass fraction 2 and fraction 3. For the evenly fractionated mass DDA method, similar numbers of precursor ions were selected in each mass fractions. Much fewer peaks were selected in the lipid rich fraction 1 and many more peaks were selected in the neuropeptide rich fraction 2 and fraction 3 than the regular DDA method. The unevenly fractionated mass DDA method further improves the number of peaks selected for MS/MS in each mass region. Figure 4b and 4c compared the spectra of regular DDA (Figure 4b) and fractionated mass DDA (Figure 4c). The precursor ions selected for MS/MS were highlighted in red and the precursor ions excluded for MS/MS were in grey. Peaks in black were not chosen by the instrument to perform MS/MS scans. Most peaks in the lipid rich mass range were selected for MS/MS in the regular DDA method, while only a few peaks were selected in the neuropeptide rich region (zoomed in spectrum). In contrast, significantly more peaks in the neuropeptide rich mass range (zoomed in spectrum) were selected for MS/MS under the fractionated mass DDA condition, which provides more useful peptide sequence information and greater peptidome coverage compared to conventional DDA condition.

By accurate mass matching to the custom-built crustacean neuropeptide database, 120 neuropeptides were putatively identified. 89 of the matches displayed on-tissue distribution overlapping with the neuronal clusters in the brain, which improves the confidence of their identities. These results were consistent with both regular multiplex-DDA-MSI and the fractionated mass multiplex-DDA-MSI methods. However, only 10 neuropeptide identifications were confirmed by MS/MS data acquired using the regular spiral setup, presumably due to the biased precursor selection without pre-separation before DDA scans. In contrast, the combination of evenly and unevenly fractionated mass range multiplex-DDA-MSI methods allowed confident identification of 39 neuropeptides with excellent sequence coverage. Details on the identified peptide family, peptide name, sequence, m/z , ppm and specific multiplex-DDA-MSI method employed can be found in Table S1.

In addition to the on-tissue characterization of known neuropeptides, the fractionated m/z multiplex-DDA method also enabled the discovery of novel neuropeptides. One novel RFamide was identified using the unevenly fractionated mass range setup, while not selected for MS/MS in other experiments. This RFamide has also been observed in our ongoing neuropeptidome characterization of *C. irroratus* using LC-ESI-MS/MS platform. As shown in Figure 5, this neuropeptide is more concentrated in the lateral antenna I neuropil and tegumentary neuropil, which are situated in the medial protocerebrum of the rock crab brain.

In summary, the unevenly fractionated mass multiplex-DDA-MSI method is most suitable for neuropeptide analysis in crustacean nervous system among the three multiplex-DDA-MSI methods. This improved performance is largely due to specific adjustment of the number of MS/MS scans according to the relative intensity and abundance of putative peptide peaks observed in a typical direct tissue MALDI mass spectrum. Therefore, the unevenly fractionated mass range multiplex-DDA-MSI method enables the acquisition of many more peptide sequences via tandem MS events while reducing the interference from other high abundance biomolecules. Moreover, because a greater number of low abundance molecular species could be selected for MS/MS analysis using this novel approach, it

provides great opportunity to discover additional novel neuropeptides that have been overlooked in previous peptidomic analysis using the traditional DDA method.

Conclusions

For the first time, multiplex MSI was coupled with DDA to achieve simultaneous identification and distribution mapping of neuropeptides in crustacean neuronal tissues. As we demonstrated in this study, traditional MSI is not amenable to direct coupling with DDA as it sacrifices spatial resolution for acquiring data dependent MS/MS scans. In contrast, the multiplex-DDA-MSI method acquires full MS scan in every raster position while obtaining DDA scans in subsequent spiral steps surrounding the main full MS step. This setup allows a continuous full MS acquisition while obtaining MS/MS information to confirm the peptide identities, which enhances the overall throughput of MSI analysis by reducing total acquisition time. Novel neuropeptides or other biomolecules can also be discovered by *de novo* sequencing from the MS/MS scans. Moreover, we introduced the concept of fractionating *m/z* range into multiple segments in multiplex-DDA-MSI acquisition to create *in situ* pseudo gas-phase fractionation of molecular species from a tissue sample before DDA analysis. This novel setup compensates to some degree for the lack of separation in MALDI-MSI based DDA experiments and significantly improves the efficiency and coverage of precursor selection and subsequent peptidome coverage. With multiplex-DDA-MSI, the spatial distributions of neuropeptides, lipids and protein fragments were mapped directly in the crustacean brain and CoG tissue sections while obtaining the structural information about these biomolecules. In total, 39 known neuropeptides were identified *in situ* from the blue crab *C. sapidus* brain tissue by the multiplex-DDA-MSI method, including a novel RFamide neuropeptide, which highlights its utility for large-scale *in situ* peptidomic analysis. In summary, the multiplex-DDA-MSI method with fractionating *m/z* range expands the capability and analytical performance of MALDI-MSI. It is capable of simultaneous distribution mapping, biomolecule identification, and novel molecule discovery. This novel platform has great potential to be widely applied to a variety of tissue types and target molecules. This work will benefit the research field of tissue imaging and stimulate future investigations of signaling biomolecules that may span a wide mass range and dynamic range.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Illustration of 9-step multiplex MSI experiment with DDA

The number indicated the sequence of spiral plate movement. A raster step size of 150 μm and a spiral step size of 50 μm were used. Steps 1, 4 and 7 were full MS scans at m/z ranges of 500–840, 840–1190 and 1190–1750 respectively. Steps 2/3, 5/6 and 8/9 were data dependent MS/MS scans of the top 2 most abundant ions detected in the previous full MS scans. The exact mass fractions for full MS scans can be varied for different experimental setup.

Figure 2. Comparisons between linear DDA MSI and spiral DDA MSI

(a, b) Illustrations of linear DDA MSI (a) and spiral DDA MSI (b), each with a step size of 50 μm . Step 1 was a full MS scan and steps 2, 3, 4 were data dependent MS/MS scans of the top 3 most abundant ions detected in step 1; (c, d) MSI result of total ion count (TIC) on CoG tissue sections for linear DDA MSI (c) and spiral DDA MSI (d); (e, f) neuropeptide (HL/IGSL/IYRamide) distribution at m/z 844.4788 $\pm$ 5ppm for linear DDA MSI (e) and spiral DDA MSI (f); (g, h) neuropeptide (VSHNNFLRFamide) distributions at m/z 1132.6010 $\pm$ 5ppm for linear DDA MSI (g) and spiral DDA MSI (h).

Figure 3. 9-step multiplex MSI results obtained from the CoG tissue of the blue crab *C. sapidus* (a) Full MS spectrum of CoG neuropeptide profile with spatial resolution of 50 μm . The spectrum was zoomed in at m/z 800–1600 and averaged over 5 scans. Annotated peaks were color coded with corresponding neuropeptide families based on accurate mass matching. Six additional peaks were identified from m/z 1600–2000, which were not shown in the spectrum. (be) annotated MS/MS spectra and MSI distributions of APSGFLGMRa (b), FDAFTTGFGHS (c), GYRKPPFNGSIFa (d) and NFDEIDRSSFG (e).

Figure 4. Comparisons among regular spiral DDA, even fraction mass DDA and uneven fraction mass DDA from the brain tissue of blue crab *C. sapidus*

(a) Comparisons of numbers of precursor ions selected by DDA under different setup within m/z ranges of 500–840, 840–1190 and 1190–1750. (b, c) Precursor ions selected for DDA (highlighted in red) under regular spiral DDA condition (b) and fraction mass DDA condition (c).

Figure 5. MS/MS spectrum and on-tissue distribution image of the novel neuropeptide obtained using fractionated mass multiplex-DDA-MSI method from the brain tissue of blue crab *C. sapidus*

Neuropeptide sequence: DL RTPALRLRFamide (m/z 1356.8223).

Table 1

Precursor ion list for targeted multiplex-MSI on blue crab CoG tissue section.

Step #	<i>m/z</i>	Sequence
1	Full scan	-
2	649.367	RYLPT
3	844.479	HL/IGSL/TYRamide
4	905.514	PSMRLRFamide
5	934.493	APSGFLGMRamide
6	1186.516	FDAFTTGFGHS
7	1198.549	NFDEIDRSGFamide
8	1204.559	TSWGKFQGSWamide+Na ⁺
9	1381.738	GYRKPPFNGSIFamide