Notes

Mass spectrometric imaging of GABA in the *Drosophila melanogaster* adult head

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Abstract

*Drosophila melanogaster* is a model organism in neurodegenerative disease research. In neurodegenerative study, the direct spatial information of neurotransmitter such as γ-aminobutyric acid (GABA) in the brain of *Drosophila melanogaster* is important to understand the role of GABA. Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) is an attractive method for direct visualization of neurotransmitters. In this paper, we describe methods to visualize GABA in the brain and head of *Drosophila melanogaster* using MALDI-IMS.

**Keywords:** Imaging mass spectrometry, *Drosophila melanogaster*, GABA, on-tissue derivatization
Introduction

*Drosophila melanogaster* is widely used in studies of neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, repeat expansion diseases and amyotrophic lateral sclerosis, based on the many advantages compared to other model organisms.\(^1\) For example, the life cycle is short (10 days), gene manipulation methods have already been established, and it is relatively inexpensive to rear *Drosophila* as well as produce new strains.

As a major inhibitory neurotransmitter, \(\gamma\)-aminobutyric acid (GABA) has important roles in many behaviors and neurodegenerative diseases.\(^2\) While measuring the neurotransmitter concentration in the whole brain is important, visualizing the localization of neurotransmitters would provide critical information about its functions. Therefore, the information about the distribution of GABA in the head of *Drosophila melanogaster* can contribute to our understanding of this neurotransmitter.

Immunohistochemistry and fluorescent imaging methods are commonly used to study the spatial distribution of GABA in heads of *Drosophila melanogaster*. These conventional methods mainly visualize either the enzymes required to produce GABA (GAD1),\(^3\) or GABA transporters,\(^4\) instead of visualizing GABA directly. Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) is an attractive alternative as a direct imaging method. GABA has already been visualized in mouse brain sections using MALDI-IMS.\(^5\) However, this method has not been applied to *Drosophila*, since there are some obstacles to visualizing GABA in the *Drosophila* head. In MALDI-IMS, it is difficult to use samples that are fixed using methods such as formalin fixation and paraffin embedment (FFPE). During the FFPE process, small molecules are washed out. On the other hand, cryosectioning the head of *Drosophila melanogaster* is challenging, as it is very small in size (less than 1 mm\(^3\)).\(^6\) Another obstacle to visualization is the difference in hardness between the head and brain. In addition,
the *Drosophila melanogaster* head is covered by hard cuticles, and its cells, which lack intermediate filaments, are softer than mammalian cells. These features make it quite difficult to generate sections of the *Drosophila* head. Moreover, the GABA detection is also difficult due to low ionization efficiency in MALDI\(^5\) and the small amount. In this study, we developed a head sectioning method of *Drosophila melanogaster* and applied on-tissue derivatization to improve the GABA ionization efficiency.

**Experimental**

**Chemicals**

The matrix α-cyano-4-hydroxycinnamic acid (CHCA) and derivatization agent 2,4-diphenyl-pyranium tetrafluoroborate (DPP-TFB), *trans*-Ferulic acid (*trans*-FA) and 4-hydroxy-3-methoxycinnamic acid (CA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). GABA, ethanol, methanol, acetonitrile, formic acid, ultrapure water, 2-propanol, triethylamine, Mayer's hematoxylin solution, 1% eosin Y solution, xylene, and Softmount were purchased from Wako (Osaka, Japan). The embedding material 4% carboxymethyl cellulose (4% CMC) was purchased from Leica Microsystems (Wetzlar, Germany).

**Fly strain and sample collection**

Canton S, a wild type strain of *Drosophila melanogaster* (Bloomington *Drosophila* Stock Center, Bloomington, IN, USA), was reared at 25°C on standard food (0.7% agar, 10% glucose, 4% dry yeast, 5% cornmeal, 3% rice bran), and female *Drosophila melanogaster* were selected for experiments.
Tissue sectioning

Drosophila heads were dissected and immediately soaked in 70% ethanol. Then, the heads were placed into a mold filled with 4% CMC, which was subsequently frozen using liquid nitrogen. The CMC blocks were stored at -20˚C for 1 hour and then sectioned to a thickness of 15 µm using a cryomicrotome (CM1950, Leica, Wetzlar, Germany). The chamber temperature and sample holder temperature were -18˚C and -16˚C, respectively. We tried two sectioning methods, using either Cryofilm type C (10) (Leica, Wetzlar, Germany) or an anti-roll plate (Leica, Wetzlar, Germany). The sections were then thaw mounted onto indium-tin-oxide (ITO)-coated glass slides (100 Ω/m² without anti-peeling coating, Matsunami Glass, Osaka, Japan). For sections acquired using the cryofilm, double-sided conductive tape (Shielding Non-woven Fabric Tape, 3M, St. Paul, MN, USA) was utilized to attach the tissue sections onto the glass slides.

Sample preparation for IMS

DPP-TFB was dissolved in methanol to prepare 10 mg/mL stock solutions. DPP-TFB solutions used for derivatization contained 6 µL of the stock solution, 69 µL of 60% methanol, and 1 µL of triethylamine. After sectioning, on-tissue derivatization was performed with 50 µL of DPP-TFB solution deposited onto each section manually using an airbrush (PS-270, GSI Creos, Tokyo, Japan). The tissue sections were then incubated in a box saturated with the vapor of 50% methanol for 60 minutes to facilitate the derivatization reaction. To acidify the tissue sections, 20 µL of 10% acetic acid was sprayed onto each section.

Another derivatization method was also evaluated to recognize GABA structural isomers. A methanol solution consisting of 11.5 mg/mL CA and 4.25 mg of trans-FA was used as another derivatization reagent. The solution was also deposited onto each section manually using an airbrush. The tissue sections were then incubated in room temperature for 10 minutes.
To detect DPP-GABA and CA-GABA, we performed imaging experiment without matrix, however obtained ion intensity is dramatically low. However, once matrix was applied, we obtained much higher peak intensity. Although this method was different from reported papers,7,8 we proactively applied matrix using following procedures.

Matrix was deposited onto the tissue by the two-step matrix application method,9 which combines sublimation and spraying of the matrix solution. The CHCA was deposited using iMLayer (Shimadzu, Kyoto, Japan) at 250°C until obtaining a thickness of 0.5 µm was achieved. Following sublimation, 50 µL of matrix solution (10 mg/mL CHCA in a solution containing 30% acetonitrile, 10% 2-propanol, and 0.1% formic acid) was sprayed onto each section with a manual airbrush.

H&E staining

The tissue sections were soaked in hematoxylin for 1 min and washed with water for 1 min. The tissue sections were then placed in 1% eosin for 3 sec and washed with water for 1 min. Tissue sections were subsequently dehydrated in ethanol for 1 min; this was repeated 3 times. Sections were then immersed in xylene twice for 1 min. The tissue was sealed with coverslips (20 × 20 mm, Matsunami Glass, Osaka, Japan) using Softmount.

Imaging mass spectrometry

Imaging mass spectrometry (IMS) analyses were performed using iMScope TRIO (Shimadzu, Kyoto, Japan). This instrument has a sample chamber to observe with a microscope and a MALDI ionization source with an Nd:YAG laser (λ = 355 nm and 1 kHz) under atmospheric pressure. The tissue surface was irradiated with the laser, with 200 shots for each data point. Mass spectra for DPP derivatization and CA derivatization were acquired in the positive ion detection mode in the mass range of m/z 100-330 and 140-270, respectively. In the
MALDI-IMS experiments, constant voltages for the sample stage and detector were applied at 3.50 kV and 2.1 kV, respectively. The laser power was kept at a constant of 25 (arbitrary unit in iMScope TRIO). To obtain a DPP-GABA and CA-GABA signal, MS/MS analysis was performed using selected precursor ions of DPP-GABA ([M]+ 318.15) and CA-GABA ([M]+ 264.12).

Results and Discussion

Optimization and evaluation of sectioning method

Generating sections of Drosophila melanogaster head with good morphology is challenging because of the small size as well as the difference in hardness between the external and internal head anatomy. For IMS, sections with good morphology are critical for obtaining accurate spatial information. To our knowledge, there is no Drosophila head sectioning method standardized for MALDI-IMS, especially for detecting small metabolites. Here, we present an optimized the sectioning method appropriate for use in MALDI-IMS.

In the field of tissue sectioning, embedding materials are commonly used to help section small samples. However, Drosophila bristles prevent the attachment of embedding material to the tissue. We observed that Drosophila head tissue could attach well to 4% CMC following a short immersion in 70% ethanol, which might be due to the solubility of cuticular lipids in a polar solvent. To prevent the tissue damage from ice crystal formation, the CMC blocks containing Drosophila heads were frozen in liquid nitrogen. We then compared two sectioning methods, one using anti-roll plate and the other using cryofilm. In both methods, we could obtain sections with good morphology by optimizing the thickness and the temperature in microtome (Fig. 1A and 1B). We found that the method using cryofilm was less technically challenging. The cryofilm could attach to the tissue section surface and preserve the morphology. Hence better sections with their morphology preserved could be obtained using
this method (Fig. 1B).

We compared peak intensity of GABA (DPP-GABA described in the next section) in the tissue sections obtained by two different sectioning methods described above. Three biological replicates were performed. Even though the method using cryofilm yielded superior tissues with well-preserved morphology, the GABA intensity was significantly lower ($p < 0.01$) (Fig. 1C). Since this cryofilm acted as an insulator, it interfered with the electric field that introduces generated ions into the mass spectrometer. Therefore, the method using an anti-roll plate without films is more suitable for generating Drosophila head sections for MALDI-IMS analysis.

Visualizing GABA in the Drosophila melanogaster brain and head by DPP derivatization

The analysis of GABA by MALDI-IMS is difficult because GABA ionization efficiency is low and the ion peak of the matrix used to facilitate analytes desorption/ionization interferes with the GABA ion peak. To solve these problems, on-tissue derivatization is commonly used. First, we developed a MALDI-IMS method to detect DPP derivatized GABA (DPP-GABA) using a standard GABA solution. This method was performed to directly visualize DPP-GABA in Drosophila brain tissue sections; unfortunately, no signal was detected. This might be due to the inhibition of derivatization or ionization by the high lipid content of the Drosophila brain.

We then optimized the derivatization reaction conditions for Drosophila melanogaster. We facilitated the derivatization reaction by performing in 50% methanol vapor. Moreover, 10% acetic acid was sprayed to acidify the tissue to ensure that derivatized GABA maintained a positive charge. Using these procedures, the peak at $m/z$ 232.11 was clearly observed (Fig. 2A) which was the peak derived from DPP-GABA. In addition, the distribution
in the brain and head of *Drosophila melanogaster* was successfully visualized using MALDI-IMS (Fig. 2B and 2C) from the obtained product ion spectrum.

Following optimization of the sectioning and derivatization method, we interpreted the distribution of GABA in the *Drosophila* head. GABA was distributed all over the brain (Fig. 2B). It is known that GABA is produced by glutamic acid decarboxylase (GAD) and it is distributed all over the brain. Moreover, when we examined the entire head section, GABA was observed in the brain surrounding tissues and very low GABA signal was detected in the *Drosophila* retina (Fig. 2C). To meet its high energy and oxygen demand, *Drosophila* brain is supported by vessel from vascular system and tracheae from tracheal system. Previous study in *Drosophila* larvae suggested that GABA is secreted into the circulating hemolymph. This phenomenon may also occur in adult *Drosophila*.

**Specific Visualization of GABA in Drosophila melanogaster brain and head by CA derivatization**

We successfully visualized GABA using DPP derivatization. However, it is impossible to distinguish GABA from its structural isomers such as 2-aminobutyric acid, 2-aminoisobutyric acid, and 3-aminobutyric acid, using DPP reagent. This is because the m/z 232.11 is the fragment ion of DPP. According to Manier *et al.*, the isomers of GABA can be distinguished by using CA as derivatization method. When the authors derivatized GABA with CA, they obtained the precursor ion of m/z 264.12 and the specific product ion of m/z 191.07. Therefore, we also applied CA as another derivatization method to support our results (Fig. 3).

In CA-GABA measurement, the obtained product ion spectrum (Fig. 3A) and distribution of m/z 264.12 >191.07 in brain and whole head are shown in Fig. 3B and Fig. 3C, respectively. As predicted, we observed similar the distribution of GABA while applying CA and DPP derivatization method.
We further examined the product ions of $m/z$ 264.12 by scanning $m/z$ 140-270 in brain tissue. No specific peaks derived from 2-aminobutyric acid and 2-aminoisobutyric acid ($m/z$ 218.2) as well as 3-aminoisobutyric acid ($m/z$ 190.09) were observed (Fig. 3A). This result indicated that 2-aminobutyric acid, 2-aminoisobutyric acid, and 3-aminobutyric acid in *Drosophila* brain either not existed or existed in very low level (under detection limit).

Together, we confirmed the specificity of GABA in MALDI-IMS analysis. Since we could not detect 2-aminobutyric acid, 2-aminoisobutyric acid, and 3-aminobutyric acid in *Drosophila* brain, both DPP and CA can be used as derivatization methods to visualize GABA using MALDI-IMS.

**Conclusions**

To the best of our knowledge, we have developed the first optimized method for visualizing GABA in the *Drosophila* head using MALDI-IMS. Sections with well-preserved morphology were obtained using our sectioning method. GABA was visualized in the head of *Drosophila melanogaster* by applying on-tissue derivatization. We observed that GABA was distributed all over the head except for in the retina. In the future, MALDI-IMS can be applied in *Drosophila* research, including in work on neurodegenerative diseases, to examine the GABA level in the brain.
References

Figure Captions

Fig. 1 Comparison between sections taken with cryofilm and an anti-roll plate.

The comparison between sections taken with cryofilm (A) and using an anti-roll plate (B).
GABA intensity was compared between the two methods (C). Scale bar is 200 μm

Fig. 2 GABA distribution in head sections of *Drosophila melanogaster*.

(A) Obtained product ion spectrum of \( m/z \) 318.15 (DPP-GABA). (B) The distribution of DPP-GABA (\( m/z \) 318.15 > 232.11) in the brain of *Drosophila melanogaster* (pitch 20 μm). (C) Signal intensity map in the whole head (pitch 15 μm). The white line indicates the area of the *Drosophila* brain. The peak at \( m/z \) 232.11 (derivatization reagent side) was mainly observed in this derivatization. Scale bars: 200 μm.

Fig. 3 CA-GABA distribution in head sections of *Drosophila melanogaster*.

(A) Obtained product ion spectrum of \( m/z \) 264.12. In this product ion mass spectrum, peak at \( m/z \) 191.07 was specific peak to separate structural isomers of GABA. The distribution of CA-GABA (\( m/z \) 264.12 > 191.07) in the brain of *Drosophila melanogaster* (pitch 20 μm) (B) and in the whole head (pitch 15 μm) (C). The white line indicates the area of the *Drosophila* brain. Scale bars are 80 μm in (B) and 90 μm in (C).
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