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Application Note

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Life Science

Visualization of GABA in Brain of Adult *Drosophila Melanogaster* by Mass Spectrometry Imaging

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Life Science

1. Introduction

The *Drosophila melanogaster* (hereinafter, *Drosophila*) is known as a model animal which is used in various fields of biology. Among its advantages as a model animal, it has a short life cycle of about 10 days, gene recombination techniques have already been established, breeding and creation of new models are inexpensive, and there are no issues from the viewpoint of animal ethics. In particular, *Drosophila* is widely used as a model organism for researches on neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS), because genetic knowledge and gene recombination techniques have been established [(1)].

Neurotransmitters play important roles in neurodegenerative diseases. For example, it is known that γ-aminobutyric acid (GABA) influences various biological behaviors and neurodegenerative diseases as an inhibitory neurotransmitter [(2)]. Investigation of not only the concentration of GABA in the brain, but also its spatial localization, is essential for determining the functions of GABA in the brain. For this reason, we believe that information on the localization of GABA in the head of *Drosophila* is critical for understanding the functions of GABA in various disease models.

Although imaging techniques utilizing immunohistochemical staining and fluorescent labeling have been used to investigate the spatial localization information of GABA in the head of *Drosophila*, these conventional methods visualize the distribution of GABA indirectly, as they mainly visualize the GABA-forming enzyme GAD1 [(3)] or GABA transporters [(4)]. Therefore, matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI), which enables direct detection of target molecules by mass spectrometry, has attracted attention in recent years as a technique for visualization of biomolecules in non-mammalian model organisms.

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Fig. 1 shows the workflow of MALDI-MSI. In this technique, the sample material is sliced, molecules in the resulting sample section are desorbed and ionized together with an ionization aid called a matrix by irradiation with a laser, and those molecules are then detected by mass spectrometry. The flow of the series of processes in laser desorption and ionization, and mass spectrometry is conducted two-dimensionally on the tissue, and a two-dimensional image showing the spatial distribution of the molecules is acquired by combining the positional information and the intensity information of the *m/z* extracted from the mass spectra. Since MALDI-MSI does not generally require fluorescent labeling or other visualization tags, and the molecules themselves are ionized and detected by mass spectrometry, this technique is considered useful for direct visualization of GABA in the head of the adult *Drosophila*. In a previous study, another group successfully visualized GABA in a mouse brain by using MALDI-MSI [(5)].

However, until now, several problems have prevented the application of MALDI-MSI to visualization of GABA in the head of the adult *Drosophila*. First, MALDI-MSI analysis of samples fixed by methods such as formalin fixation and paraffin embedment (FFPE) is difficult because the metabolites of small molecules like GABA are washed out in the process of deparaffinization. It is also difficult to prepare the cryosections generally used in MALDI-MSI analysis due to the very small size (≤ 1 mm^3) of the head of the adult *Drosophila* [(6)]. In addition to the problem of sample size, the *Drosophila* head is also covered with hard cuticles. There are no intermediate filaments in the brain cells in those hard cuticles, and because the brain cells are soft in comparison with mammalian brain cells, the hardness of the head and brain differs greatly.

Due to these features, it is extremely difficult to prepare sections of the head of the adult *Drosophila* while maintaining its morphology. Cryo-tape (Leica) is generally used to recover sections of this type of sample. However, commercially-available cryo-tape consists of an electrically nonconductive film, which greatly decreases the ion intensity at the surface of samples placed on a supporting medium of such material. The causes of this phenomenon are considered to be charge-up on the sample surface due to the presence of an insulator and disturbance of the electrical field on the sample plate.

Moreover, ionization efficiency of GABA in MALDI is low, and the amount of GABA in the *Drosophila* head is small in comparison with mammals, as can be imagined from the relative sizes of their brains. These are also considered to be obstacles to visualization of the distribution of GABA in the *Drosophila* brain. Thus, even though direct ionization and MALDI-MSI analysis of GABA in rodents' brains was possible, it is not clear whether GABA can be detected in the head of the *Drosophila*.

This Application Note presents a commentary on the technique for visualization of the spatial distribution of GABA in the brain of the *Drosophila* that was reported previously by our group [(7)]. In particular, we examined a method for preparing sections of the head of the adult *Drosophila* to solve the above-mentioned problems and proposed a technique for direct recovery of tissue sections, and also optimized the on-tissue derivatization technique to improve GABA ionization efficiency. In addition, this Application Note also presents a summary of current on-tissue derivatization methods, including their distinctive features.

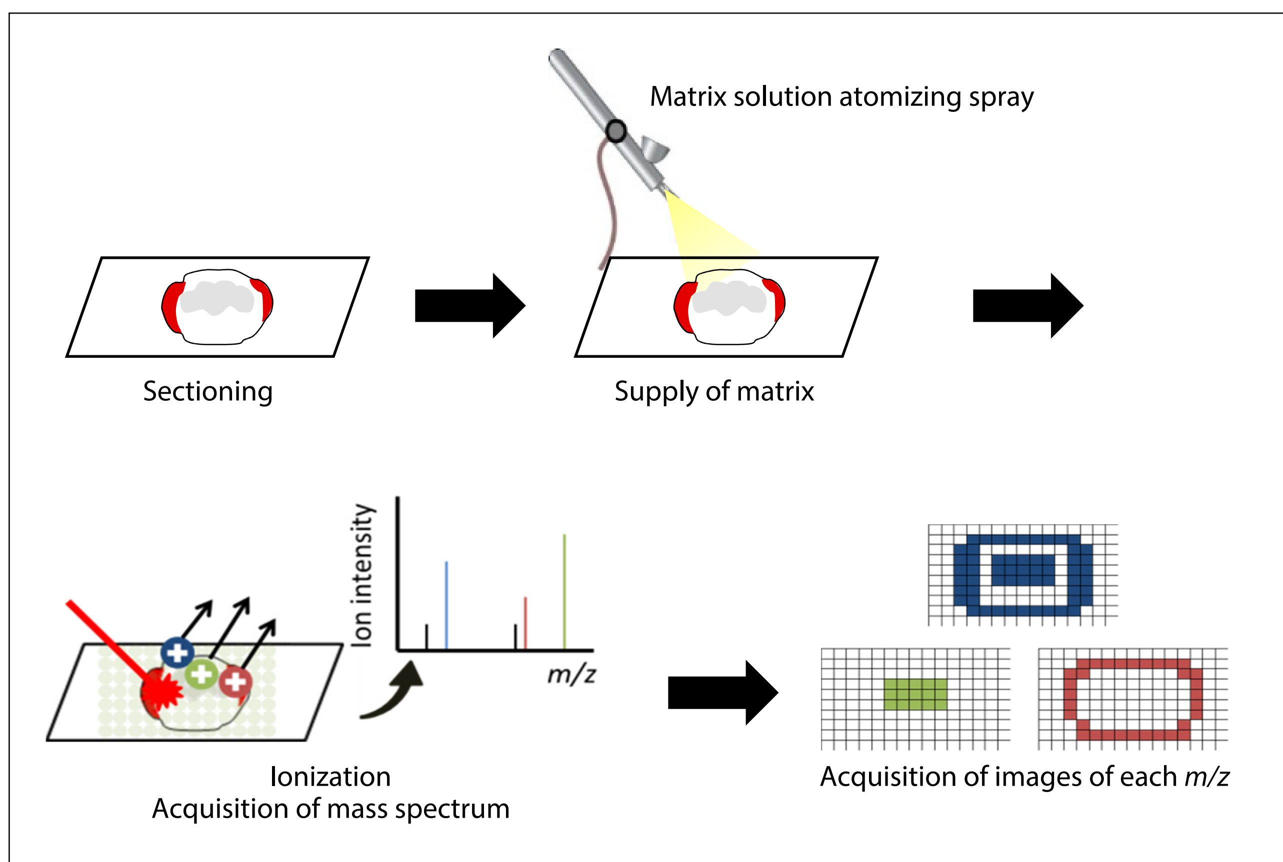


Fig. 1 Workflow of MALDI-MSI

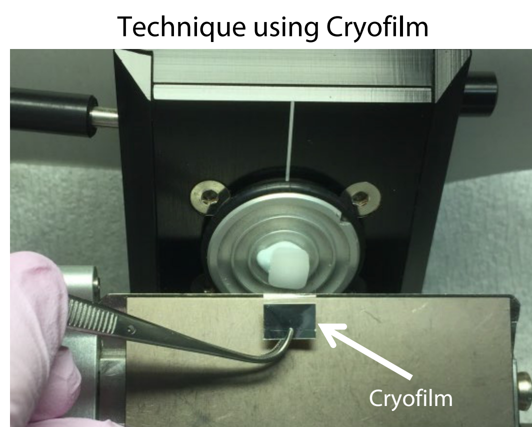
2. Sample Preparation Method

2-1. Sectioning

Drosophila heads were dissected with forceps and immediately immersed in 70% ethanol. The heads were then embedded in a disposable mold (base mold, As One Corp.) filled with 4% carboxymethyl cellulose (4% CMC). After embedding, the molds were wrapped in aluminum foil and quick-frozen using liquid nitrogen. After allowing the frozen samples to stand at -20 °C for 1 h, the samples were sectioned to a thickness of 15 µm using a cryostat (CM1950, Leica). The temperatures of the cryostat chamber and the sample holder were set to -18 °C and -16 °C, respectively.

We examined two sectioning methods, one using Cryofilm and the other using an anti-roll bar (Fig. 2). The sections prepared by these two respective techniques were mounted on glass slides with a vapor-deposited coating of indium-tin-oxide (ITO), a transparent, electrically conductive metal oxide (ITO-coated glass slides, Matsunami Glass) and dried under conditions of 40 °C for 5 min using an incubator. The sections acquired with the Cryofilm were fixed on the ITO-coated glass slides using a double-sided conductive tape (3 M).

Technique using Cryofilm



Technique using anti-roll bar

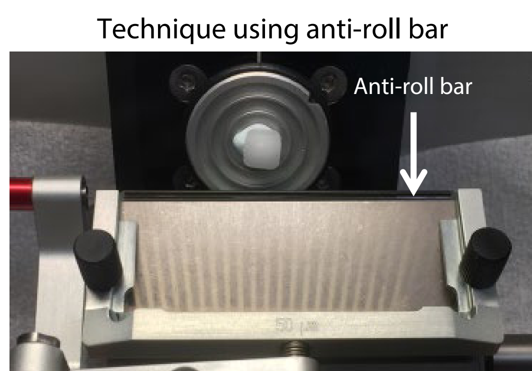


Fig. 2 Sectioning Methods

2-2. On-Tissue Derivatization

As derivatization reagents, 2,4-diphenylpyranilium tetrafluoroborate (DPP-TFB)[8] was used and, to distinguish GABA from its structural isomers, 4-hydroxy-3-methoxycinnamic acid (CA) was also used[9]. A solution of DPP-TFB adjusted to 10 mg/mL with a methanol solvent was used as the stock solution, and was stored at -30 °C until used. The DPP-TFB solution was prepared using a mixture of 600 µL of ultrapure water, 900 µL of methanol, and 1 µL of triethylamine as a solvent, and was mixed with the stock solution so as to obtain a ratio of 69 : 6. The DPP-TFB solution was spray atomized by using an airbrush (Procon BOY PS270, Creos). Spray atomizing was done while maintaining a distance of 15 cm between the nozzle and the tissue surface. 50 µL of the solution was applied to each section. After spray atomizing, the samples were allowed to stand for 60 min in a chamber filled with 50% methanol vapor to accelerate the derivatization reaction. Following derivatization, 20 µL of 10% acetic acid was sprayed on each section to acidify the tissue.

The CA solution was prepared with 11.5 mg/mL of CA and 4.25 mg/mL of *trans*-Ferulic acid (*trans*-FA) using methanol as a solvent[9]. As in the case of the DPP solution, the CA solution was also spray atomized by airbrushing. After spray atomizing, the samples were allowed to stand at room temperature for 10 min for the derivatization reaction.

2-3. Matrix Supply for DPP-GABA Detection

The matrix supply method is extremely important when attempting to visualize the biomolecules contained in extremely small samples such as *Drosophila* heads by MALDI-MSI because aggregates of matrix crystals on the tissue become artifacts. To solve this problem, we used a two-step matrix application method consisting of a first step of vapor deposition of the matrix by iMLayer™ (Fig. 3) and a second step of spraying the matrix solution[10]. After the derivatization reaction, α-cyano-4-hydroxycinnamic acid (CHCA) with a thickness of 0.5 µm was vapor-deposited on the tissue surface using the iMLayer. During vapor deposition, the temperature of the boat containing the matrix powder was set to 250 °C. In the second step after vapor deposition with the iMLayer, 50 µL of a CHCA solution was sprayed on each section with an airbrush. The CHCA solution was adjusted to 10 mg/mL using a mixture of 0.1% formic acid and 60% ultrapure water, 30% acetonitrile, and 10% 2-propanol as a solvent.

Fig. 3 iMLayer™ Used in Vapor Deposition of Matrix

2-4. MSI Analysis Conditions

An iMScope *TRIO*™ microscope (Fig. 4) was used in MALDI-MSI. In imaging of small samples like those in this work, the combination of optical image capture under the microscope and MSI is very important. The iMScope *TRIO* is effective in applications of this type. The laser used in MALDI was Nd: YAG (wavelength: 355 nm, frequency: 1 kHz). The number of laser shots was set at 200, and the cumulative number was set at 1/pixel. The laser spot diameter was 1 (approximately 10 µm), the laser intensity 25, and the laser irradiation interval was 20 µm. The sample voltage and the detector voltage were 3.5 kV and 2.1 kV, respectively. The *m/z* ranges were *m/z* 100-330 for the DPP derivatization samples and *m/z* 140-270 for the CA derivatization samples, and the MS/MS analysis was conducted in the positive ion mode. For DPP-GABA and CA-GABA, the precursor ions were set to *m/z* 318.15 and *m/z* 264.12, respectively.

Fig. 4 iMScope *TRIO*™ Used in Analysis

3. Results and Discussion

3-1. Study of Sectioning Method

Previous sections have described the difficulty of sectioning the heads of adult *Drosophila* while maintaining a good morphology, which is due to the small size of the samples and the large difference in the hardness of the brain and the outer structure of the head. In MSI, sectioning while maintaining a good morphology in the sectioning process is critical, not only for visualizing the neurotransmitters but also for acquiring accurate information on the spatial localization of molecules. Since no sectioning methods for adult *Drosophila* heads suitable for MALDI-MSI of low-molecular weight molecules have been reported until now, a sectioning method suitable for MALDI-MSI was examined in the present work.

In tissue sectioning of microscopic samples like *Drosophila*, the sample is generally embedded and frozen. However, this technique has problems, as the bristly hairs that cover the body of *Drosophila* repel the embedding material, and stable sectioning is impossible due to the gap between the sample and the embedding material. Therefore, we confirmed the possibility of embedding with tight adhesion between the sample surface and the embedding material when the sample was embedded in 4% CMC after immersion in 70% ethanol. It is thought that wetting the epidermal cuticles with 70% ethanol made it possible to embed the samples without repelling the embedding material. To avoid tissue damage caused by the formation of ice crystals, the embedded samples were quick-frozen using liquid nitrogen. Following this, a comparative study was carried out by sectioning the adult *Drosophila* heads by two different methods, one using an anti-roll bar and the other using Cryofilm. Tissue sections with a well-preserved morphology were obtained successfully with both methods by optimizing the temperature in the cryostat chamber and the temperature of the sample holder (Fig. 5A, B). In particular, preparation of sections with a good morphology was easier with the technique using the Cryofilm because the film adhered to the sample and acted as a supporting body. However, as can be understood from the comparison of the peak intensities shown in Fig. 5C, the intensity of the peak originating from DPP-GABA was significantly lower in the sample acquired using the Cryofilm.

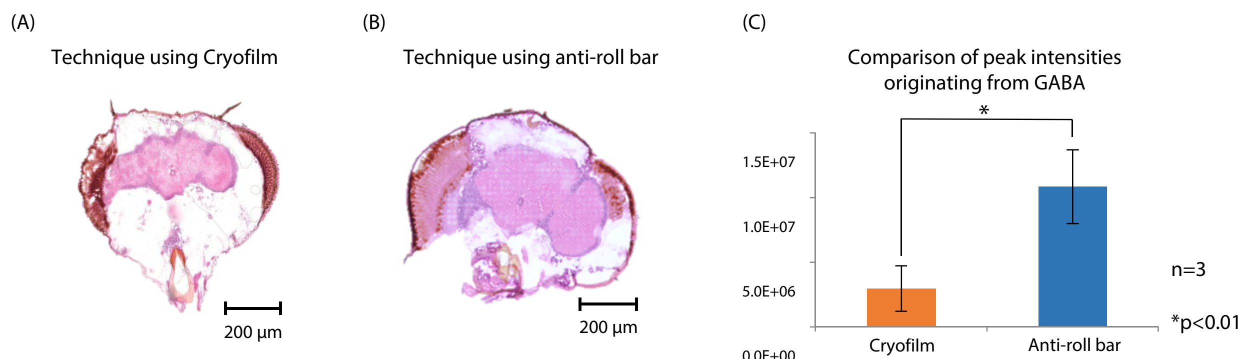


Fig. 5 Evaluation of Morphology by Sectioning Method and Evaluation of Peak Intensity Originating from GABA
(A) Section of *Drosophila* head acquired by Cryofilm technique
(B) Section of *Drosophila* head acquired by anti-roll bar technique
(C) Comparison of peak intensities originating from GABA acquired by two techniques

This difference is thought to be due to charge-up, which occurred because the Cryofilm is an electrical insulator, as noted previously, and disturbance of the electrical field caused by introduction of the formed ions into the mass separation part. Based on these results, we concluded that the technique using the anti-roll bar is the more appropriate sectioning method for MALDI-MSI of the adult *Drosophila* heads and used that method in the following experiments.

3-2. Visualization of GABA in Adult *Drosophila* Head and Brain by DPP Derivatization

Analysis of GABA in the *Drosophila* head by MALDI-MSI is generally considered difficult due to the small amount of GABA in the sample, interference by the peak originating from the matrix that supports desorption/ionization, and low GABA ionization efficiency. To solve these problems, we attempted to establish a MALDI-MSI analysis method for GABA derivatized with DPP (DPP-GABA) using a standard solution of GABA in order to enhance ionization efficiency by on-tissue derivatization. After the formulated DPP was sprayed on sections of the *Drosophila* head and the derivatization reaction proceeded at room temperature for 60 min, the matrix was supplied and visualization of the DPP-GABA was attempted. However, unlike studies with mice, adequate ion intensity could not be obtained with *Drosophila*. As one reason for this problem, it is thought that the high lipid content of the adult *Drosophila* brain may influence the derivatization reaction or ionization. In other words, further optimization is necessary in order to detect the minute amount of GABA in the fly brain.

Therefore, the conditions of the derivatization reaction were modified as follows: Derivatization was conducted in a chamber filled with 50% methanol vapor to accelerate the derivatization reaction, and the tissue was acidified by spraying with 10% acetic acid after derivatization to maintain a condition of positive ionization in the derivatized GABA.

As a result of these measures, a peak at *m/z* 232.11, i.e., the peak derived from DPP-GABA, could be clearly recognized (Fig. 6A), and the local distribution of the DPP-GABA-derived peaks in the adult *Drosophila* brain (Fig. 6B) and head (Fig. 6C) was successfully visualized by MALDI-MSI analysis. The results in Fig. 6 showed that DPP-GABA-derived signals are distributed throughout the entire *Drosophila* brain.

In *Drosophila*, it is known that GABA is formed from glutamic acid decarboxylase (GAD), and GAD is distributed throughout the entire brain. When the entire head part was analyzed, it was found that GAD also exists locally in the tissue surrounding the brain as well as the brain. To satisfy the high energy and oxygen demand of the *Drosophila* brain, the brain is supplied by the vascular and tracheal systems [11]. Previous research with *Drosophila* larvae suggested that GABA is secreted into the circulating hemolymph [12], and since this phenomenon also occurs in the adult *Drosophila*, GABA is presumably distributed throughout the brain in the adult.

3-3. Visualization of GABA in Adult *Drosophila* Head and Brain by CA Derivatization

As described in the previous section, visualization of GABA-derived signals was possible by using DPP derivatization. However, due to the existence of structural isomers of GABA, which include 2-aminobutyric acid, 2-aminoisobutyric acid, and 3-aminobutryic acid, this result alone does not support the conclusion that the distribution of GABA can be observed in isolation from its isomers. Since the peak of *m/z* 232.11 detected by MS/MS analysis, when DPP derivatization was used, is a fragment ion originating from DPP (Fig. 6A), GABA cannot be detected by distinguishing the above-mentioned structural isomers. However, according to a report by Maier *et al.*, GABA can be distinguished from its structural isomers and detected by using CA as the derivatization reagent [9].

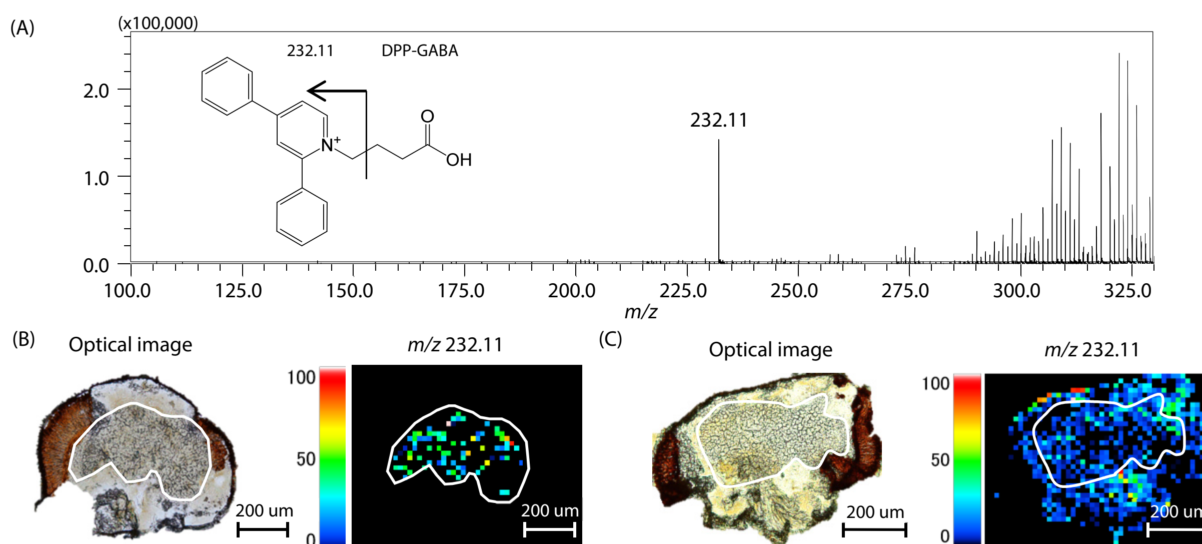


Fig. 6 Results of Mass Spectrometry Imaging of GABA in Brain and Head of *Drosophila* by DPP Derivatization
(A) Product ion spectrum obtained from brain using *m/z* 318.15 as precursor ion
(B) Distribution of DPP-GABA (*m/z* 318.15>232.11) in *Drosophila* brain
(C) Distribution of DPP-GABA in *Drosophila* head, scalebars: 200 µm

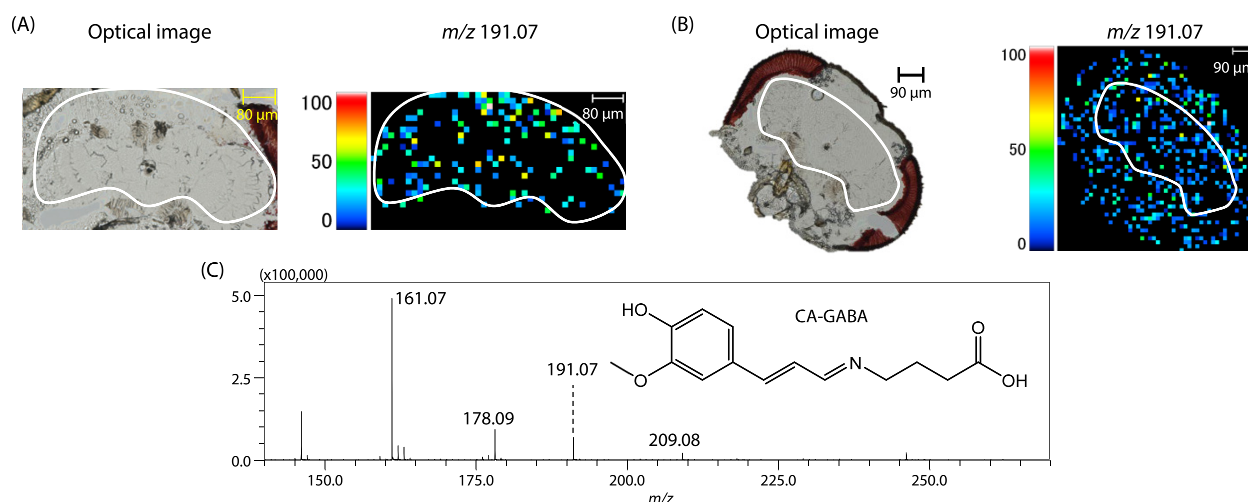


Fig. 7 Results of Mass Spectrometry Imaging of GABA in Brain and Head of *Drosophila* by CA Derivatization
(A) Distribution of CA-GABA (*m/z* 264.12>191.07) in *Drosophila* brain, scalebar: 80 µm
(B) Distribution of CA-GABA in *Drosophila* head, scalebar: 90 µm
(C) Product ion spectrum obtained from brain using *m/z* 264.12 as precursor ion
As reported in Reference 9, *m/z* 161, 178, 191, and 209 are detected

According to Reference 9, the product ion peaks of *m/z* 161, 178, 191, and 209 are detected when *m/z* 264.12 is selected as the precursor ion in the MS/MS analysis. Among these, *m/z* 191 and 209 are distinctive peaks that can be separated as structural isomers of GABA.

Therefore, the results obtained using DPP derivatization was validated, in this experiment, using the CA derivatization results. Fig. 7 shows the product ion spectrum obtained by CA-GABA analysis, together with the distribution of *m/z* 264.12>191.07 of the adult *Drosophila* brain and head. As in the DPP derivatization, GABA was also distributed throughout the entire brain and head section in the analysis by CA derivatization (Fig. 7A, B).

The product ion spectrum of *m/z* 264.12 obtained on *Drosophila* brain sections was also verified. As a result, the *m/z* 218.2 peak, which is considered to derive from 2-aminobutyric acid and 2-aminoisobutyric acid, and the *m/z* 190.09 peak, which is considered to derive from 3-aminobutyric acid, were completely absent from the detection results. Based on this result, it can be presumed that 2-aminobutyric acid, 2-aminoisobutyric acid, and 3-aminobutyric acid do not exist in the brain of the adult *Drosophila*, or they exist in infinitesimal amounts no more than the detection limit of the iMScope *TRIO*.

Based on the similarity of the distributions of DPP-TFB and CA, together with the fact that 2-aminobutyric acid, 2-aminoisobutyric acid, and 3-aminobutyric acid were not detected in the brain of the adult *Drosophila*, it can be thought that this experiment was successful in specifically visualizing the distribution of GABA in the brain of *Drosophila*. These results confirmed that the on-tissue derivatization method is essential in visualization of GABA in the head of *Drosophila* by MALDI-MSI.

4. Current Status and Distinctive Features of On-Tissue Derivatization Method

Derivatization is a technique that is generally used in GC, LC, GC-MS, and LC-MS. The purpose of use in those analytical methods is to change the polarity or volatility of the substance being measured by introducing a functional group, or to enhance resolution or detection sensitivity by introducing a functional group that emits fluorescent light. In MSI, the specimen surface is ionized directly, and the formed ions are detected without undergoing chemical separation like that in chromatography. However, as one problem of the MSI process, ionization of more-readily ionized molecules suppresses ionization of the target molecules, and thus the range of detectable molecule species are greatly reduced. One reason why many studies using MSI focus on detection of phospholipids, even today, is thought to be the fact that phospholipids are present in large quantities on the tissue surface and can be detected with high sensitivity because their molecules contain a polar group with a fixed charge. On the other hand, due to the above-mentioned ionization suppression effect, detection of the molecules directly from the tissue without derivatization is sometimes difficult with certain types of substances. In addition to the neurotransmitters examined in this paper, these include amino acids and the steroid hormones, which are medium-polarity substances. Various on-tissue derivatization methods for solving this problem have been reported recently. Table 1 shows a summary of several representative derivatization reagents.

First, in addition to the DPP and CA discussed in the present report, TAHS may be mentioned as a reagent for derivatization of amino groups [13), (14)]. TAHS enhances ionization owing to the fixed positive charge of the trimethylamine in its molecular structure. As described in Reference 14, derivatization was carried out by overnight incubation under a condition of 55 °C, after which MALDI-MSI was conducted by spraying 2,5-dihydroxybenzoic acid [(14)].

Derivatization of functional groups other than amino groups is also possible. One representative reagent is Girard's reagent T (GirT), which is used in MSI of steroid hormones [(15)].

Table 1 Examples of Derivatization Reagents Used in MSI

Structure	Name	Symbol	Object functional group	Features/References
	p-N,N,N-trimethylammonioanilyl N'-hydroxysuccinimidyl carbamate iodide	TAHS	Amino group	High reactivity. Not possible to separate structural isomers. Not for sale (requires commissioned compounding). References 13, 14
	2,4-diphenyl-pyranylium Tetrafluoroborate	DPP	Amino group	High reactivity. Not possible to separate structural isomers. No longer on sale (requires commissioned compounding). Reference 8
	4-hydroxy-3-methoxycinnamaldehyde	CA	Amino group	Possible to separate structural isomers. Reference 7
	Girard's reagent T	GirT	Carbonyl group	Mainly used in detection of steroid hormones. High reactivity with carbonyl at the C3 position. Possible to separate structural isomers. References 15, 16, 17
	4-(anthracen-9-yl)-2-fluoro-1-methylpyridin-1-ium	No published symbol	Phenolic hydroxyl group	Announced at the 2018 Annual Conference of the American Society for Mass Spectrometry. [19]

GirT has high reactivity for carbonyl groups at the C3 position, even in steroid hormones, and is effective in MSI of testosterone and corticosterone [16]-[18]. In MS/MS using GirT, mainly $[M-59]^+$ desorbed by trimethylamine is detected, and structural isomers can also be separated by further MS/MS of its peak (i.e., MS/MS/MS) (Fig. 8) [18]. As another functional group for derivatization, a reagent that derivatizes the phenol hydroxyl group has been also been announced. Although many of the details are currently unclear, as no papers concerning this reagent have been published at this point in time, this reagent reportedly displays extremely high reactivity, and the reaction is completed immediately after it is supplied to the tissue surface.

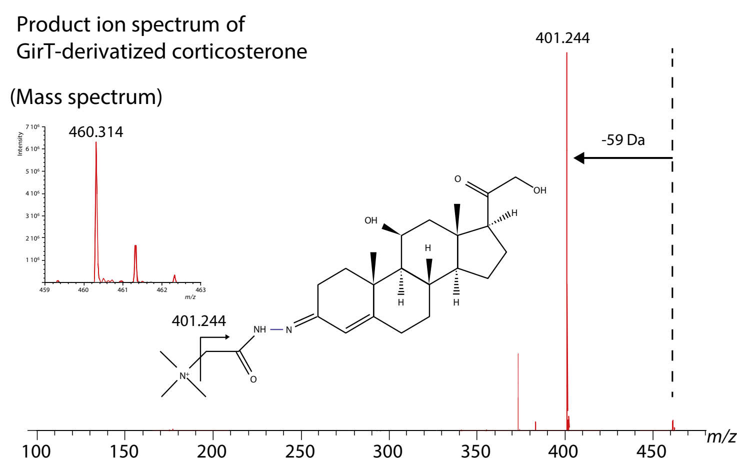


Fig. 8 Product Ion Spectrum of Corticosterone by GirT Derivatization
Detected in the mass spectrum as M^+. Desorption of trimethylamine is observed as a -59 Da peak in MS/MS.

5. Conclusion

Our group established the first method for visualization of GABA in the head of the adult *Drosophila melanogaster* by using MALDI-MSI. The dissected heads of adult *Drosophila* were embedded using 4% CMC, and these samples were quick-frozen using liquid nitrogen. We also succeeded in preparing sections of the adult *Drosophila* head with a well-preserved morphology by using an anti-roll bar. Although ionization of GABA is inherently difficult, we succeeded in visualization of the GABA in the head of adult *Drosophila* by utilizing on-tissue derivatization of GABA to enhance its ionization efficiency, and thereby confirmed that GABA is spatially distributed over the entire head of *Drosophila*. Visualization of the level of GABA in the brain of *Drosophila* as a model organism for neurodegenerative diseases by using this technique is expected in the future.

This paper also presented a summary of the on-tissue derivatization reagents now in use. With the advent of these new sample pretreatment methods, it has become possible to visualize the distribution of new biomolecules that could not be visualized until now. In the MSI field, active development of matrixes and new derivatization reagents is currently underway. In the future, our group also intends to create new sample pretreatment methods for application to various fields.

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