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### ► To cite this version:

David Touboul, Alain Brunelle. TOF-SIMS imaging of lipids on rat brain sections. Lin He. Mass Spectrometry Imaging of Small Molecules, 1203, Springer New York, pp.21-27, 2015, Methods in Molecular Biology, 978-1-4939-1356-5. 10.1007/978-1-4939-1357-2\_3 . hal-01170395

**HAL Id: hal-01170395**

**<https://hal.science/hal-01170395>**

Submitted on 2 Feb 2023

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## **TOF-SIMS Imaging of Lipids on Rat Brain Sections**

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## **Abstract**

Since several decades, Secondary Ion Mass spectrometry (SIMS) coupled to Time-of-Flight (TOF) is used for atomic or small inorganic/organic fragments imaging on different materials. With the advent of polyatomic ion sources leading to a significant increase of sensitivity in combination with a reasonable spatial resolution (1-10  $\mu\text{m}$ ), TOF-SIMS is becoming a more and more popular analytical platform for MS imaging. Even if this technique is limited to small molecules (typically below 1000 Da), it offers enough sensitivity to detect and locate various classes of lipids directly on the surface of tissue sections. This chapter will thus be dedicated to the TOF-SIMS analysis of lipids in positive and negative ion modes on rat brain tissue sections using a bismuth cluster ion source.

Keywords: Secondary Ion Mass Spectrometry, time-of-flight, mass spectrometry imaging, lipid, rat brain.

## 1. Introduction

Secondary Ion Mass spectrometry (SIMS) coupled magnetic double focusing sector field mass spectrometer was firstly used by Castaing and Slodzian in 1962 for microanalysis and mass spectrometry imaging (MSI) (1). Until the beginning of the 21<sup>st</sup> century, SIMS imaging was limited to atoms or small fragments analysis by the use of monoatomic primary ion sources ( $\text{In}^+$  or  $\text{Ga}^+$ ) even if high lateral resolution (until 10 nm) was achievable (2,3). Thanks to fundamental works in physics in the 90s (4,5), polyatomic primary ion sources have become commercially available. The first generation was based on gold clusters ( $\text{Au}_3^+$ ) allowing the detection of intact lipid species on rat brain sections in the positive and negative ion modes (6,7). At the same time, a second generation of cluster ion sources was developed. The first one was based on the vaporization of fullerene  $\text{C}_{60}$  powder and formation of singly and doubly charged  $\text{C}_{60}$  which are then focalized through an ion optic column at the sample surface. Even if the  $\text{C}_{60}$  source offers a very low surface damage and can be efficiently used for depth profiling, the best spatial resolution is restricted to 10  $\mu\text{m}$  in routine analysis (8). At the contrary, bismuth cluster ( $\text{Bi}_3^+$  and  $\text{Bi}_3^{2+}$ ) liquid metal ion gun (LMIG) offers a good compromise between sensitivity and lateral resolution (below 2  $\mu\text{m}$  in routine analysis using the high current bunched mode and down to 400 nm using the burst alignment mode) (9). The following protocol will describe how to prepare a rat brain section for TOF-SIMS imaging of lipids using a bismuth cluster ion source. A more detailed description of cluster TOF-SIMS imaging can be found in a tutorial paper (10)

## **2. Materials**

### **2.1 Chemicals**

1. Sodium pentobarbital
2. Optimal Cutting temperature (OCT) embedding medium
3. Dry ice

### **2.2 Instruments and materials**

1. TOF-SIMS IV (ION-TOF GmbH, Münster, Germany) or equivalent. The data acquisition and processing software is SurfaceLab 6.3 (ION-TOF GmbH, Münster, Germany) or later.

2. Cryostat (model CM3050-S; Leica Microsystems SA, Nanterre, France or equivalent).

3. Optical microscope (Olympus BX 51 fitted with x1.25 to x50 lenses (Olympus France SAS, Rungis, France) equipped with a Color View I camera, monitored by Cell<sup>B</sup> software (Soft Imaging System GmbH, Münster, Germany) or equivalent.

4. Desiccator.

5. Sample plate for TOF-SIMS imaging (conductive glass slide coated with indium-tin-oxide, stainless steel plate or silicon wafer).

### **3. Methods**

#### **3.1. Tissue sectioning**

All experiments on rat brains were performed in accordance with the protocols approved by the National Commission on animal experimentation and by the recommendations of the European commission DGXI.

1. Male Wistar rats of typical 400 g weight are euthanized by an intraperitoneal injection of sodium pentobarbital ( $> 65 \text{ mg.kg}^{-1}$ ).

2. The trimmed tissue blocks were immediately frozen in dry ice to prevent crack formation during freezing and stored at  $-80^\circ\text{C}$  prior to MS experiments (Note 1).

3. To fix the frozen tissue block to the cryostat adapter, you add a few drops of OCT embedding medium and quickly deposit the tissue block over it. The transparent OCT embedding medium is slowly becoming white when hardening.

4. Cut tissue section of  $8\text{-}15 \mu\text{m}$  thickness using the cryostat at  $-20^\circ\text{C}$ .

5. Deposit the tissue sections on either a conductive glass slide or stainless plate or a silicon wafer adapted to the TOF-SIMS holder (Note 2).

6. Store the samples at  $-80^\circ\text{C}$  in a box to prevent degradation by ice.

7. Before MS analysis, warm the sample at room temperature for few seconds and dry it under vacuum at a pressure of a few hPa during 30 min in a desiccator.

8. Take pictures of the sample using a microscope ( $\times 1.5$ ,  $\times 10$  and  $\times 20$ ). It is important to keep the same orientation of the sample between the microscope observation and the TOF-SIMS acquisition in order to easily correlate the images.

#### **3.2. Ion source optimization for high current bunched mode**

1. Introduce the sample holder in the mass spectrometer. Before starting the primary ion gun, the vacuum in the analysis chamber must be below  $2 \cdot 10^{-6}$  hPa. The vacuum read out is read directly on the vacuum control panel.

2. Open the Navigator, Analyzer and LMIG panel. The jpg image recorded by the microscope (or by a scanner) can be co-registered in the Navigator. The user can then navigate directly on the section and easily find small histological structures.

3. Open the Spectra and Image panel.

4. Switch on the high voltage required for the analyzer and LMIG.

5. Start the LMIG using the batch provided by the manufacturer. It allows an automatic switch on of the LMIG source. The operation takes about 10 minutes.

6. Measure the primary ion current using the Faraday cup provided on each sample holder. First of all, the primary ion beam is automatically centered in order to optimize the ion transmission through the ion optics. The ion current is then measured using the Direct Current (DC) mode. In this mode, the primary ions are neither bunched nor pulsed and all the primary ions ( $\text{Bi}^+$ ,  $\text{Bi}_3^+$ ,  $\text{Bi}_3^{2+}$ ,  $\text{Bi}_5^+$ ,  $\text{Bi}_5^{2+}$ ,  $\text{Bi}_7^+$  ...) are focused on the surface. Typical DC current between 12 and 15 nA is measured. The value of the heating current (about 2.85 A), emission current (about 1  $\mu\text{A}$ ), suppressor voltage (about 1000 V), extractor voltage (9000 V), lens source (about 3000 V) are always reported in the laboratory book in order to check any voltage deviation related to any source problem. The pulsed primary ion current is then measured for  $\text{Bi}^+$  (about 1.4 pA) and  $\text{Bi}_3^+$  (about 0.4 pA) using the pulsed mode and a cycle time analysis of 100  $\mu\text{s}$ . These values are necessary when calculating the primary ion dose density (also called 'fluence') before a MSI experiment.

7. Switch on the electron flood gun used for insulating samples.

8. In the positive ion mode, the primary ion beam is aligned with the optical images using a 'A-Grid', *i.e.* a metallic grid where the A-letter is drawn. For that purpose, X and Y-Target parameters need to be tuned in order to get a perfect match between the optical and ionic images.

9. Move the stage to the sample, adjust the Z-value, *i.e.* the distance between the sample and the extraction cone, in order to get the best sensitivity.

10. Adjust the Charge Compensation parameter directly on the sample surface. The Z value need to be checked and can vary from the 'A-grid'. For insulating samples, such as a

brain rat section, the optimization of this parameter is highly important in order not to significantly decrease the mass resolution. If needed, X and Y-Target parameters can be optimized on the tissue sample to reach a perfect match.

11. Redo steps 8 to 10 in the negative ion mode.

12. Spatial resolution can be checked by acquiring a profile on the 'A-grid'. A typical value is between 2 and 3  $\mu\text{m}$ .

12. Save all parameters before starting an imaging experiment.

### **3.3. TOF-SIMS imaging of lipids on a rat brain section**

1. Before any imaging experiments, a mass spectrum needs to be acquired on the sample surface with a very low dose in order not to damage sample surface.

2. Calibrate the mass spectrum. In the positive ion mode, the mass calibration is initially made with  $\text{H}^+$ ,  $\text{H}_2^+$ ,  $\text{H}_3^+$ ,  $\text{C}^+$ ,  $\text{CH}^+$ ,  $\text{CH}_2^+$ ,  $\text{CH}_3^+$  and  $\text{C}_2\text{H}_5^+$  ions. In the negative ion mode, the mass calibration is initially made with  $\text{H}^-$ ,  $\text{C}^-$ ,  $\text{CH}^-$ ,  $\text{CH}_2^-$ ,  $\text{CH}_3^-$ ,  $\text{C}_2^-$ ,  $\text{C}_3^-$ , and  $\text{C}_4\text{H}^-$  ions. To further improve mass accuracy, the mass calibration can be refined by adding ions of higher mass, such as fatty acid carboxylate ions and deprotonated vitamin E in the negative ion mode. Mass resolution of about 5000 at  $m/z$  500 can be achieved on the tissue section with mass accuracy of about 20 ppm over the complete mass range ( $m/z$  0-1000).

3. Some peaks of reference can be already selected in order to reconstruct the ion image during the acquisition and thus already get access to structural characterization of the tissue section. A rather complete list of lipid species already observed by TOF-SIMS on tissue section is available (**11**). Reference mass spectra of pure lipid compounds can also be found in the spectra library of SurfaceLab software.

4. Select the area of interest. When analyzing an area smaller than  $500 \times 500 \mu\text{m}^2$ , the sample is fixed and the primary ion beam is scanning the surface. For larger surfaces, a patch of  $500 \times 500 \mu\text{m}^2$  (or smaller) image is required. Before each acquisition, the primary ion dose is calculated in order to keep it below the limit of static SIMS ( $1 \times 10^{13} \text{ ions.cm}^{-2}$ ).



5. Typical TOF-SIMS imaging of lipids from a sagittal rat brain section are provided in Figure 1, as well as images of a 500  $\mu\text{m}$   $\times$  500  $\mu\text{m}$  area in Figure 2. (**12**).

#### 4. Notes

1. Formaldehyde fixation is always avoided when possible in order to prevent chemical modification of the lipid species (**13**).

2. Classical histochemistry (hematoxylin and eosin staining for example) is usually performed on adjacent tissue sections in order to visualize the different anatomical parts of the rat brain. It must be noticed that histochemistry can also be performed on the same tissue section that was used for TOF-SIMS experiments (**14**).

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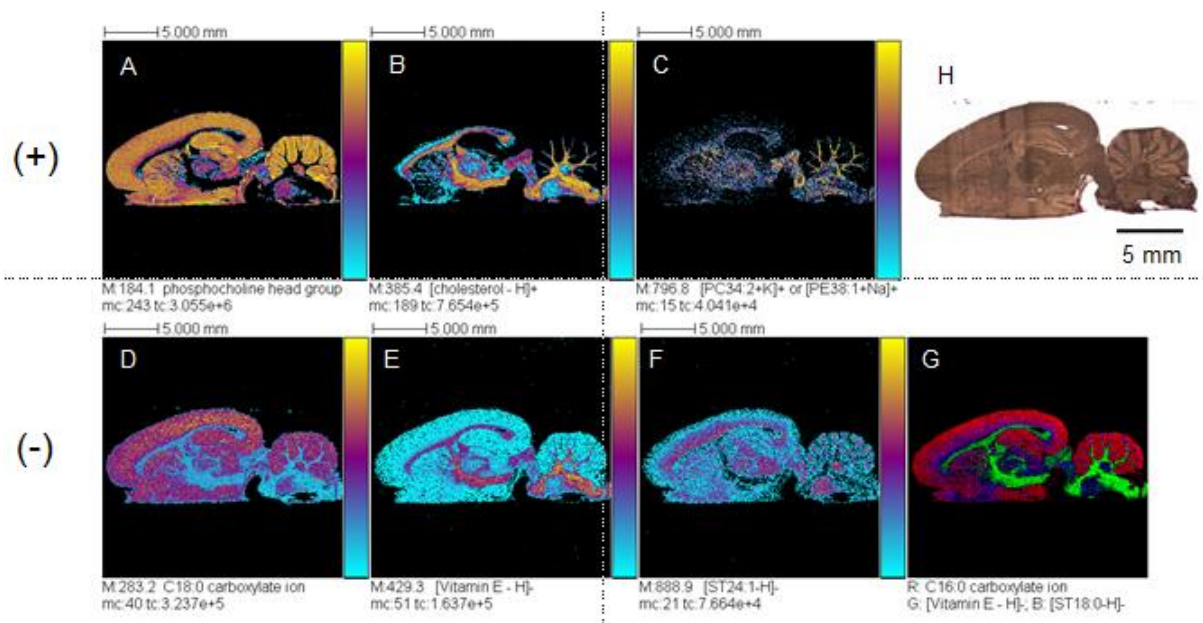


Figure 1: A to G TOF–SIMS ion images in the positive ion mode. A  $m/z$  184.1 (phosphocholine head group); B  $m/z$  385.4 ([cholesterol-H]<sup>+</sup>); C  $m/z$  796.8 ([PE 40:4+H]<sup>+</sup> or [PE38:1+Na]<sup>+</sup> or [PC34:2+K]<sup>+</sup>). D to G TOF–SIMS ion images in negative ion mode. D  $m/z$  283.2 (fatty acid carboxylate [C18:0-H]<sup>-</sup>); E  $m/z$  429.3 ([vitamin E-H]<sup>-</sup>); F  $m/z$  888.9 ([ST d18:1/24:1-H]<sup>-</sup>); G three-color overlay between TOF–SIMS ion images C16:0 carboxylate ion (red), vitamin E (green), and [ST d18:1/18:0-H]<sup>-</sup> (blue). For all TOF–SIMS images: primary ion Bi<sub>3</sub><sup>2+</sup>, 50 keV,  $3.4 \times 10^8$  ions.cm<sup>-2</sup>, area 22.4 × 22.4 μm<sup>2</sup>, 256 × 256 pixels, pixel size 87.5 × 87.5 μm<sup>2</sup>. The amplitude of the color scale corresponds to the maximum number of counts mc and could be read as [0, mc]. tc is the total number of counts recorded for the specified  $m/z$  (it is the sum of counts in all the pixels). H Optical ion image of the sagittal rat brain section (12).

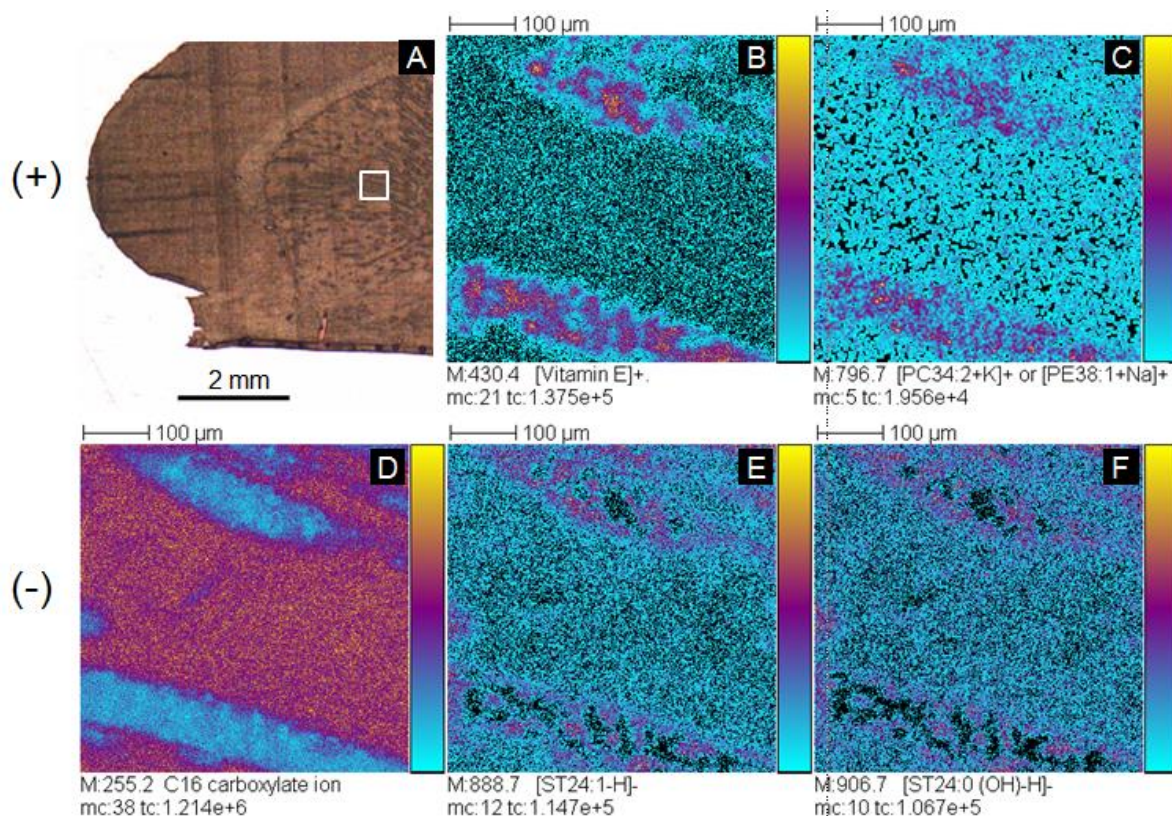


Figure 2: TOF-SIMS ion images of a sagittal rat brain section. (A) Optical picture of the sagittal rat brain section. Ions images where recorded in the square delimited in white. B, C: TOF-SIMS ion images in the positive ion mode. (B)  $m/z$  430.4 ([vitamin E]<sup>+</sup>). (C)  $m/z$  796.7 ([PE 40:4+H]<sup>+</sup> or [PE38:1+Na]<sup>+</sup> or [PC34:2+K]<sup>+</sup>). D to F: TOF-SIMS ion images in the negative ion mode. (D)  $m/z$  255.2 (C16:0 carboxylate ion). (E)  $m/z$  888.7 ([ST d18:1/24:1-H]<sup>-</sup>). (F)  $m/z$  906.7 ([ST d18:1/24:0-OH-H]<sup>-</sup>). Primary ion Bi<sub>3</sub><sup>2+</sup>, 50 keV,  $2.5 \times 10^{11}$  ions/cm<sup>2</sup>, area 500x500 μm<sup>2</sup>, 256x256 pixels, pixel size 2x2 μm<sup>2</sup>. The amplitude of the color scale corresponds to the maximum number of counts  $mc$  and could be read as [0,  $mc$ ].  $tc$  is the total number of counts recorded for the specified  $m/z$  (it is the sum of counts in all the pixels) (12).