Chapter 14

ToF-SIMS Parallel Imaging MS/MS of Lipid Species in Thin Tissue Sections

Anne Lisa Bruinen, Gregory L. Fisher, and Ron M.A. Heeren

Abstract

Unambiguous identification of detected species is essential in complex biomedical samples. To date, there are not many mass spectrometry imaging techniques that can provide both high spatial resolution and identification capabilities. A new and patented imaging tandem mass spectrometer, exploiting the unique characteristics of the *nanoTOF* II (Physical Electronics, USA) TOF-SIMS TRIFT instrument, was developed to address this.

Tandem mass spectrometry is based on the selection of precursor ions from the full secondary ion spectrum (MS^1), followed by energetic activation and fragmentation, and collection of the fragment ions to obtain a tandem MS spectrum (MS^2). The PHI *NanoTOF II* mass spectrometer is equipped with a high-energy collision induced dissociation (CID) fragmentation cell as well as a second time-of-flight analyzer developed for simultaneous ToF-SIMS and tandem MS imaging experiments.

We describe here the results of a ToF-SIMS imaging experiment on a thin tissue section of an infected zebrafish as a model organism for tuberculosis. The focus is on the obtained ion distribution plot of a fatty acid as well as its identification by tandem mass spectrometry.

Key words SIMS, Tandem MS, Imaging mass spectrometry, Lipids, Fatty acids

1 Introduction

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) analysis depends on a high energy focused ion beam that functions as a primary ion source ejecting analytes only from the top atomic monolayers of a sample surface, referred to as secondary ions. The secondary ions are separated in a time-of-flight mass analyzer based on their mass-to-charge ratio (m/z) before they hit the detector [1–3].

Traditionally, ToF-SIMS has been applied in the domain of surface physics and semiconductor industry, but over the last decade it has redirected toward biomedical applications as well. Especially in mass spectrometry imaging-based biological tissue analysis, one has to contend with a high level of complexity concerning the molecules that are found on the sample surface. In many cases, it is predictable and generally accepted which

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compounds are detected in specific samples. Although the mass resolution and mass accuracy in ToF analyzers have improved over the years, particularly for higher masses this is not sufficient for unambiguous peak identification.

Tandem mass spectrometry is considered the benchmark technique to identify and elucidate structures of unknown molecular species and distinguish isomers of which there are multiplicities in biological samples. Controlled fragmentation of ions after they are formed in the source of a mass spectrometer results in a unique fragment pattern for every compound and is therefore an extremely useful tool for identification and elucidation of molecules.

Matrix-assisted laser desorption/ionization (MALDI) is often the ionization technique of choice for tandem MS imaging in biological tissues, but has several inherent limitations compared to ToF-SIMS imaging. First, in contrast to ToF-SIMS, MALDI needs a homogeneously applied chemical matrix to absorb the laser energy, to extract and ionize compounds from the sample. This makes the sample preparation time consuming and results in the perturbation of the intrinsic molecular chemistry and loss of lateral resolution. Furthermore, photon ionization of the analytes in a MALDI ion source is very destructive and usually consumes the sample, whereas in ToF-SIMS only sub nanometer thick layers are removed from the sample surface. In addition, the typical spatial resolution of MALDI imaging is above 30 μm, ToF-SIMS imaging can reach submicron spatial resolution. Lastly, current instrument designs do not allow simultaneous collection of MS^1 and MS^2 imaging data sets.

Recently, a novel and patented PHI *NanoTOF II* TRIFT mass spectrometer was developed, equipped with a second time-of-flight analyzer designed for tandem MS imaging [4–6]. The ion optics of the first mass analyser allow selecting a precursor from the stream of generated ions to be analyzed in the tandem time-of-flight mass analyzer simultaneously. This makes the combination of surface screening with general MS imaging with targeted identification with tandem MS imaging possible (Fig. 1).

In this chapter, we show an example of a tandem TOF-SIMS imaging experiment performed on a whole-body section prepared from a diseased zebrafish. The fish was part of a study on tuberculosis and therefore infected with *Mycobacterium marinum* as a model for human tuberculosis infection. Without further preparation, the samples were analyzed in negative ion mode using a PHI *nanoTOF* II TOF-TOF mass spectrometer equipped with a liquid metal ion gun (Bi_3^+), a gas collision cell, and a second ToF detector. ToF-SIMS tandem imaging MS was used to localize and identify lipid species and fatty acids as well as the host membrane phospholipids that are believed to play an important role in the bacterial inflammation cascade.

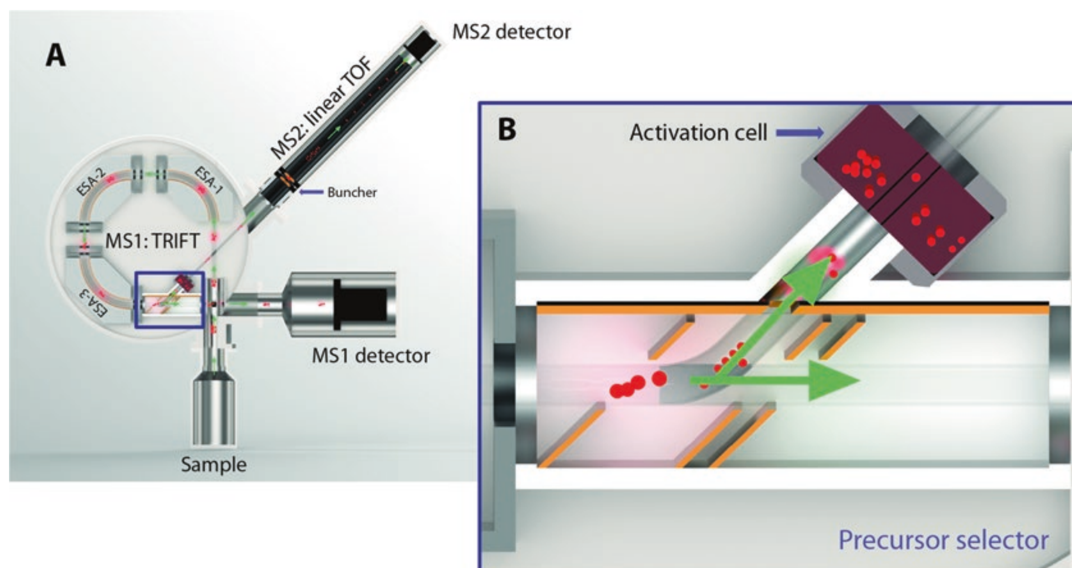

Fig. 1 (**a**) Schematic illustration of the parallel imaging MS/MS spectrometer with the MS1 (TRIFT) and the MS2 (linear TOF) analysers as well as their detectors annotated. The arrows indicate the nominal trajectories of the secondary ions (in red). The precursor selection device including the activation cell is framed in (**a**) and enlarged in (**b**)

2 Materials

2.1 Chemicals

1. Dietrich's fixative (30% ethanol, 10% formalin, 2% glacial acetic acid in deionized water).
2. Mixture of 5% carboxyl methyl cellulose (~90,000 Da polymer length) and 10% porcine gelatine, kept liquid at 37 °C.
3. Dry ice.
4. Ethanol.
5. Indium tin oxide (ITO)-coated glass slides.
6. Starfrost glass slides.
7. Acetone (at −20 °C).
8. Eosin.
9. Hematoxylin.
10. MilliQ water.
11. 95% ethanol.
12. Pure ethanol (96%).
13. Glycerol.
14. Cover slip.

2.2 Instrumentation

1. Cryotome at −35 °C.
2. Mirax slide scanner (Carl Zeiss).

3. PHI *nano*TOF II TOF-SIMS instrument (Physical Electronics, Minnesota, U.S.A.) equipped with a 30 kV Bi_n^{q+} cluster liquid metal ion gun (LMIG), a precursor selection device, a collision cell, and a parallel linear time-of-flight tube suitable to perform tandem mass spectrometry.

2.3 Software

1. The *nano*TOF MS/MS instrument is controlled by SmartSoft version 2.1.0 (PHI, USA).
2. TOF-DR version 1.1.0.1. (PHI, USA) was used for spectral and image analysis.

3 Methods

Zebrafish (*Danio rerio*) are considered a suitable model to study infectious diseases such as tuberculosis (TB) and to aid in the development of treatments [7]. *Mycobacterium marinum* bacteria cause tuberculosis in fish, and has a very similar disease process compared to *Mycobacterium tuberculosis*, which is responsible for tuberculosis infections in humans.

Foamy macrophages are loaded with lipids and use phagocytosis with the aim to inactivate the bacteria, but TB bacteria use these cells to replicate. That causes more macrophages to attack the infected ones and by that the formation of granulomas [8, 9]. The outcome of tuberculosis is different for every individual, bacteria strand, and possible treatment.

In this study, we seek to develop a way to visualize and reveal the content of tuberculosis granulomas. The mapping of molecular changes captured with mass spectrometry imaging gives more insight into the immune response of the host (zebrafish) toward the pathogen (*M. marinum*). Because the *nano*TOF system facilitates high lateral resolution molecular imaging, lipids within the granuloma structures can be visualized. Furthermore, because of the nondestructive nature of SIMS imaging, tandem MS imaging of the same sample is possible for multiple precursor ions repeatedly.

3.1 Sample Preparation

An adult zebrafish (*D. rerio*) was infected by *M. marinum* bacteria (*mptC* mutant strain [10]) and killed as part of an infection study [11].

1. Fix the fish chemically in Dietrich's fixative for >48 h at 4 °C. Fixation induces decontamination of all bacteria, which enables a safer working environment.
2. Take the fish out of the fixative and rinse it with MilliQ water a couple of times to get rid of excess fixative.
3. Embed the specimen in a mixture of 5% carboxyl methyl cellulose and 10% gelatin in a mold [12] (*see* **Note 1**).

4. Freeze the mold including the embedding material with the specimen in a 1:1 dry ice/ethanol bath. It takes about 15 min for the block to freeze, indicated by the ice turning white.
5. Leave the block in a freezer (−20 °C/−80 °C) overnight to completely solidify.
6. Prior to cutting, place the block containing the specimen in the cryotome at −35 °C for 1.5 h to adjust to the cutting temperature.
7. Fix the block to the cryotome stage with a droplet of warm 5% carboxyl methyl cellulose and 10% gelatine.
8. Cut 20 μm longitudinal tissue sections and thaw mount them on the conductive side of indium tin oxide-coated glass slides (*see* **Notes 2** and **3**).
9. Choose a tissue section collected on a conductive ITO slide showing multiple organs as well as severe infection areas for TOF-SIMS analysis. Prepare an adjacent section and thaw mount it on a Starfrost slide to have it stick better and make it suitable for histological staining.
10. Place the Starfrost slide in an acetone solution at −20 °C to fix for 5 min.
11. Perform a hematoxylin and eosin (H&E) staining on the adjacent section on a Starfrost slide to show contrast as follows:
    (a) Rinse the slide in MilliQ water.
    (b) Stain in hematoxylin solution for 30 s.
    (c) Rinse in MilliQ water for 30 s.
    (d) Wash in 95% ethanol for 30 s.
    (e) Stain in eosin for 30 s.
    (f) Rinse in pure ethanol (96%) for 2 min (two times).
    (g) Air dry the tissue.
    (h) Mount a cover slip with glycerol.
12. Scan the H&E stained slide with a Mirax slide scanner (Carl Zeiss). The image obtained from the described study is shown in Fig. 2a with annotations.

3.2 Tandem MS Imaging

The fundamentals of the modified PHI *NanoTOF II* tandem MS mass spectrometer used for this experiment are based upon the Triple Ion Focussing Time-of-flight (TRIFT) mass analyzer introduced by Schueler et al. in 1990 [13, 14]. It concerns a three-sector mass spectrometer that separates ions based on their mass-to-charge ratio (m/z). A 30 kV Bi_n^{q+} cluster liquid metal ion gun (LMIG) produces secondary ions by striking the sample surface and sputters them off.

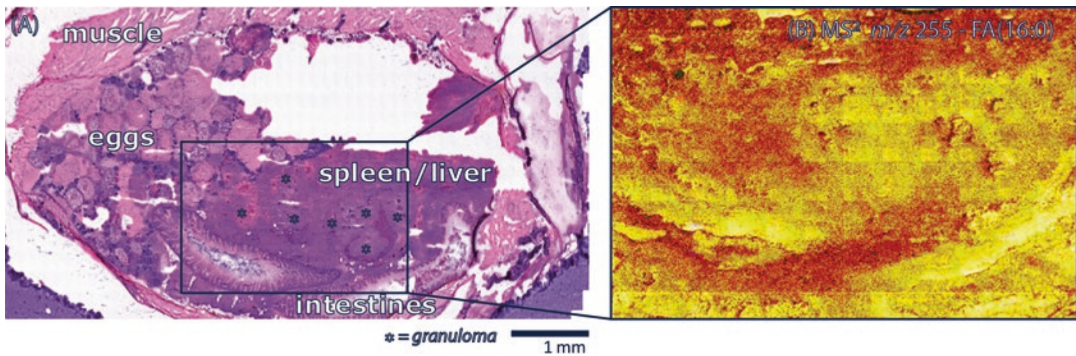

Fig. 2 (**a**) Histology of the intestine area of a zebrafish infected by Mycobacterium marinum. The different organs are annotated by their names, and several granulomatous structures appearing in the spleen and liver area are annotated by an asterisk. The box indicates the measurement region containing several granulomata from which an ion distribution plot is generated. (**b**) Logarithmic scale MS^2 mosaic map of the total ion count (TIC) of an infected zebrafish spleen area, containing the selected precursor (m /z 255) and its fragments. The dimensions are 4.8 mm × 3.2 mm

To achieve tandem MS imaging, a PHI *nanoTOF* II (Physical Electronics, USA) TOF-SIMS TRIFT analyzer was modified. The original blanker located after the existing mass separation path of the secondary ions through three electrostatic analyzers (ESAs) was replaced by a precursor selection device. This is an assembly of three electrode plates of which the two outer ones are held at ground voltage, while the middle electrode can be pulsed electrically. In the middle of every plate a small aperture is located, through which the secondary ions travel after mass-to-charge separation. The timing of the pulse of the middle electrode is crucial. To pick ions with a particular mass from the secondary ion stream, the second electrode generates a pulse exactly when the ions are located between the first outer and the middle electrode. Only then precursor ions can be deflected parallel to the electrode plates, 45° from the secondary ion stream, into the fragmentation cell and MS2 mass analyser, while the rest of the secondary ions travel through toward the MS1 detector. The precursor selection device is able to deflect ions of a specific *m/z* from 0 up to 100% of the pulses passing by, but also functions as a post ESA blanker and deflect a larger mass range.

To provide fragmentation, the deflected precursor ions are guided through a collision cell filled with argon. This collision-induced dissociation (CID) produces fragment ions, but also energy spread among them. To correct for this, a buncher constructed of two parallel grids is placed right after the collision cell. The grids are kept at ground potential, until a pulse of fragment ions is present in the space between the grids. A voltage is then applied to the first grid to accelerate the ions toward the direction of the second grid. Mass-to-charge separation of the accelerated

fragment ions is accomplished in a field free region followed by a dual multichannel plate (DMCP)-based detector, referred to as the MS2 detector. This setup is similar to the MS1 detector.

1. Optimize the 30 keV bismuth LMIG on the PHI *nanoTOF* II for molecular imaging. Select Bi_3^+ clusters and operate the gun in bunched mode for best spectral quality. A typical tuning copper grid (mesh 25 μm) on an aluminum substrate can be used to optimize the beam current as well as the image quality by fine-tuning the focussing lenses in the gun.
2. Make sure the sample bias is at the optimal voltage and the Z-height is correct as well for the specific sample by starting an acquisition. Both parameters are optimal when the secondary ion yield is highest and hit the middle of the detector. The ion count is easily visible in the SmartSoft software that is linked to the CCD camera in the detector assembly.
3. While acquiring from the sample of interest, it is very important to calibrate the MS^1 spectrum before moving toward tandem MS experiments. This can be done online in the TOF-DR software with the use of known peaks (*see* **Note 4**).
4. To perform tandem MS, open the gas leak valve to the CID activation cell to fill it with argon gas. The desired precursor peak can be selected in the SmartSoft software, thereafter the approximate values concerning the selection timing and post ESA slope are set. However, in most cases, these settings need to be fine tuned for each different m/z value. For the described experiment m/z 255.230 was selected, the post ESA slope was set at 2.0487, the picker voltage at 2780, the picker width at 0.702, and the buncher at 1300 V.
5. To be able to generate molecular images, the primary ion beam is rastered over a 400 μm × 400 μm area (tile), containing 256 × 256 pixels. The whole measured area is 4.8 mm × 3.2 mm in size, containing 12 × 8 of these tiles, exposed for five frames each.
6. The acquisition is saved as a single .raw file, containing both MS^1 and MS^2 data acquired simultaneously. In the TOF-DR software it is possible to playback a measurement and select single mass channels to generate two-dimensional ion distribution plots.

The precursor selection device was set to deflect only ions with a mass-to-charge ratio of 255 to the MS2 detector. The image of the total MS^2 ion count is shown in Fig. 2b, representing the spatial distribution of all ions with m/z 255.

The molecular fragments originating from the m/z 255 precursor are captured in the tandem MS spectrum shown in Fig. 3. Since the precursor peak is the most abundant peak in the spectrum, the mass region representing the most important fragments

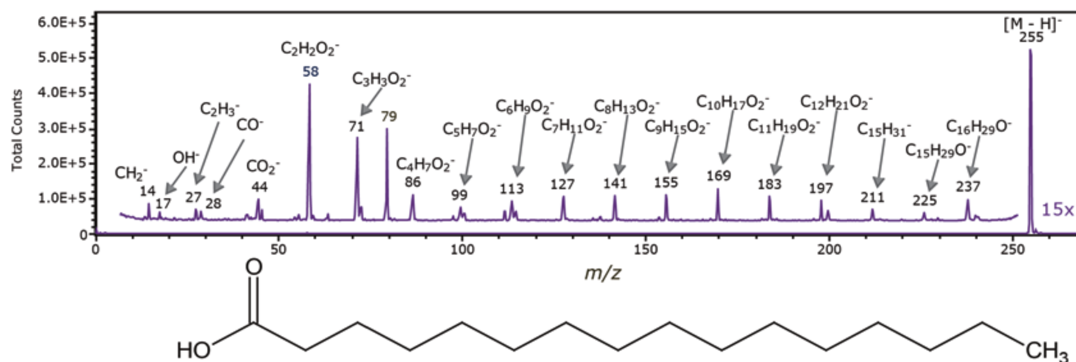

Fig. 3 CID product ion spectrum of the m /z 255 precursor produced from the surface of a thin tissue section sampled from a M. Marinum infected adult zebrafish. The precursor was identified as palmitic acid (FA (16:0)) of which the structural formula is shown below the tandem MS spectrum

is zoomed in. The typical CH_2 loss sequence shows that this spectrum undeniably originates from the long carbon chain of a fatty acid, namely palmitic acid.

4 Notes

1. Embedding of a zebrafish is carried out nicely in an ice cube tray. Make sure there is enough space around the specimen for embedding material to prevent defrosting when taken out of the freezer. To keep the fish straight, it is recommended to pour in a layer of embedding mixture first and let it solidify at room temperature. Then place the fish on its side on the top of the material and cover it with more embedding mixture.
2. Longitudinal tissue sections give a more representative overview of different organs, but the cutting of the block is easier in a vertical manner (starting at the long side of the fish) to prevent ruptures inside the tissue section. This is due to the fact that fish contain cavities filled with air, among others a swim bladder. Although 20 μm sections seem to be thick for imaging mass spectrometry, it is the optimal thickness to keep the morphology of the fish intact. Cutting a whole organism is challenging since one has to deal with a variety of tissue densities.
3. When cutting multiple consecutive tissue sections, it is recommended to leave the block to cool for about 10 min frequently. The surface temperature rises by touching it with the knife during cutting, which has a negative influence on the section quality.
4. When analyzing organic molecules from the sample surface, it is advisable to calibrate the spectrum with known organic

peaks. For biological tissue sections typical peaks are *m/z* 15.0235 (CH_3), 27.0235 (C_2H_3), 41.0391 (C_3H_5), 55.0547 (C_4H_7), 69.0704 (C_5H_9), and 86.0969 ($C_5H_{12}N$), 184.0737 ($C_5H_{15}O_4NP$). In negative mode the following *m/z* are very suitable: 15.9949 (O), 26.0031 (CN), 41.9980 (CNO), and 78.9585 (PO_3).

References

1. Pachuta SJ, Cooks RG (1987) Mechanisms in molecular SIMS. Chem Rev 87(3): 647–669
2. Castaing R, Slodzian G (1962) Microanalyse par emission ionique secondaire. J Microsc 1:31–38
3. Werner HW (1975) The use of secondary ion mass spectrometry in surface analysis. Surf Sci 47(1):301–323
4. Larson, PE, et al., (2013) Method and apparatus to provide parallel acquisition of mass spectrometry/mass spectrometry data. 2013: United States.
5. Fisher GL et al (2016) Parallel imaging MS/MS TOF-SIMS instrument. J Vac Sci Technol B 34(3):03H126
6. Fisher GL et al (2016) A new method and mass spectrometer design for TOF-SIMS parallel imaging MS/MS. Anal Chem 88(12): 6433–6440
7. van der Sar AM et al (2004) A star with stripes: zebrafish as an infection model. Trends Microbiol 12(10):451–457
8. Toossi Z (2000) The inflammatory response in mycobacterium tuberculosis infection. Arch Immunol Ther Exp (Warsz) 48:513–519
9. Adams DO (1976) The granulomatous inflammatory response. A review. Am J Pathol 84: 164–192
10. Stoop EJM et al (2013) Mannan core branching of lipo(arabino)mannan is required for mycobacterial virulence in the context of innate immunity. Cell Microbiol 15(12):2093–2108
11. van der Sar AM et al (2004) Mycobacterium marinum strains can be divided into two distinct types based on genetic diversity and virulence. Infect Immun 72(11):6306–6312
12. Nelson K et al (2013) Optimization of whole-body zebrafish sectioning methods for mass spectrometry imaging. J Biomol Tech 24(3): 119–127
13. Schueler B, Sander P, Reed DA (1990) A time-of-flight secondary ion microscope. Vacuum 41(7–9):1661–1664
14. Schueler BW (1992) Microscope imaging by time-of-flight secondary ion mass spectrometry. Microsc Microanal Microstruct 3(2-3):119–139