INDEX COPERNICUS INTERNATIONAL

Bio-Algorithms and Med-Systems

WWW.BAMSJOURNAL.COM ISSN: 1896-530X

ORIGINAL ARTICLE

Received: 12.11.2023
Accepted: 19.12.2023
Published: 31.12.2023

CITE THIS ARTICLE AS:
Skalska ME, Durak-Kozica M, "Experimental and analytical procedures for the ToF-SIMS measurement data of membranous structures", Bio-Algorithms and Med-Systems vol. 19, no. 1, pp. 64-68, 2023, DOI: 10.5604/01.3001.0054.1935

AUTHORS' CONTRIBUTION:
A – Study Design
B – Data Collection
C – Statistical Analysis
D – Data Interpretation
E – Manuscript Preparation
F – Literature Search
G – Funds Collection

CORRESPONDING AUTHOR:
Magdalena Skalska PhD; Institute of Physics, Medical Physics Department, Jagiellonian University, Krakow, Poland; Prof. S. Lojasiewicza 11, 30-348 Krakow, Poland; Phone: +48 12 664 47 30; E-mail: magda.skalska@uj.edu.pl

Experimental and analytical procedures for the ToF-SIMS measurement data of membranous structures

Magdalena E. Skalska[1,2ABCDEFG] (ORCID: 0000-0003-0069-0550), Martyna Durak-Kozica[1,2AB] (ORCID: 0000-0002-5275-6462)

[1]Department of Medical Physics, M. Smoluchowski Institute of Physics, Faculty of Physics, Astronomy and Applied Computer Science, Jagiellonian University, Krakow, Poland
[2]Centre for Theranostics, Jagiellonian University, Krakow, Poland

ABSTRACT

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a powerful analytical technique with great application potential in biomolecular matter research. SIMS measurements performed on biological samples, due to their complex structure and the content of many small and large atomic molecular compounds, suffer very rich and complex mass spectra of particles, which characterise the content and physio-chemical properties of examined samples. The proper description and understanding of features appearing in the spectra and, consequently, the final data confirming or rejecting the hypothesis put forward in the experiment, largely depend on the experimenter's correct understanding of the technique itself and its limitations, knowledge of the tested material and its appropriate preparation. These issues mean that obtaining the right answer to the questions posed in the research hypothesis requires not only the correct conduct of experiments but also the appropriate processing of post-experimental data. This study aims to demonstrate the impact of various analytical and experimental procedures applied to reach proper conclusions from TOF-SIM measurements. These are different types of data normalization, the selection of a so-called region of interest (ROI), the selection of representative secondary ions and specific quantification methods, including a combination of experimental parameters. All these aspects were checked and discussed based on the results of the analysis of pancreatic β cells placed in a PBS solution on silicon wafers.

KEYWORDS

ToF-SIMS, membranous structures, data preparation, data acquisition, secondary ion mass spectrometry experiments

INTRODUCTION

Time-of-flight secondary ion mass spectrometry (TOF-SIMS) operating in the static SIMS mode enables the emission of a variety of secondary ions, including molecular-bound ions and fragment ions, which characterize the chemical structure of sample surfaces. The processes underlying the physical basis of the SIMS technique include, generally speaking, the interactions of heavy ions with energies ranging from several to several dozen keV with the condensed phase. An ion of a heavy atom or an ionized atomic cluster bombards the tested target. As a result of this process, atoms, molecules that are the parent components of the sample, and molecular fragments are emitted from several surface layers. In addition to neutral ones, positively and negatively charged ions are also emitted from the surface. This phenomenon is called ion sputtering or ion erosion. The SIMS spectrometer records the charged components of the sputter. The obtained results allow for a comprehensive characterization of the surface of the tested sample, concerning its composition as well as its structure. The effectiveness of SIMS devices depends on the precision of the technical solutions used and the efficiency of processes in which secondary ions are produced from the tested material [1, 2].

The recorded signal coming from the analyzed surface can be presented in various ways, depending on the goals of the experiment. The data treatment is also influenced by the question of whether a given experiment examines the composition of unknown biological samples, whether samples are compared with each other or whether information about the presence of a compound of interest is important. All this will determine how the data is prepared, as well as their evaluation and visualization. It should be emphasized that thanks to spectrometric techniques such as ToF-SIMS, the result is a very large amount of data. It is up to the experimenter to select and appropriately process them to capture the essence of the results and not overwhelm the reader with excess data.

In this work, the impact of proper calibration of mass spectra, their different normalization, peak identification and ToF-SIMS data presentation and display are examined and discussed. Of course, it is not possible to discuss in detail all aspects of ToF-SIMS data processing in one text. However, in this study, we focus on what we consider to be the key aspects affecting the quality and reliability of the analysis of biological structures conducted using ToF-SIMS spectrometry.

MATERIALS AND METHODS

Cell cultures

The insulin-releasing pancreatic β-cell line (1.1B4, Merck KGaA, Sigma Aldrich, Darmstadt, Germany) was cultured in a RPMI 1640 medium (Cat. No. R0883, Merck KGaA, Darmstadt, Germany), supplemented with 10% fetal bovine serum (FBS; Cat. No. 16000036, Thermo Fisher Scientific, Waltham, MA, USA), 2 mM L-glutamine (Cat. No. G7513, Sigma-Aldrich, Darmstadt, Germany) and antibiotics – 100 µg/ml streptomycin with 100 units/ml penicillin (Cat. No. 15140122, Sigma-Aldrich, Darmstadt, Germany). The cells were grown as monolayers in an incubator under conditions of a humidified 5% CO_2 atmosphere and 37°C until they reached approximately 80% confluence. The culture was maintained by changing the medium and passaged with trypsin (Trypsin-EDTA solution, Cat. No. 25200056, Thermo Fisher Scientific, Waltham, MA, USA). After harvesting the cells from the medium they were resuspended in 1.5 ml of PBS (Cat. No. 10010023, Thermo Fisher Scientific, Waltham, MA, USA) in quadruplicates and stored at –80°C for further ToF-SIMS analysis.

Sample preparation

For ToF-SIMS measurements, silicon wafers (Cat. No. 647780, Sigma Aldrich, St. Louis, MO, USA) with dimensions of 1 x 1 cm^2 were prepared. The selection of the silicon substrate was dictated by previous studies examining the best parameters for ToF-SIMS research on biomaterials [3]. Before cell deposition, each substrate was sonicated in toluene (Cat. No. 244511, Sigma Aldrich) and ethanol (absolute, 99%, Cat. No. 396480111, POCH) for 10 minutes using an ultrasonic bath, rinsed with deionized water and then dried with N_2. The experimental material included 4 samples placed on the silicon surface with a volume of 30 µl. The concentration of deposited cells was approximately 5.3 x 10^5 cells/ml.

ToF-SIMS experiments

In the case of biological samples, ionized clusters of bismuth or fullerene (Bi_3^+, C_{60}^+) are most often used, which allow the detection of larger particles by minimizing damage to the structure being examined [4]. The experiments were performed in the so-called static mode, which is defined by the given dose density threshold of primary ions deposited on the tested surface. Measurements in this mode ensure that the surface will not be damaged and the components will not be significantly delocalized and fragmented in the sample volume. At the same time, the second operating mode of the Tof-SIMS spectrometer enables imaging of 2-D maps of secondary ions emission from the surface with a submicron spatial resolution (dynamic mode) [5].

An undoubted advantage of the ToF-SIMS technique in biological studies is the possibility of so-called comparative analysis of experimental samples with the participation of control samples. In this type of analysis there is no need to extract compounds of interest from the sample structure or label them. The essence of the analysis is a comparison of the average intensities of characteristic mass–peaks collected from several spectra of examined samples.

To obtain all spectra, the ToF-SIMS 5 instrument (ION-ToF GmbH, Münster, Germany) was used with a Bi_3^+ liquid metal ion gun (30 keV) as the primary ion source. Three well-separated surface areas of 200 × 200 μm^2 for positive ions (125 x 125 pixels) were

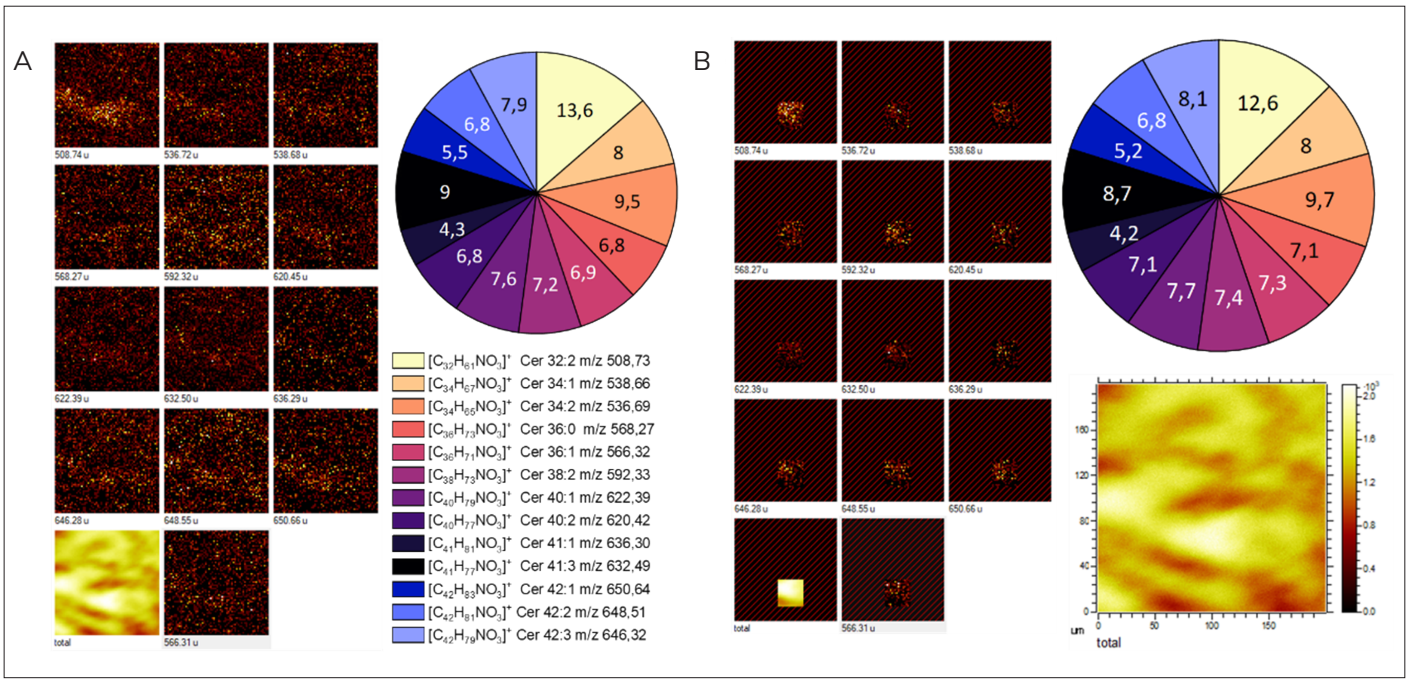

Fig. 1. Comparison of percentages calculated from the mean ion intensities of ceramides characteristic peaks from (A) the entire experimental area (200 × 200 μm^2) and (B) regions of interest (50 × 50 μm^2).

chosen from one sample, from which the secondary ions signal were recorded. The mass range of detected ions was up to 900 Da, with a mass resolution of 8,300 at *m/z* 600 (FWHM).

The comparative analysis of lipid composition was performed in the static spectrometer mode with a current equal to 1.10 pA. A low-energy electron gun was used in the interval between two pulses from the primary ion source to neutralize the charge formed on the sample surface during the bombardment. All measurements reported here were carried out at the same time and under the same conditions, enabling the comparison of biological samples.

RESULTS

Selection of the analyzed area

In this study two approaches to ToF-SIMS data analysis were tested and compared. The percentage ratio of lipids from the ceramide (CER) group was compared in two different scenarios: analyzing spectra from whole surfaces (Fig. 1A.) and analyzing selected regions of interest (Fig. 1B.). The analysis was conducted on cell samples suspended in PBS. The image of the emission of all ions (total) in Fig. 1B. showed that the analyzed surface was relatively homogeneous.

The results in the pie chart (Fig. 1A.) concern the average value from four measurements of whole surfaces with dimensions of 200 x 200 μm^2. Results in Fig. 1B. present average values from 9 regions of interest (from each 3 ROIs of analyzed surface, 3 were made surface analysis 200 x 200 μm^2).

Selection of the normalization method

There are four possible normalization methods for this technique: normalization to the total number of counts, to the total dose deposited on the surface, to the reference peak, and the sum of selected peaks. Here, the sum of selected peaks refers to the summed intensity of all peaks selected from the spectrum, which were used for further analysis.

The results presented in Fig. 2. compare three types of normalization along with raw data of the percentage of selected 13 lipids from the CER group. These were obtained from a sample of pancreatic β-cells that were cultured under normal conditions.

DISCUSSION

Mass spectrum calibration

After performing the measurements, in the first step, the obtained spectra should be calibrated. Proper calibration is crucial to correctly identify peaks of interest. The spectra included in the comparative analysis must be calibrated to the same set of peaks that are characterized by appropriate intensity and a high probability of being assigned to a specific mass. This ensures that errors in peak assignment for larger masses are minimized. Moreover, the peaks included in the calibration set

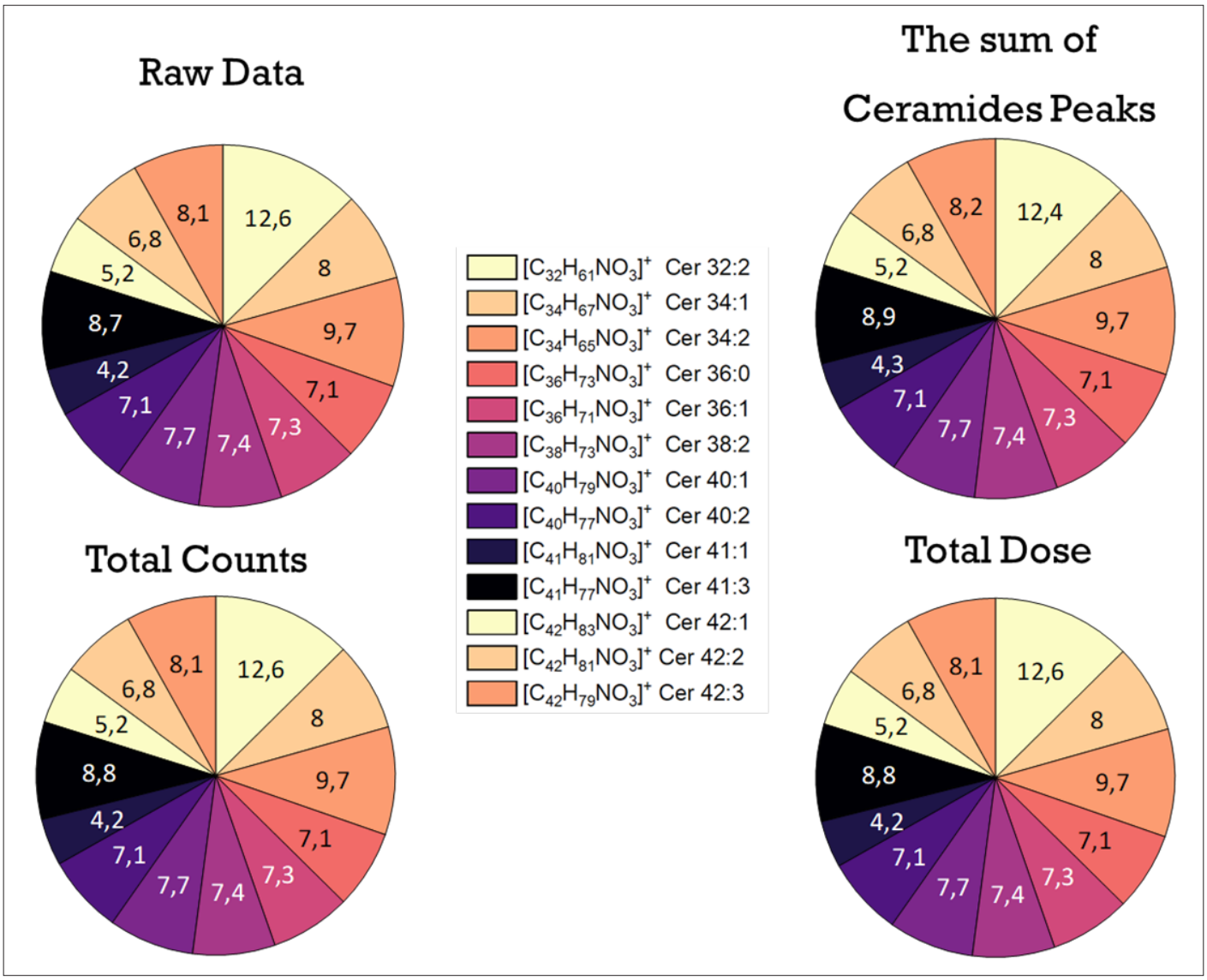

Fig. 2. Comparison of percentages calculated from the average ion intensities of ceramides characteristic peaks for different normalization methods.

should be symmetrical to avoid the influence of factors typical for this method, such as topography or sample surface loading.

For biological samples, it is worth performing a two-step calibration. Initial calibration is performed on at least three peaks occurring in the spectra of virtually all samples, these are: CH_3^+, $C_2H_3^+$ and $C_3H_5^+$ for positive polarization and CH^-, OH^- and C_2H^- for negative polarization. Then, recalibration is applied taking into account peaks with the highest possible masses for which the identity is certain. This procedure will increase the quality of the match in the higher mass part of the spectrum (>200 amu).

Selection of the analyzed area

In the literature it has been reported that it is possible to conduct a relatively quantitative assessment of changes, such as intensities for specific peaks, when measuring the surface of samples with similar characteristics or, more precisely, with analogous ionization properties [6]. A similar procedure was presented in the work by Touboul et al. [7]. According to this scheme, areas (ROI, region of interest) that are planned to be compared should be extracted from the surface sample, e.g. those that differ histologically. Then mass spectra for these areas should be obtained, and after that normalization to the same total number of counts can be taken. This approach can be used for samples that differ in various respects, for example when distinguishing samples from healthy and diseased patients [8–11]. The accuracy and correctness of the procedure increase as the number of biological and technical samples increases. In other words, when average values of normalized intensities from compared samples or areas on the surface are available for comparative analysis.

After comparing the obtained results (Fig. 1.), it is apparent that for this type of sample, i.e. cells suspended in a specific solvent (the

size of a β-pancreatic cell is approximately a diameter of 10 μm), the differences resulting from the size of the area from which the analyzed spectrum was obtained are less than 1% [12].

Selection of the normalization method

It is important to normalize all ToF-SIMS spectra from the same experiment in a consistent manner before comparing and analyzing the differences in the obtained data. The comparative analysis is based on the comparison of intensities for selected peaks, and it is important to keep in mind that the ToF-SIMS technique is a qualitative method, and the results can be influenced not only by the concentration of the tested sample but also by factors such as topography, matrix effects and instrumentation parameters. To eliminate or minimize the impact of these undesirable factors it is necessary to apply the same type of normalization to the spectra, which is adapted to the nature of the experiment.

The comparison (Fig. 2.) shows that there were no significant differences between normalization to the total number of counts of the total dose deposited and the raw data itself. The only differences that were observed were related to normalization of the sum of peak intensities for a given lipid group, but even these differences are insignificant. This indicates that there was no influence of experimental factors in this measurement series for this particular type of sample. It is important to note, however, that this statement is only valid for the biological material under consideration.

CONCLUSIONS

The ToF-SIMS technique is a highly useful method for characterizing biological samples and conducting comparative analyses. However, it is crucial to ensure that all measurements and post-experimental work are carried out properly. This requires thoughtful and repeatable preparation of biological material, as well as conducting compared measurements under identical conditions. The influence of factors such as time since preparation, temperature, pressure in the chamber, sample concentration and topography should be minimized as much as possible. Additionally, how data is treated is also significant. This involves proper calibration of the acquired spectrum, selection of appropriate polarization to acquire relatively high intensities for the examined matter, selection of the analysis area and proper normalization. All these aspects are important for obtaining reliable data to conduct a comparative analysis and reach conclusions that support or negate an experiment's hypothesis. Information obtained using the ToF-SIMS method can often complement and diversify the results obtained using other techniques in biological sciences.

ACKNOWLEDGEMENTS

This work was supported by the National Science Center (NCN), grant OPUS 17 to Prof. E. Stępień (No. 2019/33/B/NZ3/01004; 2022/45/B/NZ7/01430) and by the SciMat PRA MNS mini-grant, the qLife grant funded under the Strategic Programme Excellence Initiative at UJ.

REFERENCES

1. Vickerman JC. Secondary ion mass spectrometry-basic concepts, instrumental aspects, applications and trends. A. BENNINGHOVEN, F. G. RUDENAUER and H. W. WERNER, Wiley, New York, 1987, 1277 pages. Surf. Interface Anal. 1987;10:435.
2. Graham DJ, Gamble LJ. Back to the basics of time-of-flight secondary ion mass spectrometry data analysis of bio-related samples. II. Data processing and display. Biointerphases. 2023;18(3):031201.
3. Kamińska A, Marzec ME, Stępień EŁ. Design and Optimization of a Biosensor Surface Functionalization to Effectively Capture Urinary Extracellular Vesicles. Molecules 2021;26(16):4764.
4. Marzec ME, Wojtysiak D, Połtowicz K, Nowak J. ToF-SIMS spectrometry to observe fatty acid profiles of breast tissues in broiler chicken subjected to varied vegetable oil diet. J. Mass Spectrom. 2020;55(3):e4486.
5. Marzec ME, Wojtysiak D, Połtowicz K, Nowak J, Pedrys R. Study of cholesterol and vitamin E levels in broiler meat from different feeding regimens by TOF-SIMS. Biointerphases 2016;11:02A326.
6. Touboul D, Laprevote O, Brunelle A. Micrometric molecular histology of lipids by mass spectrometry imaging. Curr. Opin. Chem. Biol. 2011;15:725-32.
7. Touboul D, Brunelle A, Halgand F, De La Porte S, Laprévote O. Lipid imaging by gold cluster time-of-flight secondary ion mass spectrometry: Application to Duchenne muscular dystrophy. J. Lipid Res. 2005;46:1388-95.
8. Wu L, Felton JS, Wu KJJ. Applying imaging ToF-SIMS and PCA in differentiation of tissue types. Methods Mol. Biol. 2010;656:267-81.
9. Debois D, Bralet M-P, Le Naour F, Brunelle A, Laprevote O. In situ lipidomic analysis of nonalcoholic fatty liver by cluster TOF-SIMS imaging. Anal. Chem. 2009;81:2823-31.
10. Magnusson Y, Friberg P, Sjövall P, Dangardt F, Malmberg P, Chen Y. Lipid imaging of human skeletal muscle using TOF-SIMS with bismuth cluster ion as a primary ion source. Clin. Physiol. Funct. Imaging 2008;28:202-9.
11. Malmberg P, Jennische E, Nilsson D, Nygren H. High-resolution, imaging TOF-SIMS: novel applications in medical research. Anal. Bioanal. Chem. 2011; 399: 2711-18.
12. Marchetti P, Bugliani M, De Tata V, Suleiman M, Marselli L. Pancreatic Beta Cell Identity in Humans and the Role of Type 2 Diabetes. Front. Cell Dev. Biol. 2017;5:55.