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High resolution imaging by organic secondary ion mass spectrometry

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Secondary-ion mass spectrometry (SIMS) is based on the acceleration of high-energy primary ions onto a target. Secondary electrons, neutrals and ions are emitted from the target, reflecting its chemical composition. This enables simultaneous analysis and localization of target molecules, giving valuable information that is difficult or impossible to obtain with other analytical methods. The secondary ions can be extracted and detected by any type of mass analyzer. SIMS is unique in its ability to detect several target molecules simultaneously in small samples and to image their localization at subcellular resolution. The recent development of bioimaging SIMS opens up new possibilities in biotechnology and biological research with applications in biomedicine and pathology. The current development of this technique has the potential to become as important for biotechnology as the advent of the electron microscope, confocal microscope or *in situ* hybridization.

Introduction

In post-genomic biotechnology, the analysis of post-translational modification of proteins by covalent coupling to sugars and lipids will become increasingly important because such conjugates have a crucial role in protein function. Different biochemical techniques, such as thin-layer chromatography, high-performance liquid chromatography and mass spectrometry (MS), have been used to study the composition and distribution of lipids and sugars in biological tissues. All of these methods require multiple steps, including cell fractionation and lipid extraction, which necessitates a large amount of tissue. Therefore, information regarding the original spatial localization of analytes is lost.

Secondary-ion mass spectrometry (SIMS) offers the possibility of global and imaging analysis of whole tissue. The main reason why the use of this method in biology is restricted to a few laboratories, so far, has been the extensive fragmentation of molecules caused by the primary-ion sources restricting the possibility of identification of the original molecule [1].

The technical development towards a higher yield of high-mass secondary ions has followed three main lines: laser post-ionization of secondary neutrals (laser-SNMS) [2], application of a metal or other matrix that promotes ionization [3,4] and the use of polyatomic ion sources [5].

Of these lines of development, the polyatomic or cluster ion sources appears to be the most promising solution and one that is available commercially. The yield of high-mass fragments is much higher for the cluster-ion beams than for atomic-ion beams. The aim of this review is to give a background to the SIMS technique and to highlight the potential of high-resolution bioimaging time-of-flight (TOF)-SIMS in biotechnology in view of the recent developments in imaging organic TOF-SIMS.

Potential of high-resolution MS imaging in biotechnology

Imaging the distribution of molecules, analyzed as secondary ions, is not only a way of studying the spatial distribution of molecules, it also creates a new possibility of revealing by MS the origin of fragments derived from a common molecule. The localization of one fragment might give information about its possible relation to other fragments. Such analyses enable advanced studies of the biochemistry of tissues at subcellular resolution. The localization of fatty acids in biological tissues could serve as an example, showing the option of analyzing the relationship between small and large molecular fragments [6].

When localized in biological tissue, free fatty acids might stem from a large number of lipid molecules, including phospholipids, diacylglycerols (DAGs), triacylglycerols (TAGs) and sphingolipids. In traditional liquid solution-based MS, there is no possibility of determining the origin of fatty acids, even if more complex lipids are found in the same sample. In imaging TOF-SIMS, the spectra can be analyzed related to each pixel of the ion images, revealing co-localization of fatty acids with specific fragments of more complex molecules (e.g. phospholipids), thus revealing the most probable molecular origin (Figure 1).

This possibility might also be illustrated by the relationships among fatty acids, DAG and TAG in fat cells [7].

The secondary ion of DAG might originate from DAG present in the cells, by fragmentation of TAG or by fragmentation of phospholipids. The secondary ions of fatty acids might be formed by fragmentation of TAG, DAG or phospholipids. By comparing the localizations of C18, phosphocholine, DAG and TAG, we can come to some conclusions as to the localization of the original molecules in the tissue [7] (Figure 2).

According to their localization pattern, secondary-ion distributions of TAG, DAG and C18 in some areas differed from the distribution pattern of phosphocholine and were

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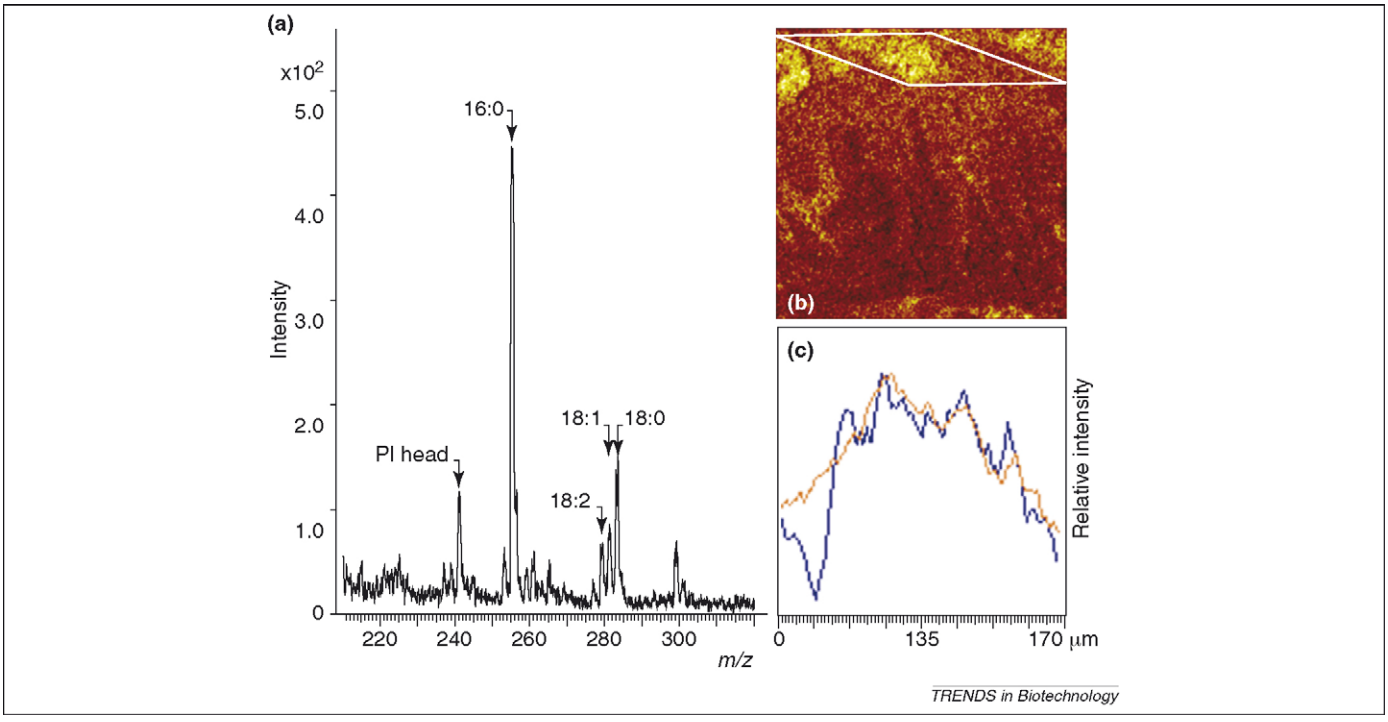

Figure 1. Localization by TOF-SIMS of fatty acids and phospholipid headgroups in the mouse intestine renders the reconstruction of a phospholipid possible by co-localization of fragments. A secondary negative ion spectrum of mouse intestine, which had been frozen in liquid nitrogen, freeze-sectioned and dried in air at room temperature, is shown. The ion spectrum was reconstructed for a region of interest at the intestinal crypt region showing high signal intensity for palmitic acid. **(a)** Highlights the positions in the mass spectrum for detected fatty acids and a peak at *m/z* 241 representing the phosphoryl inositol (PI) head group ($C_6H_{10}PO_8$). **(b)** Image showing the distribution of the secondary ion signal at *m/z* 255 with the intestinal crypt region (intestinal glands) marked in white. The marked area was used for an area intensity profile. **(c)** Area profile showing the distribution of the signal at *m/z* 255 of yellow and *m/z* 241 of blue. The intensity curves indicate co-localization of the signals in this area, indicating that the source of the fatty acid and the head group is phosphatidyl inositol. Figure reproduced from [6] with permission.

seen merged in other areas. A significant amount of DAG fragments do not originate from TAG secondary ions or from phospholipids because their localization pattern is different. These areas might contain DAG present in the cells at the time of freezing. Some cells showed secondary-ion signals specific for DAG fragments but no detectable secondary-ion signals specific for TAG. By contrast, a high proportion of C18 fatty acids could be related to TAG because their distribution pattern showed strong similarities. The experiment shows the possibility of analyzing the relationships among fatty acids, TAG and DAG in biological tissue. This kind of analysis is of potential importance in atherosclerosis research.

SIMS analysis of biosamples and its technical background

The term secondary ion refers to the desorption (or sputtering) of secondary ions from the sample as a result of bombardment with primary ions from a primary-ion source. The principle was first invented in the late 1940s and further developed in the early 1960s. Reviews on the early development of SIMS are available [8,9].

The terms static SIMS and dynamic SIMS refer to the intensity of bombardment of the sample with primary ions. In dynamic SIMS (high intensity), the sample is sputtered; in static SIMS (low intensity) the sample is not sputtered. Dynamic SIMS is used for the analysis of atom ions (Ca, Mg) or small molecular fragments, whereas static SIMS can be used for the analysis of larger fragments of organic molecules. The term organic SIMS denotes the analysis of organic molecules by static SIMS.

TOF refers to the type of mass analyzer used in most organic SIMS instruments. Imaging SIMS can also be performed using other mass analyzers, for example, quadropoles [10], however, most results published have been obtained with TOF-SIMS instruments, which were first developed in 1981 [11].

Monoatomic ion sources, such as cesium$^+$, gallium$^+$ and indium$^+$, have been used in imaging TOF-SIMS and they all share the properties of well focused beam spots, giving a spatial resolution of 100–200 nm. However, the high-energy impact of the primary ions on biological samples results in poor yield of high-mass secondary ions, complicating the identification of the original molecule [12]. The possibility of using monoatomic-ion sources in biological applications has been reviewed excellently [1,10,13]. Later studies demonstrated their use with a creative experimental design enabling studies of lipid organization through localization of fatty acids labeled with carbon isotopes [14].

The introduction of ionization promoters in the samples improved the signals of some analytes, especially that of cholesterol [15]. Surface modifications, including matrix-assisted SIMS and metal-assisted SIMS, not only enhance the yield of secondary ions in SIMS [3] but also include a preparation step that could introduce new sources of artefacts. Given the successful development of polyatomic primary-ion sources, the benefit of using ionization promoters is declining.

The ability of polyatomic-ion sources to increase the yield of secondary ions, especially of high-mass ions, was first reported in 1989 [16]. Later, bombardment with sulfurpentafluoride (SF_5) was reported to give a 10–50-fold

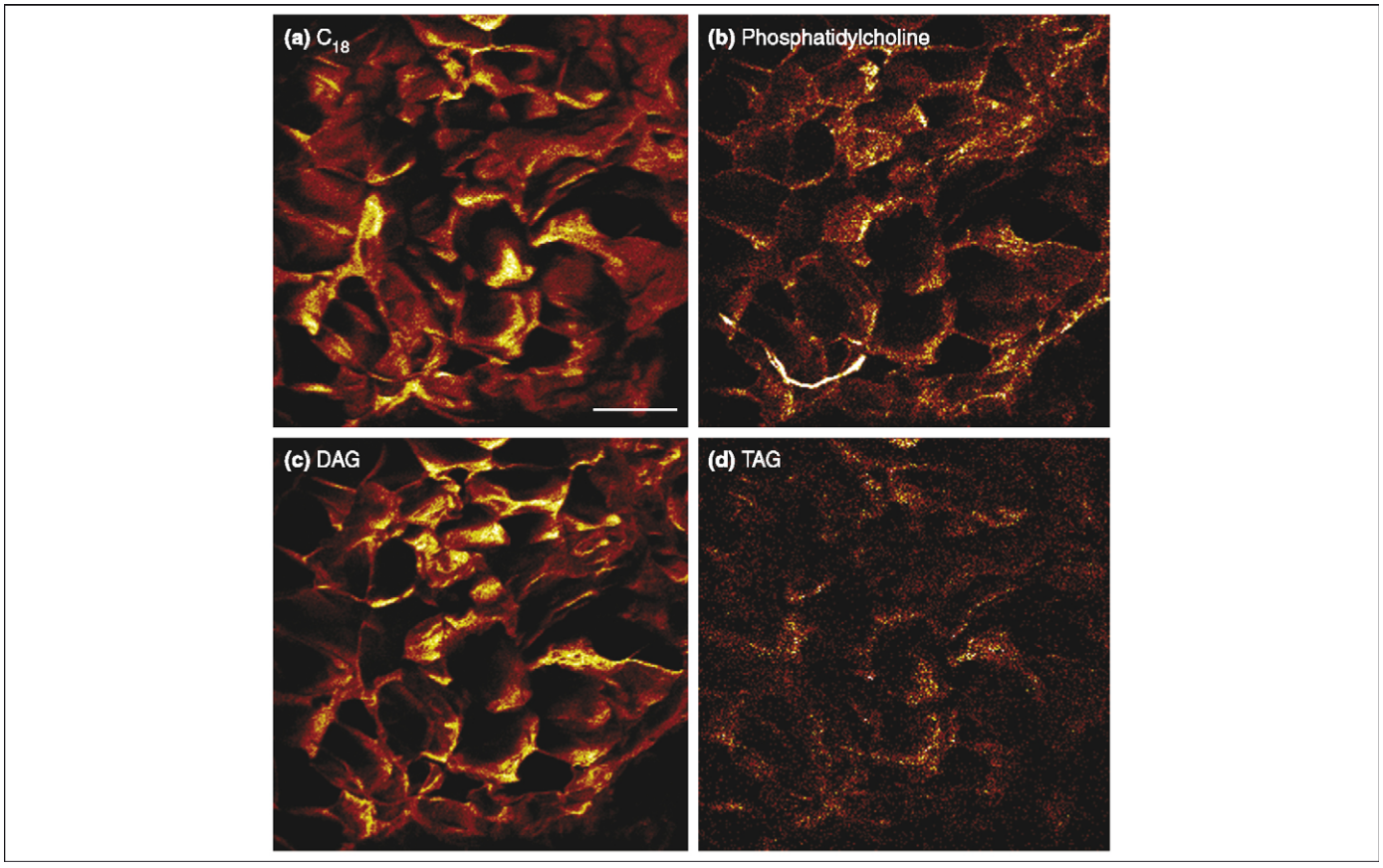

Figure 2. Localization of fatty acids, diacyl- and triacyl-glycerols in fat cells for biochemical analysis of the lipid content of fat cells from young overweight patients is shown. Image from an analysis of high-pressure frozen, freeze-fractured and freeze-dried human adipose tissue. The ion images are represented in a color scale ranging from black to red to bright yellow as shown in the image color-scale bar. Specific ion images show the distribution of secondary positive and negative ions of: **(a)** fatty acids with a C_{18} chain length. The image shows an area with large fat cells, containing lipids mainly in the lipid vacuoles of the cells. **(b)** Phosphatidylcholine head group. The image shows the localization of phosphocholine in membrane lipids of the fat cells. **(c)** Diacylglycerols (DAG). The image shows the localization of DAG in lipid membranes of the fat cells. **(d)** Triacylglycerols (TAG). The image shows a weak but significant signal of triacylglycerol in the lipid vacuoles of the fat cells. Figure reproduced from [7] with permission.

enhancement in the secondary-ion yields for molecular ions. The SF_5-primary ion can be focused to a spot, enabling molecular-ion images to be obtained at micrometer spatial resolution (i.e. low-resolution imaging). The decay in molecular-ion signal as a function of primary-ion dose observed commonly in SIMS using monoatomic primary ions is reduced, enabling depth-profiling 3D-imaging of organic layers [17]. A buckminsterfullerene (C_{60})-based primary-ion-beam system has been developed for TOF-SIMS analysis of organic materials. The resulting yield efficiencies were 30–100 times higher than those observed from gallium sources. The C_{60} source was also reported to favor the formation of high-mass secondary ions [18]. The C_{60}-ion source can also be used for depth profiling [19] and 3D imaging of organic layers and cells [20]. The ion beam of the C_{60}-ion source might be focused to a spot with a diameter of 3 μm, giving a practical image resolution in the range of 3–9 μm [18]. In a comparative study, using eight different ion sources, the possibility of using Bi_n clusters was presented [21]. As a general result, the efficiency of the secondary-ion yield improved with the mass of the monoatomic ions. A further increase was found with the use of polyatomic ions. According to this, the highest efficiencies were obtained for C_{60}, the lowest for Ga. The results reveal the potential of cluster SIMS to overcome existing limitations and to establish TOF-SIMS for new applications in the fields of biology and medicine [21]. The ion beam of the Bi_3-cluster-ion source might be focused to a spot with a diameter of 300 nm, giving an image resolution of 300–500 nm, which is in the range needed to enable analysis at subcellular resolution. In principle, the polyatomic Bi sources could be focused well below 100 nm by using the so-called burst mode. However, this would decrease the intensity to levels that would require measurement times of overnight or longer. A review of the performance of the instrument in chemical analysis is available in the literature [22].

Current issues in SIMS analysis of biosamples

Peak identification

Recent research in TOF-SIMS has demonstrated the possibility of mapping the distribution of a wide variety of compounds in biological tissue [23–28].

The current issues in TOF-SIMS analysis with a Bi-cluster ion source are the data interpretation and identification of all new peaks detected with this technique. Until 2004, only a few high-mass peaks of biological origin could be identified by TOF-SIMS, which leaves us in a situation in which much of the interpretation of spectra is still to be done. The identity of peaks can be established using reference substances [24,29]. A list of peaks identified with the help of reference spectra and of interest to

Table 1. Ion identities based on reference spectra[a] analyzed using a Bi_3^+ primary ion gun

Species	Ion	*m/z*
PC-headgroup	$C_5 H_{15} PNO_4$	184.07
Palmitoleic acid16:1	$C_{16} H_{29} O_2$	253.22
Palmitic acid 16:0	$C_{16} H_{31} O_2$	255.23
Linoleic acid	$C_{18} H_{31} O_2$	279.24
Oleic acid 18:1	$C_{18} H_{33} O_2$	281.25
Stearic acid 18:0	$C_{18} H_{35} O_2$	283.26
Cholesterol	$C_{27} H_{45}$	369.35
Cholesterol	$C_{27} H_{45} O$	385.35
7-ketocholesterol	$C_{27} H_{43} O_2$	399.33
Diacylglycerol(PP)	$C_{35} H_{67} O_4$	551.55
Diacylglycerol(OL)	$C_{37} H_{69} O_4$	577.54
Diacylglycerol(OO)	$C_{39} H_{71} O_4$	603.56
Triacylglycerol(POL)	$C_{55} H_{99} O_6$	855.75
Triacylglycerol(OOP)	$C_{55} H_{101} O_6$	857.85
Triacylglycerol(OOO)	$C_{57} H_{103} O_6$	883.78
Gal-ceramide C18:0	$C_{42} H_{81} N O_8 Na$	750.56
Gal-ceramide C24:0	$C_{48} H_{93} N O_8 Na$	834.64
Gal-ceramide Ch24:0	$C_{48} H_{93} N O_9 Na$	850.64

[a]For reference spectra and mass deviations see [7,24,29,40].

biologists is shown in Table 1. Future work, aimed at identifying peaks found after analysis of biological samples with the Bi-cluster source, might be facilitated, whereas fragments detected by TOF-SIMS (Table 1) are similar to those found by electrospray MS, in which their identity has been established.

3D imaging

Although thickness profiles and 3D imaging of solids have been used routinely, the damaging properties of the ion sources during sputtering and analysis of biological samples had been an obstacle to the development of 3D imaging of cells and tissue. Recently, Vickerman's group published 3D imaging of oocytes using C_{60} for the sputtering and analysis [20].

The C_{60}-ion source appears to be the most suitable for sputtering of organic material to penetrate deeper into the sample, whereas the Bi-cluster-ion source gives a higher image resolution when used in the burst-alignment mode. Thus, it would seem natural to combine these sources and make 3D analysis of cells using C_{60} for sputtering and the Bi-cluster-ion source for imaging TOF-SIMS at intervals. Such experiments have been performed recently and, although not in contradiction with previous published results, the resulting molecular damage remained too extensive, thus making molecular imaging difficult [30]. The same technique has been adapted for analysis of single cells, demonstrating that cellular membranes and inorganic ions can be demonstrated by molecular imaging [31] (Figure 3). The image shows the 3D distribution of sodium (blue) surrounding a cultured cell of a thyroid tumor-cell line, together with the intracellular distribution of potassium (green), phosphocholine (red) and a fragment m/z 86 (yellow). Potassium is seen contained in intracellular membrane-enclosed pools. The fragment m/z 86 is localized to the acid organelles (i.e. the endoplasmic reticulum and the lysosomes).

Frozen-hydrated samples

All processes in living cells depend on water, making analyzing cells in their water environment potentially

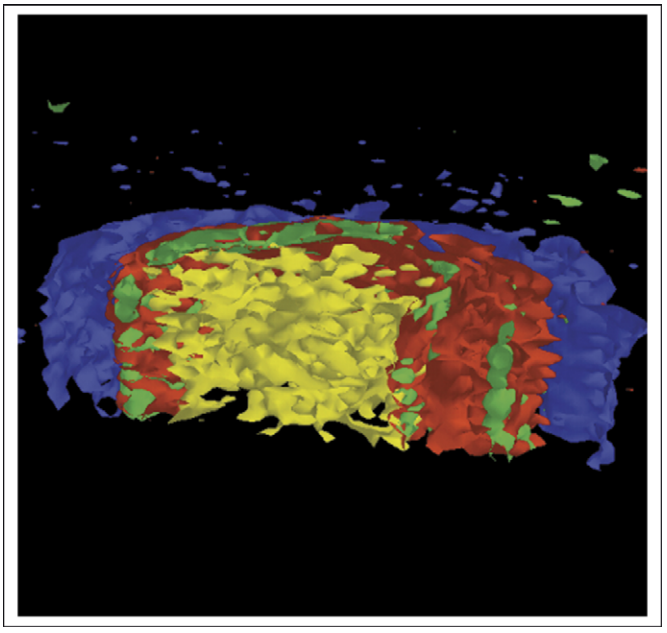

Figure 3. Analysis of a thyroid tumor-cell line that was cultured, frozen and freeze dried. The cells were sputtered sequentially with C_{60}^+ primary ion and analyzed with Bi_3^+ primary ions. The images representing sections through the cells were then stacked to form a 3D image. 3D iso-surface rendering (three color) of the ion image data cells analyzed by sequential sputtering and analysis. Na, *m/z* 23, is shown in blue; K, *m/z* 39, in green;fragment at *m/z* 86 in yellow; and the phosphocholine head group, *m/z* 184, in red. The data volume was generated from 28 single ion images, each separated by four C_{60} sputtering sequences. Figure reproduced from [31] with permission.

the most exciting application of the TOF-SIMS technique. Although a complete cold chain from freezing of samples to analysis of cooled, wet samples was published 10 years ago [32], and analysis of organic molecules in cell membranes has also been demonstrated [33], the increase in activity in this field has been small. The biotechnical development has been impressive, with the construction of an instrument dedicated to the analysis of fresh-frozen tissue [34]. This cryo-TOF-SIMS instrument contains a preparation chamber in which freeze-fracturing can be done under vacuum conditions and that can be equipped with polyatomic ion sources.

A problem with the SIMS analysis of wet frozen samples is that ice, as a polymer, will produce a plethora of secondary ions on bombardment with primary ions. This effect can be reduced by vaporizing some of the surface water but the main objective for the experiment, namely to study the interaction of ions and macromolecules with water, is compromised. If the technique of MS analysis of frozen-hydrated biological samples is made more accessible experimentally, it has the potential of starting a new age in biological research, with conceivable analysis of biomolecules in a hydrated state.

Interpretation of images

One important, maybe the most important, property of the TOF-SIMS analysis that must be kept in mind when interpreting image data is the surface sensitivity of the method. During SIMS, most of the sputtered particles originate from the first monolayer of molecules. This means, for example, that an analysis of a sample containing whole cells cultured on a surface will only reveal

secondary ions from the outer-membrane surface of the cells. The analysis will also be sensitive to the medium used, especially its content of salts, such as sodium chloride. The use of freeze-fractured material or tissue sections will solve this problem. Thus, the surface sensitivity of the instrument will increase requirements on the technique of sample preparation and will also influence the interpretation of the images obtained.

The main novel finding made by the imaging TOF-SIMS technique is the heterogeneous distribution of lipids in biological tissue [24,25,35–37]. When the heterogeneous distribution of a target molecule is detected by TOF-SIMS, three technical issues must be taken into consideration: topography, intensity versus concentration and matrix effects.

Topographical effects will influence the secondary-ion yield significantly. This will result in band broadening and decreased intensity of the peaks [38]. Topographical effects are evident in the analysis of single cells and are also created when using freeze fracturing as the preparation method. Thus, care has to be taken to include possible topographic effects in the interpretation of data.

Intensity versus concentration has its origin in the different yield of secondary ions formed by different target molecules. This effect can be addressed through the use of calibration curves of target molecules, if topographical effects and the remaining issue, the matrix effects, can be taken into account.

A matrix effect means that the signal that arises from a target molecule varies depending on the molecules that the target molecule is in contact with (the matrix). The term was coined to describe the fact that copper ions were ionized with a higher yield from copper oxide than from pure copper [8] and has been discussed as a source of nonlinearities in TOF-SIMS analysis [39]. In principle, even the matrix effect is not insurmountable if calibration curves using reference samples and the matrix of interest are available. In practice, however, this is difficult because the concentration of target molecules at the very surface must be known and only the bulk concentration of a reference sample is available typically. It is, for example, easy to realize that phase separation or different densities of constituents of a sample might cause heterogeneous distribution of target molecules at the sample surface. Again, the surface sensitivity of TOF-SIMS is important. But assuming that the matrix influence remains constant within a sample set, one could obtain relative comparison of samples.

Conclusion

The recent development in imaging TOF-SIMS has increased the potential performance of the technique in biotechnology dramatically. Several properties of TOF-SIMS make the method a versatile and valuable tool in biological research. The possibility of co-localizing a large number of biomolecules, without using molecular probes, is particularly interesting as a complement to fluorescence microscopy. The possibility of comparing experimental groups with control groups is largely unexploited by the TOF-SIMS society of today. The possibility that the design of experiments used in biology and biomedicine, with appropriate control groups, offers the possibility of comparing TOF-SIMS data from the same area in different samples, thus 'normalizing' the experiment group to the control group, offers an exciting possibility for biologists to enter the TOF-SIMS field of research.

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