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David Touboul, Felix Kollmer, Ewald Niehuis, Alain Brunelle, Olivier Laprévotte. Improvement of biological time-of-flight-secondary ion mass spectrometry imaging with a bismuth cluster ion source. *Journal of The American Society for Mass Spectrometry*, 2005, 16 (10), pp.1608-1618. 10.1016/j.jasms.2005.06.005 . hal-03985979

HAL Id: hal-03985979

<https://hal.science/hal-03985979v1>

Submitted on 13 Feb 2023

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Improvement of Biological TOF-SIMS Imaging with a Bismuth Cluster Ion source

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A new liquid metal ion gun (LMIG) filled with bismuth has been fitted to a TOF-SIMS mass spectrometer. This source provides beams of Bi_n^{q+} clusters with $n=1$ to 7 and $q=1$ and 2. The appropriate clusters have much better intensities and efficiencies than the Au_3^+ gold clusters recently used in TOF-SIMS imaging, and allow better lateral and mass resolution. The different beams delivered by this ion source have been tested for biological imaging of rat brain sections. The results show a great improvement of the imaging capabilities in terms of accessible mass range and useful lateral resolution. Secondary ion yields Y , disappearance cross sections σ , efficiencies $E=Y/\sigma$ and useful lateral resolutions ΔL have been compared using the different bismuth clusters, direct onto the surface of rat brain sections and for several positive and negative secondary ions with m/z ranging from 23 up to more than 750. The efficiency and the imaging capabilities of the different primary ions are compared by taking into account the primary ion current for reasonable acquisition times. The two best primary ions are Bi_3^+ and Bi_5^{2+} . The Bi_3^+ ion beam has a current at least 5 times larger than Au_3^+ and is therefore an excellent beam for large area imaging. Bi_5^{2+} ions exhibit large secondary ions yields and a reasonable intensity making them suitable for small area images with an excellent sensitivity and a possible useful lateral resolution <400 nm.

Among the methods aiming to determine the spatial distribution of substances in biological tissues, imaging mass spectrometry with Matrix Assisted Laser Desorption Ionization (MALDI) or with Cluster Secondary Ion Mass Spectrometry (cluster-SIMS) are two similar techniques which have several advantages. These methods do not need the use of any chemical labeling and make possible, in only one single data acquisition, the direct localization of a great variety of different biological compounds on the surface of a tissue sample. The localization of different biomarkers can consequently be correlated to their biological significance. MALDI-MS imaging was introduced a few years ago by R.M. Caprioli and co-workers [1,2] and is now utilized and also improved by several groups [3,4,5,6,7]. With commercial MALDI-TOF mass spectrometers, the lateral resolution is generally limited by the laser spot size which is on average about 50 μm . This method enables to identify directly on a tissue sample regions where proteins are located [2], but also, depending mainly on the matrix coating used, ions in different mass ranges such as phospholipids [7] or drugs [8].

Biological TOF-SIMS imaging has a long history, and has been successfully used by examining the intensities of low mass fragment ions (particularly using isotopically labeled species). The state of the art was recently reviewed by Belu *et al* [9], Pacholski and Winograd [10] and in the book of Vickerman and Briggs [11]. There are many ways to prepare biological samples, like freeze drying, sectioning and mounting in a polymer and freeze fracturing. For each of these techniques, the ionization process may be different. Nevertheless, TOF-SIMS biological imaging suffered until recently from the poor secondary molecular ion production efficiency of the traditional Gallium or Indium liquid metal ion guns (LMIGs) [12,13]. The imaging capabilities of TOF-SIMS instruments on real biological samples were generally limited to low mass ions such as the phosphocholine head group at m/z 184. A first important breakthrough has come recently by the addition of gold cluster LMIGs [14] to TOF-SIMS instruments. Mouse brain TOF-SIMS images were obtained with pixel sizes of a few micrometers or less, in positive and negative ion modes and with extended mass ranges, showing specific images of ions arising from cholesterol, fatty acids, sulfatides, phosphatidylinositols or even triglycerides [15,16]. Using model mice with Duchenne Muscular Dystrophy [17], many negative ions in degenerating and regenerating cells in

the neighborhood of a destructured area have been imaged on the surface of a muscle tissue section. Nevertheless the primary ion currents which can be obtained with focused Au_3^+ beams are still low and the data acquisition time with a reasonable primary ion dose density is still rather long, especially for large area imaging. In addition, the ions are emitted with a broad kinetic energy distribution which limits the achievable lateral resolution and mass resolution. Another possibility is the use of MALDI matrix coatings (Matrix enhanced SIMS, ME-SIMS) [18,19] in TOF-SIMS imaging. Although the use of this method certainly increases the production of some secondary ions such as cholesterol, it seems that the signal enhancement could depend on the chemical structure of the secondary ion. In addition the interpretation of the ME-SIMS spectra could be more difficult because of the presence of matrix ion peaks, which can interfere with ion peaks from the sample itself. Further, since the lateral resolution is limited to the size of the matrix crystals, generally of the order of several micrometers, the matrix deposition procedure is critical. Sample surface coating by a thin gold or silver layer [20] can increase the sensitivity of TOF-SIMS imaging. This coating method does not seem to restrict the lateral resolution, but the spectra are modified because of the metal cationization of some specific secondary ions.

In a second instrumental attempt to improve the sensitivity of TOF-SIMS, it has been recently demonstrated that a LMIG can be operated with bismuth instead of gold [21]. The author showed that the bismuth ion emission is much more intense than the gold, and that the spatial and time focusing of the bismuth cluster beams is also better. In addition, a wider variety of ion beams could be obtained, with heavy clusters having up to 7 atoms and also doubly charged ion species. Consequently, the objective of the present work has been to test this new cluster ion source for biological sample imaging.

EXPERIMENTAL SECTION

Mass Spectrometry and Imaging MS. All the experiments described in the present paper have been performed using a standard commercial TOF-SIMS IV (ION-TOF GmbH, Gievenbecker Weg 15, 48149 Münster, Germany) [22] reflectron type time-of-flight mass spectrometer located at the Institut de Chimie des Substances Naturelles at Gif-sur-Yvette (France). The primary ion source is a bismuth LMIG which delivers Bi_n^{q+} bismuth cluster ions, with $n = 1-7$ and $q = 1-2$. The kinetic energy of these ions is $25q$ keV (25 keV for singly charged ions and 50 keV for doubly charged ions). The angle of incidence is 45° .

A first setting mode of the primary ion column, which was used for most of the images shown in the present paper, ensures the three following features: a good spot size, excellent pulse duration for high mass resolution and high currents. A three lens ion column focuses the ion beam on the target plane to a spot having a typical size of ~ 2 μm . A double blanking plate system gives primary ion mass selection with repetition rates up to 50 kHz. The primary ion currents, which are measured using a Faraday cup located on the grounded sample holder, are 1 pA (Bi_1^+), 0.34 pA (Bi_3^+), 0.185 pA (Bi_3^{2+}), 0.045 pA (Bi_5^+), 0.044 pA (Bi_5^{2+}) and 0.004 pA (Bi_7^+) at 10 kHz (Figure 1). A bunching system gives pulse durations of 0.7 ns for Bi_1^+ and Bi_3^{2+} and of ~ 1.0 ns for the other clusters, which ensures sufficiently short pulses for time-of-flight analysis with a mass resolution exceeding $M/\Delta M = 10^4$ (FWHM) at $m/z > 500$.

Another operating mode can be used, which delivers beam sizes of $\sim 150-250$ nm (depending on the chosen primary ion species). In this last case, the ion beam cannot be bunched and the pulse duration is 20-100 nsec, ensuring only a nominal mass resolution. A detailed description of the different setting modes of the ion column can be found elsewhere [23].

The effective ion flight path is about 2 m, the reflectron is a single stage model (first order compensation). The secondary ions are accelerated to a kinetic energy of 2 keV and are post-accelerated to 10 keV before hitting a hybrid detector made of a single microchannel plate followed by a scintillator and a photomultiplier. Time-of-flight mass spectra are recorded using a 512 multi stops time-to-digital

converter having a minimum bin size of 50 ps. The mass calibration of the spectra was always internal, using H^+ , C^+ , CH^+ , CH_2^+ , CH_3^+ and $C_3H_2^+$ ion peaks in positive ion mode and H^- , C^- , CH^- , CH_2^- , C_2^- , C_3^- , C_4^- and C_4H^- ion peaks in negative ion mode. Mass accuracies were always better than a few ppm.

The analyzed surface can be checked at any time by the operator with the help of an integrated video camera. A low energy electron flood gun is always activated between two pulses of the primary ion beam to neutralize any charge on the surface of the samples.

For yield and damage cross section measurements, the minimum irradiation time was 1 200 seconds, over an irradiated area of $100 \times 100 \mu m^2$, ensuring a minimum primary ion dose density of 10^{12} ions. cm^{-2} .

Two modes of image acquisition have been used:

i - Images with fields of view of $256 \times 256 \mu m^2$ are performed without sample stage movement just by rastering the primary ion beam. For most of the images, the ion gun mode is the one offering the best pulse duration with a moderate focus of $\sim 2 \mu m$ and good intensities (see above). The number of pixels was consequently chosen as 256×256 in order to have pixel sizes of $1 \times 1 \mu m^2$. The number of shots per pixel, and consequently the acquisition time, were adjusted for each kind of primary ion in order to always have the same primary ion dose density of 10^{12} ions. cm^{-2} . For only one image another ion gun mode was chosen, which delivers an ion beam size of ~ 250 nm. In this case the image was acquired with a field of $55.7 \times 55.7 \mu m^2$ and the number of pixels was 256×256 , and compressed during the data processing to 128×128 pixels for a final pixel size of 435×435 nm². This pixel size value is chosen because it is close to useful lateral resolution which is described and measured below for the same ion of cholesterol.

ii - Large area analyses (18×18 mm²) have been performed using the same LMIG conditions and the so called stage scan. In this mode the sample is continuously moved by the sample stage. The repeatability of the movement is better than $5 \mu m$ and therefore does not limit the lateral resolution. In order to scan the whole surface area the primary ion beam is rastered over the area of an individual pixel. The number of pixels was 256×256 , each pixel having a size of $70 \times 70 \mu m^2$. The number of ion

beam shots per pixel and the subsequent acquisition time were adjusted for each primary ion in order to always have the same primary ion dose density of 10^9 ions.cm⁻².

The name of the compounds or the m/z value of the peak centroid, the maximal number of counts in a pixel (mc) and the total number of counts (tc) are written below each image. The color scales correspond to the interval [0, mc].

Terms and definitions. In order to compare the efficiency, for both spectroscopy and imaging purposes, of the ion generation process under the different Bi_n^{q+} bombardment, the following values were determined directly from the tissue sections: secondary ion emission yield Y , disappearance cross section σ , secondary ion generation efficiency E , specific data rate R and useful lateral resolution ΔL . The values are defined as follows [24]:

i – Secondary ion emission yields Y are calculated as the area of the peak of interest (number of detected ions) divided by the number of primary ions having impinged the surface during the acquisition of the spectrum.

ii - Disappearance cross section σ : $N(t) = N(t=0).exp(-\sigma It/Ae)$

with: $N(t)$: number of detected particles at time t ; $N(t=0)$ number of detected particles at time 0; I : primary ion current; A : bombarded area; e : elemental charge; σ is the mean area damaged by one primary ion and is a parameter depending on the respective secondary ion species under consideration.

iii - Secondary ion efficiency E : $E = Y/\sigma$, E is the number of detected secondary ions of a given species per damaged area. This value accounts for the entire analysis process, also including the analyzer transmission and the detector efficiency.

iv – Specific Data Rate for the species Bi_n^{q+} is defined as $R(Bi_n^{q+}) = Y_{Bi_n^{q+}} I_{Bi_n^{q+}}$. It is named specific because it depends on both the primary and secondary ions. This parameter is particularly important for large area analysis, while the secondary ion efficiency accounts more for small areas.

v – Relative Specific Data Rate: $R_{Bi_n^{q+}}(Bi_n^{q+}) = \frac{Y_{Bi_n^{q+}} I_{Bi_n^{q+}}}{Y_{Bi_1^+} I_{Bi_1^+}}$. For the present work, $R_{Bi_n^{q+}}(Bi_n^{q+})$ is calculated

relatively to Bi_1^+ . The higher the value the better is the primary ion species Bi_n^{q+} suited for imaging.

vi – Useful lateral resolution ΔL is the side of the minimum square area in which N secondary ions of a given mass can be desorbed and detected. This corresponds to the area ΔL^2 which has to be damaged to record N secondary ions. For $N = 4$ one obtains $\Delta L = (4/E)^{1/2}$. ΔL is related to the secondary ion emission. It is obviously sample dependent and is generally not equal to the lateral resolution, which, to the contrary depends on the beam spot size.

Sample Preparation. A rat brain was cut at a temperature of -18°C in a cryostat (model CM3050-S, Leica Microsystems SA, 86 Avenue du 18 Juin 1940, F-92563 Rueil-Malmaison, France). The tissue sections, which have a thickness of $15\ \mu\text{m}$, were immediately deposited onto a stainless steel plate and kept at -80°C . Just before analysis the plates were warmed to room temperature and dried under a pressure of a few mbar for ~ 1 h. No treatment was applied on the samples which were neither rinsed nor matrix coated. The stainless steel plates, purchased from Goodfellow (Ermine Business Park, Huntingdon PE29 6WR, England), were laminated and the composition was (15-7PH) Fe 75%, Cr 15%, Ni 7%, Mo 2.25% (weight %).

RESULTS AND DISCUSSION

Tissue sections: Yields, Disappearance Cross Sections, Efficiencies, Relative Imaging Efficiencies and Useful Lateral Resolution. The disappearance cross sections were determined by bombarding an area of $100 \times 100 \mu\text{m}^2$ of the surface of the rat brain tissue sections and monitoring the secondary ion intensity as a function of time (decay curve). From the slope of the decay curve $\ln(I)$ versus time, σ can be calculated. For the yield determination, spectra were extracted from the beginning of the irradiation (intercept of the decay curve versus time). Peak integral values were determined and yields were calculated by dividing the peak integral values by the primary ion dose. The corresponding efficiency E was determined by dividing the obtained yields by the corresponding disappearance cross sections. For each primary ion species data were taken from two freshly selected positions. It is known that the intensity of secondary ions can differ between the regions of the brain section where the data are collected [15], so we have taken care to always record the data from *inside* the *corpus callosum*. The values have been measured on the rat brain tissue sections for different positive and negative secondary ions. The results are summarised in Table 1 and Figure 2 for $[\text{M-H}]^+$ secondary ions of cholesterol (m/z 385), showing the average values over the 2 positions (other data not shown). The data could not be exactly compared to those obtained in the paper of Touboul *et al* [15] as they were recorded with two slightly different mass spectrometers and from different tissue sections (from a mouse brain in the mentioned reference and from a rat brain in the present work). Nevertheless, estimated values for Au_1^+ and Au_3^+ have been added in Figure 2, assuming that yields Y and disappearance cross sections σ must be equal for Au_1^+ and Bi_1^+ and for Au_3^+ and Bi_3^+ respectively. This extrapolation is valid because gold is an element located nearby bismuth in the periodic table (atomic numbers 79 and 83 respectively). It is known that the nuclear stopping is mainly responsible for the organic secondary ion production in the present impact energy range [25]. When comparing with the SRIM code [26] the nuclear stopping of gold and of bismuth into adipose tissue (brain tissue is not available with SRIM), it is found that the difference is never greater than 2% between 5 and 50 keV.

It has been shown by Benguerba *et al* [14] and more recently by Nagy *et al* [27] that secondary ion emission yields under the impact of Au_n^+ clusters were proportional to n^2v^2 , with n and v being the number of constituents and the velocity of the projectiles, respectively. The following discussion is supported by this important feature. In the present work, the singly charged projectiles Bi_1^+ , Bi_3^+ , Bi_5^+ and Bi_7^+ have a kinetic energy of 25 keV, while the doubly charged species Bi_3^{2+} and Bi_5^{2+} have twice the value, *i.e.* 50 keV. The secondary ion emission induced by these different projectiles can be compared, having in mind that at the same kinetic energy, the velocities range inversely to the square root of the number of constituents. Singly and doubly charged species having the same number of constituents enable to study the influence of the velocity independently of the projectile mass.

The yield increases by a factor of 8.4 when switching from Bi_1^+ to Bi_3^+ while the disappearance cross section only rises by a factor of 1.6 resulting in an efficiency enhancement by a factor of 5. Therefore, the secondary ion generation is more efficient with Bi_3^+ bombardment than with Bi_1^+ bombardment and therefore a lower primary ion dose can be used.

Yields and efficiencies for Bi_3^{2+} are unexpectedly very similar to those obtained for Bi_3^+ , although its kinetic energy is 50 keV instead of 25 keV. It can be seen in reference [28] that the energy per atom for which the secondary ion emission yield maximum occurs was not measured with precision (see for example the results with the phenylalanine and lipid ethylene glycol compounds, where it is clearly written that the solid curves were only to guide the eye). Without further experimental results, the energy value of the maximum can range between $\sim 10\text{-}15$ and $\sim 40\text{-}80$ keV per atom, likely depending on both the size of the projectile and the irradiated compound. So, a possible explanation of the similar yields for Bi_3^+ and Bi_3^{2+} could be that the velocity of a 50 keV energy Bi_3^{2+} is too high with respect to the secondary ion emission maximum.

Bi_5^+ produces lower yield and efficiency values than Bi_3^+ . This is probably due to the fact that the expected increase of yield due to the increase of projectile mass is in this case counterbalanced by the decrease of velocity at the same total kinetic energy.

On the other hand, the yield obtained with Bi_5^{2+} ions is higher than for Bi_3^+ and the disappearance cross section is lower, leading to an efficiency about 1.6 times greater than for Bi_3^+ . In this case, the increase of mass is not counterbalanced by a decrease of velocity, as Bi_5^{2+} has a velocity which is slightly higher than the one of Bi_3^+ .

The Bi_7^+ projectiles have a surprisingly low efficiency which is only the same as for Bi_1^+ . Since the primary ion current is very low the corresponding measurement time to achieve a sufficient primary ion dose density is rather long. In this case the sample damage caused by the low energy electron beam used to compensate sample charging can no longer be neglected [29].

Therefore Bi_5^{2+} ions are the best projectiles for spectroscopy measurements. This can also be illustrated by looking at the useful lateral resolutions, which are the best (377 nm) for Bi_5^{2+} , while it is ~ 500 nm for Bi_3^+ and more than 1 μm for Bi_1^+ .

In order to compare the imaging capabilities of these primary ions, it is necessary to take into account the primary ion currents in addition to the results discussed above. Indeed, a primary ion that would have the best efficiency but the smallest intensity would not be able to acquire secondary ion images in a short time. The influence of the low energy electron damage makes it even more important to achieve short measurement times. That is the reason why we have introduced the additional parameter R . This parameter takes into account both the secondary ion emission yield *and* the primary ion current. Figure 2D shows that for large area imaging purposes it is Bi_3^+ which is the best primary ion, having the highest value of R . Due to the fact that the Bi_3^+ ion intensity is about 7 times higher than it was for Au_3^+ , a secondary ion image can be recorded with the same signal intensity within a 7 times shorter acquisition time, or during equivalent acquisition times one may record an image with a 7 times signal intensity. This is particularly important when recording secondary ion images of large areas such as for an entire rat brain section (more than 3 cm^2), for which it takes rather a long time to reach a primary ion dose density higher than a few 10^9 ions. cm^{-2} . Otherwise, if only areas smaller than $500 \times 500 \mu\text{m}^2$ are considered the limited sample material becomes significant. In this case Bi_5^{2+} ions are preferable because of their particularly attractive efficiency.

A tissue section is obviously not a homogeneous surface and is consequently not best suited to precisely measure the above values, which can change significantly from one irradiated area to another. In addition, the values presented in Table 1 and Figure 2, particularly the useful lateral resolution, are significant only for the present model, which is a rat brain section. Nevertheless, the conclusions concerning the two most relevant primary ions Bi_3^+ and Bi_5^{2+} can be extrapolated to other biological or even non-biological models.

Tissue sections: Imaging. The area of the square circumscribed by the section is $18 \times 18 \text{ mm}^2$. The corresponding images (256×256 pixels) are obtained within 3.5 hours with a spatial resolution of $70 \text{ }\mu\text{m}$, 256 shots per pixel and a primary ion dose density of $10^9 \text{ Bi}_3^+ \text{ ions.cm}^{-2}$. Figure 3A to 3F displays images corresponding to the positive fragment ions of choline (m/z 86), phosphocholine head group (m/z 184), cholesterol $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$ (m/z 369) and $[\text{M}-\text{H}]^+$ (m/z 385), Vitamin E (m/z 430) and phospholipids (mean m/z 769), respectively. Different zones are precisely identified with an anatomic atlas [30]. The presence of such ions at the surface of a rat brain section has already been described and discussed in the literature [15,16]. Figures 4A to 4G show images of negative ions, which were acquired after the acquisition of the positive ion images, exactly on the same area of the same rat brain section. The image shows the spatial distributions of phosphate ions $[\text{PO}_3]^-$ (m/z 79), carboxylate ions of C16:0 (m/z 255) and of C18:0 (m/z 283) fatty acids, cholesterol $[\text{M}-\text{H}]^-$ ions (m/z 385), Vitamin E ions (m/z 429), and triglyceride ions (m/z 811 and 892). The intensity scales of these images are much higher than could be previously obtained with Au_3^+ gold clusters and equivalent acquisition times. The increase of primary ion intensity with the bismuth LMIG makes it possible to acquire such large area images in only a few hours, with a primary ion dose density leading to very good image contrast (scales of several tens or more) of secondary ions heavier than 500 Daltons, such as phospholipids or triglycerides. These images show with precision some particular structures of the brain, like the inner structure of the *gyrus dente* (Figure 4G).

One can observe from the positive ion images that the ions at m/z 184 are not only coming from the fragmentation of glycerophosphatidycholine ions, but can also come from phosphocholine itself, as the

distribution of the ions at m/z 184 and at m/z 769 (average value) are different. This is also illustrated with the color overlay shown in Figure 5. A substructure inside the *corpus callosum* can also be observed from both the positive and negative ion images. An inner line corresponds to a colocalization of C18 fatty acids – and not C16 ones – with triglycerides and phosphocholine. In the same inner line, other ions like cholesterol and Vitamin E (Figures 3C, 3D and 4D) are less abundant while mainly present in the *corpus callosum*. An area at the top center of the brain shows a particular abundance of triglyceride ions (Figure 4G), which are here localized with the C18 fatty acid ions (Figure 4C), indicating that in this region that these fatty acid are mainly coming from fragmentation of the triglycerides. The localization of fatty acids and triglycerides is also illustrated with the Figure 6A, while the Figure 6B clearly shows the differences in the spatial distribution of C16 and C18 fatty acids. A small intensity difference between the left and the right part of Figures 3 to 6 is simply due to a small non corrected height difference of the sample which slightly changed the secondary ion extraction conditions. An interpolation method has been used for a few pixels in order to correct for a temporary loss of signal.

In order to compare the capabilities of the different bismuth cluster beams for small area imaging, the following data acquisitions were made from areas of $256 \times 256 \mu\text{m}^2$ (256×256 pixels, pixel size $1 \times 1 \mu\text{m}^2$), always with the same primary ion dose density of 10^{12} ions. cm^{-2} . The images were all acquired at the edge of the *corpus callosum*, each one on a different area in order to always ensure a fresh and non irradiated zone. The primary ions chosen for the comparison were Bi_1^+ , Bi_3^+ and Bi_5^{2+} . With regard to the available pulsed primary ion intensities, the time necessary to reach 10^{12} ions. cm^{-2} was respectively: less than 4 minutes for Bi_1^+ , 10.5 minutes for Bi_3^+ and 100 minutes for Bi_5^{2+} . Figure 7 shows the resulting images for three different positive ions (m/z 184, 369 and 755-average value) and Figure 8 for two different negative ions (m/z 385 and 771-average value). It is clear that Bi_1^+ can be considered to be sufficiently efficient to acquire an image of light positive ions like those of the phosphocholine head group (m/z 184, Figure 7A) and cholesterol $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$ at m/z 369 (Figure 7B), or other ions (data not shown) like sodium (m/z 23), potassium (m/z 39), phosphocholine fragment (m/z 86). Nevertheless,

images of heavier mass to charge ratio ions like phospholipids (mean m/z 755, Figure 7C) are barely distinguished with Bi_1^+ primary ions. Bi_3^+ provides much more contrasted images for all these positive secondary ions (Figures 7D to 7F), and the intensities are further increased when using Bi_5^{2+} (Figures 7G to 7I). The same observations are reinforced in the negative ion mode, where only the secondary ions corresponding to cholesterol $[M-H]^-$ (m/z 385, Figure 8A) and Vitamin E (not shown, m/z 429) can be distinguished when using Bi_1^+ primary ions. The images corresponding to other ions like the carboxylate ions of C16:0 (m/z 255) and C18:0 (m/z 283) fatty acids or triglycerides (not shown) show little contrast and the images corresponding to the other negative ions such as m/z 771 (Figure 8B) are completely black. On the contrary the use of Bi_3^+ (Figures 8C and 8D), and furthermore the use of Bi_5^{2+} , largely increases the intensities, making all the images easily observable (Figures 8E and 8F). At this step of the discussion, one should remember that the useful lateral resolution measured with cholesterol ions is ~ 500 nm for Bi_3^+ and less than 400 nm for Bi_5^{2+} . Indeed, it appears that the accuracy of the images obtained with Bi_5^{2+} is better than with Bi_3^+ , although both recorded with the same pixel size (the pixel size, the useful lateral resolution and the beam size have the same order of magnitude, therefore the accuracy depends on these three parameters). For all the secondary ions shown the number of counts is better with Bi_5^{2+} than with Bi_3^+ . When switching from Bi_1^+ to Bi_3^+ primary ion beams, the increase of counts and of contrast is obtained at the expense of an acquisition time lengthened by a factor 2.6 (from 4 to 10.5 minutes). In the meantime, the acquisition time is further increased tenfold when changing the Bi_3^+ to Bi_5^{2+} ions. This drawback is counterbalanced by the enormous advantage of having the best efficiency and the best useful lateral resolution. It makes it possible to successfully image, for example, very thin areas at the edge of the *corpus callosum*, corresponding to only a few cells and having a thickness of less than 50 micrometers (see for example ions at m/z 385 in Figure 8E). Edgings between different regions can also be very well imaged. It can also be observed in Figure 9, as in Figure 4, that carboxylate ions of C16 and C18 fatty acids have different localizations (negative one to the other in this region) at the edge of the *corpus callosum*. Finally, Figure 10 shows that it is possible to obtain the image of ions heavier than 1000 Daltons with a pixel size of only $1 \times 1 \mu m^2$ when using Bi_5^{2+} as primary

ions. On the other hand, Figure 11 shows an image of the positive secondary ions of cholesterol obtained inside the *corpus callosum* with a pixel size of only 435x435 nm² and with Bi₃⁺ primary ions. The time necessary to obtain this image was less than 2 minutes, and the primary ion dose density was 5x10¹² ions.cm⁻². This pixel size was chosen to illustrate the values of the useful lateral resolution obtained with the same secondary ion species and in the same region of the brain.

CONCLUSION

The analytical potential of the new bismuth cluster LMIG has been explored using a model sample made of a rat brain tissue section analyzed without any preliminary washing or coating. It is shown that the increase of primary cluster ion intensity makes this ion source the most powerful when using Bi₃⁺ or Bi₅²⁺. This source has better characteristics than the gold LMIG already used in TOF-SIMS biological imaging, mainly because of the increase of primary ion intensity. While Bi₃⁺ and Au₃⁺ can provide images of comparable quality, the data acquisition can be made more rapidly with Bi_n⁺ than with Au_n⁺. Furthermore, this ion source delivers doubly charged heavy ion beams like those of Bi₅²⁺, which make it possible to reach a useful lateral resolution below 400 nanometers. Under these conditions the recourse to any sample coating, which is done at the expense of the lateral resolution, is unnecessary. In other words, this new cluster primary ion source opens the possibility to obtain images of animal or human tissue sections under excellent analytical conditions, with reasonable acquisition times, at sub-micron spatial resolution and a mass range extended to more than one thousand Daltons. This method will be useful for a large variety of biological applications involving the localization of lipids, drugs and metabolites. Furthermore, as the primary ion dose density can be maintained at a few 10⁹ ions.cm⁻², far below the static SIMS limit of 10¹³ ions.cm⁻², the damage induced to the surface can be considered to be negligible for a subsequent MALDI imaging, allowing complementary localization of peptides and proteins.

ACKNOWLEDGMENTS

D.T. is indebted to the Institut de Chimie des Substances Naturelles (CNRS) for a Ph.D. research fellowship. Ion-Tof GmbH is gratefully acknowledged for providing the bismuth LMIG. Many thanks to Colin Helliwell (ION-TOF GmbH) for his assistance when reviewing the manuscript and to Alfred Benninghoven (ION-TOF GmbH) for fruitful discussions.

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Table 1: Secondary ion yields Y , disappearance cross sections σ , ion bombardment efficiencies E , relative specific data rate $R_{\text{Bi}_i^+}$ and useful lateral resolution ΔL for Bi_n^q+ bombardment of cholesterol ($[\text{M-H}]^+$; m/z 385) in the *corpus callosum* of a rat brain section.

Primary Ion	Y	σ (cm ²)	E (cm ⁻²)	$R_{\text{Bi}_i^+}$	ΔL (nm)
Bi_1^+	8.36×10^{-05}	2.75×10^{-13}	$3.04 \times 10^{+08}$	1.00	1148
Bi_3^+	7.06×10^{-04}	4.14×10^{-13}	$1.71 \times 10^{+09}$	2.86	484
Bi_3^{2+}	6.39×10^{-04}	4.96×10^{-13}	$1.29 \times 10^{+09}$	1.41	557
Bi_5^+	3.16×10^{-04}	4.43×10^{-13}	$7.14 \times 10^{+08}$	0.17	749
Bi_5^{2+}	9.91×10^{-04}	3.52×10^{-13}	$2.81 \times 10^{+09}$	0.52	377
Bi_7^+	4.06×10^{-04}	1.92×10^{-12}	$2.12 \times 10^{+08}$	1.94×10^{-2}	1374

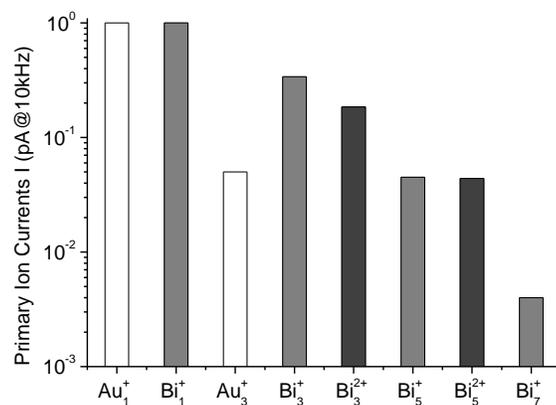


Figure 1: Bismuth cluster ion currents, measured using a Faraday cup located on the grounded sample holder at 10 kHz. The measured values have been divided by 2 for the doubly charged ions, in order to show the number of clusters per second. Gray bars are for singly charged ions, black bars for doubly charged ones. The white bars indicate the gold cluster ion currents that can be obtained under the same conditions.

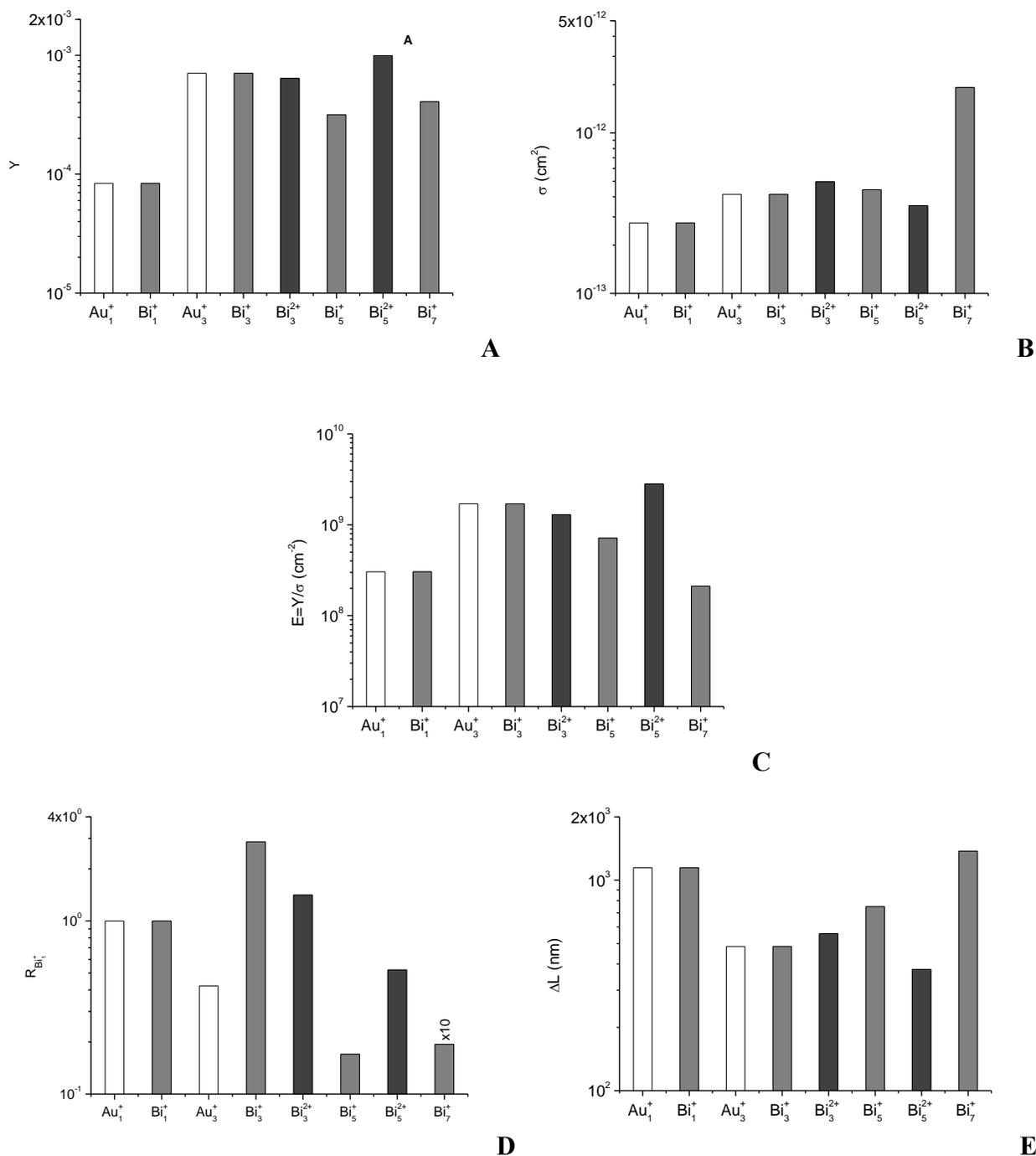


Figure 2: **A:** Secondary ion yields Y ; **B:** disappearance cross sections σ ; **C:** efficiencies E ; **D:** relative specific data rate; $R_{Bi_i^+}$, **E:** useful lateral resolutions ΔL , all measured for the $[M-H]^+$ ions of cholesterol in the *corpus callosum* of a rat brain tissue section and under Bi_n^{q+} primary ion bombardment. Gray bars are for singly charged ions, black bars for doubly charged ones. Estimated values for Au clusters are shown with white bars for comparison.

Field of view: 18000.0 x 18000.0 μm^2

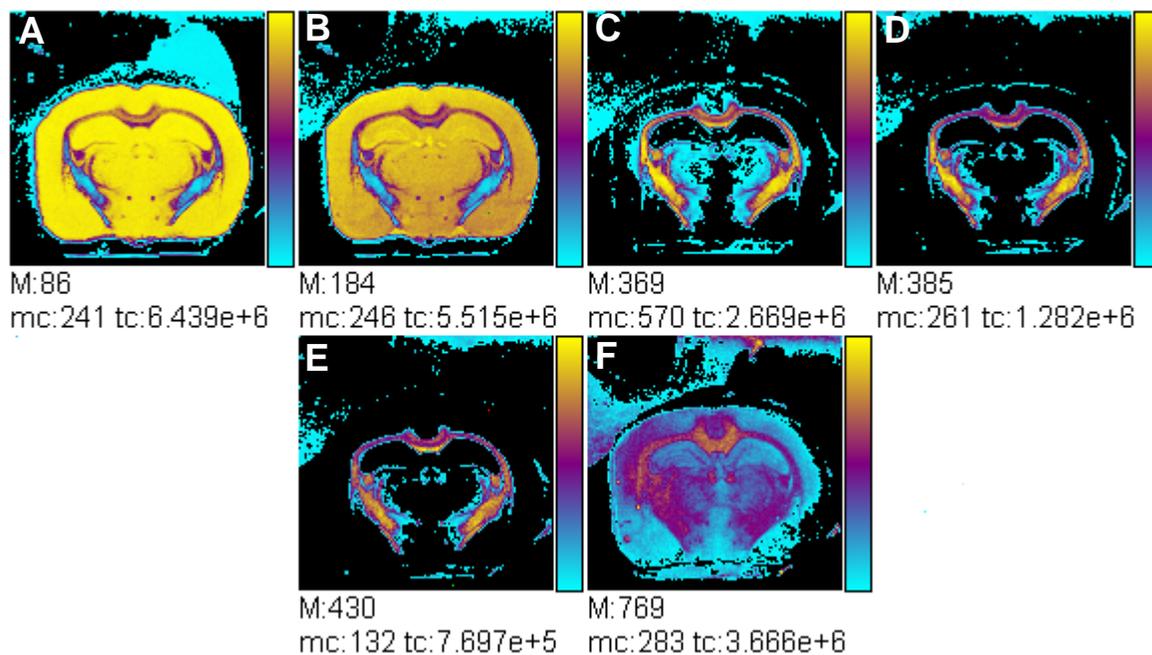


Figure 3: Positive secondary ion images obtained from a rat brain section under the irradiation of Bi_3^+ primary ions. 256x256 pixels, pixel size $70 \times 70 \mu\text{m}^2$. See text for details.

Field of view: 18000.0 x 18000.0 μm^2

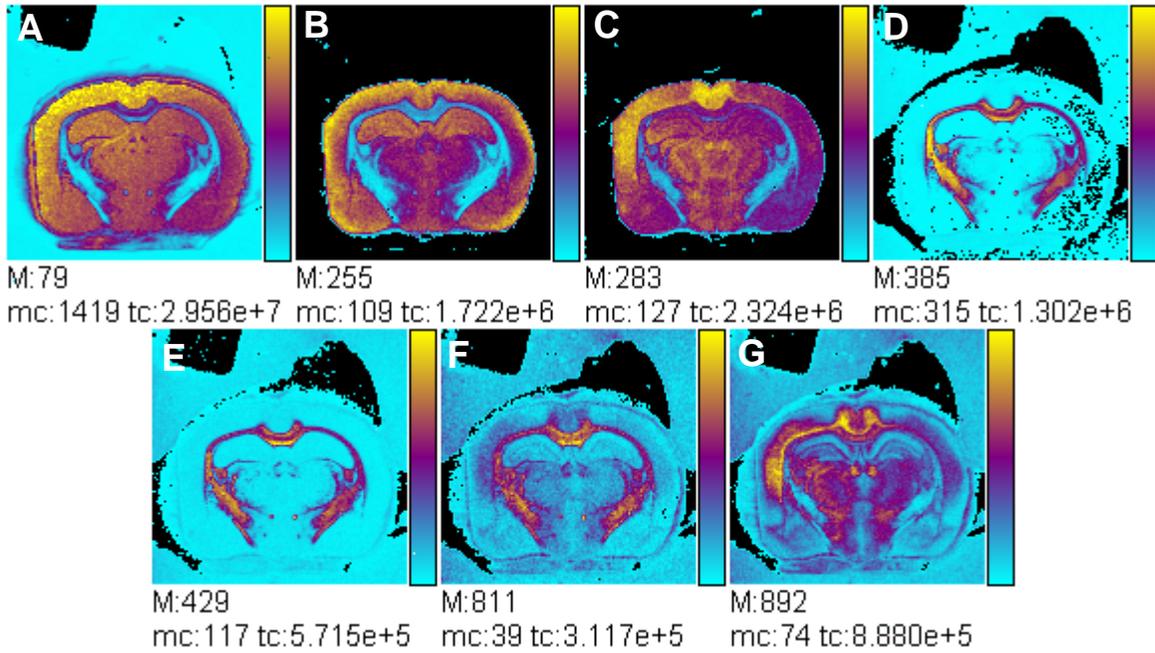
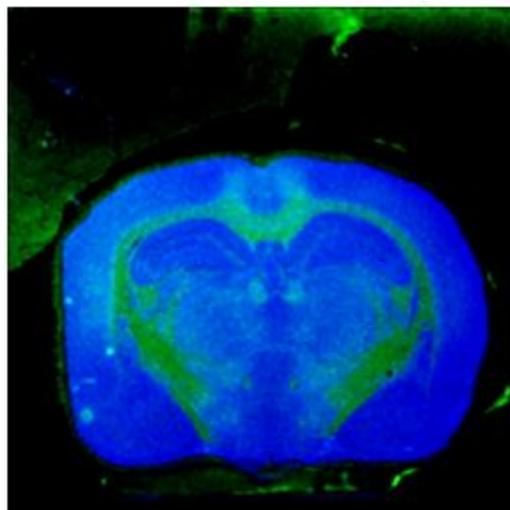


Figure 4: Negative secondary ion images obtained from a rat brain section under the irradiation of Bi_3^+ primary ions. 256x256 pixels, pixel size 70x70 μm^2 . See text for details. This image has been acquired from the same tissue section that the image of Figure 3.

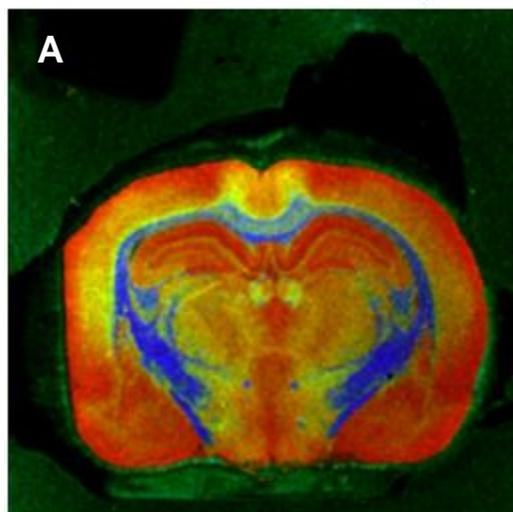
Field of view: 18000.0 × 18000.0 μm^2



Blue=184
Green=769

Figure 5: Color overlay showing the different localizations of the positive ions at m/z 184 (blue) and m/z 769 (green). Same data acquisition as for Figure 3.

Field of view: 18000.0 × 18000.0 μm²



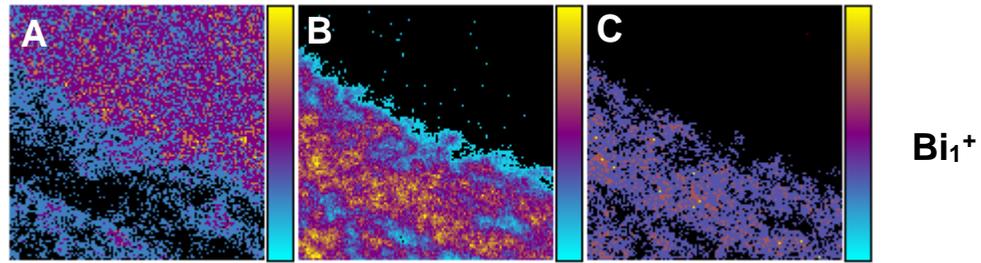
red=(255+283)
green=892 blue=771



red=283
blue=255

Figure 6: Color overlays showing the different localizations of the following negative ions: A: m/z (255+283) (red), m/z 892 (green) and m/z 771 (blue); B: m/z 255 (blue) and m/z 283 (red). Same data acquisition as for Figure 4.

Field of view: 256.0 x 256.0 μm^2



M:184

mc:8 tc:1.099e+5

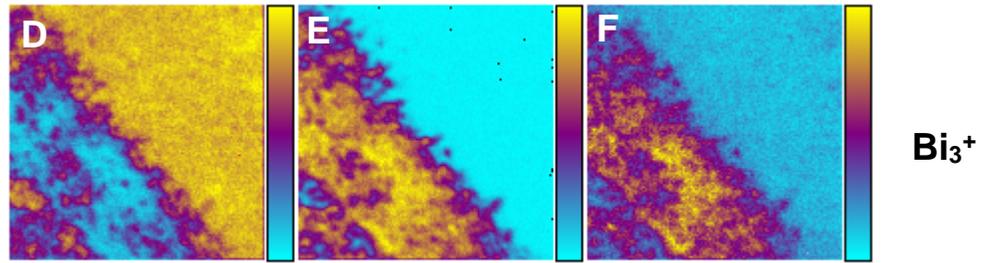
M:369

mc:16 tc:1.924e+5

M:755

mc:7 tc:4.690e+4

Field of view: 255.9 x 255.9 μm^2



M:184

mc:44 tc:1.658e+6

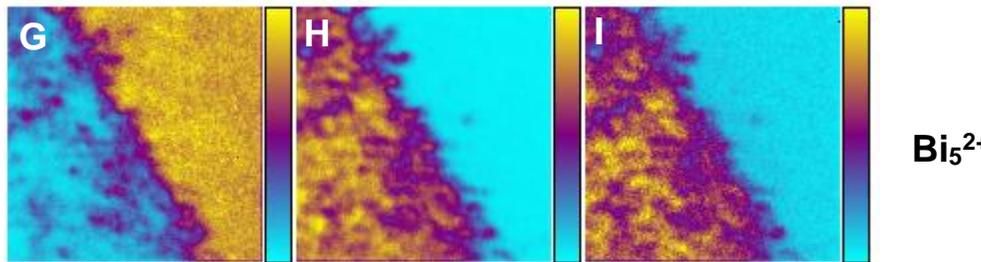
M:369

mc:118 tc:2.366e+6

M:755

mc:45 tc:7.969e+5

Field of view: 256.0 x 256.0 μm^2



M:184

mc:91 tc:2.654e+6

M:369

mc:190 tc:4.205e+6

M:755

mc:61 tc:1.168e+6

Figure 7: Positive secondary ion images obtained under the irradiation of Bi_1^+ , Bi_3^+ and Bi_5^{2+} primary ions and with the same primary ion dose density of 10^{12} ions. cm^{-2} . The *corpus callosum* is on the left part of the images. 256x256 pixels, pixel size 1x1 μm^2

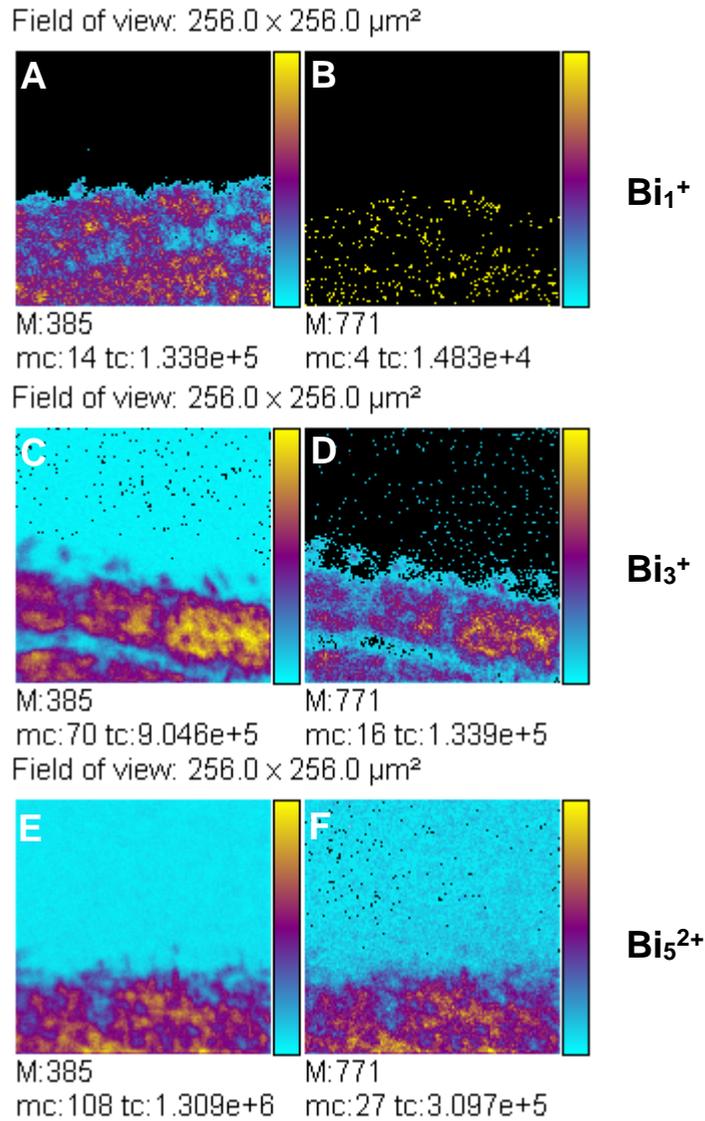
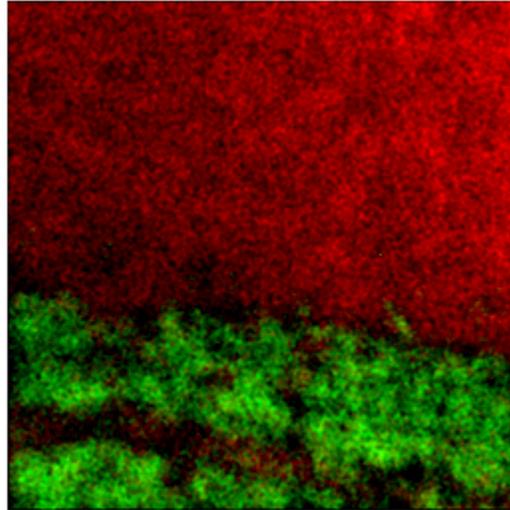


Figure 8: Negative secondary ion images obtained under the irradiation of Bi_1^+ , Bi_3^+ and Bi_5^{2+} primary ions and with the same primary ion dose density of 10^{12} ions. cm^{-2} . The *corpus callosum* is on the bottom part of the images. 256×256 pixels, pixel size $1 \times 1 \mu\text{m}^2$

Field of view: 256.0 × 256.0 μm^2



red=255
green=283

Figure 9: Two color overlay showing the different localizations of the negative ions at m/z 255 (red) and m/z 283 (green). The *corpus callosum* is at the bottom part of the image. Same data acquisition as for Figures 8C-8D (Bi_3^+ primary ions, 256x256 pixels, pixel size 1x1 μm^2).

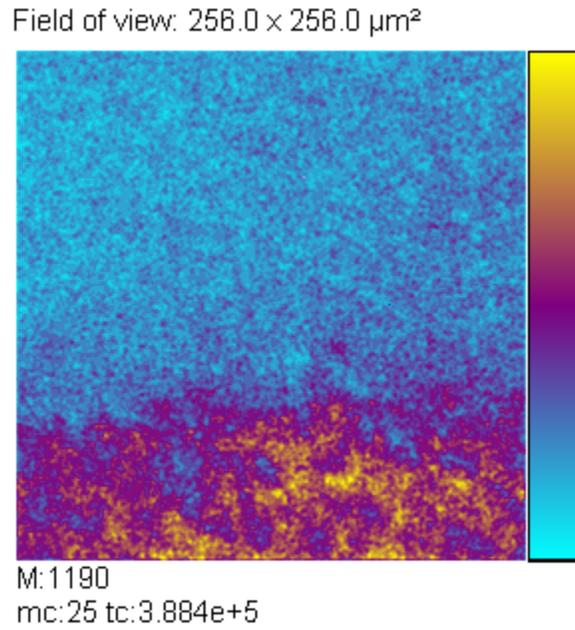
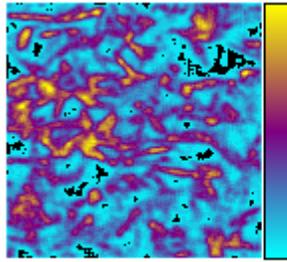


Figure 10: Negative secondary ion (m/z 1190) image obtained under the irradiation of Bi_5^{2+} primary ions. The *corpus callosum* is on the bottom part of the images. 256x256 pixels, pixel size 1x1 μm². Same data acquisition as for Figures 8E-8F.

Field of view: $55.7 \times 55.7 \mu\text{m}^2$



M:385

mc:29 tc:1.082e+5

Figure 11: Positive secondary ion image of the cholesterol ions obtained inside the *corpus callosum* under the irradiation of Bi_3^+ primary ions and with a primary ion dose density of 5.10^{12} ions. cm^{-2} .
128x128 pixels, pixel size $435 \times 435 \text{ nm}^2$