Analytical procedure for mapping the distribution of $^{10}$B and $^{99}$Tc markers in cryo-sections of animal tissue samples by secondary ion mass spectrometry

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A B S T R A C T
The development of a complete, standard analytical procedure for a quantitative use of secondary ion mass spectrometry to map the distribution in animal tissues of exogenous isotopes presents difficulties inherently related to sample preparation and preservation, as well as to the specific application being considered. We have tested in two very different cases a procedure based on the cryo-preparation of samples and calibration standards. The applications under investigation were the mapping of $^{10}$B in mouse brain tissue, with relevance to the boron neutron capture therapy, and of the perfusion tracer $^{99}$Tc in mouse heart tissue, with relevance to the study of microcirculation and cardiovascular pathologies. Scanning electron microscopy and inductively coupled mass spectrometry analysis were used as reference techniques for secondary ion mass spectrometry images and analyte measurements, respectively. Cryo-preparation of tissue sections for ion microscopy proved to be simple and efficient (in terms of structural and chemical integrity) for both brain and heart samples derived from fresh organs. This technique, however, turned out to be reliable only on the brain tissue when applied to the preparation of standards, which required chemical fixation of portions of organs. Brain and heart tissues showed a totally different response to chemical fixation, from both a structural and an analytical point of view. On the one hand, we were able to estimate a relative sensitivity factor for $^{10}$B in the cryo-sectioned brain matrix; on the other hand, even without the possibility of an absolute quantification of the $^{99}$Tc signal and notwithstanding the presence of an isobaric interference, secondary ion mass spectrometry mapping however proved to be capable to resolve the specific response of the cardiac tissue to the perfusion mechanism.

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1. Introduction

Molecular imaging is a valuable tool to understand the mechanism of action of drugs and how diagnostic agents like radiopharmaceuticals and contrast media are distributed inside a target tissue. For example, knowing how radioligands used for diagnostics or therapy are distributed in human tissue is an essential prerequisite for dosimetry studies in nuclear medicine. Furthermore, development of molecular biology and personalized medicine also calls for high-sensitivity and high-specificity tools to assess variations in cell and tissue composition following the exposure to noxae and drugs, or the onset of pathologies.

Molecular imaging mainly relies on positron emission tomography, magnetic resonance spectroscopy, and infrared spectromicroscopy. Imaging of peptides and ion distributions using matrix-assisted laser-desorption-ionization mass spectrometry is developing as a powerful tool in proteomics and metabolomics [1]. Among the imaging/microprobing techniques, secondary ion mass spectrometry (SIMS) is however the only one capable to image practically all the elements in the periodic table – including stable and radioactive isotopes – with high sensitivity and spatial resolution (typically at the μm scale).

SIMS, first developed by Castaign and Slodzian in the sixties [2,3], is based on the progressive sputtering of the most superficial molecular layers of a solid specimen, by means of a primary ion beam focused onto the surface. The secondary ions produced are characteristic of the atomic composition of the area analyzed. After an extraction/acceleration stage,
they are energy-filtered, separated by means of a mass spectrometer, focused and finally detected.

SIMS has opened new horizons in the field of imaging techniques for biological investigations [4,5], as a first-hand tool to track selected endogenous/exogenous elements. It can also be used as a powerful probe in connection with tracing techniques. These are based on the use, within the biological system under investigation, of isotopic species which do not occur naturally in the sample, such as artificially-produced radioactive isotopes, or are introduced at a higher enrichment with respect to their natural abundance.

Although the high elemental sensitivity, a good spatial resolution and an excellent depth resolution make SIMS the ideal tool for the study of the elemental distribution in animal cells and tissues, several analytical problems have prevented quantitative imaging of biological samples from becoming routine. It is worth mentioning, for example, the influence of local (i.e. position-dependent) matrix effects. But sample preparation undoubtedly represents the most challenging aspect of SIMS analysis on biological matrices [6,7].

The ideal methodology should preserve the chemical and structural integrity of the matrix, as well as provide a flat and conductive sample. This has been the object of several works over the last 20 years [8–10]. As a widely accepted protocol, tissue sections are applied on a conductive substrate and the water content, which is a characteristic of any biological matrix, is eliminated trying to avoid structural damage [11,12]. Cryogenic preparations, which involve cryo-sectioning and freeze-drying, have been proved to be more reliable than solvent substitution and resin-embedding as far as chemical preservation is concerned [13].

This study focuses on the development and testing of a complete analytical procedure, based on the cryo-preparation of samples and calibration standards, aimed at using SIMS as a quantitative technique to map the distribution in tissues of exogenous and enriched isotopes relevant to nuclear medicine, drug development and pathophysiological issues. We have investigated two potential applications: 1) a quantitative approach to the mapping of $^{10}$B distribution in brain tissue, with relevance to the boron neutron capture therapy (BNCT) [14], and 2) the detection of $^{99m}$Tc in heart tissue sections, which represents an important stepping stone for the modeling and understanding of microcirculation and cardiovascular pathologies [15]. Scanning electron microscopy (SEM) and inductively coupled plasma mass spectrometry (ICP-MS) have been used as reference techniques for SIMS images and analytic measurements.

1.1. Boron neutron capture therapy

Quantitative localization of boron in cerebral tissue has a strong relevance to the progress of BNCT, a novel strategy for the treatment of highly aggressive brain tumors and melanoma.

BNCT relies on initial targeting of tumor cells by an appropriate chemical compound tagged with $^{10}$B – a stable isotope of boron having a high interaction cross section with thermal neutrons – which preferentially concentrates in tumor cells [16]. By the effect of irradiation of the tumor site by neutrons, the $^{10}$B nucleus absorbs a low-energy neutron and decays into an energetic short-range $\alpha$-particle and a Li ion which deposit most of their energy within the cell containing the parent $^{10}$B nucleus. Therefore, if a higher concentration of $^{10}$B occurs in tumor cells with respect to normal tissues, a concomitantly higher dose will be delivered to the tumor during neutron irradiation.

The selectivity of boron carriers towards the tumor cells is a key factor for the clinical application of this therapy [17]. SIMS has been indicated [18,19] as a suitable technique to obtain the quantitative localization of boron in cerebral tissue and the measurement of the $^{10}$B/$^{11}$B ratio [20,21], which allow to assess the effectiveness of different candidates as $^{10}$B carrier drugs [22]. In this work, we have studied the localization of $^{10}$B-enriched 4-Borono-L-phenylalanine (BPA), used in the most stable form of adduct with fructose (f-BPA), which have been already applied in patients for BNCT treatment [23].

1.2. Imaging and modeling of myocardial perfusion

$^{99m}$Tc is a $\gamma$-emitter (half-life of 6 h) widely utilized in nuclear medicine as a labeling agent of radiopharmaceuticals. $^{99m}$Tc decays to $^{99}$Tc, a long-lived radioisotope with a half-life of 2.13 $\cdot$ $10^5$ yr; therefore, the radiopharmaceuticals used in nuclear medicine contain both species in a variable proportion. Among the $^{99m}$Tc-labeled radiopharmaceuticals, bis(N-ethoxy,N-ethyl)dithiocarbamato nitride technetium-99 m ($^{99m}$Tc-N-OEt) is used in nuclear cardiology as myocardial blood-flow tracer thanks to the fact that the tracer is extracted by the tissue in proportion to the local blood flow. It has been also suggested as an alternative to $^{201}$Tl for imaging by single-photon-emission computed tomography in the assessment of coronary artery disease and myocardial viability [15]. In this study, $^{99}$Tc-N-OEt has been used – via a perfusion technique – as a “probe” for the study of myocardial microcirculation by ion imaging.

The perfusion of the isolated heart of small mammals is an experimental technique which offers an effective reproduction of the cardiac functioning, both in ischemic and unaffected conditions. This is performed according to the so-called Langendorff model [24], see Fig. 1. Mapping the distribution of a tracer in the tissue provides important indications for the study of the perfusion mechanism, which in turn is fundamental for the understanding of many related pathologies. The tissue we wanted to investigate in this work is the possibility of assessing the blood flow in a very small scale of coronary microcirculation.

While the concentration of $^{99m}$Tc should be kept as large as possible in order to be detected by a $\gamma$-camera, in SIMS analysis this is not necessary since both $^{99}$Tc and $^{99m}$Tc can be detected, giving the same signal. Moreover, the measurement time is no longer limited by the short half-life of $^{99m}$Tc. Artifacts and matrix effects, as well as mass interferences, are however a major problem of every SIMS analysis and must be taken into account. Previous works exist about SIMS analysis of the $^{99}$Tc distribution in cells [25–27], and report the existence of a polyatomic interference which arises from the biological matrix. Mass resolution of or above 5000 is typically referred (depending on the sample) to detect the $^{99}$Tc specific signal. We report that such a high resolution cannot be achieved in the imaging of fresh cryo-fixed tissue; nevertheless, our results indicate that the distribution of $^{99}$Tc can still be analyzed by means of SIMS even at a lower resolution, and can provide new insights into myocardial perfusion imaging in the isolated heart model.

2. Experimental

We analyzed tissue sections obtained from mouse organs such as heart or brain, in order to investigate the distribution of two isotopes, $^{10}$B and $^{99}$Tc respectively, which were relevant to different biomedical applications.

Male Wistar rats weighing 275–300 g (Harlan, Italy) were used to study the brain distribution of $^{10}$B and male C57BL/6 mice weighing 28–32 g (Harlan, Italy) were used to investigate the heart uptake of a $^{99}$Tc-conjugated tracer.

Experimental protocols have been approved by the Animal Care Committee of the Italian Ministry of Health and they conform to the “Guiding Principles for Research Involving Animals and Human Beings”, approved by the American Physiological Society.

2.1. Sample preparation

2.1.1. $^{10}$B-containing cerebral samples

Investigation of the $^{10}$B distribution in brain tissue was conducted on rats whose right carotid artery was cannulated with a
30-gage cannula under anesthesia with pentobarbitone sodium (40 mg/kg ip). Then, a solution of \(^{10B}\)-enriched 4-Borono-L-phenylalanine (Boron Biological Inc.) complexed to fructose (Sigma-Aldrich) \cite{28} was infused for 20 min at the dose of 500 mg/kg body weight. Rats still under deep anesthesia were sacrificed 180 min after boron administration by a lethal dose of barbitone. The brains were excised, immediately frozen in liquid nitrogen and stored at \(-80^\circ C\). As a reference sample, the brain of a not infused animal was used.

### 2.1.2. \(^{99}\)Tc-containing cardiac samples

To investigate the \(^{99}\)Tc distribution we used the ex vivo beating heart model according to Langendorff \cite{24}, in conjunction to infusion with the flow tracer \(^{99}\)Tc-N-NOEt. Briefly, the mice were heparinized (500 U im) 10 min prior to inducing anesthesia with pentobarbitone sodium (40 mg/kg ip). The hearts were excised and transferred to a modified, non-recirculating Langendorff apparatus where they were perfused with a Krebs–Henseleit bicarbonate (KHB) solution (kept at 37 °C and pre-equilibrated with 95% O₂ and 5% CO₂ at pH 7.4) at a constant pressure of 65 mmHg, and allowed to beat spontaneously. The apparatus was equipped with two side-arms in the perfusion pathways, located close to the heart inlet, consenting to add to the main perfusion line an accessory infusion line for the administration of \(^{99}\)Tc-N-NOEt (Fig. 1). The flow tracer solution was freshly prepared daily and a 240 ml bolus was infused for 2 min at the concentration of 0.17 mM. The infusion time and the tracer concentration were derived from theoretical computation on the estimated volume of perfused myocardial tissue and its drug extraction power. At the end of the experiment the hearts were rapidly frozen in liquid nitrogen and stored at \(-80^\circ C\). The hearts perfused with KHB solution but not infused with \(^{99}\)Tc-N-NOEt were used as control.

### 2.1.3. Preparation of tissue samples for SIMS and SEM analysis

For both SIMS and SEM analyses, the organs were excised and frozen, and sections were obtained using a cryo-microtome. Cutting operations were carried out at \(-25^\circ C\). The sections were picked up onto conductive substrates (indium foil coupled to a 5-mm thick aluminum stub) and, taking extreme care in preventing them from defrosting, transferred to a freeze-dryer (VirTis BenchTop 6.6). Freeze-drying was performed at \(-53^\circ C\) and 0.9 psi pressure. After this operation, the samples reached room temperature, but had to be kept in a desiccator in order to avoid rehydration.

### 2.1.4. Preparation of calibration standards

Standard samples were prepared by means of a procedure similar to the one described by Oyedepo et al. \cite{29}.

Portions of the mouse brain fixed in paraformaldehyde were transferred to a Phosphate Buffer Solution (PBS) containing F-BPA in the following nominal concentrations: 0, 40, 250, 400 and 800 ppm. They were kept in these solutions for 48 h, the soaking process being enhanced by a sonic bath. Fixation was required in order to avoid deterioration of the tissue during the soaking stage. Formaldehyde traces were eliminated through a PBS wash. Tissues were then frozen and sections were cut. The remaining part of every organ, after the sectioning operation, was kept frozen in order to detect the bulk \(^{10B}\) concentration by ICP-MS.

The same procedure was applied to the heart samples, which were fixed and then soaked in PBS solution containing \(^{99}\)Tc (nitrate) at the following concentrations: 0, 10, 100, 300 ppm. Cryo-sections were prepared as described above and the remaining part of every sectioned organ was kept for ICP-MS bulk analysis.

### 2.1.5. Samples for ICP-MS analysis

In order to determine the total concentration of the tracer, the frozen organ portions were freeze-dried and digested by means of a microwave digester (Anton Paar MULTIWISE) and ultra pure acids. To a portion of 0.5 g of sample, we added 0.5 ml HCl (35% V/V), 3 ml HNO₃ (65% V/V) and 2 ml H₂O₂ (31% V/V). A 35-min cycle was applied (increasing power from 500 to 1000 W and temperature up to 280 °C). A limpid solution was obtained, and then evaporated by means of an evaporator rotor 6EVAP (Anton Paar MULTIWISE). 1 ml HNO₃ 1% was added to the residue, and diluted to a volume of 100 ml again with HNO₃ 1%. This diluted solution was analyzed by ICP-MS.
2.2. Instrumental

2.2.1. SEM microscopy

Every In-plated tissue section was initially analyzed by means of SEM microscopy. Images of the whole section were obtained in secondary-electron mode, with a magnifying factor of 15. In this way the most relevant details of the sections were isolated, to undergo further magnification (up to 240×).

Two different instruments were used: a Tescan Vega TS5130LS, working at 30 kV, with 50 μA emission current intensity, and a SEM Philips 515, working at 25 kV.

2.2.2. SIMS

The instrument we used is a CAMECA IMS-6F (CAMECA, France), equipped with an O₂²⁻ primary ion source (duoplasmatron). The mass resolution ranges from 300 to 20,000. The maximum lateral resolution is 0.5 μm, while the analyzed area can be varied from 1.5 μm to 400 μm in diameter. The IMS-6F is a so-called “ion microscope” and can be further operated as a microprobe, providing two different modes of localizing the surface distribution of the elements.

In the microscope mode the sample is illuminated by a broad primary ion beam so that each point of the surface can be considered as a source of secondary ions. In this mode, “direct” ion images are obtained by means of a microchannel plate coupled with a fluorescent screen and a Resistive Anode Encoder (RAE). The microchannel plate converts secondary ions to electrons and the RAE generates a pulse for each event, determining the spatial position of the deposited charge. It is possible, this way, to form an ion map with a lateral resolution limited by the diameter of the microchannels.

In the microprobe mode a narrow illuminating beam is used, which can be rastered over the analyzed area. In this mode it is possible to acquire “scanning” ion images that have a higher lateral resolution, a quality directly related to the primary beam diameter. Detection of the secondary ions is performed, depending on the beam intensity, by means of a Faraday Cup (FC) or an Electron Multiplier (EM), resulting in a very high dynamic range for the ion counting. Count rates in the range of 5 · 10⁵–5 · 10⁶cps and 1 · 10⁻¹–1 · 10⁶cps are measured by FC and EM, respectively, the overlap of the intensity ranges allowing to combine both detectors within the same analysis (the secondary beam is automatically diverted to the FC if the output exceeds 2 · 10⁶cps).

All the ion maps collected in our study were obtained via the detection of positive secondary ions in the “scanning mode” by using an O₂²⁻ primary ion beam, accelerated to 10 kV and scanned over a 250 × 250 μm² area. The primary beam diameter was about 3–5 μm.

Before the acquisition of each ion map, a pre-sputtering of the sample surface was operated: for 5 min, a 50 nA primary beam was scanned over a 500 × 500 μm² area, to thin the zone involved in the subsequent analysis was surely included.

23Na and 39K ion maps were obtained by using an 80 pA primary beam and cumulating 10 repeated acquisitions, each one lasting 1 s. At the same time, with the same primary beam intensity, also the maps of 12C (major element in the biological matrix) and ¹¹⁵In (from the conductive substrate) were acquired, in 10 cycles of 20 s and 5 s, respectively. ⁹⁹Tc maps were recorded at 50 nA primary current, in 10 cycles of 20 s. ¹⁰B maps were recorded at 10 nA primary current, in 10 cycles of 20 s.

2.2.3. SIMS image processing

In order to extend the CAMECA IMS-6F capability of image elaboration, a computer program has been developed. This program can access the raw data files acquired by the instrument, making available the punctual EM signal intensities (expressed in counts) of every secondary species inside a selected area of the original ion map. This way, the EM signals at two mass values can be compared over the same zone and their ratio can be calculated. These data can then be exported to a conventional spreadsheet for further analysis.

2.2.4. ICP-MS analysis

All ICP-MS measurements were carried out in a double focusing sector field ICP-MS (Element 2, Thermo Finnigan MAT GmbH, Bremen, Germany). Element 2 is equipped with a self-aspiring micro-concentric nebulizer, a Scott-type spray chamber, a Fassel torch and a 27 MHz generator. Instrumental settings and optimized measurement parameters for boron isotopes are given in Table 1. Isotopic measurements were carried out using only electric scanning (E-scanning), at low (M/ΔM = 300) and medium resolution (M/ΔM = 4000) settings for Tc and B, respectively.

3. Results and discussion

3.1. Preservation of tissue

The preparation of tissue samples for SIMS analysis by means of cryo-sectioning and freeze-drying of fresh organs is known to be the most suitable methodology in order to preserve the elemental distribution in living tissues [30–33]. Using no fixative chemical agents, no cryo-protectants, and no coating of the sections prior to SIMS analysis, as instead suggested by other authors [34], the chemical composition is kept as close as possible to the one of the original biological matrix.

We experienced this procedure to be excellent for our samples, which could be analyzed by means of SIMS as well as SEM, with the possibility of an easy comparison between the two kinds of images. The samples exhibited the same morphology under SIMS and SEM imaging; an example is provided in Fig. 2.

As far as the preservation of the elemental distribution is concerned, the K/Na concentration ratio can be used as an index of preservation of living tissues [35,36]. Inside the cell this ratio is supposed to be around 8, while outside the cytoplasm it is normally reversed, with Na being the predominant element. From the visual interpretation of the SIMS images of our samples, by means of the color scale, the intensity of the ³⁹K⁺ signal – after the removal of the most superficial layers of the tissue sections (pre-sputtering, see Section 2.2.2) – was usually up to a factor of

<table>
<thead>
<tr>
<th>Table 1 HR-ICP-MS instrument settings and scanning conditions.</th>
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<tbody>
<tr>
<td>Sample introduction system and instrumental operating conditions</td>
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<tr>
<td>Nebulizer</td>
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<tr>
<td>Spray chamber</td>
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<tr>
<td>Sampling cone</td>
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<tr>
<td>Skinner cone</td>
</tr>
<tr>
<td>RF Power</td>
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<tr>
<td>Plasma gas flow rate (l/min)</td>
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<tr>
<td>Auxiliary gas flow rate (l/min)</td>
</tr>
<tr>
<td>Nebulizer gas flow rate (l/min)</td>
</tr>
<tr>
<td>Measurement conditions</td>
</tr>
<tr>
<td>Resolution (10 s valley definition)</td>
</tr>
<tr>
<td>Acquisition mode</td>
</tr>
<tr>
<td>Magnet settling time (s)</td>
</tr>
<tr>
<td>Magnet mass</td>
</tr>
<tr>
<td>Mass range (amu)</td>
</tr>
<tr>
<td>¹⁰B</td>
</tr>
<tr>
<td>¹¹B</td>
</tr>
<tr>
<td>⁹⁹Tc</td>
</tr>
</tbody>
</table>
10 higher than the $^{23}$Na$^+$ intensity, as shown in Fig. 3. This can be taken as an evidence of preservation of the original elemental distribution in the living tissue. The ratio of the punctual signal intensities was found slightly different in samples coming from different organs, ranging on the average between 4 and 8.

It should be mentioned, however, that for tissue sections obtained from different organs, the K/Na ratio was observed varying between 5 and 15 by Arlinghaus et al. [37]. Nevertheless, as stated by other authors [38] and generally accepted in micro-imaging study of this kind, Na and K are not supposed to be redistributing themselves inside the tissue section during the phases of the sample preparation here adopted, at least not at the micrometric scale.

3.2. Boron in brain tissue

The study of the $^{10}$B distribution in the brain tissue was performed on samples coming from organs of animals infused with f-BPA and on standard samples obtained by chemically fixed organs soaked in standard BPA solutions. All the sections were analyzed by means of SEM and SIMS, while the remaining part of every organ underwent ICP-MS analysis.

SEM images show that the tissue was compact and morphologically preserved under both kinds of preparation (Fig. 4). In particular, the fixed tissue does not seem damaged or fractured, although fixation and slam-freezing are quite aggressive procedures (Fig. 4a).

SIMS ion maps from both chemically fixed and soaked cerebral tissues show that $^{10}$B was distributed homogeneously through the sample, coherently to the $^{12}$C distribution which indicates the presence of the biological matrix (Fig. 5).

ICP-MS analysis was performed in order to obtain the $^{10}$B concentration value averaged over the whole organ mass. The $^{10}$B concentration in the standard solution was correlated to the $^{10}$B concentration detected in the corresponding standard sample. As the samples were analyzed once they had been dehydrated, the concentration in the living tissue was rescaled taking into account an 80% water content. The fit of the $^{10}$B concentration in tissue as a function of the $^{10}$B concentration in solution shows a linear uptake of the tissue (Fig. 6). By increasing the concentration of the standard solution by 1 ppm, one can expect to obtain an increase by 0.4 ppm in the tissue. In addition, an infused-animal (i.e. non-fixed) sample, labeled “BB”, was also analyzed.

![Fig. 2. SEM and SIMS images of a heart tissue section, showing the same morphology of the detail. a) SEM image (60× magnification). The flat, uniform area is an uncovered zone of the In substrate. b) $^{12}$C$^+$ ion map by SIMS (scale of brightness). The dark areas are clearly distinguishable as not covered by the tissue.](image-url)
All the ICP-MS results are reported in Table 2 and were used to validate SIMS analysis, in order to obtain a calibration factor for $^{10}$B in the cryo-sectioned cerebral tissue. After Ausserer et al. [39], we adopted the following definition of relative sensitivity factor (RSF) for $^{10}$B with respect to the matrix reference element $^{12}$C:

$$\text{RSF}_{^{10}B/^{12}C} = \frac{I_{^{10}B}}{I_{^{12}C}} / c_{^{10}B}$$

(1)

where $I$ indicates the signal intensity and $c_{^{10}B}$ is the $^{10}$B bulk concentration.\(^1\) The following procedure was applied in order to obtain an acceptable statistical significativeness for RSF.

For every sample of the set analyzed by ICP-MS, SIMS images of a tissue section were acquired on 10 different spots. Over each spot, both $^{10}$B$^+$ and $^{12}$C$^+$ maps were recorded, each map being the result of the accumulation of 10 cycles of acquisition. $^{10}$B$^+$ signal intensities were always normalized to the $^{12}$C$^+$ intensities, used as an internal standard. Hence, the mean value of this ratio was calculated over all the spots (see Table 2). In this way, data originated from different samples could be compared. Matching the $^{10}$B concentration by ICP-MS with the $^{10}$B/$^{12}$C ratio by SIMS, a good linearity was observed (Fig. 7). Vertical error bars in the graph represent the standard deviation of the $^{10}$B/$^{12}$C ratio, as reported in Table 2, and take also implicitly into account the statistical variability due to possible local matrix effects over the 10 analyzed spots; horizontal error bars represent a 10% uncertainty conservatively associated to the ICP-MS output. In general, highly matrix-specific. In effect, as it is evident in Fig. 7, the point relating to the sample BB (solid point) matches the trend of the fixed samples (open points).

Finally, the fit returned the value RSF$_{^{10}B/^{12}C} = (1.27 \pm 0.08) \cdot 10^{-2}$ ppm$^{-1}$ (2$\sigma$ standard error), which we assumed as a practical sensitivity factor for $^{10}$B in the cryo-sectioned mouse brain tissue, regardless of the differences in sample preparation required by the two modalities of B uptake (viz. infusion or soaking in solution).

3.3. Technetium in the heart tissue

The same preparation methodology applied to the brain tissue was used to prepare standard heart tissue samples, with the purpose of providing a quantitative significance to the results of the imaging of the $^{99}$Tc distribution after Langendorff perfusion. The difference in the kind of matrix, however, caused significant problems during the production of the tissue sections. The chemical fixation in formaldehyde, prior to soaking in standard solution of $^{99}$Tc, lessened the elasticity of the heart tissue. Moreover, the morphology of the organ itself, with all its cavities, made the matrix more difficult to cut after freezing. The sections resulted severely damaged, with fractures, while phenomena of general shrinkage were observed in the tissue, as it is evident from the comparison between SEM images of sections of non-fixed (i.e. perfused) and fixed hearts (Figs. 8 and 9, respectively). In addition, no correlation could be retrieved between ICP-MS and SIMS data in fixed samples.

On the other hand, some interesting observations could be drawn regarding the signal at mass 99 in SIMS maps over Langendorff-perfused samples. In particular, we found a not homogeneous distribution of $^{99}$Tc in these samples, as expected by the effect of the perfusion mechanism in different zones of the organ. The presence of an isobaric interference, however, had to be taken into account. Even without the possibility of an absolute quantification of the $^{99}$Tc signal, the mapping provided indications on certain specific conditions to apply in Langendorff model perfusion experiments in order to improve the analytical response of the cardiac tissue.

3.3.1. Langendorff-perfused samples

The SIMS maps recorded on tissue sections obtained from Langendorff-perfused hearts showed a well preserved tissue. The distribution of mass-99 was coherent to the distribution of C, Na and K, but was not homogeneous in the tissue, as shown in Fig. 10.

At mass resolution 300, the $^{99}$Tc signal was found affected by isobaric interferences, as expected [25,26]. The interference covered a spectral region at mass values higher than the $^{99}$Tc one (98.906 amu).
and made the ion map homogeneous over the whole tissue section (with the exception of the uncovered zones of the In substrate).

As no chemical treatment was applied during the preparation, no external chemical substances had been introduced in the sample. Furthermore, the pre-sputtering of the sample surface eliminated also the possibility of a severe environmental contamination. Consequently, the interference was probably due to a polyatomic species that originated from the recombination of secondary ions of the most common matrix elements: carbon, hydrogen and oxygen.

Applying the mass resolution of 5000 that has been used in other works [25,26] to separate the $^{99}\text{Tc}$ signal required a more intense primary beam that would have destroyed our sample. Nevertheless, according to our spectra as well as to the ones reported in the literature, the most significant contribution comparable to the $^{99}\text{Tc}$ mass was peaked around mass 99.023. This could be indeed separated even at a mass resolution of 1000. We were actually able to reach a mass resolution of 1600 by using a 1 µA primary beam, which resulted in a sputtering rate still so high

Fig. 5. SIMS ion maps of $^{10}\text{B}^+$ and $^{12}\text{C}^+$ in brain tissue samples (scale of brightness). a) Chemically fixed brain, soaked in f-BPA solution. b) Fresh frozen brain sample from an animal infused with f-BPA solution.

Fig. 6. Linear least squares fitting of $^{10}\text{B}$ mean concentration detected in the tissue by ICP-MS vs. $^{10}\text{B}$ concentration of the solution where the fixed organs were soaked. The equation of the fitting function and the coefficient of determination, $R^2$, are reported.

Table 2
SIMS and ICP-MS results on $^{10}\text{B}$-containing brain tissue samples.

<table>
<thead>
<tr>
<th>Sample label</th>
<th>Type of tissue preparation</th>
<th>$^{10}\text{B}$ conc. by ICP-MS (ppm)</th>
<th>Uncertainty (±10%, ppm)</th>
<th>Ratio $^{10}\text{B}$/ $^{12}\text{C}$ by SIMS</th>
<th>St. dev.</th>
<th>RSD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>Fixed</td>
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<td>0.0001</td>
<td>$&lt;1 \cdot 10^{-5}$</td>
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<td></td>
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<tr>
<td>B1</td>
<td>Fixed</td>
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<td>0.1</td>
<td>0.11</td>
<td>0.02</td>
<td>19</td>
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<tr>
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<td>B3</td>
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<td>Fixed</td>
<td>180</td>
<td>20</td>
<td>2.4</td>
<td>0.4</td>
<td>16</td>
</tr>
</tbody>
</table>

RSD%: Relative Standard Deviation (%).
that no good image acquisition was possible. The resulting mass spectrum is reported in Fig. 11. As one can notice, the $^{99}$Tc signal intensity is about one order of magnitude higher than that of the matrix-based interference. This typically occurred all over the sample. This implies that the map of the mass-99 signal substantially reproduces the $^{99}$Tc distribution even in low-resolution images.

These mass-99 maps showed a not very intense signal (around $10^3$ cps). ICP-MS analysis showed that the concentration of $^{99}$Tc in our Langendorff-perfused samples varied between 13 and 18 ppm ($\pm 10\%$) in the dehydrated tissue. In view of practical applications, the extent of the $^{99}$Tc concentration in the perfused organs represents a key factor to distinguish variations in the distribution of the tracer over different zones of the sample, especially on account of the interference. The concentration of $^{99}$Tc in the tissue should be kept at a level sufficient to give a signal intense enough either i) to be mapped even at a mass resolution higher than that we used (e.g. 1000) or ii) compared to which the matrix interference signal (supposedly independent of the $^{99}$Tc concentration) is negligible. In the present study, a practical difficulty in achieving a higher $^{99}$Tc concentration in the samples was experienced. The main limitation was the concentration of $^{99}$Tc-NOEt available in its original formulation (0.17 mM). In a mouse heart perfused in Langendorff configuration at physiological perfusion pressure, the volume of the $^{99}$Tc-NOEt bolus has to be limited at about one tenth of the overall perfusion flow (1.2–1.5 ml/min, varying with the heart weight) in order to maintain the percentage of ethanol (necessary for $^{99}$Tc-NOEt solubility) below 2%, due to physiological reasons.

The problem in achieving a good mass resolution without the fast deterioration of the sample due to continuous sputtering may be overcome by using a Time-Of-Flight (TOF) equipment with a pulsed primary ion beam, which would also provide a higher sensitivity and allow detection of molecular fragments with higher mass (e.g., fragments of the $^{99}$Tc-NOEt molecule). The analysis of our tissue samples by TOF-SIMS would be a natural follow-up of the present study.

The interesting observation of a not homogeneous distribution of $^{99}$Tc over the heart sections, depending on the perfusion condition of the heart, could be significant for physiopathological issues and will be addressed in future studies.

4. Conclusions

The development of an analytical methodology for a quantitative use of SIMS to map the distribution in tissues of exogenous and enriched isotopes presents some difficulties inherently related to sample preservation, SIMS sensitivity, as well as to the specific application under consideration. We tested a procedure based on the cryo-preparation of samples and standards in two very different applications, namely the mapping of $^{10}$B distribution in the mouse brain tissue, with relevance to BNCT, and the detection of $^{99}$Tc in the mouse heart tissue, following Langendorff perfusion, with relevance to coronary microcirculation. The main findings of our work are here summarized.

The procedure for tissue sectioning has been proved to be simple and efficient: cryo-sections derived from fresh organs showed good structural and chemical preservation in the case of both heart and brain samples. The procedure itself required little manipulation of the sample, ensuring fewer artifacts during the preparation. Furthermore, the use of In substrates allowed to obtain both SEM and SIMS images without having recourse to any gold or carbon coating of the sample.

The procedure we adopted for the preparation of standard specimens for semiquantitative analysis was similar to the previous one, but required the additional step of chemical fixation of organ portions, prior to their soaking in solutions with a known concentration of the analyte. We experienced this technique does not have the same effect on different kinds of tissue matrix. For brain samples, however, we

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**Fig. 7.** Linear least squares fitting of the ratio $^{10}$B/$^{12}$C in tissue sections by SIMS vs. the $^{10}$B mean concentration by ICP-MS (see Table 2). The solid point corresponds to the sample BB, from a non-fixed brain; all the other points refer to fixed samples. The equation of the fitting function and the $R^2$ value are reported.

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**Fig. 8.** SEM images of a section of a non-fixed heart tissue (from the left, 15× and 120× magnification).
noticed no critical aspect; the fixed tissue was again well preserved under the structural point of view, even though no cryo-protectors had been used in the fixative solution. These samples showed a linear uptake from the f-BPA standard solution as well as a good correlation between SIMS and ICP-MS measurements, in terms of $^{10}$B concentration. This finding made it possible to estimate a simplified RSF for $^{10}$B (with respect to $^{12}$C) in the cryo-sectioned brain tissue. The comparison between standard fixed samples and fresh samples also showed that the fixation of the tissue did not cause a significantly different response from the matrix, in terms of ion imaging and RSF evaluation.

On the other hand, heart samples showed a totally different response to chemical fixation. The procedure for the preparation of standard samples that was reliable on brain tissue did not produce the same results on heart sections, for reasons due, very probably, to the extreme fragility of the fixed cardiac tissue. Serious structural damages were caused during the slam-freezing process, preventing the sections from being representative.

Non-fixed heart samples prepared by means of the Langendorff model perfusion showed a very low $^{99}$Tc signal in SIMS ion maps. This signal was also affected by a mass interference stemming from the organic matrix itself. A mass resolution of 1600 allowed us to separate this contribution. The very low tracer concentration available, restricted by the Langendorff perfusion conditions themselves, did not allow, however, to carry out imaging at high mass resolution. Even without the possibility of an absolute quantification of the Tc signal and notwithstanding the presence of an isobaric interference, SIMS mapping however proved to be capable to resolve the specific response of the cardiac tissue to the perfusion mechanism (resulting in a not homogeneous distribution of $^{99}$Tc), even at a low mass resolution (300).

In conclusion, we optimized the analytical procedure for a semi-quantitative measurement of the $^{10}$B distribution in the brain tissue, whereas further improvements in tissue preservation and tracer feature are still needed to obtain analog results in the detection of $^{99}$Tc in the tissue of the Langendorff-perfused heart.

![Fig. 9. SEM images of a section of a fixed heart tissue (from the left, 30× and 60× magnification). The sample appears severely damaged, with fractures.](image)

![Fig. 10. SIMS images of a Langendorff-perfused heart sample: ion maps of $^{23}$Na$^+$, $^{39}$K$^+$ and $^{99}$Tc$^+$ (common color scale, a.u.) showing a proper K/Na ratio and a not homogeneous distribution of Tc in the tissue.](image)
Fig. 11. IMS mass spectrum recorded over a Langendorff-perfused heart tissue section, at mass resolution 1600. The $^{99}$Tc signal is clearly resolved from a polyatomic interference arising from the biological matrix.

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