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TECHNICAL NOTE

# Gold internal standard correction for elemental imaging of soft tissue sections by LA-ICP-MS: element distribution in eye microstructures

**Ioana Konz · Beatriz Fernández · M. Luisa Fernández · Rosario Pereiro · Héctor González · Lydia Álvarez · Miguel Coca-Prados · Alfredo Sanz-Medel**

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**Abstract** Laser ablation coupled to inductively coupled plasma mass spectrometry has been developed for the elemental imaging of Mg, Fe and Cu distribution in histological tissue sections of fixed eyes, embedded in paraffin, from human donors (cadavers). This work presents the development of a novel internal standard correction methodology based on the deposition of a homogeneous thin gold film on the tissue surface and the use of the $^{197}Au^{+}$ signal as internal standard. Sample preparation (tissue section thickness) and laser conditions were carefully optimized, and internal normalisation using $^{197}Au^{+}$ was compared with $^{13}C^{+}$ correction for imaging applications. $^{24}Mg^{+}$, $^{56}Fe^{+}$ and $^{63}Cu^{+}$ distributions were investigated in histological sections of the anterior segment of the eye (including the iris, ciliary body, cornea and trabecular meshwork) and were shown to be heterogeneously distributed along those tissue structures. Reproducibility was assessed by imaging different human eye sections from the same donor and from ten different eyes from adult normal donors, which showed that similar spatial maps were obtained and therefore demonstrate the analytical potential of using $^{197}Au^{+}$ as internal standard. The proposed analytical approach could offer a robust tool with great practical interest for clinical studies, e.g. to investigate trace element distribution of metals and their alterations in ocular diseases.

**Keywords** Laser ablation · Biological samples · Mass spectrometry/ICP-MS

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I. Konz · B. Fernández (✉) · M. L. Fernández · R. Pereiro · A. Sanz-Medel (✉)
Department of Physical and Analytical Chemistry, Faculty of Chemistry, University of Oviedo, Julian Clavería 8, 33006 Oviedo, Spain
e-mail: fernandezbeatriz@uniovi.es
e-mail: asm@uniovi.es

H. González · L. Álvarez · M. Coca-Prados
Fundación de Investigación Oftalmológica, Instituto Oftalmológico Fernández-Vega, 33012 Oviedo, Spain

## Introduction

Bio-imaging analytical techniques with adequate spatial resolution are today of crucial interest in life science studies to achieve a deeper understanding of the role of metals in biological systems [1, 2]. In this vein, the use of laser ablation coupled to inductively coupled plasma mass spectrometry (LA-ICP-MS) has demonstrated a great potential for spatially resolved analysis of heteroatoms (especially metals) in different types of tissues, including mouse kidney and heart [3, 4], human lymph nodes and respiratory tissues [5, 6], liver biopsy, breast cancer or prostate tissues [7, 8] and brain sections [9, 10]. In general, such micro-local analysis is performed to study the accumulation of certain heteroatoms in the regions of interest as well as to compare the differences of the elements' distribution between non-pathogenic and pathogenic tissues.

Both in qualitative and quantitative elemental imaging by LA-ICP-MS, different internal standards (IS) have been investigated to account for matrix effects as well as for variations in ablated mass, transported mass and instrumental drift normally present in laser-based analysis techniques [11]. More conventional approaches in elemental bio-imaging applications employ the $^{13}C^{+}$ signal for internal normalisation [5, 9, 10]. However, Frick et al. [12] have recently performed a detailed study of the ablation of carbon-containing matrices demonstrating the formation of two individual phases, a gaseous carbon-containing species and a carbon-containing particle phase. Such fundamental

findings line up with the hypothesis that carbon might not be a suitable IS even if a close matrix matching is performed. Austin et al. [13] proposed the use of thin polymeric films spiked with Ru and Y solutions as IS. The tissue sample can be placed on top of a thin film containing the adequate IS, but the complete ablation of the sample, including the polymeric film, has to be performed to ensure reproducible analysis. Moreover, a tedious and time-consuming step is added to the sample preparation process.

This work presents the development of a new internal standard correction methodology for qualitative elemental imaging by LA-ICP-MS using ocular tissue sections as model. The proposed strategy is based on the deposition of a homogeneous thin gold film on the tissue surface and the use of $^{197}Au^{+}$ signal as IS for normalisation. A structurally complex biological sample (eye section from a paraffin-embedded human eye of normal donors) was used as model tissue, and the bio-metal distribution ($^{24}Mg^{+}$, $^{56}Fe^{+}$ and $^{63}Cu^{+}$) was investigated by LA-ICP-MS in the structures of the anterior segment of the eye. Reproducibility studies with samples from both the same donor and different donor eyes were performed to investigate the potential of the novel IS correction approach with $^{197}Au^{+}$ as the internal standard signal.

## Experimental

*Standards, reagents and samples* Chemicals and reagents used were of analytical grade. All solutions were prepared using distilled deionized water (18.2 $M\Omega cm^{-1}$) obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Iron, copper, magnesium and gold standard solutions (1,000 $\mu g mL^{-1}$) used for ICP-MS tuning were purchased from Merck (Darmstadt, Germany). All gases used for ICP-MS and LA analyses (argon and helium, each 99.999 % purity) were purchased from Air Liquide (Spain).

Ten eyes from normal donors (cadavers) were obtained 24 h post-mortem through the National Disease Research Interchange (Philadelphia, PA, USA). The procedures conformed to the tenets of the Declaration of Helsinki. Eyes were fixed in 10 % formalin and paraffin embedded (FFPE) following a conventional protocol [14]. The authors are well aware of the fact that this sample preparation might distort the initial metal concentration due to leaching of the metals from the tissue into the formalin solution or following paraffin embedding [2]. Nevertheless, this sample preparation was necessary in our case in order to ensure the sample's integrity. Sections through the eye bulbs in FFPE blocks (thicknesses between 10 and 160 μm) were prepared using a Microm HM550 cryostat (Thermo Fisher Scientific, Walldorf, Germany), mounted on microscope glass slides and dried for 24 h at 37 °C.

### Instrumentation

*LA-ICP-MS* Element-specific detection of C, Mg, Fe, Cu and Au in ocular tissue sections was carried out using a double-focusing sector field ICP-MS (Element 2, Thermo Fisher Scientific, Bremen, Germany) at medium mass resolution ($R$=4,000) to avoid spectral interferences on the determination of the sought elements. For laser ablation analyses, a CETAC LSX-213 laser system (Cetac Technologies, Omaha, NE, USA) was employed. The optimized experimental parameters used for LA-ICP-MS measurements are summarized in Table 1. More details about the LA-ICP-MS system, other employed instrumentation as well as experimental conditions are provided as electronic supplementary material.

### Procedures

*Thin film Au deposition* Thin sections (15 μm) of paraffin-embedded human eye tissues were metallized for 40 s with gold. The total duration of the thin film deposition was below 5 min, and no alteration was observed in the samples. Such metal coating was performed with a Balzers SCD 004 Sputter Coating Unit (Balzers, Bal Tec AG, Fürstentum, Lichtenstein) at the intensity current level of 20 mA under Ar atmosphere ($7\times10^{-2}$ atm). The Au layer thickness (9±1 nm) was determined on cross-sectioned witness samples by using a mechanical step profilometer (XP1–Ambios Technology, Santa Cruz, CA, USA).

**Table 1** Operating conditions of the ICP-MS and laser ablation systems

| | |
|---|---|
| ICP-MS | Thermo element 2 |
| RF power | 1,330 W |
| Cooling gas | 15.5 $L min^{-1}$ |
| Auxiliary gas | 0.8 $L min^{-1}$ |
| Nebuliser gas (Ar) | 0.9 $L min^{-1}$ |
| Cones | Ni (skimmer and sampler) |
| Isotopes | $^{13}C$, $^{24}Mg$, $^{56}Fe$, $^{63}Cu$, $^{197}Au$ |
| Sample time | 0.01 s |
| Mass window | 100 % |
| Samples per peak | 10 |
| Scan time per pass | 1.3 s |
| LA System | CETAC LSX-213 |
| Output laser energy | 100 % (5.6 mJ) |
| Repetition rate | 20 Hz |
| Spot diameter | 10 μm |
| Scan speed | 6.5 $\mu m s^{-1}$ |
| Ablation mode | Single line scan |
| Carrier gas (He) | 1.0 $L min^{-1}$ |

## Results and discussion

*Optimization of sample thickness* A basic requirement for a reliable bio-imaging analysis by LA-ICP-MS is the complete ablation of the sample matrix [15]. This task is even more challenging when inhomogeneous tissues with varying local distributions in the low micrometre range, such as ocular tissues, are investigated. In order to ensure the complete ablation of the sample and, therefore, to overcome a likely inhomogeneous and irreproducible ablation process, different section thicknesses between 10 and 160 μm were investigated. A detailed explanation about the studies carried out is collected as electronic supplementary material. The craters formed in the samples after LA-ICP-MS analysis were subsequently visualized by confocal laser scanning microscopy generating 3D images of the samples' surface (Fig S1, Electronic supplementary material). As can be seen in Fig. S2 (Electronic supplementary material), complete ablation of the sample could be only achieved in the case of tissue sections below 20 μm. Based on these results, all further imaging studies were performed with human eye tissue sections of 15-μm thickness.

*Evaluation of thin gold film for internal standard correction* The major source of error in imaging studies by LA-ICP-MS is usually related to inhomogeneities between different tissue structures. Therefore, the use of an effective IS correcting for matrix effects as well as variations in mass ablated, mass transported and instrumental drift is a must for reliable results. In order to experimentally demonstrate that at the optimized experimental parameters the signal intensity measured for $^{197}Au^+$ is stable, a glass reference material (NIST612) covered with a thin gold film was analysed. The signal intensities for the target elements and the internal standard were plotted as a function of time as well as the ratio of the target elements vs. $^{197}Au^+$ showing in all cases stable signals (data not shown). The temporal relative standard deviation was calculated to be below 20 %, presenting slight differences depending on the analysed element and/or measuring in low or medium resolution of the mass spectrometer.

*Comparison of results for elemental distributions without normalisation, with $^{13}C^+$ and with $^{197}Au^+$ as internal standards* A sample area of about 10 $mm^2$ of human eye tissue sections was selected containing different tissue structures of ophthalmological interest, such as ciliary body, ciliary muscle, iris, cornea and the outflow system, including trabecular meshwork (see Fig. 1a). Tissue structures differ very much in morphology, molecular composition and, as a consequence, also in water and solid matter content. Figure 1b shows the qualitative elemental distributions obtained by LA-ICP-MS for $^{24}Mg^+$, $^{56}Fe^+$ and $^{63}Cu^+$: with gas blank-corrected signal intensities (left), net signals corrected using $^{13}C^+$ (middle) and corrected with $^{197}Au^+$ (right) as IS. As can be seen in the left (gas blank-corrected) images, the three elements seemed to be present to different extents in the ocular tissue, while being totally absent in the paraffin matrix. Here, it should be pointed out that images of tissue sections without the Au layer on top showed the same elemental distributions for $^{24}Mg^+$, $^{56}Fe^+$ and $^{63}Cu^+$ (data not shown) than tissues with the Au layer on top. Nevertheless, significant increase in ablation efficiency was observed with the top Au layer. The use of internal standardization at this point is critical to ensure that the observed signal intensities are not artefacts due to matrix effects, as well as variations in ablated and transported mass or due to instrumental drifts. When the obtained signal intensities were corrected with the corresponding $^{13}C^+$ signal intensity (middle column), a drastic loss in structure definition (elemental contrast) was evident. Comparing the elemental distributions obtained after correction with $^{13}C^+$ signal with those obtained by correction with $^{197}Au^+$ signal (right), apparent conclusions from images are different: with $^{197}Au^+$ signal as IS, $^{24}Mg^+$ and $^{63}Cu^+$ seemed to be present in the cornea and ciliary muscle, while $^{56}Fe^+$ was clearly identified in the ciliary region. Such different conclusions for the elemental distributions of $^{24}Mg^+$, $^{56}Fe^+$ and $^{63}Cu^+$ in the tissue depending on the IS selected ($^{13}C^+$ or $^{197}Au^+$) raised the question of which of the obtained results reflect the real presence of the sought elements in the analysed zone of the ocular tissue section. To answer that key question, a closer look was taken to the elemental distributions obtained by LA-ICP-MS for $^{13}C^+$ and $^{197}Au^+$, and the results are included as electronic supplementary material. It was demonstrated that changes observed in signal intensities for $^{197}Au^+$ were not driven by selective accumulation of the gold in the different structures of ocular tissues but by changes in the ablation process itself, which depends on the sample composition at every structure.

*Reproducibility studies* After careful evaluation of the optimum laser ablation parameters and plasma conditions for imaging studies, the next step was a closer evaluation of the proposed Au-based normalisation strategy. Due to the lack of certified reference materials of biological tissues for elemental imaging purposes and the lack of information on distribution of metals in histological tissue sections, reproducibility studies through intra- and inter-donor comparisons were performed in order to assess the analytical potential of our approach using $^{197}Au^+$ as IS. It should be stressed that due to the long analysis time needed for obtaining high-resolution images (~15 h), the IS has to correct not only for matrix effects or tissue inhomogeneities within one analysis but also for instrumental drifts. Thus, elemental distribution maps of three ocular tissue sections of the same patient were performed firstly. Figure 2 shows the images obtained for $^{24}Mg^+$, $^{56}Fe^+$ and $^{63}Cu^+$ of three adjacent tissue sections after LA-ICP-MS analysis. The upper row represents the gas blank-corrected signals of the sought elements and the lower row, the same

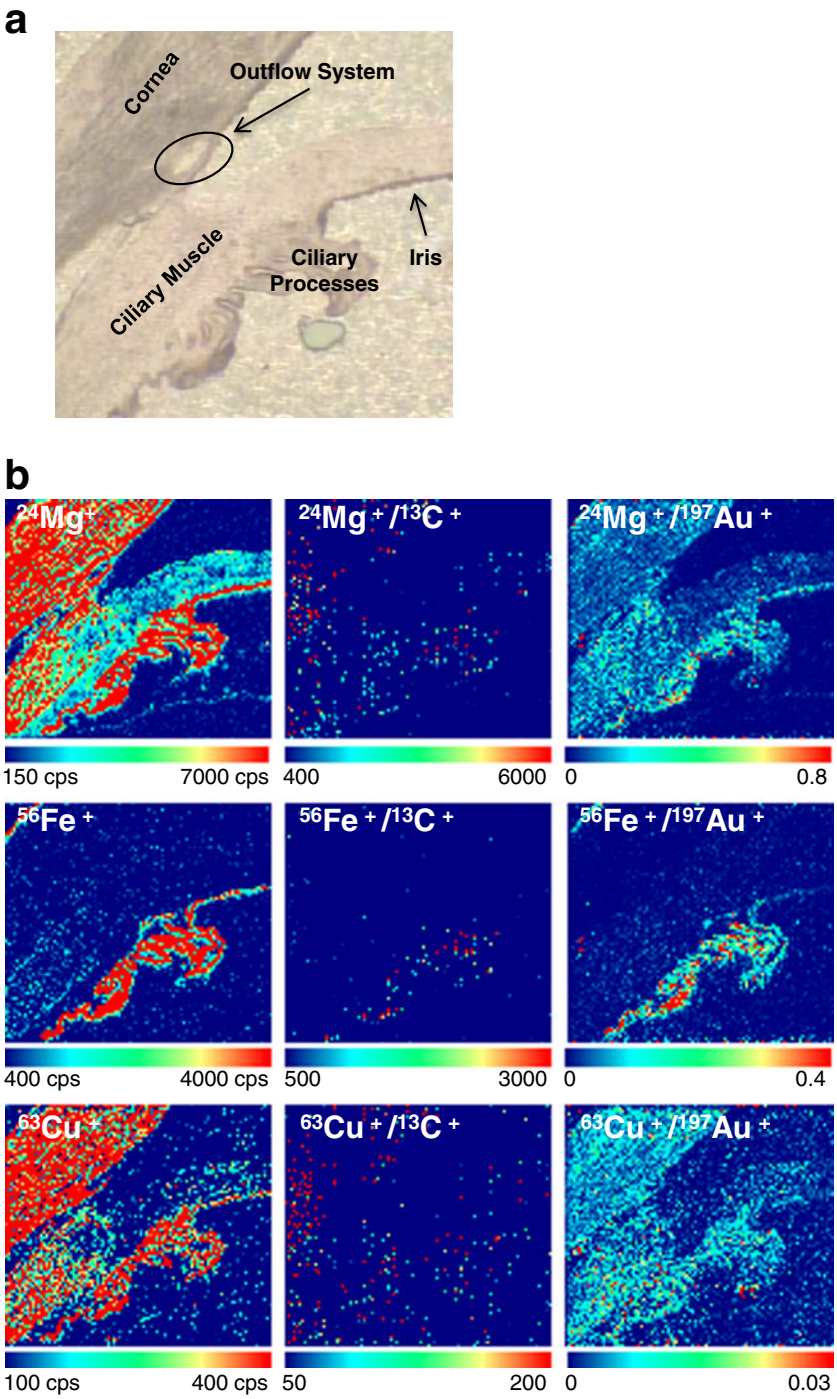


**Fig. 1** **a** Histological image of a paraffin-embedded human eye tissue section of the anterior segment, analysed by LA-ICP-MS, showing the different structures of ophthalmological interest; **b** elemental distribution of $^{24}Mg^+$, $^{56}Fe^+$ and $^{63}Cu^+$ in the tissue section measured by LA-ICP-MS without internal standard correction (*left*), with $^{13}C^+$ (*middle*) and with $^{197}Au^+$ (*right*) as IS

images after IS correction with $^{197}Au^+$. As can be seen for the upper row (uncorrected images), differences in absolute signal intensities of one order of magnitude in the case of $^{24}Mg^+$, one and a half for $^{56}Fe^+$ and up to two orders of magnitude for $^{63}Cu^+$ could be observed for the three tissue sections scrutinized, which could lead to a distorted interpretation of the results. The great differences observed in intensities can be explained, at least partially, by daily variations in the laser ablation and ICP-MS systems, apart from real elemental differences in the three individual tissue sections. Such element-independent response variations could be compensated by applying the proposed approach of IS correction with $^{197}Au^+$. As can be seen in Fig. 2, differences in relative signal intensities were reduced drastically. In other words, using such IS methodology, the reproducibility of LA-ICP-MS analysis can be significantly improved allowing more reliable comparative studies among different laboratories.

Next, the study was extended to the comparison of $^{24}Mg^+$, $^{56}Fe^+$ and $^{63}Cu^+$ elemental distribution in sections of ten different eyes from normal donors. As an example, Fig. 3

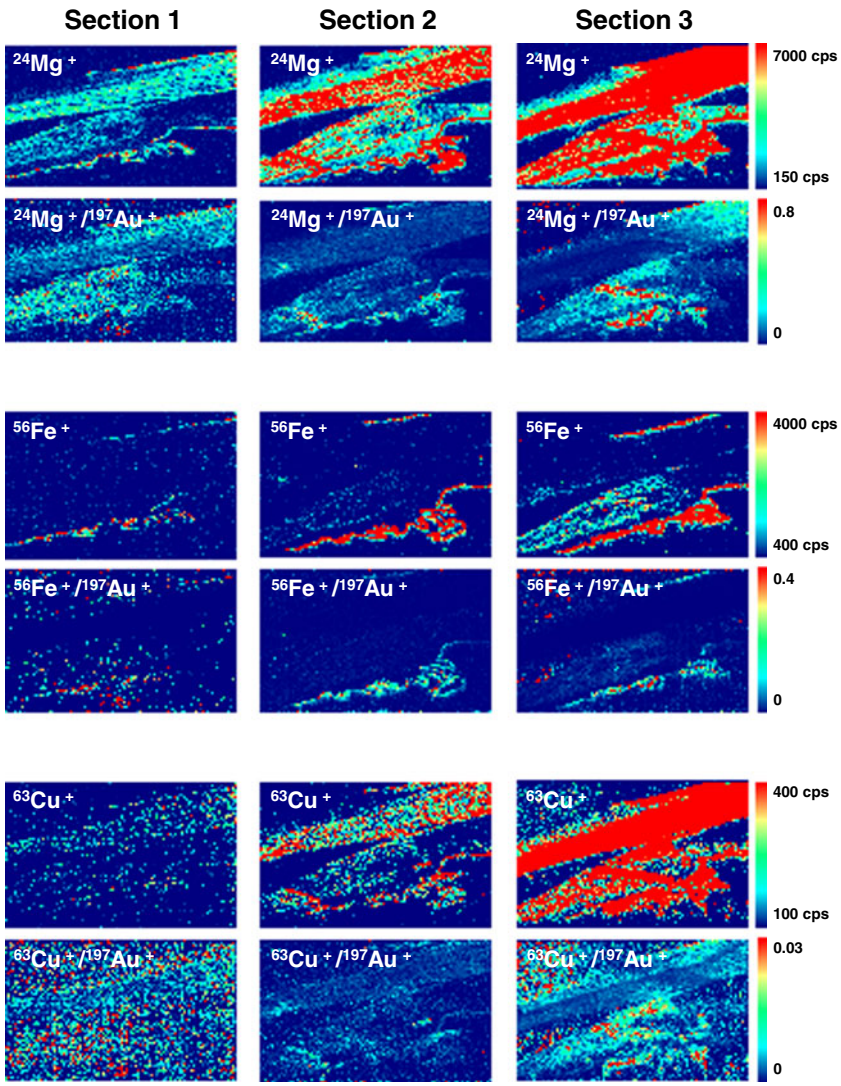


**Fig. 2** Elemental distribution of $^{24}Mg^+$, $^{56}Fe^+$ and $^{63}Cu^+$ in three ocular tissue sections (similar to those shown in Fig. 1a) of the same donor (cadaver) measured by LA-ICP-MS without internal standard correction (blank-corrected signals) and using $^{197}Au^+$ as IS (*upper* and *lower row*, respectively)

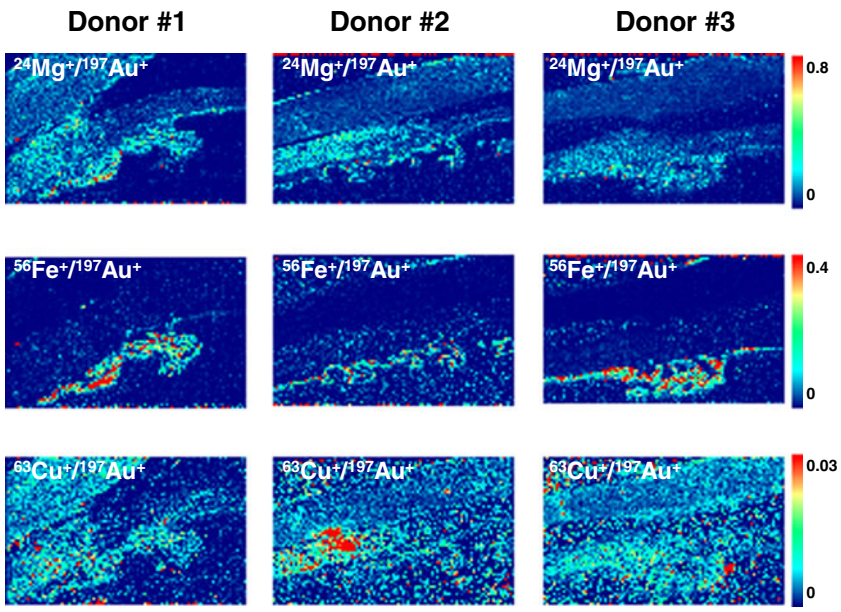


**Fig. 3** Elemental distribution of $^{24}Mg^+$, $^{56}Fe^+$ and $^{63}Cu^+$ in human eye sections (similar to those shown in Fig. 1a) of three normal donors measured by LA-ICP-MS with corrected signal intensities using $^{197}Au^+$ as IS

collects images obtained by LA-ICP-MS for eye sections from three normal donors. Although slight differences between the elemental images can be observed, they could be attributed mainly to biological differences in elemental concentrations, characteristic for each individual. In any case, it should be stressed that the observed reproducibility in terms of relative signal intensities is still maintained. As can be seen in Fig. 3, $^{24}Mg^{+}$, $^{56}Fe^{+}$ and $^{63}Cu^{+}$ were not equally distributed along ocular structures: $^{56}Fe^{+}$ was found to be concentrated in the ciliary body, while $^{24}Mg^{+}$ and $^{63}Cu^{+}$ were mainly present in the ciliary body and muscle.

## Conclusions

A new internal standard correction analytical strategy has been developed which affords more precise LA-ICP-MS imaging studies, and its performance has been checked for the first images of metal distributions in ocular tissue sections. The novel normalisation approach proposed here is based on the simple deposition of a thin and homogeneous gold film on the surface of the tissue section to be analysed and the use of the $^{197}Au^{+}$ signal as internal standard. This time-effective strategy offers the possibility for more robust direct and simultaneous qualitative determination of element distribution in tissue structures (having different compositions and morphology). Such strategy has been successfully checked here to improve the imaging of Mg, Fe and Cu in ocular tissue sections. In this work, the technique has been applied to different normal eye donors, and elemental images of the eye outflow system, iris, ciliary body and cornea regions have been obtained (see Figs. 2 and 3). To the best of our knowledge, those pictures represent the first published images of the likely distribution of the essential metals Mg, Fe and Cu in human eye microstructures.

Therefore, this work warrants further research to uncover Au as IS for LA-ICP-MS imaging and its application in biomedical research (e.g. more reliable comparative studies of normal and pathogenic tissues by IS elemental imaging can be anticipated).

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