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REVIEW

Quantitative mapping of specific proteins in biological tissues by laser ablation–ICP-MS using exogenous labels: aspects to be considered

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Abstract

Laser ablation (LA) coupled with inductively coupled plasma mass spectrometry (ICP-MS) is a versatile tool for direct trace elemental and isotopic analysis of solids. The development of new strategies for quantitative elemental mapping of biological tissues is one of the growing research areas in LA-ICP-MS. On the other hand, the latest advances are related to obtaining not only the elemental distribution of heteroatoms but also molecular information. In this vein, mapping of specific proteins in biological tissues can be done with LA-ICP-MS by use of metal-labelled immunoprobes. However, although LA-ICP-MS is, in principle, a quantitative technique, critical requirements should be met for absolute quantification of protein distribution. In this review, progress based on the use of metal-labelled antibodies for LA-ICP-MS mapping of specific proteins is reported. Critical requirements to obtain absolute quantitative mapping of specific proteins by LA-ICP-MS are highlighted. Additionally, illustrative examples of the advances made so far with LA-ICP-MS are provided.

Keywords Immunohistochemistry · Antibody labelling · Laser ablation · Inductively coupled plasm mass spectrometry · Protein quantification

Introduction

Increasing research effort is being addressed to the study of proteins to understand their biological functions. The great analytical challenge in this area is to combine specificity (biological samples are complex matrices), high sensitivity (the species of interest can be at very low concentrations) and absolute quantitative information (i.e. the capability to assess not only differential protein expression between two samples but also protein absolute quantities) [1, 2]. Optical spectroscopy and mass spectrometry (MS) techniques offer interesting capabilities in this field for liquid samples and for chemical imaging of solid specimens [3, 4].

In particular, molecular MS techniques with soft-ionization sources, such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), are commonly used in protein analysis. However, although several technical and methodological improvements have been developed to remove or circumvent limitations [5, 6], MALDI-MS and ESI-MS are typically not considered for absolute quantification purposes. On the other hand, elemental MS, and particularly the inductively coupled plasma (ICP) ion source, can facilitate quantitative research in the life sciences. Low detection limits, wide linear dynamic range, elemental specificity, multielement (and multi-isotope) analysis and virtual species- and matrix-independent ionization are among the main analytical features of ICP-MS. Such performances are of particular interest to obtain absolute concentrations of biomolecules in complex samples without the need for specific standards [1, 7].

Absolute protein quantification by ICP-MS can be done by direct measurement of many proteins through naturally present heteroatoms such as S, Se, P and metal atoms; however, in this case the protein sought must be carefully isolated from the

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sample matrix. Additionally, exogenous elemental (or isotopic) labelling of biomolecules makes almost all proteins detectable by ICP-MS. I [8], Fe [9], Hg [10] or lanthanides [11, 12] have been used to directly label the analyte for ICP-MS quantification, with different degree of success; such direct labelling strategies require the careful isolation of a given target biomolecule with appropriate separation techniques (e.g. liquid chromatography or gel electrophoresis) before the detection step. As a step forward, pioneering experiments at the beginning of this century [13–15] were performed using specific immunochemical reactions between the species sought and a metal-labelled antibody. Although the approach is not ideal because it has the typical drawbacks related to the use of antibodies [16], it is fair to say that it has opened new perspectives in specific and multiplexed protein quantification by ICP-MS [17–20]. Single metal chelates [21], polymers containing several chelates [22] and metal nanoparticles (NPs) [14, 23] have been proposed as chemical reporters to label the antibody to be used in the immunochemical reaction.

In the case of chemical imaging by ICP-MS, maps of specific proteins in biological tissues can be obtained by laser ablation (LA) sampling after an immunohistochemistry (IHC) protocol with metal-labelled antibodies [24]. LA-ICP-MS is currently regarded as a versatile MS tool for trace (element and isotopic) direct analysis of solids, offering quantitative capabilities [25]. Moreover, LA-ICP-MS allows high spatial resolution (typically higher than 1 μm) and, with the development of faster ablation cells [26, 27], well-resolved images of the specimen can be obtained in rather short times. In addition, the use of mass analysers with quasi-simultaneous spectral acquisition capabilities, such as time-of-flight (TOF) analysers, allows multielemental (hyperspectral) imaging [28, 29]; unfortunately, the sensitivity of TOF-MS analysers is not as high as that of other MS systems, particularly for lighter elements. LA-ICP-MS is being used for a wide range of different analyses, and a main area of interesting applications of LA-ICP-MS lies in the biomedical field [25, 30–32].

Several approaches based on the use of metal-labelled antibodies have been developed for simultaneous mapping of specific proteins by LA-ICP-MS, constituting an interesting alternative to commonly used fluorescence detection. Fluorescent labels with different emission lines have made possible multiplexed fluorescence analysis. Unfortunately, the development of highly multiplexed assays is hampered by the dye bandwidth (i.e. the potential spectral overlap), the difficulty in measuring simultaneously targets that differ in abundance by an order of magnitude or more and the risks of dye fluorescence quenching. In the case of biological tissues and cells, IHC imaging based on fluorescence measurements is, in addition, limited by sample autofluorescence, signal scattering and lack of robust quantification procedures [33, 34].

However, although the expectations for specific protein mapping by LA-ICP-MS are high, some issues should be addressed to achieve absolute quantification of the specific proteins within the biological tissue. In the following sections we summarize the challenges to be faced for absolute quantitative mapping as well as the advances made so far with LA-ICP-MS.

Critical requirements for absolute quantitative mapping of specific proteins by LA-ICP-MS

Quantitative elemental information on heteroatoms naturally present in biological tissues can be achieved by LA-ICP-MS by use of appropriate calibration standards (e.g. homogenized and spiked tissues, agarose gels, gelatin and polymeric films) [25, 30–35]. However, the analytical concept is more complex in the case of proteins. Mapping of specific proteins by LA-ICP-MS requires the use of proper labelled antibodies and the development of IHC protocols [36]. In principle, such protocols can be similar to those used in conventional immunofluorescence and immunoperoxidase staining, except that the label (chemical reporter) should contain an element (or isotope) detectable by ICP-MS. In addition, special care should be taken when conventional IHC protocols are used because the microchemical composition can be altered during the sample preparation procedure (e.g. formalin fixation and paraffin embedding) [3, 37].

Figure 1 shows the steps of two general IHC protocols for LA-ICP-MS mapping of biomolecules. Tissue sections (a few microns thick) from formalin-fixed paraffin-embedded blocks are mounted on IHC microscope slides. After paraffin removal, a surfactant is added to the slides. A further washing step is necessary, and then a blocking agent solution is added for a certain time. Then, depending on the procedure chosen, two different protocols can be followed:

(a) The labelled primary antibody is added and incubated. This is followed by washing step, and the specimen is then ready for LA-ICP-MS measurement.
(b) Incubation with a primary antibody is followed by a washing step, and then a labelled secondary antibody (which recognizes the unlabelled primary antibody) is added and incubated; this is followed by a final washing step, and the specimen is then submitted to LA-ICP-MS analysis.

Both protocols have inherent advantages: labelled primary antibodies offer higher specificity and greater potential for multiplexing, whereas the use of labelled secondary antibodies allows higher sensitivity and they may be used as a generic platform [3].

Studies aiming at quantitative mapping of biomolecules in tissue sections by LA-ICP-MS are still scarce. The use of a

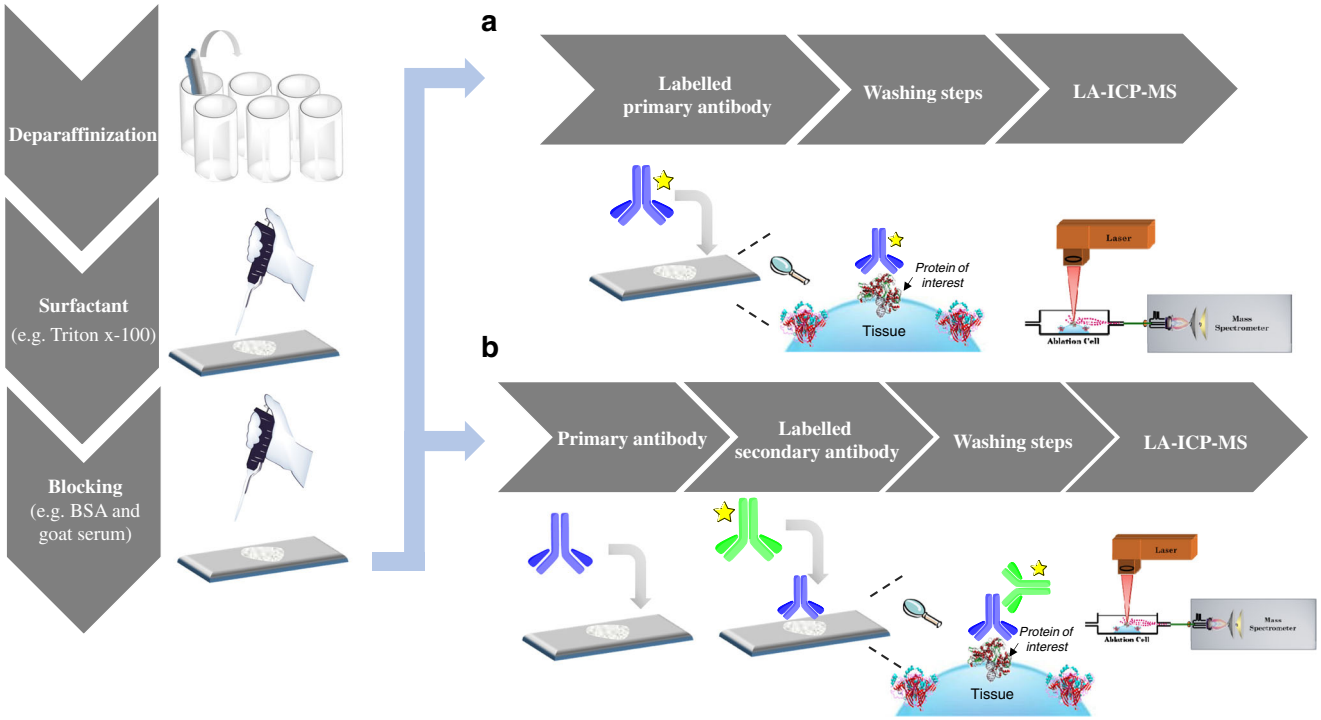

Fig. 1 Main steps of immunohistochemistry protocols required for laser ablation–inductively coupled plasma mass spectrometry (LA-ICP-MS) analysis of biological tissue sections: primary antibody labelled with the selected elemental/isotopic label (a), and secondary antibody labelled with the selected elemental/isotopic label (b). BSA bovine serum albumin

homogeneously distributed internal standard should correct the data for variation in laser power, changes in transport, drift in the plasma sensitivity and the ablated mass introduced into the ICP-MS system. Several internal standardization strategies have been proposed within this context, such as iodination to correct data for tissue inhomogeneities [38]; unfortunately, iodine has some limitations such as a long wash out (memory effects). Also, labelling with an Ir intercalator that targets nuclei has been proposed [39]; unfortunately, this strategy can be used only for low-resolution imaging because at the 5-μm-resolution level certain structures in the intensity are recognized. Printing of metal-spiked inks onto tissue sections has also been proposed for internal standardization [40]. Moreover, Mueller et al. [41] presented an interesting approach using lanthanide-labelled antibodies for quantitative immunoimaging of single eukaryotic cells; the calculation of an exact labelling degree and the correlation factor between the amount of lanthanide per cell and the amount of antibody–antigen complex were reported as critical aspects for further optimization.

In this context, there is still a lack of reliable and universal quantification strategies for protein images within tissues by LA-ICP-MS after IHC protocols. Ten general points ("golden rules") are identified in Table 1 which should be carefully considered in the development of a method for absolute quantitative mapping of specific proteins in biological tissues by elemental MS. First, it is necessary to keep in mind that to avoid risks of signal background from the sample, it is recommended that the elements (or isotopes) used for labelling should not be present at detectable concentrations in the biological tissue. Rare earth elements, Au, Ag, Pt and Ir are examples of elements of choice. The use of monoisotopic elements such as Au will allow higher amplification with cheaper labels. Conversely, isotopically enriched stable elements will be more expensive but would allow higher multiplexing capabilities.

The next aspect to be considered is related to the label size. Small labels should be used to prevent size impediments, since big labels have a higher risk of blocking active sites in the antibody or will make the bioconjugate too big to properly penetrate the tissue section. In contrast, to get signal amplification (i.e. high sensitivity for protein detection) a high number of atoms of the element (or isotope) per label is required. Therefore, a compromise is needed regarding label size: the optimum labels will be those having a high ratio of the number of atoms of the element (or isotope) sought per label size. Another point related to label requirements is to know, as accurately as possible, the number of atoms of the detected element (or isotope) per label. Moreover, the concentration of the label in the solution for antibody labelling should be known.

Regarding the bioconjugation of the metal label with the antibody, it is necessary to keep in mind that the experimental conditions should be as mild as possible to prevent risks related to antibody degradation. Moreover, binding properties of

Table 1. Requirements and steps to follow for absolute quantitative mapping of specific proteins in biological tissues by elemental mass spectrometry

Requirements		Steps to follow
1	Elements not detected in the biological tissue to be analysed are recommended for labelling	It should be checked that the element to be used as a chemical reporter is not present at detectable levels in the native tissue
2	Compromise between label size and amplification provided	A label should be selected that provides high amplification but that is small enough to not affect the recognition capabilities of the antibody
3	Number of atoms of the detected element (or isotope) per label should be known, as well as the concentration of the label in the antibody labelling procedure	Combination of studies with TEM and ICP-MS can provide the number of atoms per label as well as the concentration of the label in the labelling solution
4	Antibody labelling conditions as mild as possible	A search should be conducted for efficient labelling conditions that do not affect antibody reactivity
5	Reproducible antibody labelling procedures producing stable bonds between label and protein	Experiments should be done to check reproducibility. Also, the stability of the bond should be investigated with time and in different media
6	Unlabelled antibodies should not be present after the antibody labelling process, or at least the labelling yield should be known	To achieve this, it is convenient to use an excess of the label in the antibody labelling procedure. Also, different strategies can be studied for removal of the excess of unconjugated antibody (e.g. by chromatography or ultrafiltration)
7	Aggregation of labelled antibodies should be avoided	This should be checked with gel electrophoresis, fluorescence microscopy and electron microscopy techniques
8	The number of labels per available antibody should be known	The stoichiometry should be determined by ICP-MS and ELISA
9	Quantitative immunohistochemistry processes	The labelled antibody should be in excess and the interaction with the specimen should be maintained for sufficient time
10	Detection system with high sensitivity, structure-independent response and wide dynamic linear range	A detection system such as an LA-ICP-MS system should be used

ICP-MS inductively coupled plasma mass spectrometry, *TEM* transmission electron microscopy

antibodies may be altered during labelling. Therefore, labelling compromise conditions are required to achieve effective labelling without affecting the binding properties and the specificity of the antibody (i.e. it is convenient to use physiological labelling conditions). One of the critical points is the labelling yield. If the labelling is not complete (close to 100% yield), at least the labelling degree must be reproducible and must be known for accurate quantification. Also, the labelled antibody should preferably be stable under different pH conditions and salt concentrations.

Once the labelling of the antibody has occurred, unconjugated antibodies should not be present in the solution used for IHC (their presence will produce incorrect results since antibodies not detectable by ICP-MS can be connected with the analyte sought). This can be achieved by use of the antibody as a limiting reactant in the procedure for labelling the antibody, or by removal of the excess of unconjugated antibody (e.g. by chromatography or ultrafiltration) before the IHC protocol. On the other hand, care should be taken to avoid aggregation of the labelled antibodies. Gel electrophoresis, fluorescence microscopy and electron microscopy techniques can be used to assess aggregation. In addition, the number of labels per available antibody (stoichiometry) should be known. For such a purpose, both the label concentration in the purified bioconjugate solution and the available antibody concentration should be defined. Here, it is important to highlight that the determination of total protein per bioconjugate unit will not provide enough information because during the bioconjugation process some antibody molecules could be partially degraded or impeded for interaction with the analyte. Therefore, the immunoreactivity of the labelled antibody should be calculated with an ELISA experiment. On the other hand, the concentration of the heteroatom label in the labelled antibody can be calculated, for example, by ICP-MS.

Regarding the IHC procedure and to facilitate a quantitative interaction between the labelled antibody and the target molecule in the biological tissue, the sample should be thin enough (i.e. a few microns) to ensure full labelled antibody penetration in the sample tissue. A probably more important factor is that the labelled antibody should be in excess and the interaction

with the specimen should be maintained for sufficient time (overnight incubation is typically used to ensure complete tissue penetration and the commonest dilution for antibodies is in the range from 1:1000 to 1:5000). Finally, the excess (unreacted) of labelled antibody must be efficiently removed.

Finally, the use of an elemental mass detection system with high sensitivity, structure-independent response and wide dynamic linear range (i.e. LA-ICP-MS system) will be most convenient. Here, special attention should be paid to proper calibration using suitable matrix-matched standards and, preferably, internal standardization as well.

Labels used for protein mapping of specific proteins by LA-ICP-MS

Proteins contain several reactive groups which can be used as targets for modifications by chemical labelling [42, 43]. For example, amino groups react with isothiocyanate and *N*-hydroxysuccinimide functionalities. For labelling thiol groups of reduced cysteine residues, maleimides and halogenacetamides can be used. Carboxyl-reactive chemical groups in biomolecular probes can also be used for labelling through carbodiimide chemistry by cross-linking carboxylic acids to primary amines. Click chemistry can be used for labelling after artificial introduction of azide or alkyne moieties into the proteins. Several strategies and label types have been proposed for heteroatom labelling in immunoassays. We briefly review these, focusing on those reported with LA-ICP-MS detection.

Most LA-ICP-MS applications deal with labels containing only one detectable element/isotope. Diethylenetriaminepentaacetic acid (DTPA) (Fig. 2a) and the macrocycle 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (Fig. 2b) [21] form strong complexes with metals and can be used as antibody labels. High multiplexing and quantification capabilities are the main advantages resulting from such combination. To be used as labels, they should contain, additionally, a reactive group which binds covalently to antibodies. Furthermore, labels with a high number of detectable elements/isotopes (e.g. polymers, NPs) have also been proposed, aiming at signal amplification and, therefore, increasing sensitivity for protein detection.

Labels with one detectable element/isotope atom

Single metal chelates, and specially the macrocyclic DOTA coordinated with heteroatoms (typically lanthanide ions which have high complexation affinities for DOTA and low natural background in biological samples), have been widely used for elemental/isotopic labelling [21, 44]. As indicated already, to be of use for antibody labelling they should contain a reactive residue. Various bifunctional DOTA molecules have been developed. Among the groups most commonly used for such purposes are isothiocyanatobenzyl (Bz-SCN) and maleimide (see Fig. 2c and d) [21, 45, 46]. Depending on the linking chemistry, an average of between one and four detectable atoms per antibody are introduced [20].

Bz-SCN-DOTA lanthanide complexes [47], which bind to lysine residues, have been applied in several LA-ICP-MS applications, such as the multiparametric analysis of cytochrome P450 isoenzymes after electrophoretic separation and blotting [48], or the multiplexed IHC detection of the tumour markers human epidermal growth factor receptor 2 (HER2), cytokeratin 7 (CK7) and mucin 1 (MUC1) in breast cancer tissue by labelling anti-Her2, anti-CK7 and anti-MUC1 with Ho, Tm and Tb Bz-SCN-DOTA complexes (see Fig. 3) [44]. Unfortunately, it has been shown that Bz-SCN-DOTA do not lead to one kind of labelled antibody molecule. Instead, a label distribution and a high percentage of unmodified molecules (the reported labelling yield is as low as 0.03) could occur [49].

Another common labelling strategy involves a maleimide linker, which binds covalently to sulfhydryl residues (such as maleimido-monoamide-DOTA [50] and metal-coded affinity tags [45, 51]). In this case, reduction of disulfide bridges is a necessary step. For a suitable labelling, the challenge is to reduce as many disulfide bonds of the antibody as possible to produce reaction sites for the maleimide group without affecting the binding capability of the antibody [49]. Application examples of maleimide-DOTA-related compounds include the multiplexed analysis of plant thylakoid proteins by Western blots [50] and the development of a multiparametric microarray for simultaneous analysis of eight different cytochromes [51]. The labelling efficiency is higher than in the case of Bz-SCN-DOTA [49]; however, severe risks of high complexity of the metal-labelled antibody have been reported. Antibody fragments of different sizes and labelling degrees were obtained following the chemistry required for maleimide-DOTA labelling [52]. This prevents the development of a quantification procedure because the calculation of antibody molecules in the sample is not possible. As an alternative, a labelling concept resulting in one label per antibody has been proposed. The strategy is based on the previous modification of small antibody binding domains, $C2_{Fc}$ and $C2_{Fab}$, with a metal maleimide-DOTA compound. Antibodies are then labelled with these modified C2 domains by mixing and their being subjected to far-UV light [53].

Highly amplified label methods for protein analysis by LA-ICP-MS

Polymeric labels containing several metal chelates of a given metal or isotope provide noticeable signal amplification [22, 28, 29, 54]. Here, we highlight the commercially available MAXPAR® labelling kits containing DTPA chelating compounds. Two polymer types are commercially available: the 7-

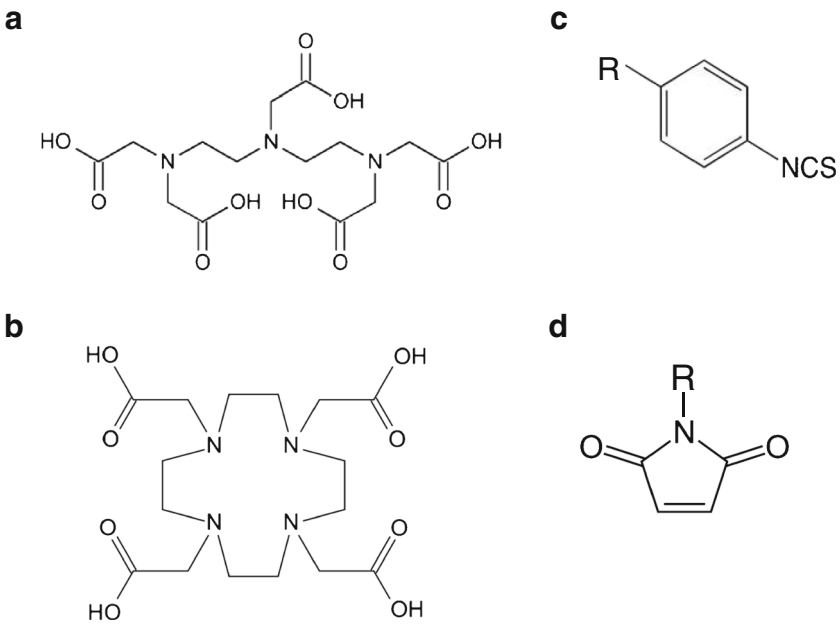

Fig. 2 Chemical formulas of the chelating compounds diethylenetriaminepentaacetic acid (DTPA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and of groups frequency used for linking to antibody residues used in combination with laser ablation–inductively coupled plasma mass spectrometry detection: **a** DTPA, **b** DOTA, **c** isothiocyanatobenzyl, and **d** maleimide

nm-long linear X8 polymer containing about 22 chelators per polymer and the branched DN3 polymer with 5-nm diameter and containing about 16 chelators per polymer. The metal polymeric label is bound to the antibody through a maleimide linker, by disulfide reduction. Typically, four or five such polymers will be conjugated to each antibody [19].

These polymers have been successfully used as antibody labels in varied applications, mostly in combination with ICP-TOF-MS for mass cytometry [19], where it has shown outstanding value. In addition, the strategy has been coupled with LA for imaging mass cytometry [28] and for highly multiplexed imaging of tissues at subcellular resolution. Here, it is worth highlighting the pioneering work of Giesen et al. [29] where simultaneous imaging of 32 target biomolecules (proteins and protein modifications) was achieved with polymers containing rare earths as reporters on antibodies. However, the polymeric non-metallic part of these labels gives room for improvement of the ratio of "number of detectable metal atoms per label size". Moreover, the risk of non-specific interactions has been reported in the case of IHC procedures in biological tissues [49].

Finally, antibodies labelled with metal NPs have proved to be another interesting strategy for immunoassay detection in liquid samples by ICP-MS [14, 23]. In the case of LA-ICP-MS, the use of antibodies labelled with metallic NPs is still scarce. For instance, 10-nm Au NPs were used in blotting membranes after protein separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [55]. Bioimaging of breast cancer biomarkers using secondary antibody labelled with 5-nm Au NPs was performed [56]. In addition, this protein labelling approach, based on the use of a secondary IgG antibody (labelled in this case with 10-nm Au NPs), was used for bioimaging tyrosine hydroxylase (rate-limiting enzyme in dopamine biosynthesis) in mouse brain [57, 58].

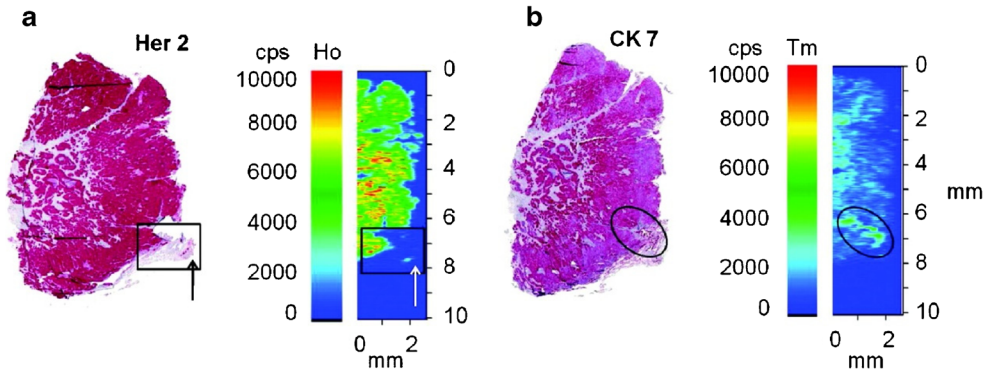

Fig. 3 Immunohistochemical staining of 3-μm-thick breast cancer tissue sections, positive for **a** human epidermal growth factor receptor 2 (Her 2) and **b** cytokeratin 7 (CK 7). Laser ablation–inductively coupled plasma mass spectrometry images are shown on the left in **a** and **b**. Laser spot size 200 μm. Characteristic microscopic findings are highlighted by black frames. (From [42] with permission of the American Chemical Society. Copyright 2011)

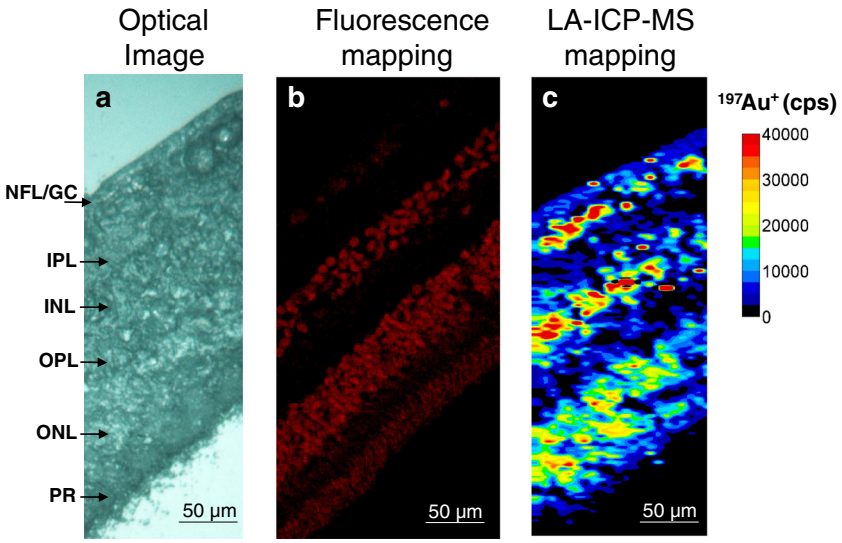

Fig. 4 Dual study of the distribution of metallothioneins 1 and 2 (MT1/2) in neurosensory retina of an eye tissue section after immunohistochemistry with Au nanocluster bioconjugate (MT1/2) by fluorescence detection and laser ablation–inductively coupled plasma mass spectrometry (LA-ICP-MS). **a** Transmission image for the analysed area, **b** Au nanocluster–antibody bioconjugate fluorescence obtained by confocal microscopy and **c** qualitative image obtained by LA-ICP-MS (4-µm spot size) for $^{197}Au^{+}$ (i.e. MT1/2) distribution. (From reference [24] with permission of Springer. Copyright 2018). NFL/GC nerve fiber layer/ganglion cells, IPL inner plexiform layer, INL inner nuclear layer, OPL outer plexiform layer, ONL outer nuclear layer, PR photoreceptors

Recent advances in nanotechnology have introduced a new class of fluorescent labels, named "fluorescent metal nanoclusters" (NCs), with sizes between 0.2 and 3 nm (this type of nanostructure is characterized by sizes comparable to the Fermi wavelength of electrons and, therefore, they can exhibit molecule-like properties) [59]. These NCs are composed of a few to several hundred metal atoms, and the surface of the nanostructure can be tailored with selected groups for different chemistries [60]. Au NCs exhibit strong fluorescence, good photostability and biocompatibility. Therefore, they constitute a promising alternative to more conventional luminescent markers used in the bioanalytical field [61]. Moreover, these small labels can provide signal amplification as elemental labels in LA-ICP-MS bioimaging. In addition, there will be no accessibility restrictions to the target protein within the tissue (this is one of the problems associated with larger labels). Finally, Au NCs will allow sequential bimodal detection (fluorescence detection by microscopy and elemental/isotopic detection by LA-ICP-MS) in the same sample spot. Recently, the proof of concept was demonstrated for the mapping of metallothioneins in the human retina (see Fig. 4) using water-soluble Au NCs with an average size of 2.7 nm and more than 500 Au atoms per label [24]. In such an example, carboxylic groups of the Au NC surface were bioconjugated with specific primary antibodies by carbodiimide coupling. This format offers advantages as compared with the use of a labelled secondary antibody (labelled with Au NPs) as described above [56–58], such as higher multiplexing capabilities with use of a different type of metal NC per specific primary antibody. Moreover, this format can be further extended to NCs of other metals, such as Ag [62] or Pt [63]. This opens the door for the synthesis of isotopically enriched metal NCs (thus increasing the palette of chemical reporters for antibody labelling compared with the monoisotopic Au NCs).

Conclusions

There is no doubt that the combination of IHC with spectroscopic and MS techniques offers great possibilities for quantitative imaging of specific proteins in biological tissues, with detailed aid in experimental design provided in previous reviews [3].

In particular, the combination of LA-ICP-MS with IHC is of great interest for chemical imaging of specific proteins. For example, in this context it is worth pointing out that the use of antibodies labelled with metal isotopes (e.g. lanthanides) and rapid-response ablation cells combined with ICP-TOF-MS allows amazing hyperspectral capabilities for fast multiprotein imaging [29, 38].

Any increase in spatial resolution with LA-ICP-MS implies lower sensitivity. Therefore, labels providing signal amplification (e.g. several metal atoms per label) are most convenient to obtain highly resolved images, such as MAXPAR® labels and metal NCs. Here, studies should aim at selecting a compromise between the number of metal atoms and label size: labels with a high number of metal atoms will provide high signal amplification, but the limit will be given by steric impediments with the antibody. On the other hand, we highlight the interest in developing labels for multimodal imaging detection (e.g. fluorescence and MS detection), allowing

complementary analytical information to be obtained [24, 64, 65]. Additionally, although fluorescence measurements are more prone to troubling effects (e.g. autofluorescence and signal scattering), they are typically cheaper and faster than conventional LA-ICP-MS measurements. Therefore, these dual probes can serve both for optimization of the method and for a first test of the samples after the IHC protocol before measurement by LA-ICP-MS.

LA-ICP-MS is an intrinsically quantitative technique. However, it is not easy to obtain absolute quantitative information for protein maps by LA-ICP-MS after an IHC protocol because different requirements (considered in the previous sections) must be fulfilled during the whole procedure. Probably the most critical one to achieve a reliable absolute quantitative IHC image by LA-ICP-MS is to ensure a controlled chemistry for antibody metal labelling. In this vein, new, interesting approaches have been recently proposed [53, 66] but further intense collaborative work between specialists in different fields would be of high value to achieve more progresses in this direction.

Finally, it is worth mentioning that research in this field can be extended to other cutting-edge studies, such as the elucidation of mechanisms related to antibody–drug conjugates for oncology therapeutics [67, 68], as well as for nucleic acid (DNA and RNA) assays [69].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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