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 to ICP-MS is regarded as 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, latest advances are related to obtaining not only elemental distribution of heteroatoms but also molecular information. In this vein, mapping of specific proteins in biological tissues can be carried out with LA-ICP-MS by using 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 are tackled. Critical requirements to obtain absolute quantitative mapping of specific proteins by LA-ICP-MS are highlighted. Additionally, illustrative examples with the advances carried out so far using LA-ICP-MS are collected.

Keywords: immunohistochemistry, antibody labelling, laser ablation, ICP-MS, protein quantification

Introduction

Nowadays increasing research effort is addressed towards the study of proteins to understand their biological funtions. 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 concentration levels) 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 within 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- 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 the quantitative research in life sciences. Low detection limits, wide linear dynamic range, elemental specificity, multi-element (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 of specific standards [1,7].

Absolute protein quantification using ICP-MS can be carried out by direct measurement of many proteins through naturally present heteroatoms such as S, Se, P and metals; however, in this case it is required that the sought protein is carefully isolated from the sample matrix. Additionally, exogenous elemental (or isotopic) labelling of biomolecules makes almost all proteins ICP-MS-detectable. 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) prior to the detection step. As a step forward, pioneering experiments at the beginning of this century [13-15] were carried out making use of specific immunochemical reactions between the sought species 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 using 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 >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 the time of flight (TOF), allows for multielemental (hyperspectral) imaging [28,29]; unfortunately, sensitivity of TOFMS analysers is not as high as compared with other MS systems, particularly for lighter elements. Nowadays, LA-ICP-MS is being used for a wide range of different analysis, 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 enabled multiplexed fluorescence analysis. Unfortunately, the development of highly multiplexed assays is hampered by the dyes 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 expectations for LA-ICP-MS specific protein mapping are high, some issues should be addressed to achieve absolute quantification of the specific proteins maps within the biological tissue. Below are collected a summary of the challenges to face for absolute quantitative mapping as well as the advances carried out so far using LA-ICP-MS.

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

Quantitative elemental information of heteroatoms naturally present in biological tissues can be achieved by LA-ICP-MS by resorting to 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 than those used in conventional immunofluorescence and immunoperoxidase staining, excepting that the label (chemical reporter) should contain an element (or isotope) detectable by ICP-MS. In addition, special care should be taken when using conventional IHC protocols, 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 μm 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 period of time. Then, depending of the selected procedure, two different protocols (a or b) can be followed: (a) The labelled primary antibody is added and incubated. After incubation, a washing step is done and the specimen is then ready for LA-ICP-MS measurement. (b) After incubation with a primary antibody, a washing step is performed and then a labelled secondary antibody (which recognizes the unlabelled primary antibody) is added and incubated; a final washing step is carried out, and the specimen is then submitted to LA-ICP-MS analysis. Each of both alternatives has inherent advantages: labelled primary antibodies offer higher specificity and greater potential for multiplexing, while the use of labelled secondary antibodies allows for higher sensitivity and may be used as generic platform [3].

Studies aiming at quantitative mapping of biomolecules in tissue sections by LA-ICP-MS are still scarce. The use of a homogeneously distributed internal standard should correct for variation in laser power, changes in transport, drift in the plasma sensitivity as well as for the ablated mass introduced into the ICP-MS. Several internal standardisation strategies have been proposed within this context, such as iodination to

correct 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 only used for low resolution imaging because at the 5 μm resolution level certain structures in the intensity were recognized. Printing of metal spiked inks onto tissue sections has been also proposed for internal standardization [40]. Moreover, Mueller *et al.* [41] presented an interesting approach, using lanthanide-labelled antibodies, for quantitative immuno-imaging of single eukaryotic cells; the calculation of an exact labelling degree as well as the correlation factor between amount of lanthanide per cell and 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 of protein images within tissues by LA-ICP-MS after IHC protocols. In Table 1 are identified ten general points ("golden rules") 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 or Ir are examples of elements of choice. The use of monoisotopic elements such as Au will allow for higher amplification with cheaper labels. Conversely, isotopically enriched stable elements will be more expensive but would allow for higher multiplexing capabilities.

The next aspect to be considered is related to the label size. Small labels should be employed to prevent size impediments, since big labels have higher risk of blocking active sites in the antibody or will make the bioconjugate too big to properly penetrate in the tissue section. On the opposite, to get signal amplification (i.e., high sensitivity for protein detection) it is required a high number of atoms of the element (or isotope) per label. Therefore, a compromise should be taken regarding label size: optimum labels will be those having a high ratio of number of atoms of the sought element (or isotope) per label size. Another point related with 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 procedure of the metal label with the antibody, it is necessary to keep in mind that experimental conditions should be as mild as possible to prevent risks related to antibody degradation. Moreover, binding properties of

antibodies may be altered during labelling. Therefore, labelling compromise conditions are required to get 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 taken place, unconjugated antibodies should not be present in the solution employed for IHC (their presence will produce incorrect results since antibodies not detectable by ICP-MS can be connected with the sought analyte). This can be achieved by using the antibody as limiting reactant in the labelling procedure of the antibody, or by removing the excess of unconjugated antibody (e.g. by chromatography or ultrafiltration) prior to 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 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, 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 during a time long enough (overnight incubation is typically used to ensure complete tissue penetration and the most common dilution for antibodies is in the range of 1:1000 to 1:5000). Finally, the excess (unreacted) of labelled antibody must be efficiently removed.

To end with, the use of an elemental mass detection system with high sensitivity, structure-independent response and wide dynamic linear range (i.e. LA-ICP-MS) 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 (NHS) functionalities. For labelling thiol groups of reduced cysteine residues, maleinimides and halogenacetamides can be used. Carboxyl-reactive chemical groups in biomolecular probes can be used also for labelling through carbodiimide chemistry by crosslinking carboxylic acids to primary amines. Click-chemistry can be employed 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. Below they will be briefly reviewed focusing in those published with LA-ICP-MS detection.

Most LA-ICP-MS applications deal with labels containing only one detectable element/isotope. Diethylenetriaminepentaacetic acid (DTPA) (Figure 2a) and the macrocycle 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (Figure 2b) [21] form strong complexes with metals and can be used as antibody labels. High multiplexing and quantification capabilities are main advantages brought about by 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 been also proposed aiming at signal amplification and, therefore, to increase 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 to DOTA and low natural background in biological samples), have been widely employed for elemental/isotopic labelling [21,44]. As indicated above, to be of use for antibody labelling they should contain a reactive residue. Varied bi-functional DOTA molecules have been developed. Among the most commonly groups employed for such purposes are isothiocyanatobenzyl (bz-SCN) and

maleimide (see Figures 2c and 2d) [21,45,46]. Depending on the linking chemistry, an average between 1 and 4 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 multi-parametric analysis of cytochrome P450 isoenzymes after electrophoretic separation and blotting [48], or the multiplexed IHC detection of Her 2, CK7 and MUC1 tumour markers in breast cancer tissue by labelling anti-Her 2, anti-CK7, and anti-MUC1 with Ho, Tm, and Tb Bz-SCN-DOTA complexes (see Figure 3) [44]. Unfortunately, it has been shown that bz-SCN-DOTA do not leads to one kind of labelled antibody molecule. Instead, a label distribution and a high percentage of unmodified molecules (reported labelling yield is as low as 0.03) could occur [49].

Another common labelling strategy resorts to a maleimide linker, which binds covalently to sulfhydryl residues (such as maleimido-mono-amide-DOTA [50] and the Metal-code affinity tags or MeCATs [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 on western blots [50] and the development of a multi-parametric microarray for simultaneous analysis of 8 different cytochromes [51]. Labelling efficiency is higher than in the case of bz-SCN-DOTA [49], however, it has been reported severe risks of high complexity of the metal-labelled antibody. In fact, it has been shown that 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 alternative, it has been proposed a labelling concept resulting in one label per antibody. 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 subjecting them to far-UV light [53].

Highly-amplified label methodologies 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, it should be highlighted the commercially

available MAXPAR^{TM} labelling kits containing DTPA chelating compounds. Nowadays two polymer types are commercially available: the 7 nm-long linear X8 polymer containing about 22 chelators/polymer and the branched DN3 polymer with 5 nm-diameter and containing about 16 chelators/polymer. The metal polymeric label is bound to the antibody through a maleimide linker, using disulfide reduction. Typically, 4-5 of 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-TOFMS for mass cytometry [19] where it has shown an outstanding value. In addition, the strategy has been coupled to LA for imaging mass cytometry [28] and for highly multiplexed imaging of tissues at subcellular resolution. Here, it is worth to highlight the pioneering work by Giesen *et al.* [29] where simultaneous imaging of 32 target biomolecules (proteins and protein modifications) was achieved using polymers containing rare earths as reporters on antibodies. However, the polymeric non-metallic part of these labels gives room for improvement of the ratio "number of detectable metal atoms per label size". Moreover, risk of non-specific interactions has been reported in the case of the IHC procedures in biological tissues [49].

Finally, antibodies labelled with metal NPs have proved to be another interesting strategy for immunoassays 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 size Au NPs were employed in blotting membranes after protein separation by SDS-PAGE [55]. Bioimaging experiments were carried out for breast cancer biomarkers using 5 nm Au NPs labelled secondary antibody [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 employed for bioimaging tyrosine hydroxylase (rate-limiting enzyme in dopamine biosynthesis) in mice brain [57,58].

Recent advances in nanotechnology have introduced a new class of fluorescent labels, named as fluorescent metal nanoclusters (NCs), with sizes between 0.2 and 3 nm (this type of nanostructures 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]. Gold nanoclusters (Au NCs) exhibit strong fluorescence, good photostability and bio-compatibility. 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 for sequential bimodal detection (fluorescence by microscopy and elemental/isotopic by LA-ICP-MS) in the same sample spot. Recently, the proof of concept has been demonstrated for the mapping of metallothioneins in the human retina (see Figure 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 example, carboxylic groups of the AuNCs surface were bioconjugated with specific primary antibodies by carbodiimide coupling. This format offers advantages as compared to the use of a secondary antibody labelled (with Au NPs) above described [56-58], such as higher multiplexing capabilities using a different type of metal NC per specific primary Ab. Moreover, this format can be further extended to nanoclusters 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 to 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 offers great interest for chemical imaging of specific proteins. For example, within this context it is worth to point out that the use of antibodies labelled with metal isotopes (e.g. lanthanides) and rapid-response ablation cells combined with ICP-TOFMS allows for amazing hyperspectral capabilities for fast multi-protein imaging [2938].

As it is known, 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 the MAXPAR^{TM} and the metal NCs. Here, studies should aim at selecting a compromise between 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, it should be highlighted the interest

of developing labels for multimodal imaging detection (e.g. fluorescence and MS), allowing to obtain complementary analytical information [24,64,65]. Additionally, though 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. 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, to obtain absolute quantitative information of protein maps by LA-ICP-MS after an IHC protocol is not an easy task because different requirements (considered in the previous sections) should be fulfilled along 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 it would be of high value a further intense collaborative work between specialists of different fields to achieve more progresses in this direction.

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

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LEGENDS OF FIGURES

Figure 1. Main steps of IHC protocols required for LA-ICP-MS analysis of biological tissue sections. (a) Primary antibody labelled with the selected elemental/isotopic label, and (b) Secondary antibody labelled with the selected elemental/isotopic label.

Figure 2. Chemical formula of DTPA and DOTA chelating compounds and of frequent group-types for linking to antibody residues used in combination with LA-ICP-MS detection. (a) DTPA, (b) DOTA, (c) Bz-SCN, and (d) Maleimide.

Figure 3. Immunohistochemical staining of 3 μm thick breast cancer tissue sections, positive for (a) Her 2, and (b) CK 7. LA-ICP-MS images are observed at the left of Figures a and b. Laser spot size 200 μm. Characteristic microscopic findings are highlighted by black frames. From reference [44] with permission of American Chemical Society. Copyright © 2011.

Figure 4. Dual study of MT1/2 distribution in neurosensory retina of eye tissue section after immunohistochemistry with AuNC bioconjugate (MT 1/2) by fluorescence and LA-ICP-MS. a) Transmission image for the analysed area, b) AuNCs-Antibody bioconjugate fluorescence 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.

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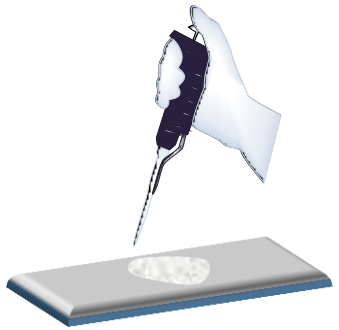
Table 1. Requirements and steps to follow for absolute quantitative mapping of specific proteins in biological tissues by elemental mass spectrometry.

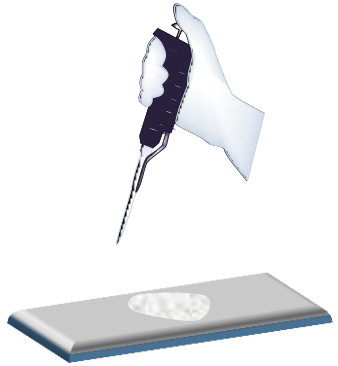
Requirements		Steps to follow
1	Elements not detected in the biological tissue to be analysed are recommended for labelling	It should be checked that the element to be used as chemical reporter is not present at detectable levels in the native tissue.
2	Compromise between label size and amplification provided	It should be selected a label providing high amplification, but 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 procedure of antibody labelling	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	It should be searched for efficient labelling conditions not affecting the antibody reactivity.
5	Reproducible antibody labelling procedures producing stable bonds between label and protein	Experiments should be done to check reproducibility. Also, stability of the bond should be investigated along time and under 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 labelling procedure of the antibody. Also, different strategies can be studied for removing 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 kept during a time long enough.
10	Detection system with high sensitivity, structure-independent response and wide dynamic linear range	A detection system such as laser ablation coupled to ICP-MS should be used.

Deparaffinization

Surfactant
(e.g. Triton x-100)

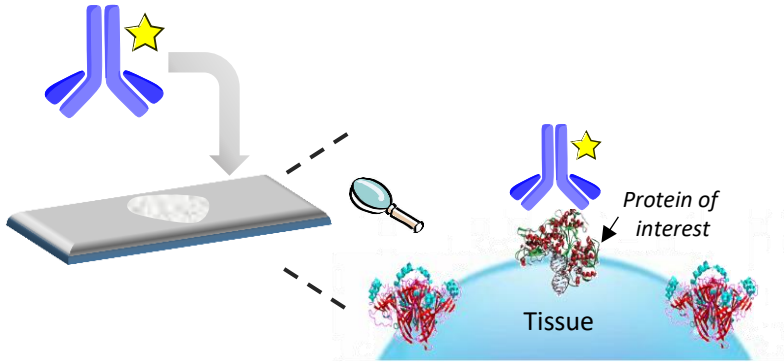


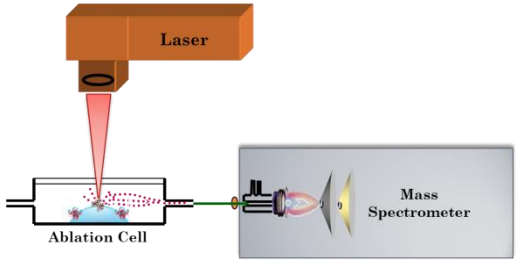
Blocking
(e.g. BSA and goat serum)



a)

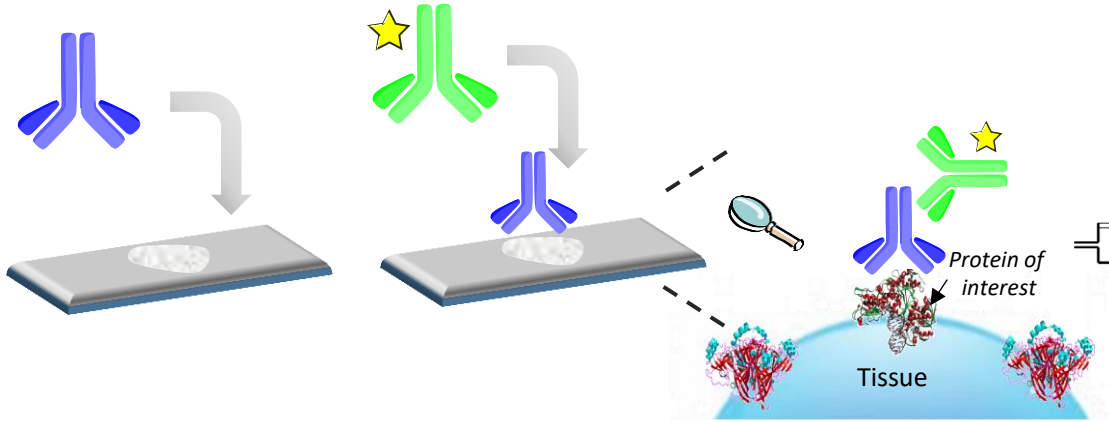
Labelled primary antibody → Washing steps → LA-ICP-MS





b)

Primary antibody → Labelled secondary antibody → Washing steps → LA-ICP-MS



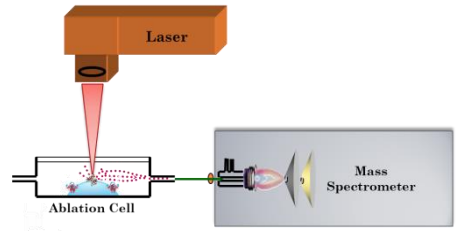
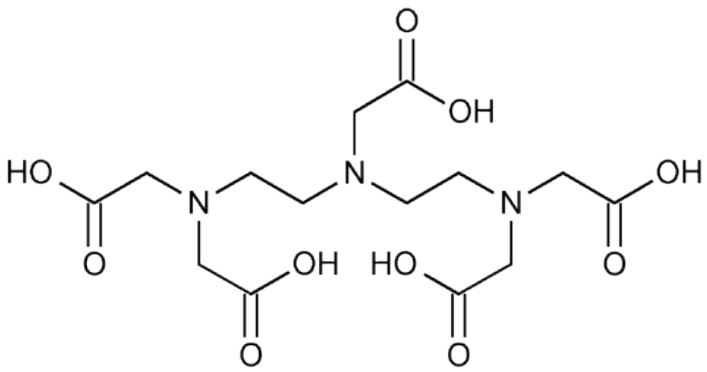
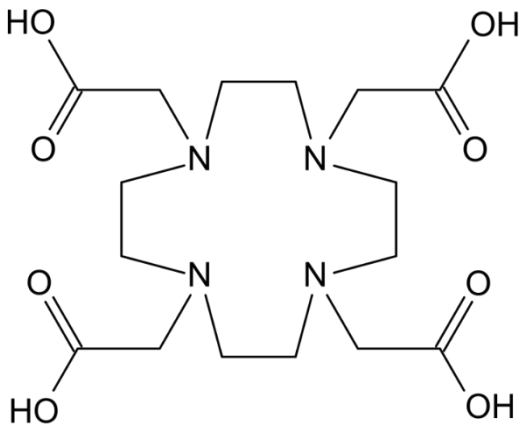


Figure 1

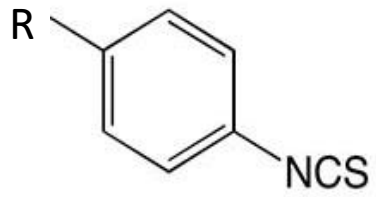
a)



b)



c)



d)

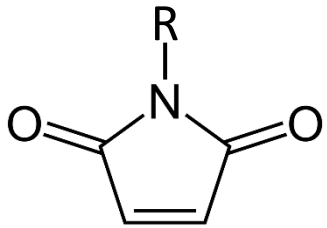


Figure 2

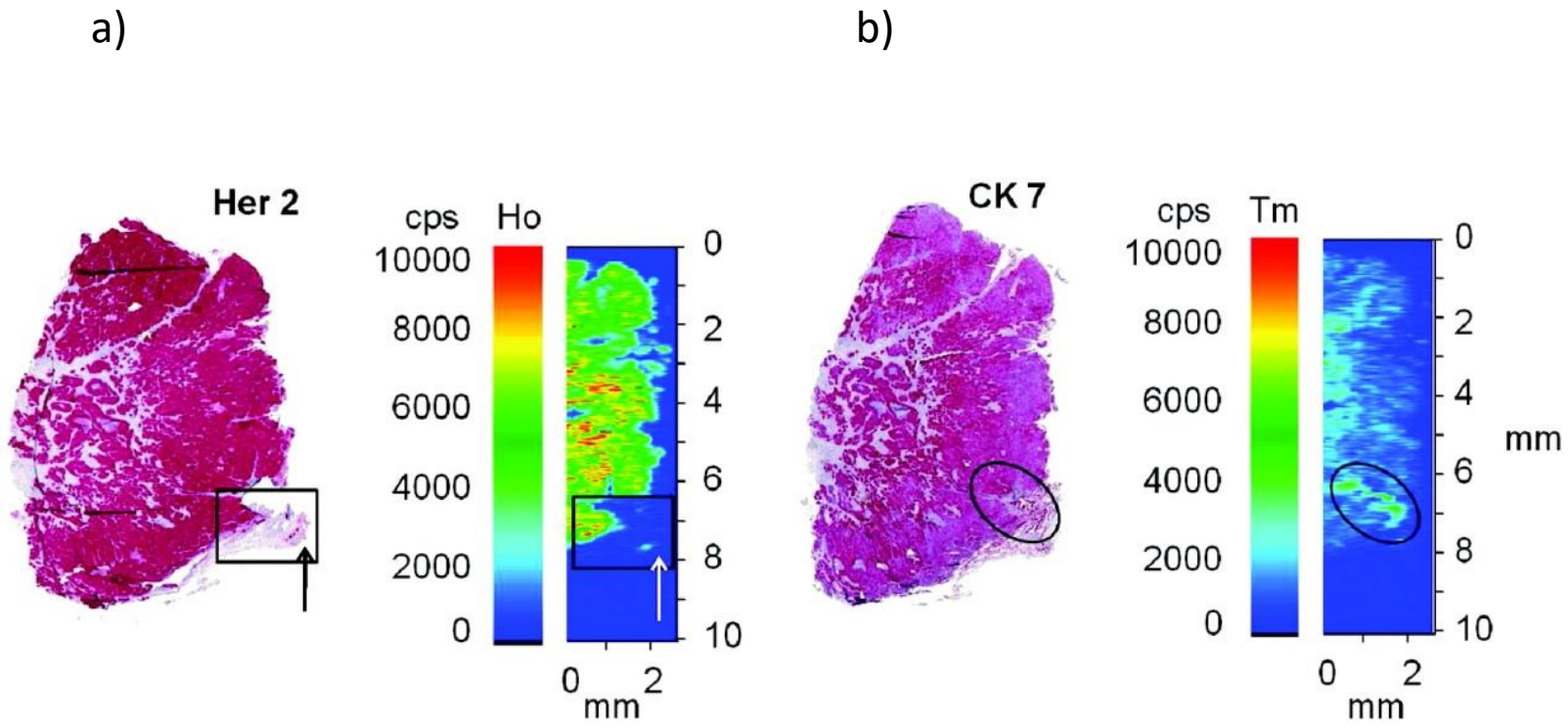

a)
b)
Her 2
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Figure 3

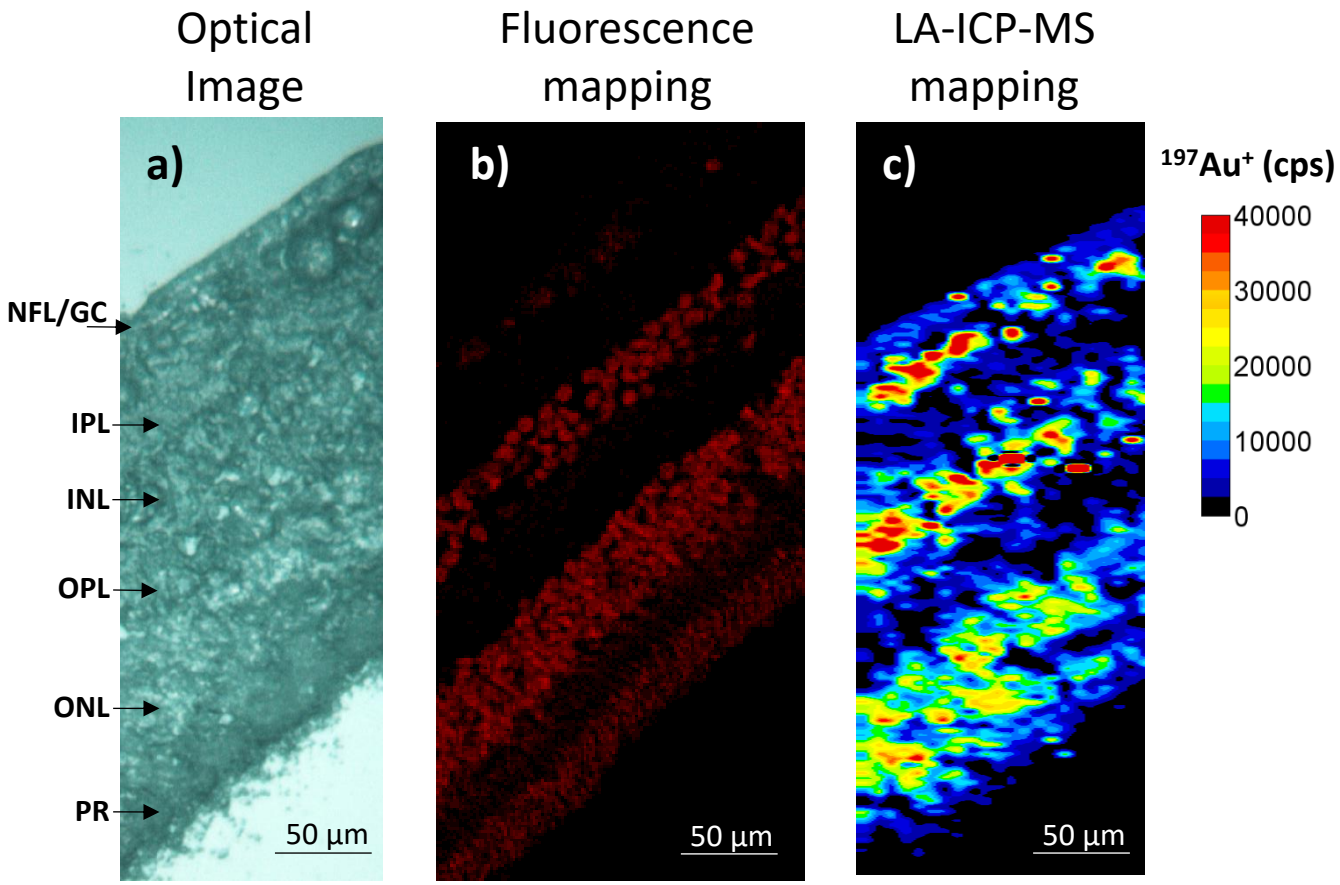
Optical Image
Fluorescence mapping
LA-ICP-MS mapping
a)
b)
c)
NFL/GC
IPL
INL
OPL
ONL
PR
50 µm
50 µm
50 µm
197Au+ (cps)
40000
30000
20000
10000
0

Figure 4