

Imaging of metals and metal-containing species in biological tissues and on gels by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS): A new analytical strategy for applications in life sciences*

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Abstract: Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has become established as a very efficient and sensitive trace, ultratrace, and surface analytical technique in the life sciences. We have developed a new analytical imaging technique using LA-ICP-MS to study element distribution in biological tissues. Nowadays, imaging LA-ICP-MS using double-focusing sector field (LA-ICP-SFMS) or quadrupole-based mass spectrometers (LA-ICP-QMS) can be applied as an exciting tool providing new information on the pathophysiology, pharmacology, and toxicology of elements of interest in biological systems. The quantitative determination of elements (e.g., Cu, Fe, Zn, Se, and others) in biological tissues is of growing interest especially in brain research (e.g., for studying neurodegenerative diseases such as Alzheimer's or Parkinson's disease). LA-ICP-SFMS was employed to produce images of detailed regionally specific element distributions in thin tissue sections of different sizes (such as control human or rat brain tissues or tumor regions). In addition, imaging MS using LA-ICP-QMS was applied to study the uptake and transport of nutrient and toxic elements in plant tissues.

Besides the quantitative imaging of essential and toxic elements in tissues, powerful analytical techniques are also required for the determination and characterization of phosphoproteins and metal-containing proteins within a large pool of proteins, after electrophoretic separation [e.g., blue native polyacrylamide gel electrophoresis (BN-PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)] into 1D and 2D gels. LA-ICP-MS was used to detect metalloproteins in protein bands of 1D gels or protein spots separated after 2D gel electrophoresis (2D-GE). In addition to elemental determination by LA-ICP-MS, matrix-assisted laser desorption/ionization, or MALDI-MS, was employed to identify metal-containing proteins.

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Recent progress will be discussed in applying LA-ICP-MS in the life sciences, including the imaging of thin slices of tissue and applications in proteome analysis in combination with MALDI-MS to investigate phosphoproteins and metal-containing proteins.

Keywords: biological tissues; imaging mass spectrometry; LA-ICP-MS; metal distribution; element distribution.

INTRODUCTION

Mass spectrometry (MS) is one of the most important analytical techniques used today for the determination of element concentration, for surface and isotope analysis, and for structural analysis of organic and bioorganic compounds due to its very high sensitivity and low detection limits [1]. The analysis of element distribution (imaging or mapping) in thin sections of biological tissues is of increasing interest in the life sciences. The determination of essential metals (e.g., Cu, Zn, Fe, Ni, Mn, Mo, Mg, Ca, Na, K, Cr, B, and others), metalloids (e.g., Se) or non-metals (e.g., C, S, O, N, P, I, Cl, and S) of vital importance in biological systems is a main issue in analytical chemistry. Whereas a deficit of essential elements often results in deficiency diseases, too high concentrations can be toxic to the living organism. In addition, certain toxic elements (e.g., Cd, Pb, Hg, U, Th, and others) influence the biological processes in living organisms. Because all nutrient and toxic elements are in general inhomogeneously distributed in biological tissues, imaging studies require powerful analytical techniques with both good spatial resolution and high signal/noise ratio (low detection limits). Commonly used methods in biological and medical research for the visualization of metal distribution in tissues are specific chemical stains, immunohistochemical staining (tags) techniques, and radiolabels for visualizing and identifying metal and molecular tags, which, however, do not provide multielement capability and high sensitivity. Other non-mass-spectrometric surface analytical techniques, such as scanning electron microscopy with energy-dispersive X-ray analysis (SEM-EDX), proton-induced X-ray emission (PIXE), or autoradiography, are generally not sensitive enough for trace metal imaging [2].

Nowadays, imaging MS is one of the greatest challenges combining the multielemental measurement capability of mass spectrometers with the surface sampling process of material using focused laser or ion beam. Imaging MS techniques enable the distribution of the elements and isotopes to be analyzed using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) [1–7] in the investigated tissues and also small molecules or even large molecules (like proteins) for biomedical research. Among the various imaging MS techniques, matrix-assisted laser desorption/ionization (MALDI)-MS [8–11] and also the well-established secondary ion mass spectrometry (SIMS) [12–14] are employed to study the molecule distribution in biological systems. As an inorganic MS technique, SIMS allows primarily the imaging of elements and isotopes in the area investigated.

Consequently, the existing imaging MS techniques can be divided into those involving the imaging of metals using inorganic MS techniques (LA-ICP-MS, SIMS) and the imaging of molecules (including future applications of imaging of metal-containing biomolecules) by MALDI-MS and in part SIMS—the latter for the analysis of small molecules of biological and pharmacological relevance with $m/z < 1000$. Pioneering work on organic ion imaging—of small molecules and large biomolecules up to the m/z range over 100 000 Da within biological systems using imaging MALDI-MS (MALDI-IMS)—has been performed by Caprioli's group [8,12]. At present, the distribution of hundreds of unknown compounds in a single measurement can be determined by MALDI-IMS. Interest in MALDI-IMS has grown rapidly, enhanced by the recent introduction of commercial instrumentation and devices for sample preparation and data acquisition and analysis [10]. Tissue samples are typically thin sections, 5–20 μm thick, that have been spray-coated or micro-spotted (e.g., using a robotic picoliter volume spotter) with MALDI matrix. Due to matrix spotting and as a function of the laser-based system employed, a spatial resolution of about 20 μm is achieved in routine measurements. Fresh section cuts from sample tissue were analyzed after matrix application whereby partial series of mass spectra (images

were produced from measurements of ion intensity as a function of x and y coordinates) with selected m/z ratio (different molecules) were obtained.

Imaging MS techniques of metals and metal-containing species by SIMS have been well known in life science studies for many years. Commercial TOF-SIMS instruments are already available for imaging MS measurements and software for evaluating the analytical data. Using SIMS, it is possible to produce images of the distribution of metals and small molecules in tissue with a spatial resolution in the low μm range and below. The use of liquid metal ion guns (such as Ga^+ , Bi_n^+ , Au_3^+ with primary ion energies of several keV), which cause fragmentation of most molecular species larger than 1000 Da, is advantageous. However, the quantification of the images obtained is difficult due to inherent matrix effects of up to six orders of magnitude [1]. A real problem is the lack of suitable matrix-matched reference materials for imaging MS. Small areas of interest up to $500 \times 500 \mu\text{m}$ were mostly analyzed by SIMS.

Due to serious problems with the quantification of SIMS images, LA-ICP-MS has been developed as the method of choice for the imaging of elements in thin cross-sections of biological tissues. At present, LA-ICP-MS is the most sensitive technique for imaging of metals, metalloids, and non-metals on biological tissues [15–17]. A direct LA of thin sections of tissue from 10–100 μm is possible, which means no additional matrix for LA (compared to MALDI-MS) is required. Detection limits of imaging LA-ICP-MS were observed at the sub $\mu\text{g g}^{-1}$ down to the low ng g^{-1} range. LA-ICP-MS (compared to SIMS) is very advantageous due to the relatively low instrumental costs and high sample throughput, high sensitivity, accuracy, and precision of the analytical data. Due to fewer matrix effects, the quantification of analytical data in LA-ICP-MS is relatively simple compared to SIMS if suitable matrix-matched standard reference materials are available because no charging-up effects occur. The limits of detection (in the ng g^{-1} range) are in general lower than in SIMS [18]. LA-ICP-MS can be employed as a sensitive inorganic MS technique for the imaging of essential and toxic metals (such as Cu, Zn, Pb, Th, and U—often at trace concentration level) and also for the mapping of metalloids (Se) and non-metals (P, S, C, I) in microtome thin tissue sections (optimum at 20 μm thickness) of tissues (e.g., of rat or human brains). Since no suitable certified standard reference materials for quantification procedures are available, different calibration strategies were developed. For quantitative LA-ICP-MS analysis on biological tissues, well-defined matrix-matched laboratory standards can be prepared and employed [2,3,19]. Furthermore, solution-based calibration by inserting a micro-nebulizer into the LA chamber was created for quantification purposes [2]. By means of MS imaging analysis using LA-ICP-MS inhomogeneous (often layered), site-specific metal distributions can be obtained in tissue sections (as demonstrated, e.g., in human brain tissues for the hippocampus, insular region, region around the precentral sulcus) or in tumor-infected regions in human and rat brain [2,3,6,7,19]. The quantitative imaging of elements and biomolecules using LA-ICP-MS in thin tissue slices or on biological surfaces was developed for special medical applications (to the study of neurodegenerative diseases) [19–21]. Significant progress has been made in the development and application of all three imaging MS techniques, MALDI-MS [11,12,22,23], SIMS, and LA-ICP-MS [14,24,25], for the determination of the quantitative distribution of organic compounds (including biomolecules), element species and elements on biological tissues to solve unanswered questions in the life sciences [2,19].

The combined determination of molecular or elemental changes is associated with disease progression studied by MALDI-MS and LA-ICP-MS. For example, a significant metal depletion was found in tumors compared to control tissues [1,19]. The distribution elemental profiles for essential metals such as Fe, Cu, Zn, Mn, Mo, Co, and Ni through the cross-section of analyzed tissue (e.g., brain, liver, kidney but also in single cells and cell organelles, etc.) can reveal unique information on the tissues investigated. For instance, unknown regions in the analyzed biological tissues with the enrichment/depletion of metals of interest provide information about many open questions in different areas of biological and medical research in the life sciences.

In addition to the advantages of LA-ICP-MS (high sensitivity, low detection limits of metals, and easy quantification capability), this inorganic MS technique allows fast screening of 1D and 2D gels in

order to detect metals, metalloids, and non-metals in protein bands or protein spots separated by 1D and 2D gel electrophoresis (GE), respectively. It will be demonstrated that LA-ICP-MS as an elemental imaging MS technique with multielement capability and excellent detection limits and MALDI-MS as a biomolecular MS technique complement each other well and can be used for an accurate quantification of elemental images and identification of biomolecules whereby structural information and molecular weight (MW) determination are provided by biomolecular MS.

The main aim of this paper is to describe advanced analytical MS techniques with imaging capability in order to perform microlocal analysis on biological tissues. It will be demonstrated that the combination of LA-ICP-MS and MALDI-MS can solve analytical problems in the life sciences.

EXPERIMENTAL

In LA-ICP-MS, mostly a commercial LA system using a Nd:YAG laser (e.g., from NewWave UP 213, UP 266, Fremont, CA, or from CETAC Technologies, Omaha, NE, LSX 200, LSX 213, LSX 500, LSX 3000 at 266, 213, and 193 nm wavelength) is coupled to a quadrupole-based inductively coupled plasma mass spectrometer (ICP-QMS) without or with collision cell or to a double-focusing sector field ICP-MS with single ion collection (ICP-SFMS) or multiple ion collectors (MC-ICP-MS) [1]. Possible experimental arrangements of LA-ICP-MS using different types of ICP-MS are illustrated in Fig. 1. LA-MC-ICP-MS with multiple ion collection (not shown in Fig. 1) could be employed advantageously for the precise and accurate imaging of isotopes of selected elements in biological systems.

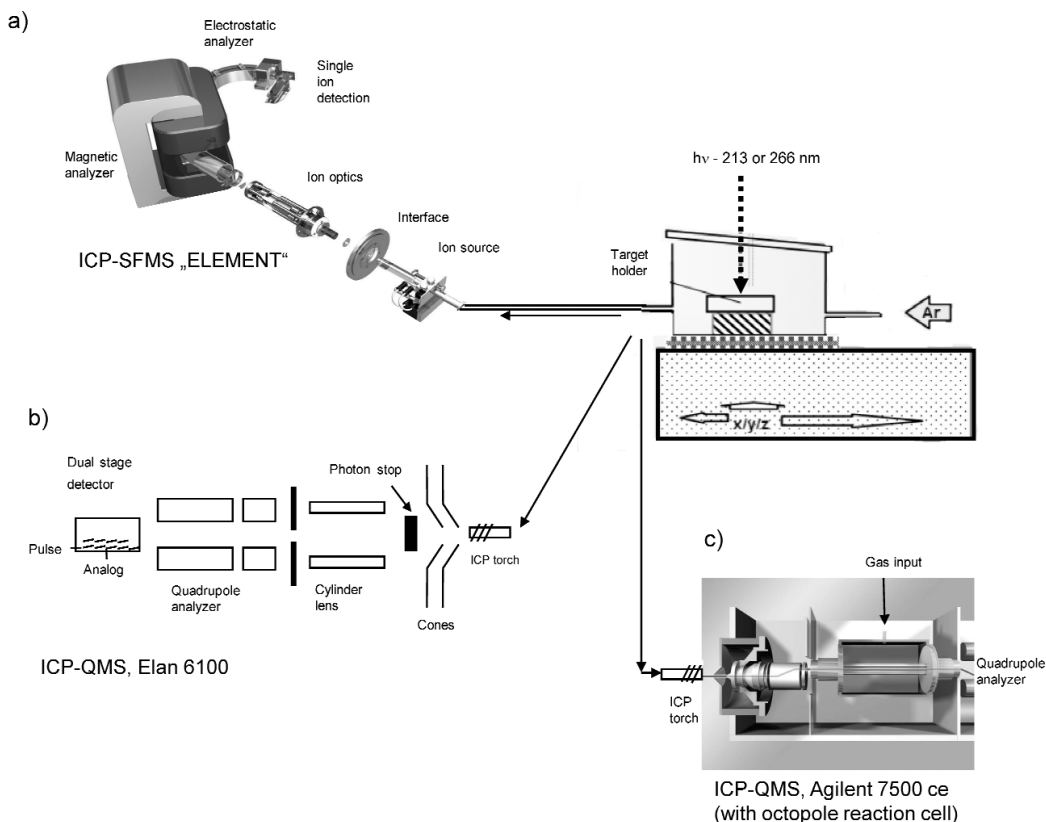


Fig. 1 Schematic of LA-ICP-MS using (a) double-focusing SF ICP-MS, Element, from Thermo Fisher Scientific, (b) ICP-QMS Elan 6100 without collision cell, Perkin Elmer Sciex, and (c) ICP-QMS with octopole collision cell, Agilent.

Modern commercial LA systems support the mapping of elements in small- but also large-size tissues [1]. However, no commercial software is available up to now for the evaluation of analytical LA-ICP-MS data, therefore home-made imaging software was developed in different laboratories.

The diameter of the laser crater can be varied during imaging of the thin tissue section between 5 and about 200 μm . Because no matrix is applied in LA-ICP-MS compared to MALDI-MS, the spatial resolution of LA-ICP-MS can be improved in the low μm range. The measurement time for imaging LA-ICP-MS of biological tissues (up to several hours) depends on the size of the tissue area analyzed or gel section selected and the laser scan speed applied (varied from 20 up to 100 $\mu\text{m/s}$). To obtain high-resolution images, the spot size and laser scan speed should be optimized.

APPLICATION FIELDS OF IMAGING MASS SPECTROMETRY

New analytical strategies for the life sciences consist of the combined application of element MS techniques such as LA-ICP-MS and biomolecular MS such as ESI- or MALDI-MS [20,21,26,27]. In Fig. 2, the combination of imaging MS by LA-ICP-MS and proteome analysis is shown schematically. The new analytical strategy starts by imaging thin sections of tissues to detect firstly metals, metalloids, and non-metals in order to study neurodegenerative diseases or tumors growth via the quantitative (mostly abnormal) metal distribution of essential elements (Cu, Zn, Fe, Se) or toxic metals (Pb, Cd). In the second step by proteome analysis, after the separation of proteins from the selected region of interest in 1D or 2D gels, metalloproteins and phosphoproteins were detected by LA-ICP-MS by the new powerful and sensitive screening technique whereas the proteins were identified after MALDI- or ESI-MS.

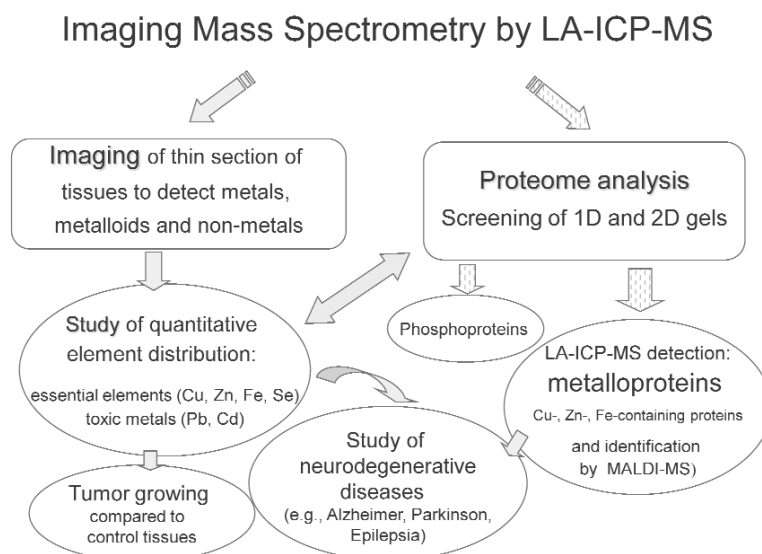


Fig. 2 Application fields of imaging MS by LA-ICP-MS on biological tissues in combination with proteome studies.

For example, recently an analytical procedure for the quantitative imaging of Se in thin sections of biological tissues (slugs) by LA-ICP-SFMS was created in the author's laboratory [25]. Higher concentrations of Se were found in the skin and gut compared to other parts of the slugs. The maximum natural Se concentration in slug tissues was observed to be up to 25 $\mu\text{g g}^{-1}$. Parallel to imaging studies, small sections of slug tissues were investigated after digestion by ICP-QMS [28] and proteins were separated after GE (1D blue native, or BN) to detect seleno- and metalloproteins. In these experiments,

three new Zn-containing proteins at 75, 100, and 150 kDa in the slug's digesting gland were detected [29].

Imaging of brain tissues by LA-ICP-MS

Images of C, Cu, and Zn measured on a thin section of rat brain tissue by LA-ICP-QMS (Agilent 7500 ce, NewWave UP 266) obtained in the routine mode are summarized in Figs. 3b, 4b, and 4d. The shape and structure of these LA-ICP-MS images are in good agreement with the photograph of the tissue from a similar rat brain stained with Cresyl violet (see Fig. 3a). The LA-ICP-MS images demonstrate that the distribution of the two metals and one abundant non-metal is quite different. The $^{13}\text{C}^+$ and $^{64}\text{Zn}^+$ images also clearly demonstrate the localization of the hippocampus in the rat brain. An interesting multi-layered structure of the cortex is especially visible in the $^{63}\text{Cu}^+$ image. Furthermore, in all three images (Figs. 3b, 4b, and 4d) the vertical blue line in the left hemisphere demonstrates the correctness of the images obtained. In these blue lines, the material of the tissue was previously ablated by a "line scan LA-ICP-MS measurement". The results of the line scan analysis are summarized in Figs. 4a and 4c. In the first investigation by LA-ICP-MS from line scan measurements, evidence of the layered structure of tissue can be demonstrated, but in general, 2- (or 3-) dimensional images yielded more information on element distribution. Images of P, S, Fe, Cu, Zn, and C in a thin section of the rat brain control and tumor tissue was compared by Zoriy et al. [7]. The growth of tumor tissue via the element distribution and the change of water content were first studied by imaging LA-ICP-MS. A depletion of P, S, and Fe was detected in tumor tissues [7]. A low carbon content in the studied tumor region correlates with a relatively high water content.

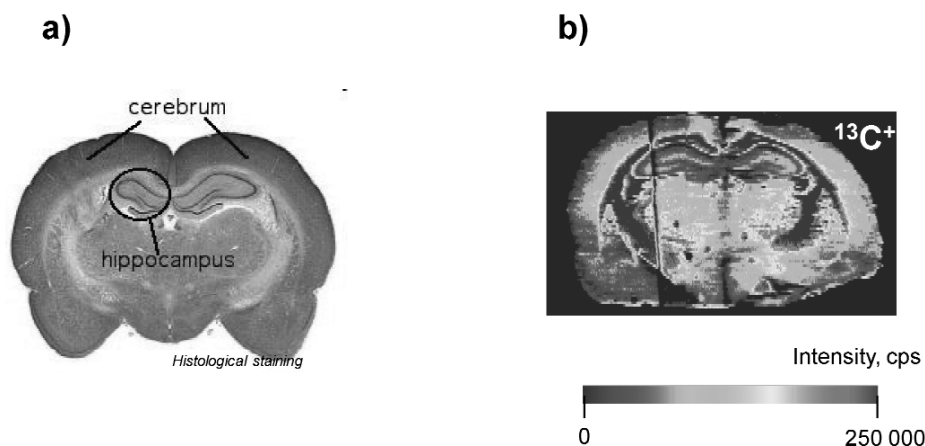


Fig. 3 Image of (b) $^{13}\text{C}^+$ of control rat brain measured by LA-ICP-QMS (Agilent 7500 ce and NewWave UP 266) compared with (a) a histological stain tissue.

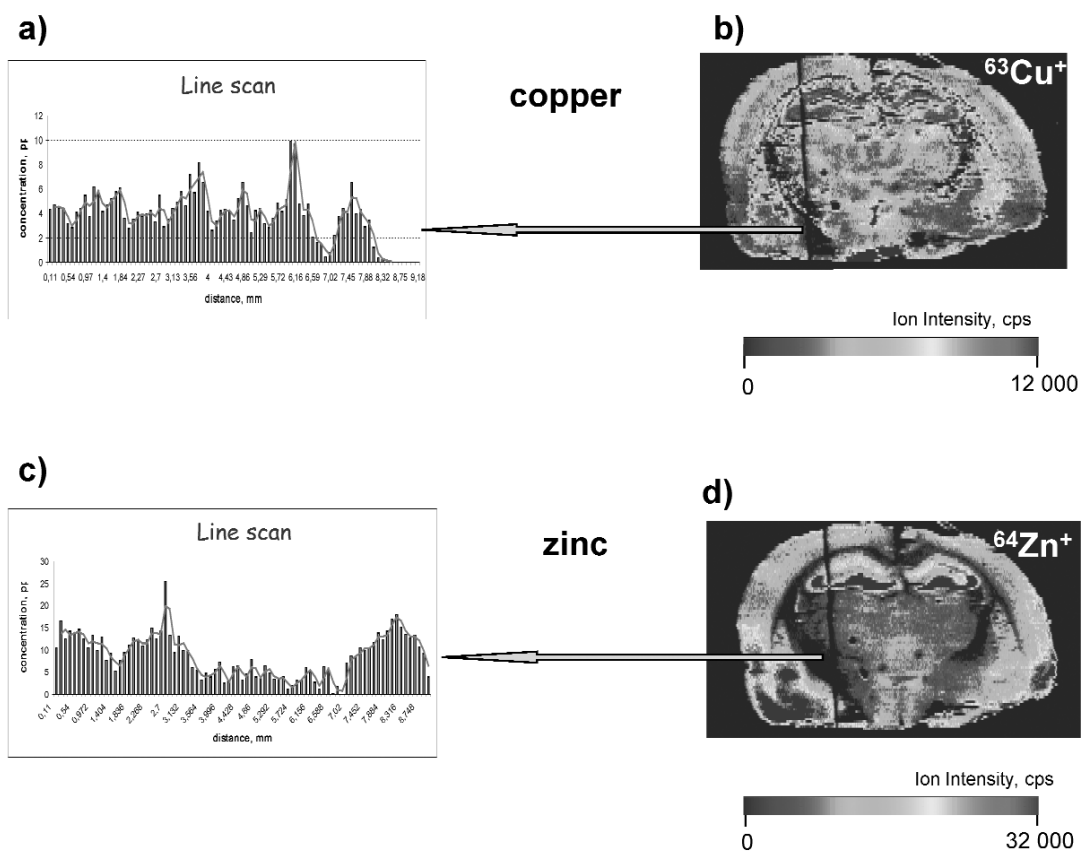


Fig. 4 Images of (b) $^{63}\text{Cu}^+$ and (d) $^{64}\text{Zn}^+$ and line scan measurements of (a) Cu and (c) Zn of control rat brain using LA-ICP-QMS (Agilent 7500 ce and NewWave UP 266).

The new strategy of imaging MS in combination with proteome analysis is demonstrated on human brain (hippocampus) in Fig. 5 [3]. After cryo-cutting of brain tissues, thin sections of human brain were investigated by LA-ICP-SFMS. Cu and Zn distribution in human hippocampus was quantitatively determined (using matrix-matched synthetic laboratory standards for quantification of the analytical data). Selected regions of human brain were selected for further studies after 2D-GE in order to detect metal-containing proteins and phosphoproteins.

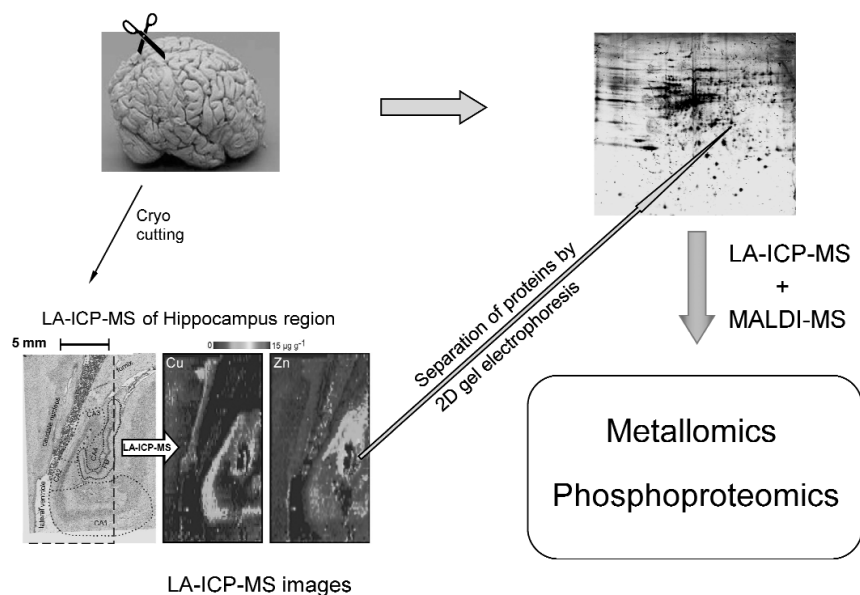


Fig. 5 Combination of imaging MS by LA-ICP-MS and proteome studies on human brain samples (hippocampus).

Detection of metalloproteins by LA-ICP-MS imaging of 2D gels

2D-GE is a powerful separation technique, which allows the separation of thousands of proteins. The high-resolution capability of 2D-GE can be explained by the independent protein separation steps in the first and second dimensions. The first dimension of 2D-GE is isoelectric focusing (IEF) whereby the individual proteins of a protein mixture move to their isoelectric point in a pH gradient. Thereby, the proteins lose their net charge and their electrophoretic mobility. In the second dimension, these proteins are separated orthogonally by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to their MW. The separated proteins in 2D gels are visualized by several staining techniques, for example, with a Coomassie Blue [30], or a silver staining [31]. To detect metals and/or phosphorus in separated protein spots, LA-ICP-MS was applied.

A powerful and sensitive gel screening technique using LA-ICP-SFMS of P, S, Cu, Zn, and Fe in proteins and the identification of phosphorylated protein structures by MALDI-FTICR-MS (matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry) was developed in the author's laboratory [26]. Yeast mitochondria membrane protein complexes were separated in their native state in the first dimension and their subunit composition resolved by SDS-PAGE in the second dimension (BN/SDS 2D-PAGE). The proteins were marked by the silver staining technique (compared to Coomassie staining). P, S, and selected essential metals (Cu, Zn, and Fe) were detected in a short analysis time with detection limits determined in the gel blank of 0.22 or 8.2 $\mu\text{g g}^{-1}$ for P and Cu, respectively, for more than 60 mitochondrial protein spots in 2D gels. For quantification purposes, S was used as an internal standard element. Furthermore, the identification of phosphorylated subunits (Atp1p and Atp2p) of yeast mitochondrial ATPase was carried out by a combination of LA-ICP-MS with MALDI-FTICR-MS (BN/SDS-PAGE). The results were confirmed by Western blot analysis using antibodies directed against phosphorylated amino acids. The combination of LA-ICP-MS and MALDI-FTICR-MS with BN/SDS-PAGE provides a fast and sensitive tool for the structure analysis of phosphorus and metal-containing subunits of membrane protein complexes [27].

Figure 6 demonstrates the results of structure proteome analysis by MALDI-FTICR-MS in the mass range of 1100–3300 Da on a selected protein spot from a 2D gel of human brain (somatic motor cortex) [20]. The protein spot (Fig. 6) cut out from 2D SDS-PAGE gel [20] was identified after tryptic

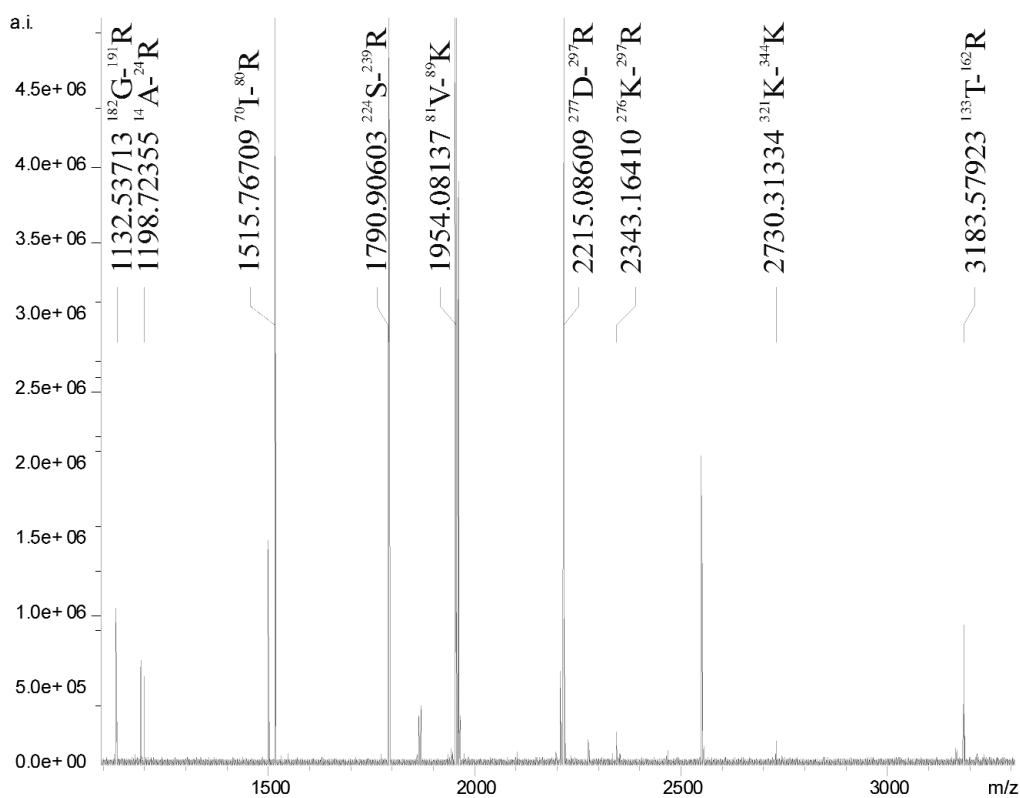


Fig. 6 MALDI-FTICR-MS of ACTB protein (*H. sapiens*, somatomotor cortex).

digestion by high-resolution MALDI-MS using an FTICR instrument with a high score of 126 as the ACTB protein (*Homo sapiens*). Using LA-ICP-SFMS measurements on 2D gel also a low P and Zn content was detected in this protein spot. Due to the lower sensitivity of the MALDI-MS, P- and Zn-containing peptides could not be detected.

A new strategy proposed in the author's laboratory is to image the protein spots in 2D gel. The imaging technique allows a better presentation of the metal distribution in proteins. This is demonstrated for Cu- and Zn-binding proteins in a 2D BN-PAGE gel of rat kidney water extract [29]. In Fig. 7, the lateral distribution of Zn and Cu on 2D BN-PAGE gel are compared with a photograph of a silver-stained gel section. Of several visible well-separated protein spots, the two metals were detected by LA-ICP-MS only in two spots as shown in Figs. 7a and 7c. The identification of metal-containing protein spots will be performed in future studies by MALDI-MS.

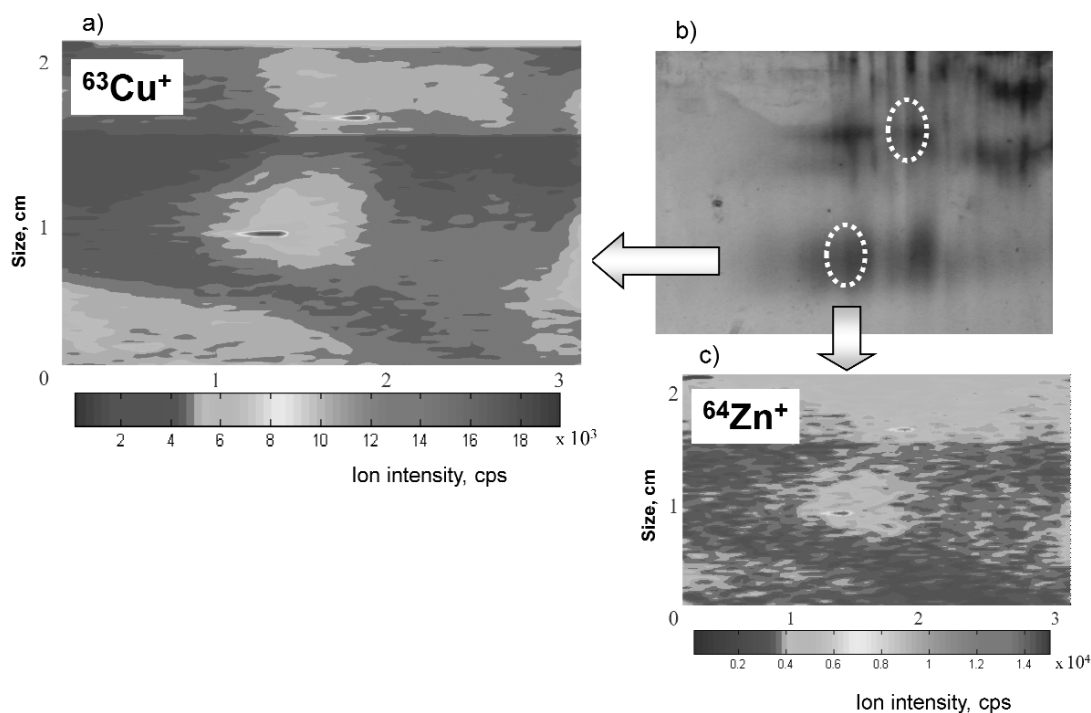


Fig. 7 Detection of (a) Cu- and (c) Zn-containing proteins on (b) a 2D BN-PAGE gel (rat kidney water extract) by imaging of gel section using LA-ICP-SFMS.

Imaging of plant tissues by LA-ICP-MS

In studying the nutrient uptake and the transport of nutrients in plants, the imaging technique was also applied to determine lateral elements of heavy elements in plant tissues. Similar to brain tissue imaging, the analysis of metal distribution in leaves is performed by LA-ICP-MS on 30- μm thin leaf sections with a laser spot size of 50 μm . The LA-ICP-MS images of Mg and Fe as nutrient metals are summarized in Fig. 8. The structure and shape of the tobacco leaf, especially the localization of several veins in the leaf investigated, are demonstrated well by LA-ICP-MS measurements. The highest ion intensity for Mg of up to 10^6 cps was found at the epidermis of the main vein, whereas in the interior of the main vein (vascular tissue) the Mg content drops significantly. Similar to Mg, higher amounts of Fe are distributed at the epidermis of the main vein and on the margin. In addition, high metal ion intensities were measured for Cu (not shown here), Mg, and Pb at the petiole, but the concentrations decrease from the bottom to tip of the leaf. This experimental finding demonstrates the path of metal uptake via the petiole and main veins. The lowest metal contents were observed in the mesophyll of the leaf. Together with nutrient elements, toxic metals like Cd and Pb were detected in the investigated leaf with higher ion intensities for Pb compared to Cd. The highest metal concentration was detected on the epidermis of the middle leaf vein. The analytical data can be quantified by using certified standard reference materials (e.g., Apple leaves SRM NIST 1515) or synthetic matrix-matched laboratory standards applying a similar preparation procedure to that described in [2,3]. This was done together with distribution studies of essential elements in *Elsholtzia splendens* leaves. In these experiments fresh leaves were measured directly by LA-ICP-QMS (Agilent 7500 ce and NewWave UP 266) without sample preparation. The quantitative images (with concentration scales in $\mu\text{g g}^{-1}$) of essential elements such as K, Mg, and Mn are illustrated in Fig. 9. K, Mg, and Mn in the leaf veins can be clearly observed, indicating the importance of the vein in transporting macro-essential elements. In further studies, several parts of the leaves

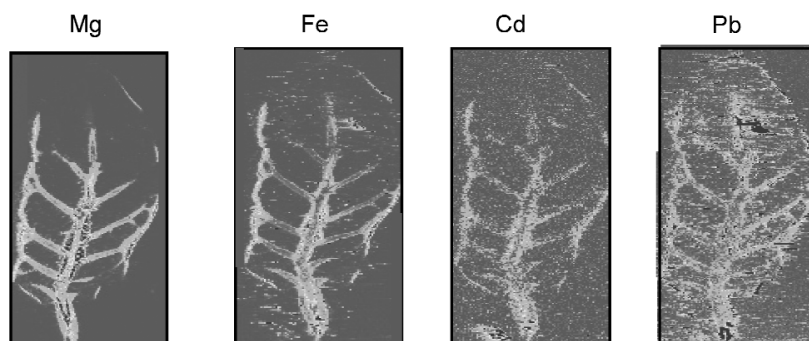


Fig. 8 LA-ICP-QMS images of $^{24}\text{Mg}^+$, $^{57}\text{Fe}^+$, $^{114}\text{Cd}^+$, and $^{204}\text{Pb}^+$ in tobacco leaf.

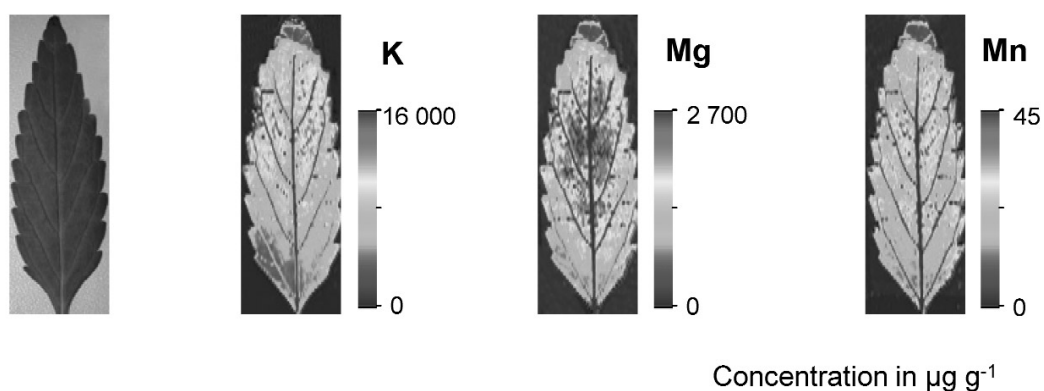


Fig. 9 Quantitative images of K, Mg, and Mn in *E. splendens* leaf measured by LA-ICP-QMS.

were subjected to biomolecular MS to understand the binding of metals in plants. In future work, the binding of metals to biomolecules will be studied after GE by MALDI/ESI-MS.

Newly developed imaging techniques using LA-ICP-MS in combination with MALDI-MS offer not only new capabilities providing both elemental and biomolecular information for the analysis of tissue samples but also for the development of novel array-based bio-chips.

CONCLUSION

Imaging MS techniques using LA-ICP-MS were developed for imaging the distribution of metals and non-metals in thin sections of biological tissues. Quantitative imaging analysis of essential and toxic elements in biological tissues allows studies to be performed of element distribution, transport processes, bioavailability, and possible contamination.

The results of imaging MS using LA-ICP-MS in combination with biomolecular MS provide novel information on the distribution of elements and element species in biological tissues and the identification of protein structures.

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