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Review

Bioimaging of metals in brain tissue from micrometre to nanometre scale by laser ablation inductively coupled plasma mass spectrometry: State of the art and perspectives

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ABSTRACT

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) with multi-element capability is well established for the trace and ultratrace analysis of metals, metalloids and selected non-metals (such as C, P, S) in biological and clinical samples. Nowadays LA-ICP-MS is employed as a sensitive elemental mass spectrometric technique for the imaging of metals and non-metals in microtome thin tissue sections, especially for the determination of element concentrations at the trace and ultratrace level in selected small brain regions. This article discusses the state of the art of bioimaging of metals in thin brain tissue sections by LA-ICP-MS with spatial resolution at the micrometre scale and prospects for developing quantitative techniques at nanometre range.

Contents

1. Introduction

Bioimaging analytical techniques are today of key interest in life science studies and have been rapidly growing in biology and medicine [1–3]. Over the past decades, several (non-mass spectrometric) bioimaging techniques such as scanning electron microscopy with energy-dispersive X-ray analysis (SEM-EDX) [4], energy-filtering transmission electron microscopy (EFTEM) [5], quantitative positron emission tomography (PET) [6,7], nuclear magnetic resonance imaging (MRI), proton-induced X-ray emission (PIXE) [8] or nano-X-ray fluorescence (nanoXRF) e.g., at a synchrotron radiation facility [9], or immunohistochemical staining of tissues using fluorescence microscopy [10] have been developed and applied to visualize structures and to study elemental

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and molecular distribution in tissues. To an increasing extent, mass spectrometric analytical methods (including instrumentation, sample preparation, whole analytical procedures and imaging software) are being improved and employed for imaging studies of biological systems to provide information on various classes of biomolecules (such as proteins, metabolites and lipids) and metals on tissues with spatial resolution at the micrometre scale. Of the mass spectrometric techniques, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) for imaging of biomolecules [11–18] or secondary ion mass spectrometry (SIMS) for mapping of elements and molecules are widely distributed and have been applied for many years [3,19–25]. Commercial time-of-flight SIMS (ToF-SIMS) [26,27] and double-focusing sector field instruments with single and multiple ion collection [28] are available for valuable imaging mass spectrometric measurements with dedicated software for evaluating the analytical data. Whereas SIMS using bismuth, gold or carbon cluster ion bombardment with spatial resolution of a few micrometres and below is applied to an increasing extent for biomedical applications, especially for analysing relatively small biomolecules (<1000 Da) in cells and tissues [29], there is a serious problem in analysing large biomolecules due to fractionation effects and in determining the quantitative metal distribution in tissue due to huge matrix effects.

The scientific community has begun to focus more on the relevance of metals in the neurosciences during the first 10 years of the 21st century [30]. Today, the distribution analysis of metals is increasingly an object of basic research and development in a variety of institutions throughout the world (Sheffield University [31], University of Aberdeen [32,33], Forschungszentrum Jülich [2,34], Utah University [35], Boston University, University of Technology, Sydney [36] and others). Especially sensitive and elemental- and isotopic-specific laser-induced analytical techniques have been developed.

A significant laser-induced technique in elemental mass spectrometry is LA-ICP-MS (laser ablation inductively coupled plasma mass spectrometry). LA-ICP-MS uses the evaporation of sample material by a laser beam in an argon atmosphere under normal pressure. The ablated material is transported with an argon stream in inductively coupled plasma and ionized in argon plasma. For mass spectrometric imaging studies by LA-ICP-MS, various commercially available laser ablation systems mostly using a Nd:YAG laser (e.g., from NewWave, Fremont, CA, or from CETAC Technologies, Omaha, NE, working at wavelengths of 266 and 213 nm) are applied. Because as a soft material biological tissue is easy to ablate from a glass substrate a Nd:YAG laser with a wavelength of 266 nm is sufficient for complete evaporation of sample. The spot size and laser scan speed were optimized to obtain highly spatially resolved images. In LA-ICP-MS, different types of mass spectrometers mostly quadrupole mass spectrometers are applied. Quadrupole mass spectrometers work very robust and stable over many hours, which is an important precondition for time-consuming imaging studies on biological tissues. LA-ICP-SFMS with sector fields offers the highest sensitivity achievable (approximately 1 order of magnitude more sensitive compared to LA-ICP-QMS) for imaging of selected trace elements and can be applied at higher mass resolution if interference problems occur. In our imaging studies with LA-ICP-MS, we used laser ablation systems (NewWave and CETAC) coupled to ICP-QMS from Agilent Technologies, Tokyo, Japan (with octopole reaction cell), Elan 6100 or to double-focusing sector field ICP-SFMS (with reverse Nier–Johnson geometry) Element from Thermo Fisher Scientific, Bremen, Germany.

It should be noted that LA-ICP-MS is already the most important laser-induced analytical technique in inorganic mass spectrometry applied for materials research (high-purity materials, ceramics and microelectronic applications), determination of long-lived radionuclides or geological research due to the advantage of direct sampling by focused laser beam [1]. The limits of detection (LODs) for the determination of trace metals (bulk analysis) depend on the laser parameters, laser power density, wavelength of laser beam, the matrix composition and the figures of merit of ICP-MS used. The LODs of metals measured by LA-ICP-MS via the direct multi-element trace analysis of solid samples including biological materials varied from $\mu g\,g^{-1}$ down to sub-$ng\,g^{-1}$ range. With its ability to provide microscale information this technique can be employed not only as a sensitive elemental mass spectrometric technique for the determination of element concentration at the trace and ultratrace level, but also for the imaging (mapping) of elements in different materials, like geological samples [37], single zircon grains for U–Pb age dating, for isotope analysis of single nuclear uranium oxide particles [38] or in material sciences [39]. Nowadays, LA-ICP-MS is employed for imaging of metals (such as Cu, Zn, Fe, Mn, Ni, Cr, Na, K, Mg, Cd and Pb) but also for selected non-metals in microtome thin tissue section. In addition, it is the only technique which allows precise isotope analysis (very important for tracer experiments in kinetic studies using enriched stable isotopes or for application of the isotope dilution technique) [40–42].

Table 1
Figures or merit of LA-ICP-MS imaging.

Advantages
Simultaneous determination of almost trace and minor elements in biological tissue
Thin tissue sections can measured directly (no additional sample preparation is required)
Complete ablation of biological sample (thickness of section < 100 μm) on glass substrate and following no fraction effects occur
Only small samples are required
Low relative limits of detection (LODs); 0.001–1 $\mu g\,g^{-1}$
Spatial resolution: 5–200 μm
Low contamination danger
Analytical data are easy to quantify if homogeneous matrix-matched standards are available
Precision of trace metal distribution 5–10%
Limits
Isobaric interferences of atomic ions of analyte with isobaric atomic (e.g., $^{40}Ca^+$ and $^{40}Ar^+$) of polyatomic ions (e.g., $^{41}K^+$ and $^{40}ArH^+$) at same nominal mass
Standard reference materials with similar matrix composition for quantification are required

Main features of LA-ICP-MS in respect to advantages and drawbacks are summarized in Table 1. The interference problem in ICP-MS and LA-ICP-MS has been discussed in selected papers [1,43–46]. Trace element analysis using LA-ICP-MS does not require involved interference corrections inherent in SIMS analysis. In general in LA-ICP-MS (under dry plasma condition) lower interference problems compared to ICP-MS (under wet plasma condition) were observed. For example iron can be measured in biological tissue sections by LA-ICP-MS at m/z – 56 due to a constant background formed by ArO^+ ions (this background signal is then substrated). The second limit of LA-ICP-MS is the need of a suitable reference material for quantification of analytical data. Different reliable quantification strategies for the LA-ICP-MS data using well-prepared homogeneous matrix-matched laboratory standards or solution-based calibration in bioimaging of metals in tissues are established and employed [47–49].

In recent years, LA-ICP-MS with multi-element capability has been developed and successfully applied as a powerful imaging (mapping) technique to produce quantitative images of detailed regionally specific metal distributions in thin tissue sections of human or rodent brain as demonstrated, for example, in human brain for the hippocampus) or in tumour-invaded regions or control brain [50–53] or of cancer biomarkers [54].

1.1. Motivation of quantitative bioimaging of metals in brain tissue

Metal ions play a key role in the origin and generation of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases [30,55]. Interaction between redox-active metal ions and proteins can lead to damage of critical biological systems and may initiate a cascade of events leading to oxidative damage, neurodegeneration and cell death [55]. With an ageing population continually growing worldwide, neurodegenerative diseases have become a major concern, and represent a social, human and economic burden. An estimated 37 million people worldwide live with dementia—with Alzheimer's disease (AD) causing the majority of cases. The estimated prevalence in industrial countries is 1.5% for Alzheimer's patients and 0.2% for Parkinson's disease (PD). In addition, neurodegenerative diseases overlap with the symptomatic conditions of epilepsy and depression with a prevalence of 0.7% and 2%, and are thus amongst the leading contributors to the global burden of disease [56].

To further underline the link between metals, neurotoxicity and neurodegeneration, it should be recall that we all are continuously exposed to small but significant amounts of toxic metals. These metals can accumulate in our tissues, poison our bodies and can lead to serious health problems. Poisoning by toxic heavy metals such as As, Pb, Cd or Hg, even in very low amounts at ng g^{-1} levels, can cause permanent damage to the brain and nervous system. We are exposed everyday to traces of toxic metals like arsenic, cadmium and also aluminium (as a neurotoxin) and nickel in food, drinking water, and pollution in air (e.g., in cigarette smoke) and these metals accumulate in different parts of the human body including the brain.

For example, the accumulation of anthropogenic arsenic in the human body with increasing age is discussed by Dani [57]. Important sources of anthropogenic arsenic are gold ores in hard rock mines and fossil fuels such as coal and oil. Chronic arsenic poisoning has the potential to cause dementias (like Alzheimer's diseases). With increasing environmental concentrations of arsenic – in the 7–18 ppm range – in topsoils an exponential rise in the prevalence and mortality of Alzheimer's disease and other dementias of the old age in European countries was observed [58]. The arsenic contamination in different brain regions is unknown.

1.2. Why do we need quantitative metal distribution analysis for the brain?

The role of metal ions in neurodegenerative diseases in brain is discussed in several studies [30,59–61]. From the literature is known that:

(a) Small amounts of metal ions such as Ca, Fe, Cu, Zn, Mn, Mg, Co and Mo are required for the growth and function of the brain. Certain trace metals protect against many diseases and reactive oxygen species (ROS). These essential metals are critical as catalysts, second messengers, gene expression regulators, and as cofactors for enzymes (e.g. Cu/Zn superoxide dismutase plays an important role in antioxidative defence and cellular oxygen metabolism and protects against oxidative injury) [29,30].

(b) Nutritional and genetically induced deficiencies of essential metals result in neurological disease. An excess of essential metals such as Fe, Cu, and Zn in the brain also results in significant neurological impairment by the stabilization of abnormal proteins (such as beta amyloid), oxidation of ROS-scavenging enzymes, and lipid peroxidation. In most major neurodegenerative diseases (e.g. Alzheimer's disease—AD, Parkinson's disease—PD, Wilson's disease), abnormal metal deposition has been observed within specific areas of the brain [31–33]. As examples, the Alzheimer Aβ-peptide, the central player and target of vaccinations, is a fragment of a copper efflux pump, which binds copper itself and is enhanced in its aggregation by copper. The formation of α-synuclein protofibrils, a central step in PD, is substantially catalysed by copper. Neuromelanin, a polymer with high iron content, is assumed to be a cellular susceptibility factor in PD [62].

(c) Age-related increases in Al, Cu, Fe and Zn occur within the brain and may contribute to senile neurodegeneration. Increased deposition of a variety of metals in brain has been described in Alzheimer's disease, Parkinson's disease and Wilson's disease [63,64]. Zn(II) and Cu(II) inhibit the amyloid-β ($A\beta_{42}$) peptide fibrillization and initiate formation of non-fibrillar aggregates of $A\beta_{42}$ in Alzheimer's diseases. The mechanisms for assembly and fibrillogenesis of $A\beta_{42}$ in the presence of Zn(II) and Cu(II) was studied by Tõugu et al. [65] Metal chelators including metallothioneins prevent metal-induced $A\beta_{42}$ aggregation. Miller et al. [63] described the accumulation of Zn and Cu with amyloid-β plaques using synchrotron X-ray imaging. Recently, at the Brookhaven National Synchrotron Light Source the same author imaged Zn, Cu, Fe and Ca distribution in amyloid plaques (PSAPP) mice which bind less metal than plaques in human Alzheimer's disease. Specifically, when compared to surrounding tissue, there was approximately a 908% increase in Zn, 1171% increase in Cu and 573% increase in Fe. Human plaques showed 339% increase in Zn, 466% increase in Cu and 177% increase in Fe compared to the control surrounding tissue [66]. We observed in Parkinson's diseased brain (MPTP mouse model) compared to control an increasing of Fe content (+40%) in the interpendicular nucleus from 4.5 μg g^{-1} (control) to 6.2 μg g^{-1} (PD) by bioimaging LA-ICP-MS [67] Furthermore, a significant increases of Cu concentrations (+40%) in the periventricular zone from 8.9 μg g^{-1} (control) to 12.3 μg g^{-1} (PD) was found. In addition, the increased deposition of metals has been described in Huntington's disease, amyotrophic lateral sclerosis, Friedreich's ataxia, prion diseases, central nervous system (CNS) malaria, stroke and intracranial hemorrhage.

(d) The transport of trace metals into the brain is strictly depending on the brain barrier systems, i.e., the blood–brain barrier (BBB) and blood–cerebrospinal fluid (CSF) barrier. Furthermore, metal homeostasis in the brain is highly regulated at the level of BBB, the blood–CSF barrier, and probably also at the CSF–brain barrier [34]. As metal ions do not diffuse across neural barriers, specific transporters exist at multiple sites within the BBB, choroid plexus and other regions in order to ensure adequate delivery of these metals to the brain and removal of excess metals from the brain. Iron uptake transport occurs via the transferrin receptor and ferroportin.

(e) Copper is an essential element that serves as a cofactor of various enzymes. Copper transporters in the brain include Ctr1, ATP7A and ATP7B transporters. Zinc transport occurs via the SLC30 family (ZnT1, 3, 4, and 6) and the SLC39 family (zip 1, 7, and 14). Non-selective transporters also exist (DMT-1, SLC11A2, A3), which may transport many divalent metal ions, including Fe, Cu and Zn. Metal transporters are expressed in neurons, synaptic vesicles, blood vessels and capillaries, and are enriched in specific regions of the brain, such as the hippocampus and choroid plexus. For example, ZnT3 is found in some glutamatergic synaptic vesicles of the hippocampus.

(f) The regulatory control of metal transporters in the brain is only incompletely understood. Nevertheless, since small amounts of metal ions are required for protection from ROS, and since excess metals result in oxidative injury, oxidative triggers may play an important role in the expression of metal transporters.

(g) Deficiencies of essential elements can result in several deficiency diseases, while high concentrations even of essential

elements can be toxic. On the other hand, the occurrence of toxic elements (e.g. Cd, As, Cr, Pb, Tl, Hg, U, Th, etc.) can disturb certain vital biological processes in living organisms. However, growing data indicate that cerebral dysregulation of metals, as well as oxidative stress, contribute to the pathologies of neurodegenerative diseases. The distribution of metals in deposits (plaques) in the brain of a person suffering from a neurodegenerative disorder, which can result in the deterioration of nerve paths and nerve cell loss, is widely unknown.

(h) Some pharmacologically active compounds contain rare noble metals like Pt. In this case, studies of their absorption, distribution, metabolism and elimination (ADME) can be performed using imaging LA-ICP-MS thus avoiding radiocarbon [^{14}C] labelling. Nowadays, therapeutic drug monitoring or studies of contamination with toxic metals over periods of months is possible by the LA-ICP-MS analysis of single hair strands. We recently imaged Pt in mouse brain tumours, kidneys and single human hairs after cis-platinum treatment [35–37]. By single hair analysis maximum concentrations of Pt found along the hair strands were 26.9 ± 5.3, 14.7 ± 3.3, 20.9 ± 3.9 and $26.1 \pm 3.8\ \mu g\ g^{-1}$, which correspond to four treatment of cisplatin administered to the patient at 3-week intervals were observed. The platinum distribution found in the analysed hair may contribute to the optimization of cisplatin therapy.

In particular, a series of new Pt compounds is targeted with respect to brain tumours.

(i) There is currently an increasing interest in the development of next-generation contrast media for magnetic resonance imaging (MRI), which bind selectively to molecular target structures. For this purpose, antibodies, proteins or small organic compounds are labelled with lanthanides, such as gadolinium, europium or lutetium, and also with superparamagnetic iron nanoparticles.

The microlocal distribution of metals in the brain and the formation of metalloproteins in the diseased brain compared with the normal brain are largely unknown. Knowledge today mainly relies on measurements of metal concentration of tissue homogenates. Thus, microlocal elemental analytical techniques for solid state samples are urgently needed in order to permit the quantification of elements on a microscopic scale in relation to protein specification on tissue samples. In order to understand the pathophysiology of metalloproteins, metal metabolism and metal-containing deposits (plaques) and, finally, to facilitate therapeutic interventions, knowledge of the metal distribution in the diseased brain compared to controls is fundamental.

LA-ICP-MS can be applied for imaging of metals in the brain at a scale of 150–5 μm spatial resolution. Novel studies on the nanometre scale (e.g., of individual single cells or cell organelles) will lead to the understanding of several signalling pathways and are currently of great importance in all fields of the life sciences. Therefore the aim of present and future work is to develop novel tools for quantitative imaging of metals integrated in the framework of high-end analytical techniques available to the neuroscience community.

2. State of the art of bioimaging of metals in tissues by LA-ICP-MS

Fig. 1 illustrates the entire analytical procedure from sample preparation by cryocutting thin tissue sections via the bioimaging procedure in LA-ICP-MS, including the scanning (line by line) by a focused laser beam to measure ion intensities of analyte ions as a function of time and for a final evaluation of the data in order to obtain quantitative images of metals. This figure shows the Cu and Fe distribution in mouse brain (Parkinson's disease). In general, the images of different metals obtained by LA-ICP-MS correlated with immunostained and autoradiographic images (not shown here) and

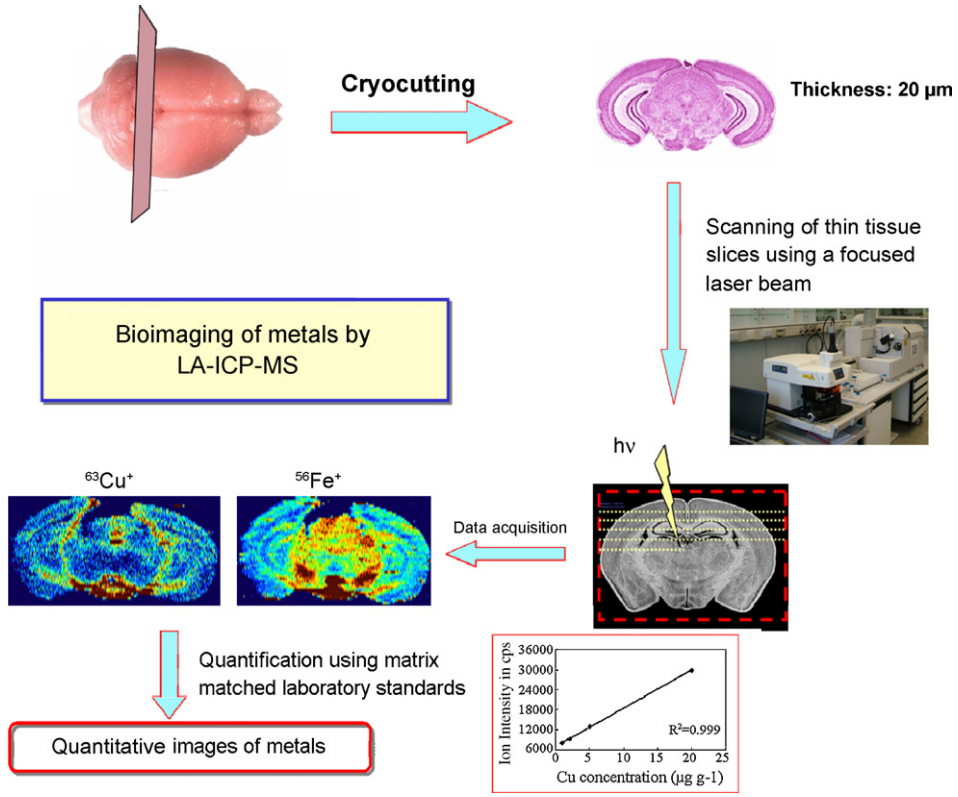

Fig. 1. Workflow of bioimaging of tissue by the newly developed LA-ICP-MS technique from sample preparation by cryocutting of thin tissue sections, measurements, generation of images and quantification of analytical data.

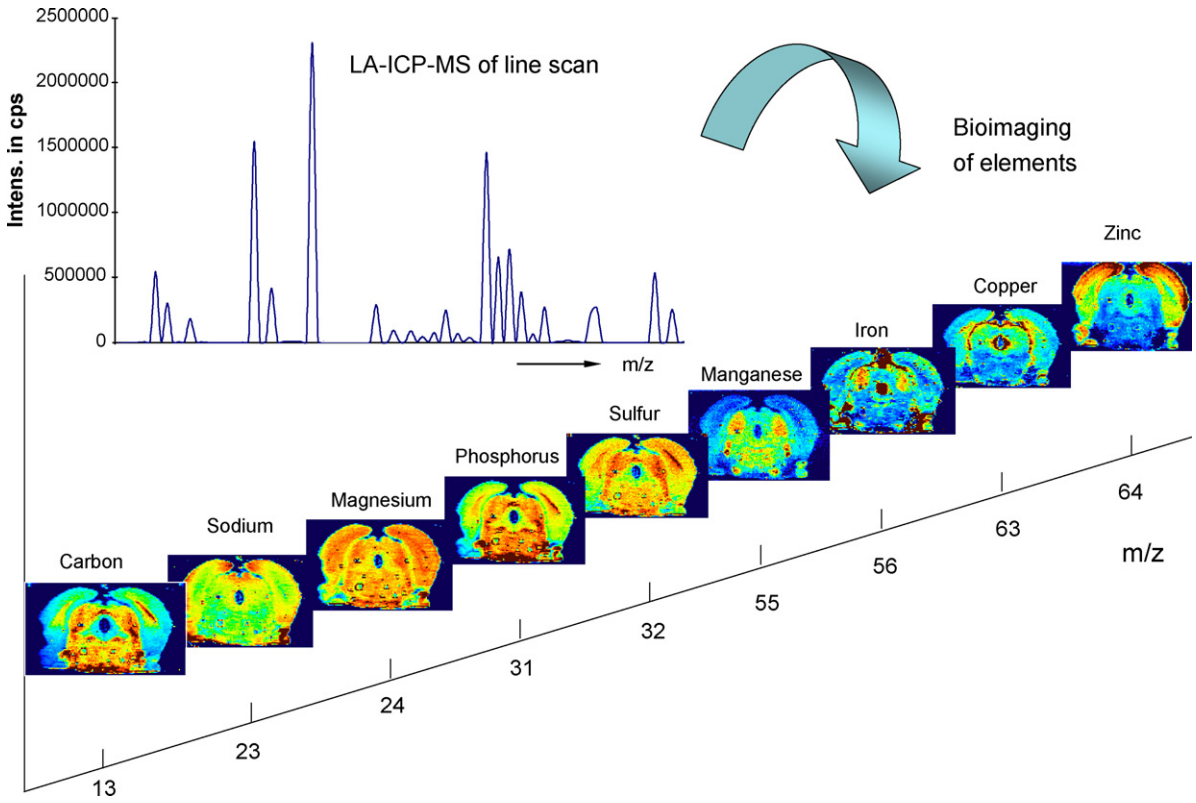

Fig. 2. Mass spectrum of a line scan (top left) and images of selected metals and non-metals measured by LA-ICP-MS in routine mode.

microphotographs of the investigated slice. A mass spectrum of one line scan measured by LA-ICP-MS and images of selected metals and non-metals in a rat brain sections are summarized in Fig. 2. Each elemental image shows a different distribution of the analyte investigated with similarities for the non-metals carbon, sulphur and phosphorus but not for the metals. The structure of C, S, and P measured by LA-ICP-MS illustrates the localization of the white matter, the metals are located rather in grey matter of brain. Microlocal analysis identified substructures of interest (e.g., corpus callosum, substancia nigra, hippocampus). In the images of metals in Fig. 2 enrichment of Zn in the cortex of Mn and Fe in colliculus interferior and Fe in aqueduct was found. The reproducibility of the developed analytical bioimaging techniques for five neighbouring sections of human brain using LA-ICP-MS ranged between 5 and 8%, the limits of detection were in the low ng g^{-1} (ppb) range. Details of reproducibility studies are described in [50].

2.1. Quantification procedure

Quantitative images of elements were obtained in our laboratory mainly using prepared matrix-matched laboratory standards for calibration as demonstrated in several previous papers [1,47,48]. In general, five laboratory synthetic standard solutions with all elements of interest (Cu, Zn, Fe, Pb, Cd, U, etc.) and well-defined concentrations were prepared. Five slices of the same biological tissue (*each of about* 0.65 g) were spiked with selected standard solutions (final concentration of Cu, Zn, Fe in brain tissue: 50, 20, 10, 5, 1 μg g^{-1} and of Pb, Cd and U: one order of magnitude lower). An additional slice was not spiked and was used for blank correction. The spiked biological tissues were properly mixed and centrifuged at 5000 rpm for 5 min. Samples were then frozen below a temperature of −50 °C and cut with a microtome into thin sections with a thickness of 20 μm and placed onto the glass substrate. These synthetic laboratory standards prepared for the calibration of LA-ICP-MS images were measured together with biological tissues in the same measurement cycles and consequently ideal matrix matching was obtained. Matrix-matching standards were used to constitute calibration curves, whereas the regression coefficient of the attained calibration curves was typically >0.9 for all analytes investigated.

Other approaches for quantification of images of tissue were studied in author's lab [47,48,67–70]. The quantification of metal distribution in a thin slice of the snail tissue was compared using different strategies: by one-point calibration and via matrix-matched laboratory standards using several biological materials (BCR 278, snail tissue, and rat brain). Synthetic laboratory standards were prepared from certified reference material mussel tissue BRC 278 doped with trace elements with defined concentrations [69]. In addition, the solution-based calibration using a micronebulizer, which was inserted directly into the laser ablation chamber, was developed for quantification of analytical data and for validation of synthetic laboratory standards [47]. This arrangement allows an easy, accurate and precise quantification by on-line isotope dilution using a defined standard solution with an isotope-enriched tracer nebulized to the laser-ablated sample material. An ideal matrix matching in LA-ICP-MS is therefore obtained during the measurement [48]. This technique is not used in routine imaging of tissue due to higher experimental effort.

2.2. Selected applications of bioimaging LA-ICP-MS on brain tissues

Advanced quantitative bioimaging techniques are employed in the author's BrainMet (BrainMet – Bioimaging of Metals in Brain and Metallomics) laboratory for routine measurements on diseased brain sections compared to controls. The LA-ICP-MS technique was

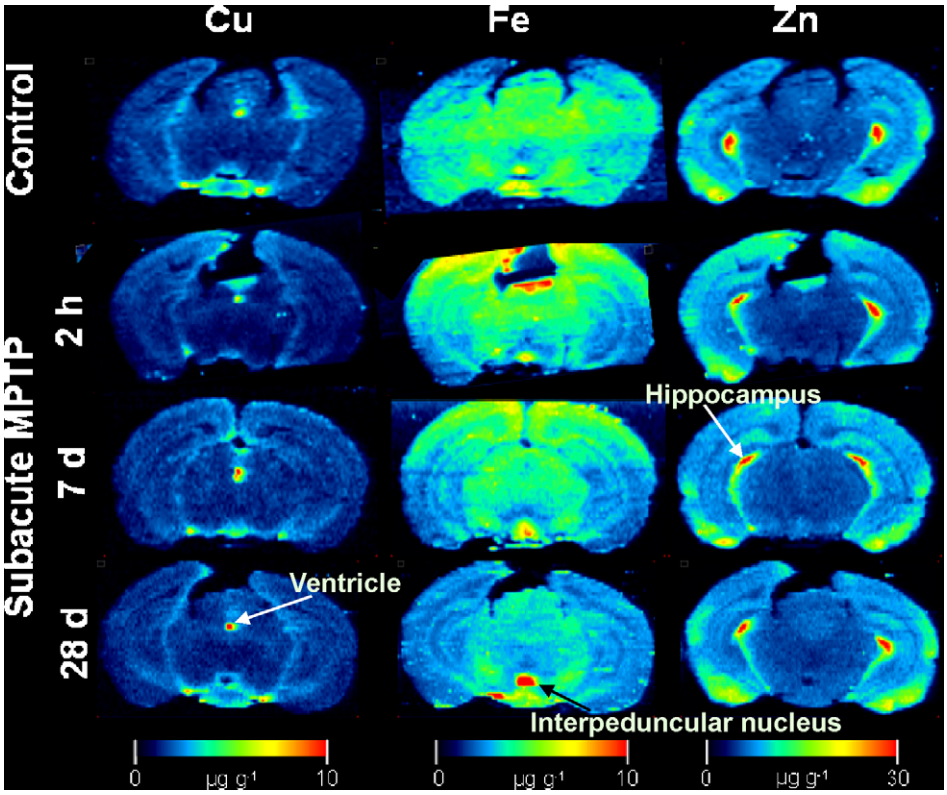

Fig. 3. Copper distribution in the mouse brain 2 h, 7 d and 28 d after treatment with MPTP – a neurotoxin that causes Parkinsonism – compared to controls (Ctrl) measured by imaging LA-ICP-MS [67].

developed into an emerging routine technique for metal imaging and extended to produce large series of quantitative maps of selected metals in thin native mouse brain sections. We analysed 40 mice brain slices from 19 animals (wild-type C57B16 male mice) to study the kinetics of Parkinson's diseases (PD). Fig. 3 shows quantitative LA-ICP-MS images of Cu, Zn and Fe in brain sections from mice treated with the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin), a model of Parkinson's disease, 2 h, 7 d and 28 d after the last injection compared to controls (Ctrl). Clear effects of enrichment of Cu in the ventricle, Fe in the interpeduncular nucleus and Zn in the hippocampus area of Parkinson's are observed in diseased mouse brain compared to the control brain. Details of these investigations are described by Matusch et al. [67].

In other experiments we studied the change in essential metal distribution during aging by a combination of high-resolution autoradiography (in cooperation with K. Morton from Utah University, Salt Lake City) and the LA-ICP-MS. Fig. 4 illustrates the copper distribution measured by LA-ICP-MS in young and old mice and a comparison to the copper image measured by high-resolution autoradiography [35]. The spatial resolution of LA-ICP-MS images compared to autoradiography is higher. Using LA-ICP-MS the total Cu content and distribution in the brain in young (2-month-old) compared to old (14-month-old) mice was measured [35] A decrease of copper in the brain parenchyma was observed. The experimental findings are confirmed by the autoradiographic measurements studying the active uptake of ^{67}Cu into brain by Morton and co-workers [35]. Bioimaging of the other essential metals (Zn and Fe) measured by LA-ICP-MS showed a significant enrichment of Zn in the CA3 region of the hippocampus and in the cortex, of iron in the thalamus and the CA1 regions of the hippocampus. It is known that Fe catalyses the formation of reactive oxygen species. Increased Fe levels in brain may contribute to age-related neurodegeneration [34].

In Fig. 5, one example is presented of the bioimaging of selected essential metals and non-metals in mouse brain hippocampus (dimension: ~3 mm × 3 mm) measured by the established LA-ICP-

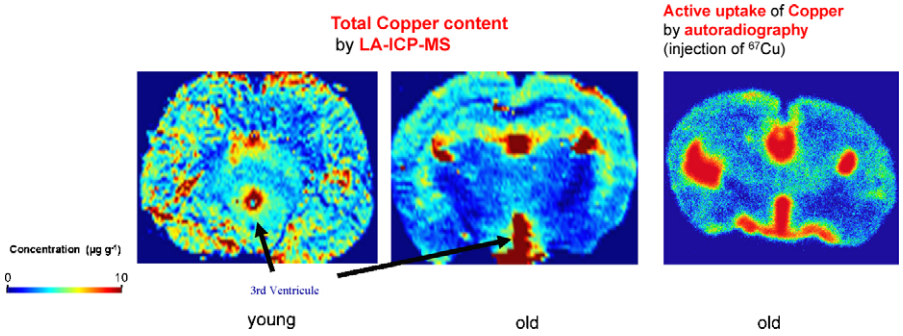

Fig. 4. Comparison of copper images of mice brain sections from 2- to 14-month-old mice measured by LA-ICP-MS. With aging the Cu content in the brain parenchyma decreases (brain samples from Prof. K. Morton, University of Utah Hospitals and Clinics, Salt Lake City).

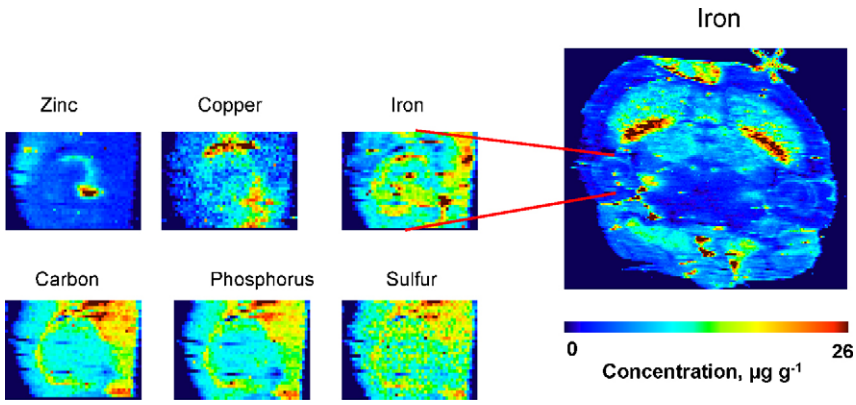

Fig. 5. Metal and non-metal distributions in mouse brain hippocampus and Fe image in whole mouse brain section measured by LA-ICP-MS at spatial resolution of 100 μm.

MS technique and the Fe image in the whole brain section. All measurements at a spatial resolution of 100 μm illustrate the possibilities of the powerful imaging tool with multi-elemental capability. The shape and structure of these LA-ICP-MS images are always in good agreement with the photograph of the tissue from a similar rat brain stained with cresyl violet (not shown in this figure). The measured images reflect the metal distribution which correlates strongly with the structure. In order to illustrate smaller structures in brain LA-ICP-MS measurements of selected essential metals were performed at 12 μm spatial resolution on human hippocampus (see Fig. 6). The spatial resolution of imaging technique using LA-ICP-MS was validated by measurement of the ablated line width using a light optical microscope.

Imaging LA-ICP-MS of tissues (like rat brain) incubated with heavy metals (e.g., U and Nd) or compounds of interest provides an informative, versatile tool for toxicological research allowing the differentiation of critical substructures within organs [71]. Using the developed technique rapid evaluation of entire classes of toxins is possible.

The existing bioimaging techniques were employed not only in brain research but also for the study or distribution analysis of metals in other organs (like kidney) and in plant tissues [53,68,70] and animal samples (e.g., selenium, mercury, lead and cadmium distribution was first illustrated on slug and snail tissues [69,72], platinum was imaged in 14 μm sections of kidneys from a mouse

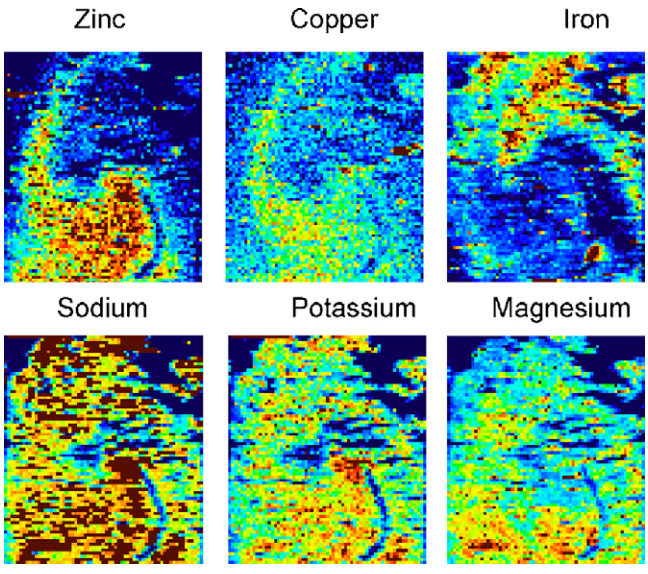

Fig. 6. Metal images in human brain hippocampus measured at spatial resolution of 12 μm.

treated with cisplatin) [73]. Recently, LA-ICP-MS was applied for metal imaging of 2D gels for the detection of metalloproteins in rat kidney after electrophoretic separation [74]. Further applications and new analytical strategies in life sciences are discussed in [75]. These few examples of bioimaging metals in tissues and gels demonstrate the different possibilities of the newly established analytical technique with a spatial resolution at the μm scale. In any case quantitative bioimaging of metals in smaller biological specimens such as single nerve cells and cell organelles is not yet possible using laser-induced mass spectrometric techniques.

However, in spite of all the advantages of the LA-ICP-MS technique its application in some analytical tasks is limited by the restriction of focusing the laser beam due to the diffraction feature of the light, and therefore, a finite lateral resolution. In some cases the spatial resolution of 5 μm would be insufficient, for example, for the analysis of the fine structures of small regions of biological tissues and single cells. This is the new challenge for the next generation of novel more powerful analytical tools and new strategies for looking at the smallest regions of the brain on the nanometre scale.

Based on the established bioimaging techniques on tissue, the development, implementation and dissemination of new nano-laser ablation mass spectrometric imaging techniques (nano-LA-ICP-MS) are required in order to measure the quantitative metal distribution in selected regions of the brain (e.g., in the hippocampus, in cortex layers or nerve cells, cell organelles or on synapses) and metal-containing aggregates (plaques) in the brain on the nanometre scale for fundamental studies of functionality in the biochemical and signalling pathways in a convergent manner [16,17].

3. Instrumental developments of nano-LA-ICP-MS techniques

3.1. Insertion of a thin Ag needle into a laser ablation chamber for a better spatial resolution of nano-LA-ICP-MS (to 50 nm) and combination with atomic force microscopy (AFM)

The basic idea of further improving the spatial resolution of laser ablation in the nanometre (nm) scale (to 50 nm) was the insertion of a thin Ag needle into a defocused laser beam using the near-field effect in laser ablation [76]. The tip of the thin needle acts like a "nanomagnifier" (like the "antenna effect"—a similar principle to the "lightning conductor" on a house). Photons are focused on the tip of the needle due to the near-field effect, the focusing is 300× better than the best focusing lens and a strong field enhancement was observed.

Different laboratories are intensively investigating the exploitation of the near-field effect for laser ablation. In contrast to others (using glass fibre coated with Al without field enhancement) we

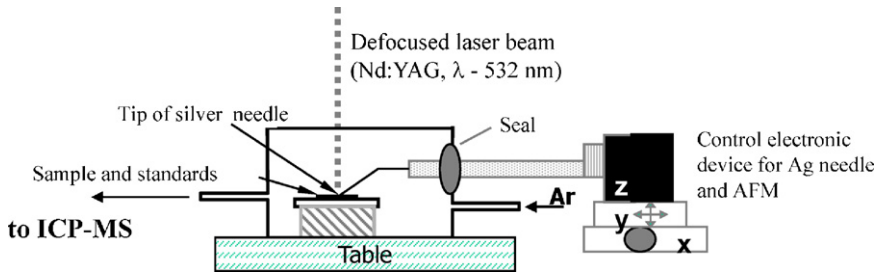

Fig. 7. Combination of elemental mass spectrometry for bioimaging of metals in human brain tissue by LA-ICP-MS from micrometre to nanometre scale with biomolecular mass spectrometry by MALDI/ESI-MS.

favoured the robust apertureless mode on the thin tip of needle because of strong field enhancement (TIP Apex acts like a Mie–Rayleigh scattering particle). We established the electrochemical etching of appropriate very sharp silver tips and presented the first evidence of this setup in elemental and isotopic analysis while ablating and analysing nanometre scale spots from gels and biological samples [77]. For ablating of small sample under the near-field conditions the control of the tip-to-sample distance is critical. This can be realized by different feedback mechanisms. In a first attempt, we controlled the tunnel current between the tip, only samples with good electrical conductivity were analysed [77–80]. At present we are developing an approach for measuring non-conductive materials (small biological tissues and single cells) with a spatial resolution in the nanometre range.

The schematic of the laser ablation chamber with the thin Ag (or Au) needle for the nano-laser ablation apparatus is shown in Fig. 7. The small laser ablation chamber will be mounted on the vibration-damped table. This experimental arrangement will allow us to study fundamentals, to develop and standardize the nano-LA-ICP-MS technique for the elemental and isotopic characterization of samples in nanometre dimensions. In order to provide feedback control of the tip also applicable to non-conducting samples we now aim to implement a constant force feedback very similar to the feedback mechanism used in AFM. A high-tech experimental arrangement integrating a microscale cantilever to measure atomic forces will also allow us to analyse the sample surface in the AFM mode. Nano-laser ablation coupled to a very sensitive ICP mass spectrometer (double-focusing sector field ICP-MS Element, Thermo Fisher Scientific) in combination with AFM was proposed in a German patent [81]. Furthermore, it is recommended that some features will be beneficial for the future improvement of nano-LA-ICP-MS. This includes the vibration-damped table, the high-performance optical microscope necessary for the observation of all micro-manipulations and of the ablation process and the comparatively large operating distance. Therefore, the small transparent laser ablation chamber with all the necessary adapters based on established and proven laser ablation chambers will be inserted into our existing apparatus described in [80]. In the experimental arrangement, the thin silver (or gold) needle is mounted on an electroacoustic transducer (quartz glass), which in combination with the control electronics allows the tip to be positioned in an *x*–*y* and *z* direction for the laser ablation of the biological sample in the near field. The control electronic device with an electroacoustic transducer for the thin Ag (or Au) needle will be designed and constructed on the basis of existing equipment for nano-LA-ICP-MS in author's laboratory by a small innovative company in Saxony (Anfatec Instruments, Oelsnitz), which supplies nano-devices for measurements, science and technology especially for surface science, mainly comprising scanning probe microscopes and related components.

The present research concerns the development of nano-LA-ICP-MS for the smallest specimens, complemented by the combination with atomic force microscopy yielding structural information even at atomic resolution. Near-field enhanced LA-ICP-MS will allow an in situ two-dimensional mapping of elemental concentration and isotope ratios to be made in the smallest structural details such as inclusion bodies, plaques, deposits of protein precipitates, subcellular compartments of single cells. By means of isotope ratio measurements following the in vivo application of stable isotope-enriched tracers it would, for example, be possible to discriminate protein deposits of different ages in situ.

Based on currently ongoing work, the ablation procedure will be intensively studied in more detail in order to further understand the basic physical principles of near-field laser ablation mechanisms, as well as to improve performance.

3.2. Combination of nano-bioimaging of metals in brain with other established biomedical imaging techniques and metallomics studies for functional speciation of metalloproteins

Metallomics is a new emerging field addressing the role, uptake, transport and storage of trace metals essential for protein functions. 30% of the human proteome consists of metalloproteins. Protein metal binding sites are responsible for catalysing important biological processes in nature, such as photosynthesis, respiration, water oxidation, molecular oxygen reduction and nitrogen fixation [82].

As an elemental mass spectrometric technique, LA-ICP-MS has mostly been employed to detect a metal bound to a protein and MALDI/ESI-MS to elucidate the structure, dynamics and function of a metal-protein complex. The combination of bioimaging LA-ICP-MS of metals with proteomic studies using biomolecular mass spectrometry allows the identification of metal-containing proteins and also phosphoproteins (with a spatial resolution on the hundred micrometre scale) and has been demonstrated in several papers [34,40–42,74,83–85]. The combination of elemental mass spectrometry for bioimaging of metals in human brain tissue by LA-ICP-MS from the micrometre to nanometre scale with biomolecular mass spectrometry by MALDI/ESI-MS is illustrated in Fig. 8. This novel analytical strategy starts from elemental (metal and non-metal) imaging of thin sections of tissues: in our example, a human brain hippocampus was analysed by LA-ICP-MS with respect to essential metals (Cu, Zn) and a toxic metal (Pb). The quantification of analytical data was performed using synthetic matrix-matched laboratory standards in the described manner. In the second step, a small region of interest is selected for further analysis at the nanometre scale using nano-LA-ICP-MS. Once the results of the distribution analysis of metals have been determined in the sample at the micrometre to nanometre scale, it is then possible to use e.g., a laser microdissection apparatus to cut out selected analogous areas of the tissue in which metals or phosphorous have been detected (e.g. plaques) and to use these for further analysis of the metalloproteins and/or phosphoproteins.

In the next step, proteins are separated from the selected region of interest by one-dimensional or two-dimensional

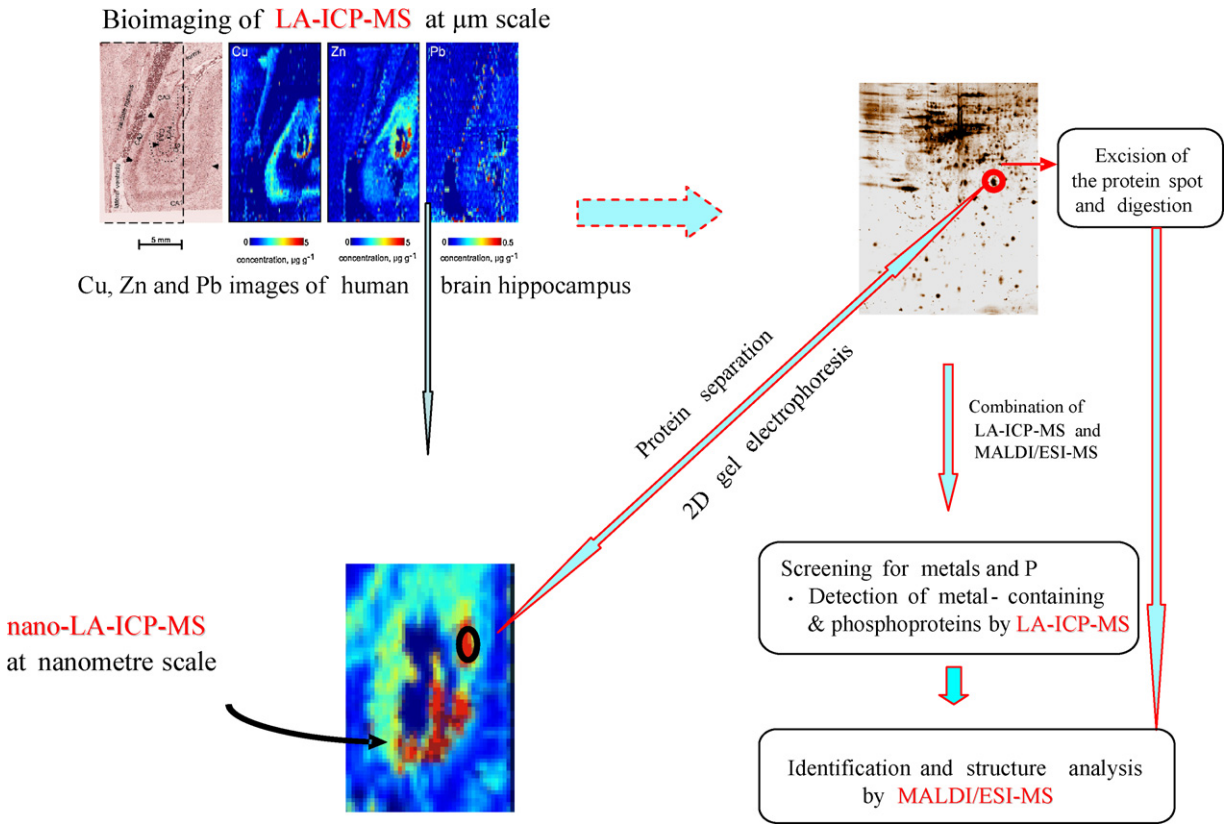

Fig. 8. Combination of bioimaging and metallomic studies using nano-BrainMet techniques.

gel electrophoresis. Metalloproteins and phosphoproteins were detected in gels by the developed LA-ICP-MS after screening of separated protein spots as previously described [34,74]. The separated proteins in gels containing metals (or phosphorus) were thus cut out from a second gel produced under exactly the same conditions, and after tryptic digestion the proteins were identified and the sequence determined by MALDI- or ESI-MS. Metal-containing proteins can also be detected in a two-dimensional gel after the electrophoretic separation of proteins (SDS or Blue Native PAGE) by the LA-ICP-MS imaging technique as demonstrated recently for the detection of metalloproteins in rat kidney separated in their native state in the first and second dimension by blue native gel electrophoresis (BN-PAGE) [74].

The nano-LA-ICP-MS techniques together with established bioimaging techniques on brain tissues using LA-ICP-MS in combination with metallomic studies will allow a new dimension of fundamental brain research to understand the pathophysiology of metalloproteins, metal metabolism and metal-containing deposits, and, finally, to facilitate therapeutic interventions, through knowledge of the quantitative total metal distribution in diseased brain compared to controls.

4. Possible applications to novel bioimaging approaches in brain research

The development of instrumentation and methodology goes hand in hand with basic research and high-tech applications, as demonstrated in the recent Parkinson's study at the routine level yielded valid and statistically robust results on 40 mice brain slices [67]. Practical issues like handling of the data sets and selection of appropriate samples will be dealt with and at the same time applications of the described nano-bioimaging techniques (including the improvement of quantification strategies in bioimaging mass spectrometry) can be focused on new different areas (e.g., Huntington's or Wilson's diseased tissues compared to controls including the study of metal-containing plaques and metalloproteins). Bioimaging techniques were applied in author's laboratory also to study human epilepsy tissues, depression (e.g., Li in brain after treatment with Li-containing drugs), photo-induced thrombosis (Watson stroke model), aging studies on rat and mouse brain, cancer growth and especially to monitor diseases treatment using metal-containing drugs.

However, since these methods are largely restricted to structural or functional assessments they provide no or very limited spatially resolved chemical information. The term molecular imaging has been used so far for methods which indirectly visualize molecules via specific binding of labelled tracers typically in the setting of single- or dual-tracer experiments. Especially molecular imaging mass spectrometry using MALDI-MS [11,13,14,16,17] has been employed to visualize biomolecules. These techniques do not allow the quantitative distribution analysis of metals at trace concentration level and isotope analysis for tracer experiments in the objects being studied. A key interest in life sciences is distribution analysis of metals in small biological specimens, which remains a challenging task in analytical chemistry.

LA-ICP-MS allows all metals, metalloids (e.g., Se, As, Ge, Sb) to be measured as well as difficult-to-analyse non-metals (like C, S, P, Cl, I and others). Native biological samples (thin tissue sections) can be analysed without preparation (compared to MALDI-MS). The analysis of paraffin embedded or formalin fixed tissue section is not recommended. Serious contamination of ion source, sampler, skimmer and ion optics were observed by the analysis of paraffin embedded samples whereas treatment of samples with formalin result is significant loss of certain metals (like Zn). The field of view is in the range of 0.5–200 cm^2, and entire human brain hemispheres have been imaged [1]. The resulting measurement is the average

through the entire slice thickness between 10 and 100 μm and not only through some atomic layers on the surface as is the case for SIMS. The dynamical quantitative range spans five orders of magnitude, its sensitivity is outstanding for heavy elements, and real multi-element measurements with tens of elements (35 or more in our routine mode) in a single scan are possible. Isotope ratios can be measured with high precision and accuracy. The developed bioimaging technique is comparatively fast (1 h per cm^2), relatively cheap compared to very expensive synchrotron facilities or also SIMS instrumentation and can be transferred to any laboratory. One main problem is that no commercial instrumentation for imaging with user-friendly software exists. An improvement of instrumentation is required in respect to increase the repetition frequency of laser systems (100 Hz to 50 kHz), to develop new more effective laser ablation chambers and more sensitive, stable and robust mass spectrometers to improve the limit of the detection at the $ng\,g^{-1}$ (and below) range. Furthermore, high spatial resolution, high precision scanning, a reliable video control for precise sample navigation in the area investigated from μm^2 to several cm^2 and fast imaging acquisition are required. The quantitative distribution analysis of metals on brain tissue has been described only rarely in the literature up to now due to lack of established techniques and quantification procedures available. Therefore, new sets of matrix-matched laboratory standards for quantification of analytical data should be prepared or standard-free analytical techniques have to be developed. In addition, there is a need to develop techniques for 3D analysis in order to construct metal distribution in a whole rat or mouse brain.

In addition, a further combination of elemental and biomolecular mass spectrometry and other established biomedical imaging techniques (such as autoradiography, PET, MRI, histochemistry and others) will be sufficient to give insights into the brain and give us the opportunity to study neurodegenerative diseases from a different emerging point of view. By combining the bioimaging LA-ICP-MS techniques with established biomedical imaging techniques (immunohistochemistry, autoradiography, magnet resonance imaging (MRI) and others) and metallomics, novel and relevant information on the identity, quantity and function of metalloproteins on nanometre structures is obtained. This opens up new emerging application fields in brain research for a better understanding of the disease mechanisms underlying neurodegenerative disorders, in the development and therapeutic monitoring of metal-containing drugs and in the development of novel targeted diagnostic agents for molecular imaging.

Recently, we witnessed an explosion of interest in the use of nanomaterials in assays as protein markers for many diseases and this has become a crucial issue in life science research. Over the past few years, the development and application of several imaging mass spectrometric techniques has grown rapidly in biology and medicine. In addition, depression, schizophrenia and bipolar diseases, treatment with lithium-containing drugs and the distribution of Li in brain after treatment (studies of brain tissues are in progress), photo-induced thrombosis, Watson stroke model (a correlation of metal enrichment with *cis*-4-[18F]fluoro-D-proline uptake in stroke region and caudate nucleus was detected) [86], and aging studies illustrating the changing metal distribution in the brain [34,35] are main future topics in the application of LA-ICP-MS. Furthermore, cancer growth and especially the monitoring of treatment by quantitative bioimaging LA-ICP-MS on a micrometre to nanometre scale will be a main topic for future studies in brain research. This includes the diagnosis of treatment by brain biopsies using advanced bioimaging techniques, including studies of biomarkers and metal-containing drugs and the development of next-generation biomarkers (e.g., antibodies, proteins or small organic compounds labelled with strong paramagnetic lanthanides, such as gadolinium, europium or lutetium as new contrast agents for nuclear magnetic resonance imaging). The development of advanced mass spectrometric techniques will enable us to image single cells, to monitor the incorporation of nanoparticles (e.g. in the context of developing new molecular probes for MRI), to perform kinetic studies at the nano-scale using stable isotopes. Thus, this novel research tool will yield critical insights into neurons and assist in probing the mechanisms of neuronal disease.

5. Conclusions

Current work focuses on the development, implementation and dissemination of bioimaging techniques from the micrometre to the nanometre scale by LA-ICP-MS based on the established quantitative bioimaging techniques for direct quantitative 2D and 3D bioimaging of metals in the brain. Developing quantitative imaging techniques is a main issue in the improvement of the spatial resolution for single-cell analysis and cell organelles. For example, the insertion of a thin Ag needle in the laser ablation chamber (applying the near-field enhancement effect at the tip of the needle) will help to further improve the spatial resolution of LA-ICP-MS in the 50 nm range. A combination of nano-bioimaging of metals in brain with other established biomedical imaging techniques and metallomic studies will help to elucidate metalloproteins.

The new bioimaging techniques will provide a highly inter- and multidisciplinary bridge between chemistry, physics and engineering and allow fundamentally novel biomedical studies to decipher biochemical and signalling pathways. Neurologists, physicians, toxicologists, microbiochemists will benefit from this powerful diagnostic tool for disease monitoring and treatment. Looking at the smallest regions of brain tissue, even at single cells, will pioneer fundamental and applied research for the development of neuroprotective therapies of aging, inflammation and neurodegenerative diseases.

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