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Scaling down the bioimaging of metals by laser microdissection inductively coupled plasma mass spectrometry (LMD-ICP-MS)

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ABSTRACT

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has been established as a powerful quantitative elemental imaging technique in routine mode for biological tissue with a spatial resolution of 12–160 μm. Several applications necessitate an improved spatial resolution of LA-ICP-MS at the low micrometre scale and below. To achieve the improvement of spatial resolution of LA-ICP-MS we created a new experimental arrangement by coupling a laser microdissection system (LMD) used for laser ablation of tissue with a sensitive quadrupole-based inductively coupled plasma mass spectrometer for the subsequent analysis of ablated material. A flat laser ablation chamber made of glass was inserted into the LMD, fitted to the microscope slide with the specimen. The biological tissue fixed on the glass slide was ablated using the focused solid-state Nd:YAG laser of the LMD. The laser ablated material was transported by argon as carrier gas into the inductively coupled plasma of the mass spectrometer and analysed according to the mass-to-charge ratio. Using this novel LMD-ICP-MS arrangement, in initial experiments ion signals of $^{63}Cu^+$ and $^{65}Cu^+$ were measured from a 30-μm-thick cryosection impregnated with a droplet of a Cu solution. A spatial resolution of about 3 μm was obtained using the modified LMD system coupled to the ICP-MS. Laser-induced mass spectrometric measurements of metal distributions can be performed together with simultaneous inspection of the tissue section via the microscope of the LMD and be combined with other modalities of the LMD system. In future, a more powerful laser in the LMD apparatus will allow ablation down to the sub-micrometre scale to study the elemental distribution in small tissue sections.

1. Introduction

Metal ions and metalloproteins play a key role in many biological processes, in ageing and in the origin of neurodegenerative diseases like Alzheimer's, Parkinson's diseases and Wilson's syndrome. Abnormal metal-containing deposits such as beta amyloid plaques and Lewy bodies are the neuropathological hallmarks of Alzheimer's and Parkinson's diseases, respectively. Metal ions critically influence misfolding and toxicity of proteins such as beta amyloid fragments, tau and α-synuclein [1–6]. This exemplifies that in the biomedical sciences there is also a growing interest in quantitatively assessing total metal concentrations within small structures in native tissue in situ. Extra-neuronal objects of interest are atherosclerotic deposits within blood vessels and the fate of smoke and dust particles in the lung. In the pharmaceutical sciences, the broad field of controlled delivery systems like diverse microcrystalline suspensions or nanoparticles and target-specific multimodal probes containing lanthanides or nanoparticles involve a plethora of applications for microlocal element analysis at the lower micrometre and nanometre scales.

Element-specific imaging techniques like EFTEM (energy-filtering transmission electron microscopy) [7], scanning electron microscopy with energy-dispersive X-ray analysis (SEM-EDX) [8], micro-XRF (X-ray fluorescence analysis) [9] or SIMS (secondary ion mass spectrometry) [10–12], cannot be applied to a series of open questions from the medicine, whereas synchrotron radiation XRF (SXRF) [13,14] and proton-induced X-ray emission (PIXE) [1] have extremely restricted availability. Several of these element analytical techniques provide a low dynamical range and encounter difficulties in quantifying analytical data. Furthermore, these imag-

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ing techniques (except SIMS) cannot analyse isotopes, which are interesting for studying the kinetics of metal metabolism in diseases using isotope-enriched tracers or for the application of the isotope dilution technique.

Among the existing analytical techniques for studying the metal and non-metal distribution in tissue, 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 life science studies [5,15–18]. Quantitative LA-ICP-MS imaging of native biological tissues (rodent and human brain to study the metalloarchitecture in brain sections and neurodegenerative diseases) has been established as a routine technique in the BrainMet laboratory at Forschungszentrum Jülich (http://www.brainmet.com) during the last five years [19–21]. For the quantification of elemental images homogenized matrix-matched laboratory standards have been prepared and employed. This technique enables the measurement of elemental distributions from the mg g^{-1} to ng g^{-1} range in 10–40 μm thick cryosections. The spatial resolution of bioimaging LA-ICP-MS using commercially available laser ablation systems with solid-state lasers (Nd-YAG lasers at 266 and 213 nm wavelength, e.g., from NewWave UP 266 and UP 213 or from Cetac LSX 200 or 213) varied between 200 and 50 μm and has been restricted to approximately 5–10 μm due to limitations in the laser optics of commercial laser ablation systems.

Two analytical strategies have been pursued in order to improve the spatial resolution. One uses the near-field effect in laser ablation at a nanometre-scaled tip of a silver needle – so-called near-field laser ablation (NF-LA) – that is combined to a sensitive double focusing sector field ICP-MS (ICP-SFMS). The principles and application of NF-LA-ICP-MS have been described and discussed elsewhere [22–26].

Another innovative technique at low micrometre and sub-micrometre resolution would be the enhancement of a commercial laser microdissection (LMD) apparatus with a powerful solid-state laser to a sample introduction (laser ablation) system for a sensitive ICP-MS. The aim of this paper is a proof of concept of extending an LMD to form a laser ablation system coupled to an ICP-MS for thin tissue section analysis on a glass substrate with increased spatial resolution compared to conventional LA-ICP-MS.

1.1. A novel LMD-ICP-MS tool for the quantitative bioimaging of metals in small biological specimens

Laser microdissection (LMD) is a microscope-based technique using a high-precision laser beam applied via the microscope objective to selectively isolate specific cell types, individual cells, or cell organelles from embedded, frozen or fresh tissue sections. LMD is utilized in life science areas like drug discovery, pathology, forensics, medical diagnostics, food and environmental analysis as well as in medical research [27]. One of the aims in the conventional microdissection mode is maximum preservation of the selected sample area [28]. High-precision instruments for laser microdissection, applied for analysis in the fields of proteomics, genomics, bioarrays and biochips, offer highly resolved observation optics in combination with the option of cutting out previously identified and defined areas of a sample using a highly focused laser beam guided precisely over the sample. Specimens are mounted on a special PET membrane (e.g., 1.4 μm thickness) slide or directly on a microscope (glass) slide and placed on the table of the LMD apparatus.

The new idea was now to use an LMD apparatus with a high spatial resolution as laser ablation system together with a sensitive ICP-MS for elemental and isotope analysis on tissue. An LMD system using inverted microscopes is well suited for insertion of a laser ablation chamber because of the open space on top of the microscope stage. Brain samples may be much larger than the field of view of a microscope, therefore an LMD system with a fixed laser beam (e.g., SmartCut Plus system from MMI Molecular Machines and Industries, Zurich, Switzerland) where the sample is moved with a motorized microscope stage is preferred to investigate large areas of interest. A flat laser ablation chamber has to be constructed and mounted directly onto the glass slide with the specimen and connected to ICP-MS. The microscope stage control software can easily be modified in such a manner that laser ablation can then be performed directly online together with a quantitative and time-correlated determination of the distribution of elements in sample areas by LMD-ICP-MS. Images can be obtained than via a defined laser ablation process of the analysed area (the "line scan modus" – ablation line by line). For further studies on metallomics, preferentially by LMD, a corresponding analogue area can be cut out and analysed. After tryptic digestion of the cut-out tissue, for example, biomolecular mass spectrometry is used to analyse the tissue in terms of the structural determination of metal-binding proteins and/or phosphoproteins.

Extending LMD to form a laser ablation system and coupling it to a sensitive ICP mass spectrometer was proposed by Becker and Salber [29]. The advantage of this experimental arrangement is that a high-resolution microscope of LMD with significantly better resolution and higher precision compared to those in commercial laser ablation (LA) systems allows us to observe and select the small specific brain structures which can be ablated by the focused laser beam in general with a spatial resolution down to the low micrometre scale and below.

2. Experimental

2.1. Insertion of a flat laser ablation chamber into the laser microdissection apparatus (LMD) and coupling to ICP-MS

Because of the pivotal device of this study an appropriate laser ablation chamber was constructed that fits the object holder on the sample stage of the LMD microscope. The photograph of the laser ablation chamber (schematic diagram, see on right side in Fig. 1) before and after the insertion into the SmartCut Plus LMD (MMI Molecular Machines and Industries, Zurich, Switzerland) is shown in Fig. 1 a and b, respectively. The rectangular laser ablation chamber was constructed of borosilicate glass (Duran®, Schott AG, Mainz, Germany) with the inner dimensions 50 mm × 20 mm × 4 mm. The individual 76 mm × 26 mm microscope glass slide (StarFrost, Braunschweig, Germany) containing the sample section served as bottom and was sealed using a silicon foil or vacuum grease.

The laser ablation chamber was coupled to a quadrupole-based ICP-MS with hexapole collision cell (XSeries2, Thermo Fisher Scientific, Bremen, Germany) using standard Tygon tubes. The basic experimental setup is illustrated in the figure of Appendix B.

In our experimental arrangement, a Nd:YAG solid-state laser of LMD for laser ablation of biological tissue was employed at a wavelength of 355 nm with a repetition rate of 5 kHz. This repetition rate is significantly higher compared to commercial laser ablation systems of 20 Hz. The 500 ps laser pulses and precise optics produce a beam spot size of 1 μm and below, providing exceptional cutting and/or laser ablation accuracy. The LMD control software permits microlocal analysis within predefined single points, straight lines, free-hand traced lines and in a raster modus using a set of parallels.

2.2. Measurement procedure of biological tissue

The sample selected for this preliminary experiment was a 30 μm thick native cryosection of a block of mouse brain

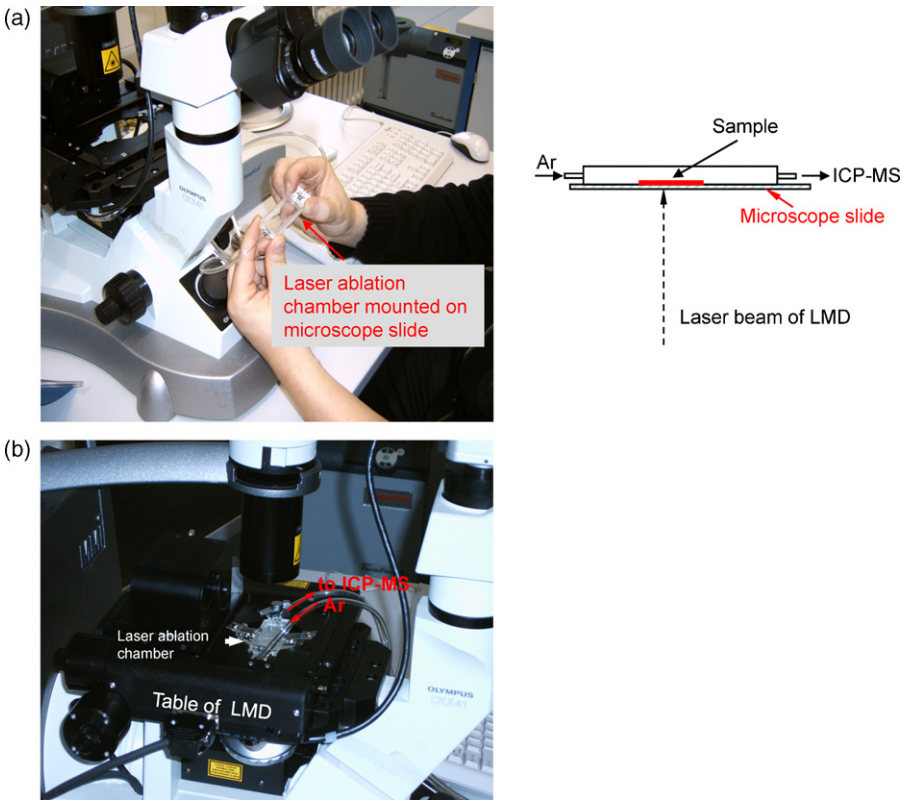

Fig. 1. (a) The laser ablation chamber was mounted on the glass slide bearing the sample and constituting the bottom (see schematic on the right side) will be inserted in the LMD apparatus (MMI SmartCut Plus, Molecular Machines and Industries, Zurich, Switzerland). (b) Photograph of laser ablation chamber the mounted on the table of LMD.

homogenates containing segments with 0, 4, 8, 12, 16, 20 $\mu g\,g^{-1}$ added Cu, Zn and Fe [21]. By preliminary LMD-ICP-MS experiments on the 20 $\mu g\,g^{-1}$ brain segment no remarkable ion signals for these 3 metals were observed. Therefore to increase microlocal concentration profiles, this section was impregnated by a droplet of 1000 ppm Cu solution (Merck, Darmstadt, Germany) of 6 mm diameter.

The specimen was visually examined using the existing high-resolution optics of the microscope in the LMD apparatus at a magnification of 400–1000. An area was selected for ablation by the focused laser beam of the LMD and trace metal analysis using ICP mass spectrometry. The solid-state Nd:YAG laser in the LMD apparatus was focused on the surface of the tissue section. Ablation was carried out in the line scan modus of the LMD. An ablation of tissue with a spatial resolution up to 1.5 μm was possible. The experimental parameters of laser ablation using LMD-ICP-MS compared to conventional LA-ICP-MS are summarized in Table 1.

2.3. Verification of results

Microphotographs of the sample after ablation were obtained using the digital monochrome camera integrated in the MMI SmartCut Plus and a standard stereomicroscope with transmitted light illumination. In order to estimate Cu concentrations throughout the droplet area the sample was imaged using LA-ICP-MS as established in author's laboratory while coupling the commercial NewWave UP 266 ablation system to the Thermo XSeries2 ICP-QMS.

3. Results and discussion

To test the experimental LMD-ICP-MS arrangement, material was ablated at different spatial resolutions (3–5 μm) from a 30-μm-thick cryosection of brain homogenate containing a dried copper droplet of 6 mm diameter (Fig. 2). In the centre of this droplet two line scans of 3 μm width are shown, on the bottom a

Table 1
Optimized experimental parameters of LMD-ICP-MS compared to conventional LA-ICP-MS.

ICP mass spectrometer	Thermo Fisher Scientific XSeries 2	
RF power	1500 W	
Carrier gas flow rate	1.2 L min^{-1}	
Mass resolution (m/Δm)	300	
Laser ablation system	MMI SmartCut Plus with inserted LA chamber	NewWave UP 266
Laser	Nd:YAG	Nd:YAG
Wavelength	355 nm	266 nm
Ablation mode	Line scanning and free-hand line	Line scanning and imaging (line by line)
Laser pulse duration	0.5 ns	20 ns
Repetition frequency	5 kHz	20 Hz
Laser pulse energy	1 μJ	120 mJ
Laser beam diameter	between 3 and 5 μm	160 μm
Scan speed	40 μm s^{-1}	50 μm s^{-1}

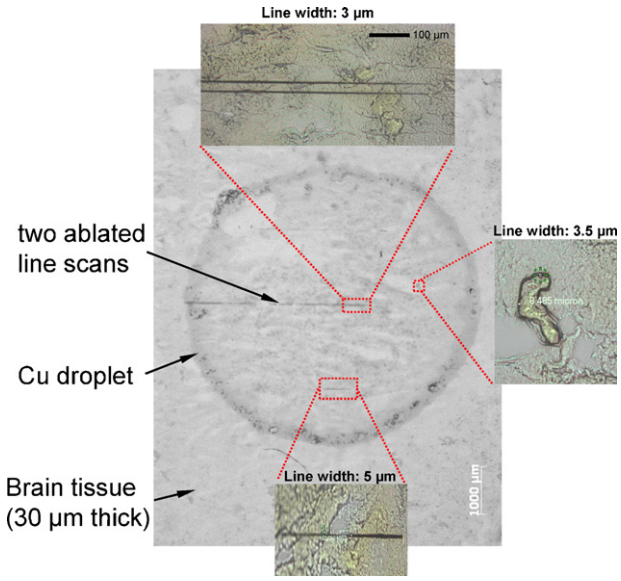

Fig. 2. Photograph of the 30 µm cryosection of brain homogenate impregnated with a Cu droplet (diameter – 6 mm) after LMD laser ablation. Two parallel lines of 3 µm width were ablated in the middle of droplet, a 5 µm wide line at the bottom and a 3.5 µm wide free-hand line on the right. In addition, these areas are shown at higher magnification (c.f. scale bars).

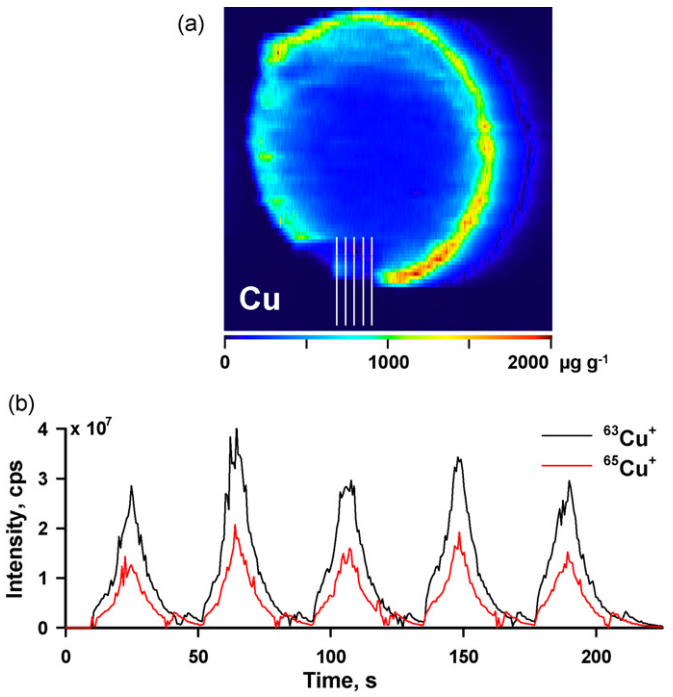

Fig. 3. Quantitative Cu image of the droplet (a) on a tissue section (thickness 30 µm) measured by conventional LA-ICP-MS with 160 µm spatial resolution. $^{63}Cu^+$ and $^{65}Cu^+$ ion intensities (b) for 5 line scans measured on the border of the Cu droplet (marked in a).

shorter line scan (5 µm width) and on the right a free-hand line of 3.5 µm width. Line widths were verified by light microscopy underlining the feasibility of spatial resolutions in the range of 3–5 µm. Due to the very low pulse energy of the LMD Nd:YAG laser of 1 µJ ablation across the thickness of the section was incomplete. We conclude that there is additional potential for increasing the ion intensities by replacement of a more powerful solid-state laser in the LMD system.

However, we demonstrated for the first time the laser-induced ablation of biological tissue using the new LMD-ICP-MS configuration with spatial resolution in the low micrometre range.

To characterize the Cu distribution within the droplet, this area was studied by the established bioimaging LA-ICP-MS technique in the BrainMet lab [1,11] at a spatial resolution of 160 µm (other experimental parameters are summarized in Table 1). The corresponding Cu image of a dried droplet of the tissue section is illustrated in Fig. 3. The highest enrichment on the drops edge (see also Fig. 2) was observed. The Cu concentration profile was estimated using the homogeneous brain lab standard segments present on the glass slide assessed by line-scan measurements. By LA-ICP-MS imaging of the droplet, an inhomogeneous distribution of analyte was found with lowest concentration in the middle of the droplet and the highest Cu concentration at the border. The shadow to the right of the maximum concentration in the droplet (in Fig. 3) indicates an artefact during laser ablation (from left to right) owing to memory effects. The quantitation of the measured data was performed using homogeneous brain lab standards on a glass slide [18]. In the middle of the droplet, the concentration varied between 500 and 600 µg g^{-1}, whereas the maximum Cu concentration at the border of the droplet was about 2000 µg g^{-1}. This concentration profile was further confirmed by measurement of five line scans on the border of the Cu droplet (marked in Fig. 3a and shown in Fig. 3b). By means of the line scan measurements, an average Cu concentration at the border of droplet was determined as about 2200 µg g^{-1}. Since the surface of Cu droplet is not homogeneously distributed on the tissue (visible using the microscope, see Figs. 2 and 3), the relative standard deviation (RSD) of the averaged concentrations is very high (~100%).

Fig. 4(a and b) illustrates transient signals of $^{63}Cu^+$ and $^{65}Cu^+$, measured using a quadrupole ICP mass spectrometer, while ablating tissue in the area of the Cu droplet via the focused laser beam of the LMD in the line scan modus with spatial resolutions of 3 µm (A), 5 µm (B) and 3.5 µm in the free-hand modus (C). Maximum ion intensities of 10^4 cps for $^{63}Cu^+$ were observed in each measurement. The ion intensity varied strong due to the inhomogeneous distribution of tissue and Cu. Low ion intensities in the line scan measurement in Fig. 4 B can be explained by holes in the tissue under the droplet (as visible in the microphotographs in Fig. 2). The $^{63}Cu/^{65}Cu$ isotope ratios measured by LMD-ICP-MS, see bottom (c) of Fig. 4A–C, were found to be close to the IUPAC table value of 2.24.

The analytical LMD-ICP-MS technique developed here enables for the first time laser ablation thin line scans of 3 µm and below (line scans with 1.5 µm spatial resolution were observed) in tissue sections and a simultaneous inspection of the sample via the high-precision microscope of the LMD. Straight parallel lines and curved lines were ablated on a structure of interest (Fig. 2). The distribution of Cu along the line scans was confirmed by LA-ICP-MS imaging in routine mode using the New Wave U266 ablation system. The limit of detection (LOD) of LMD-ICP-MS for Cu (for 3 µm line scan) of the actual setup was 190 µg g^{-1}. In comparison the LOD of conventional LA-ICP-MS on the Cu droplet was 4 µg g^{-1} at a spatial resolution of 160 µm.

One has to keep in mind that these LMD-ICP-MS results presented here were obtained using a special laser with low pulse energy (1 µJ) intended for the use in classical LMD experiments that must avoid laser mediated collateral damage of biomolecules such as RNA and DNA. Using a picosecond laser with a pulse energy >100 mJ in the LMD system will enable a complete ablation of the 30-µm-thick tissue section with improvement of spatial resolution and limit of detection for microanalytical investigations on biological samples. Applications include a continuous full-line raster imaging mode using a spot size as a compromise between spatial resolution and sensitivity and a discontinuous mode assessing

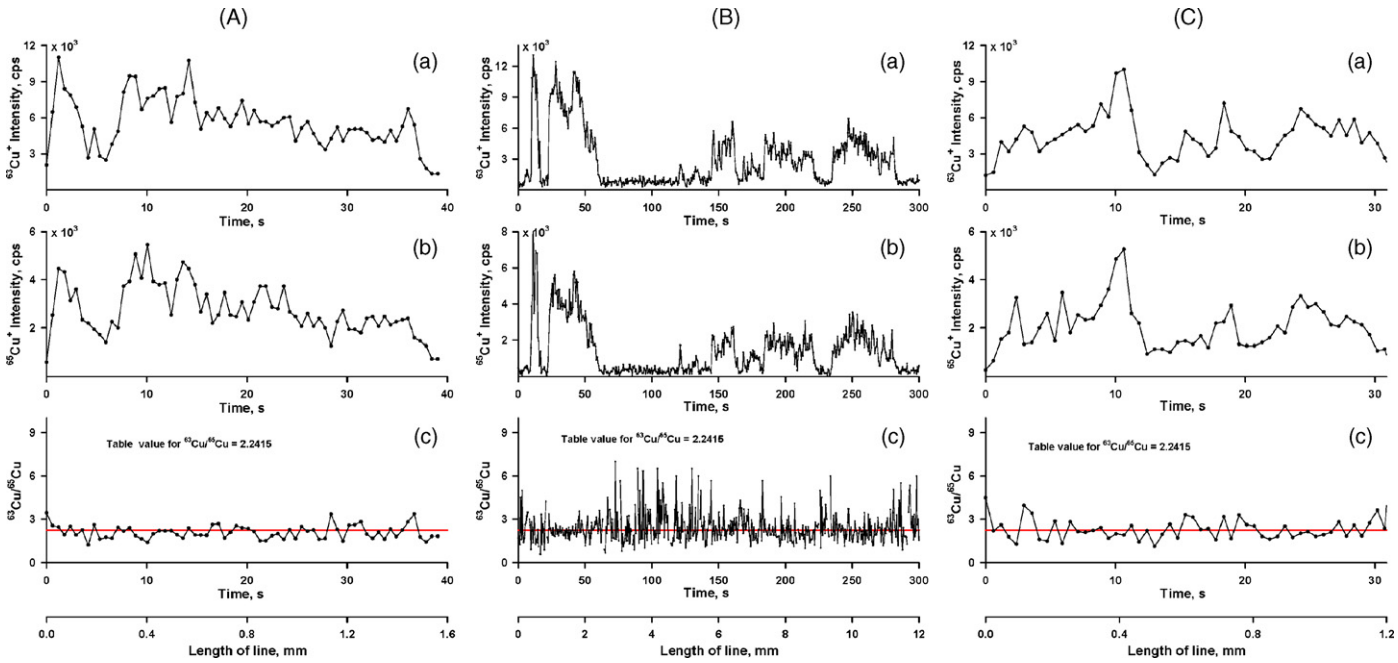

Fig. 4. Ion intensities of $^{63}Cu^+$ and $^{65}Cu^+$ and $^{63}Cu/^{65}Cu$ isotope ratio measured by LMD-ICP-MS in the line-scan modus with spatial resolution of (A) 5 μm, (B) 3 μm and in the free-hand modus with spatial resolution of (C) 3.5 μm.

objects of interest with high element concentrations at high resolution down to the ≈300 nm scale.

A further improvement of the spatial resolution by one order of magnitude up to the 30 nm scale will be possible by inserting a thin needle close to the sample surface in the laser ablation chamber and the application of the near-field effect in laser ablation of LMD apparatus as proposed in the German Patent application by Becker [30].

4. Conclusion

A novel analytical tool was designed for the bioimaging of metals in thin tissue sections at a spatial resolution at the low micrometre scale by a combination of a laser microdissection system with an inserted laser ablation chamber coupled to an ICP mass spectrometer. Biological tissue was ablated using the focused solid-state laser of the LMD. The LMD-ICP-MS technique with a more powerful laser will offer practicable quantification strategies for the determination of the chemical composition of the analysed sample with regard to metal (and non-metal) contents as developed for established LA-ICP-MS imaging in routine mode. The LMD-ICP-MS arrangement will open up new fields of bioimaging metals and non-metals in small tissular and cellular substructures with high spatial resolution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.03.013.

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