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Imaging of Cu, Zn, Pb and U in human brain tumor resections by laser ablation inductively coupled plasma mass spectrometry

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Abstract

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) was used to produce images of element distribution in 20 μm thin tissue sections of primary human brain tumors (glioblastoma multiforme—GBM) and adjacent non-neoplastic brain tissue. The sample surface was scanned (raster area ~1 cm^2) with a focused laser beam (wavelength 266 nm, diameter of laser crater 50 μm, and laser power density 1×10^9 W cm^{-2}). The laser ablation system was coupled to a double-focusing sector field ICP-SFMS. Ion intensities of $^{63}Cu^+$, $^{64}Zn^+$, $^{208}Pb^+$, and $^{238}U^+$ were measured by LA-ICP-MS within the tumor area and the surrounding region invaded by GBM as well as in control tissue. The quantitative determination of copper, zinc, lead and uranium distribution in brain tissues by LA-ICP-MS was performed using prepared matrix-matched laboratory standards doped with these elements of interest. The limits of detection (LODs) obtained for Cu and Zn were 0.34 and 0.14 μg g^{-1}, respectively, while LODs of 12.5 and 6.9 ng g^{-1} were determined for Pb and U.

The concentration and distribution of selected elements are compared between the control tissues and regions affected by GBM. A correlation was found between LA-ICP-MS and receptor-autoradiographic results. As receptor-autoradiographic techniques, a labeling for A_1AR and the pBR was employed.

Regarding the A_1AR, we used the specific A_1 adenosine receptor (A_1AR)–ligand, ^{3}H-CPFPX [^{3}H-cyclopentyl-3-(3-fluoropropyl)-1-propylxanthine], which has been shown to specifically label the invasive zone around GBMs. The peripheral benzodiazepine receptor was labeled with ^{3}H-Pk11195 [^{3}H-1-(2-chlorphenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline-carboxamide].

Keywords: Human brain; Glioblastoma multiforme; Imaging; Laser ablation inductively coupled plasma mass spectrometry; Receptor autoradiography

1. Introduction

Determination of element concentrations as well as their distribution in different kinds of biological tissues (e.g., brain, liver, plant section, etc.) is a challenging task in analytical chemistry and is of great importance in different areas of biological research [1–5]. The deficit of essential elements within tissues (e.g., Fe, Cu, Se, Zn, Mn, Mo, Co, Ni) results in diseases. Metals can also catalyze cytotoxic reactions leading to DNA modification (such as the Fenton reaction) or toxic processes at high concentrations that might be involved during tumor progression. Determination of other elements, such as Pb and U, could be of additional importance in biological and medical research as Pb and other divalent cations of heavy metals have been shown to impair calcium-channel proteins and affect neuronal axons and neurotransmitter-release. All these elements are involved in various biological processes, and their distribution and quantity has been analyzed in different kinds of tracer [4–6]. Thus, the element concentration in tissue is of interest, but also their precise spatial distribution (mapping or imaging) in organs, tissues or even single cells [2,5]. At present, a number of analytical techniques are available for imaging of elements in biological tissues [7–10]. Some of these techniques offer excellent spatial resolution but poor detection limits and vice versa. However, for imaging studies of biological tissue a powerful analytical technique with both good spatial resolution and high sensitivity is necessary. Commonly used methods in biological and medical research for the visualization of element distribution in tissues

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are histochemical staining techniques [11], which however do not provide multielement capability. Other surface analytical techniques, such as scanning electron microscopy with energy dispersive X-ray analysis (SEM-EDX) [7], proton-induced X-ray emission (PIXE) [8] or autoradiography [12] are generally not sensitive enough for trace analysis. Using secondary ion mass spectrometry (SIMS), it is possible to produce ion images of the distribution of chemical elements in tissue with a spatial resolution in the low μm and sub-μm range, but quantification is difficult due to inherent matrix effects of up to six orders of magnitude [13–15].

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has been established in many analytical laboratories worldwide as a powerful surface and bulk analytical technique for the determination of element concentrations as well as their spatial distribution in the analyzed sample. LA-ICP-MS possesses high sensitivity, fewer matrix effects (compared to SIMS), requires no or only simple sample preparation and allows easy quantification procedures [16–18]. If no suitable standard reference material is available, several strategies have been developed for quantification purposes in LA-ICP-MS including a preparation of matrix-matched laboratory standards [19,20] or the use of solution-based calibration [21,22]. Due to these advantages, LA-ICP-MS is mostly used as a trace analytical technique with multielement capability for the bulk analysis of solid inorganic matrices and for isotope ratio measurements [16–18,23]. LA-ICP-MS has been applied to an increasing extent for element distribution analysis (including analysis of trace elements) in biological samples and medical tissues. Recently, we applied LA-ICP-MS for imaging of elements (such as P, S, Cu, Zn, Th and U) to microtome sections of rat [24] and human brains (hippocampus) [20]. The mass spectrometric analysis yielded an inhomogeneous, site-specific distribution for P, S, Cu, and Zn in 20 μm thin brain sections of the human brain's hippocampus suggesting a physiological role of these elements. In contrast, Th and U are more homogeneously distributed at a low-ng g^{-1} concentration level. Furthermore, LA-ICP-MS was extensively used for the determination of elements concentrations in selected protein spots derived from 2D gel electrophoresis [4,25,26]. Hutchinson et al. [27] used LA-ICP-MS for qualitative imaging of β-amyloid protein in brains of a transgenic mouse model of Alzheimer's disease. A correlation between the Aβ deposits and the concentration of trace elements was found.

The aim of the present study was to develop an analytical technique for quantitative imaging of element distribution (such as Cu, Zn, Pb and U) in histological sections of glioblastoma multiforme, the most common primary human brain tumor. For quantification purposes, prepared matrix-matched laboratory standards were applied. The results of the measurements were compared with the tumor area and the invasive zone determined by receptor-autoradiography for ^{3}H-Pk11195 and ^{3}H-CPFPX in adjacent slices.

2. Experimental

2.1. Instrumentation

A double focusing sector field ICP-MS (ICP-SFMS, ELEMENT, Thermo Electron Corporation, Bremen, Germany) coupled to a laser ablation system CETAC LSX 200 (Cetac Technologies Inc., Omaha, NE, USA) was used to produce images of Cu, Zn, Pb and U distributions in thin cross-sections of human glioblastoma tumors (thickness of slices—20 μm). Laser ablation of biological tissue was performed using a Nd:YAG laser (wavelength, 266 nm; repetition frequency, 20 Hz; spot diameter, 50 μm; laser power density, 1×10^{9} W cm^{-2}). Schematic

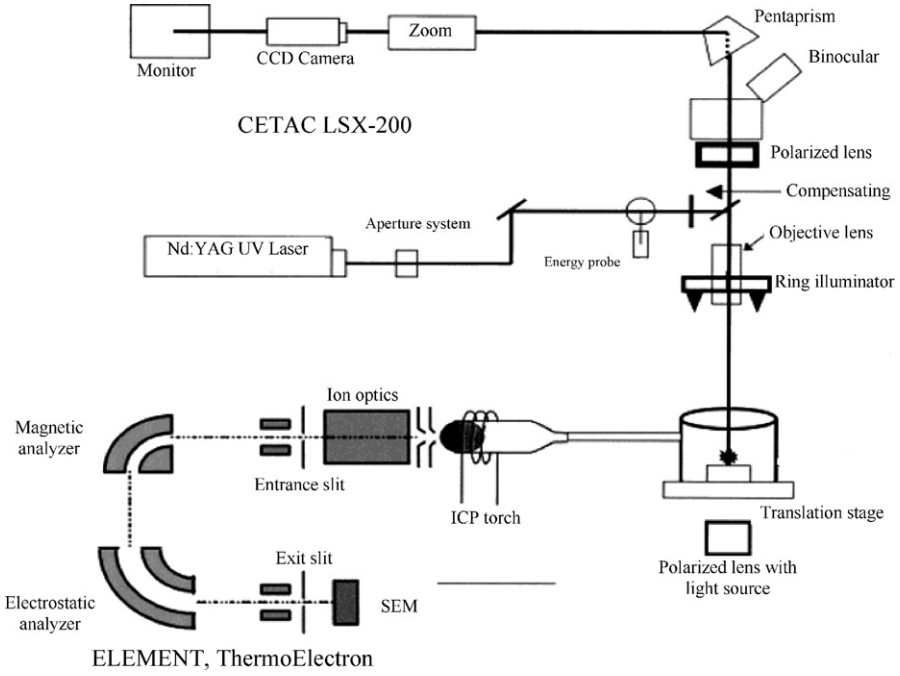

Fig. 1. Schematic arrangement of LA-ICP-MS for imaging element distribution in thin cross-section of human brain samples.

arrangement of the instrumentation used is presented in Fig. 1. The ablated material was transported by argon as a carrier gas into the inductively coupled plasma (ICP). The ions formed in the ICP were extracted in the sector field mass spectrometer and separated according to their mass-to-charge ratios. The ICP torch was shielded with a grounded platinum electrode (Guard-Electrode, Thermo Electron Corporation). All measurements were performed in the low mass resolution mode of ICP-SFMS. Further details about the instrumentation used can be found elsewhere [21,23,28].

2.2. Samples and sample preparation

Twenty micrometer thick frozen serial sections were prepared from three different human glioblastoma specimens (designated GBM-I, GBM-II and GBM-III) in a Leica 3010 cryostat microtome at −20 °C.

Results presented here are from representative measurements of glioblastoma multiforme grade III (GBM-III).

The size of the samples was approximately 1 cm^2. Following sectioning, the tissue was mounted to sodium-free glass slides, air-dried and stored. The first section containing the GBM tumor was used for adenosine A_1 receptor autoradiography (labeling of the receptors by ^{3}H-CPFPX), the following section was used for cell body staining with Cresyl-violet, the third section was used for receptor autoradiography of the peripheral benzodiazepine binding site (labeling of the receptors by ^{3}H-PK 11195), the fourth section was investigated by LA-ICP-MS and the last section in a group of serial sections was subjected to ^{3}H-CPFPX autoradiography.

Finally, following LA-ICP-MS analysis, the remaining section was stained with Prussian blue to detect hemorrhagic deposits.

2.3. Preparation of synthetic laboratory standards for LA-ICP-MS measurements of brain samples

Matrix-matched laboratory standards with well-defined concentrations of the elements of interest were prepared for the calibration of analytical data. For this purpose, three slices of the same brain tissue (each ∼0.65 g) were spiked with 100 μl of standard solutions containing known concentrations of selected elements (Cu, Zn, Pb and U). The final concentrations in the brain tissue after spiking were 10, 5, and 1 μg g^{-1} for Cu and Zn and 0.1, 0.05, and 0.01 μg g^{-1} for Pb and U. The fourth brain slice was spiked with 100 μl of 2% HNO_3 and was used for blank correction. All brain samples were carefully homogenized and centrifuged for 5 min. After that, samples were frozen at a temperature of −50 °C. Frozen matrix-matched synthetic laboratory standards of human brain tissues from the hippocampus were cut into sections 20 μm in thickness and placed onto the glass substrate.

2.4. Measurement procedure

The experimental parameters of LA-ICP-MS were optimized with respect to the maximum ion intensity of $^{63}Cu^+$ using the laboratory standard with a concentration of copper of 5 μg g^{-1}. Maximum ion intensity was observed at a carrier gas flow rate of 1.1 l min^{-1} for the transport of ablated material to the ICP-MS. Thin brain tissue sections were investigated by LA-ICP-MS with respect to the element distribution of Cu, Zn, Pb and U. The optimized experimental parameters for the analytical method are summarized in Table 1.

Table 1
Optimized operating conditions of developed LA-ICP-MS procedure for determination of lateral distribution of Cu, Zn, Pb and U in thin cross-section of human brain tissues

Laser ablation system	LA-ICP-SFMS, CETAC LSX-200
Wavelength of Nd–YAG laser (nm)	266
Laser power density (W cm^{-2})	1×10^9
Laser scan speed (μm s^{-1})	30
Number of lines per analyzed sample	100–120
Repetition frequency (Hz)	20
Laser beam diameter (μm)	50
Inductively coupled plasma mass spectrometer	Element (Finnigan)
RF power (W)	1200
Cooling gas flow rate (l min^{-1})	18
Auxiliary gas flow rate (l min^{-1})	0.65
Nebulizer gas flow rate (l min^{-1})	1.1
Extraction lens potential (V)	2000
Sampler cone	Nickel, 1.1 mm orifice diameter
Skimmer cone	Nickel, 0.9 mm orifice diameter
Mass resolution ($m/\Delta m$)	300
Mass window (%)	10
Runs	12000–14000
Passes	1
Scanning mode	Peak hopping
Analysis time (h)	5–6

To obtain two-dimensional images of element distribution, the region of interest was systematically screened (line by line). The spot size of laser craters was 50 μm. In total, 100 lines were scanned through the analyzed cross-section. ICP-MS data files for each analyzed brain section were converted into txt file and were used to produce 2D images of element distribution. The images were plotted using programming script in MATLAB® 6.5 computing software. Further details about the measurements procedure used are described elsewhere [20,23,24].

2.5. Autoradiographic procedures

The sections were exposed to a ligand for the peripheral benzodiazepine–receptor, ^{3}H-PK11195 [^{3}H-(1-(2-chlorphenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline-carboxamide)], at 100 pM – a concentration that equals approximately the dissociation-constant (K_D). This receptor is known to be upregulated in gliomas [29]. Non-specific binding was determined in control sections in which the tritiated ligand competed with an excess of the unlabeled ligand PK11195.

Other sections were studied autoradiographically with ^{3}H-CPFPX [^{3}H-8-cyclopentyl-3-(-3-fluoropropyl)-1-propylxanthine], a specific marker for the adenosine A_1 receptor at 4 nM (K_D = 4.4 nM). Non-specific binding was determined with an excess of cold R-PIA (R-PIA (R (−)-*N*-(2-phenylisopropyl)-

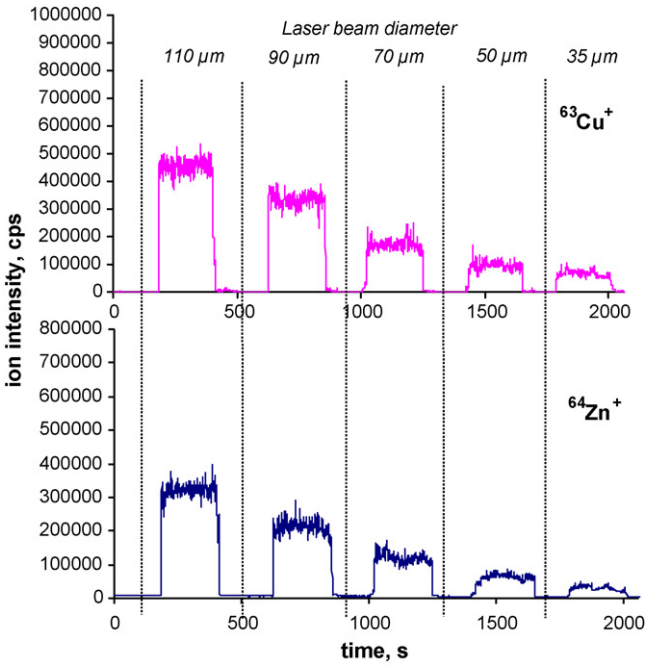

Fig. 2. Dependences of ICP-MS signal intensities of (a) $^{63}Cu^+$ and (b) $^{64}Zn^+$ on the laser beam diameter studied using synthetically prepared homogeneous brain laboratory standard doped with 10 μg g^{-1} of Cu and Zn.

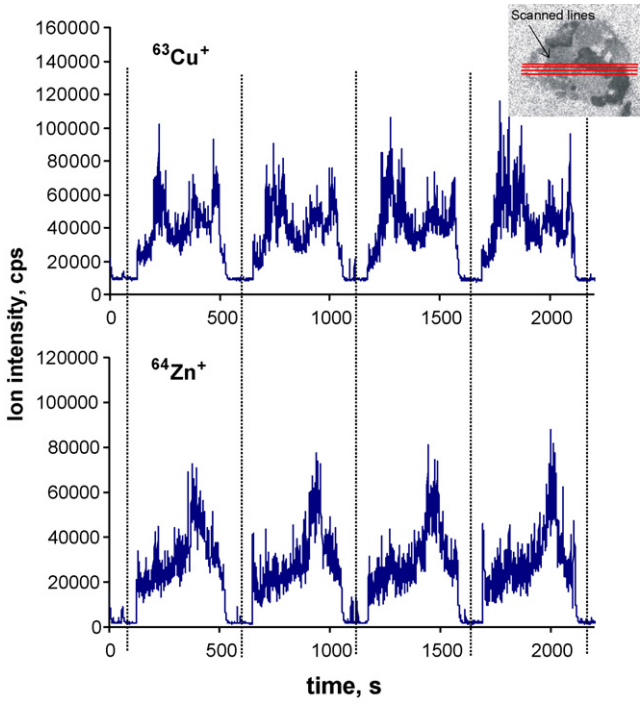

Fig. 3. Ion intensities of $^{63}Cu^+$ and $^{64}Zn^+$ in four line scans of sections through a human GBM (sample GBM-III) and control brain tissue. The red lines in the inset indicate the position in the scanned tissue from which copper and zinc distributions were obtained.

adenosine), a synthetic and specific A_1AR-agonist. ^{3}H-CPFPX and ^{18}F-CPFPX have been reported as to be a marker of invasive zones around glioblastomas *in vivo* in the rat and in GBM patients, respectively [11].

Quantification of analytical data was done by employing a BAS reader and the AIDA software (Raytest, Freiburg, Germany). Plates were scaled to an appropriate ^{3}H-tissue standard (Amersham, Freiburg, Germany).

3. Results and discussion

3.1. LA-ICP-MS procedure

Variation of the laser beam diameter has a direct influence on the amount of ablated material. Since the energy density of the laser remains constant during ablation, it can be expected that the laser crater size is directly related to the measured ion intensity. On the other hand, using a smaller laser beam diameter (resulting in a smaller laser crater) better spatial resolution was achieved. However, under such conditions, a relatively small amount of material is transported to the ICP, which will result in a deterioration of the detection limits of elements. In addition, the smaller laser beam diameter would lead to smaller step sizes (distance between the scanned lines) of the method and, therefore, longer analysis time. To find the optimal laser spot size for the present work, the dependences of ICP-MS signal intensities on the laser beam diameter were studied using homogeneous synthetically prepared brain standard doped with 10 μg g^{-1} of Cu and Zn (see Fig. 2). With increasing of ablated areas an improvement in signal intensities (the square low) was measured. For $^{63}Cu^+$, ion intensities of 1.9×10^5 and 4.6×10^5 cps, and for $^{64}Zn^+$, ion intensities of 1.3×10^5 and 3.2×10^5 cps were observed at a diameter of the laser beam of 70 and 110 μm, respectively. It was also found that an optimum laser beam diameter of 50 μm would provide adequate spatial resolution as well as sufficient sensitivity for all measured elements in the present study. Under such experimental conditions, the limits of detection (3δ of the blank ion intensity divided by sensitivity of the analyte) were calculated to be 0.34 and 0.14 μg g^{-1} for Cu and Zn, respectively. The precisions (R.S.D.—relative standard deviation) for the selected single line scans were in the range of 20–30%.

3.2. Single line scans measurements on brain tissue sections

Glioblastoma as well as control human brain tissue sections were scanned by the LA-ICP-MS procedure using the single line mode. In Fig. 3 the $^{63}Cu^+$ and $^{64}Zn^+$ intensities are shown as a function of time corresponding to four single line scans through the GBM and control brain regions. In contrast to Fig. 2, where the ablation of homogenized lab standard is performed, the changes in ion intensities demonstrate an inhomogeneous distribution for both elements in the line scans (marked in red). In both figures, the time scale correlates with a defined distance (∼1 cm) on the investigated tissue sample scanned slowly by a focused laser beam. The element distributions determined in the brain tissue within the neighboring scans show, in general, similar profiles for each of the scanned elements. Fig. 3 also shows that Cu and Zn are not equally distributed in the scanned region. For instance, in line 4 (time about 1800 s) a relatively

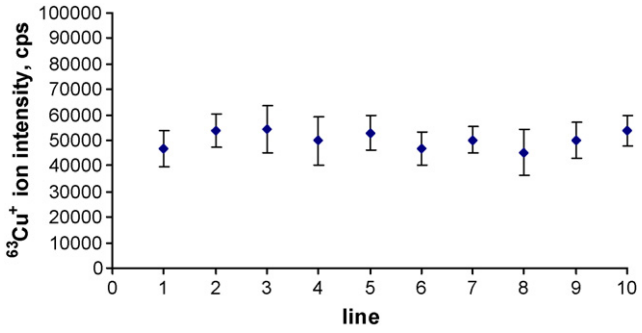

Fig. 4. $^{63}Cu^+$ ion intensities for 10 line scans (length: each 300 μm) measured by the LA-ICP-MS procedure developed at different locations of synthetically prepared laboratory brain standard containing 5 μg g^{-1} Cu.

high ion intensity of the $^{63}Cu^+$ (<100,000 cps) was observed in comparison to the average value of $^{63}Cu^+$ within the scanned line (41,548 cps), while in the same region (time about 1800 s) the average intensity for the $^{64}Zn^+$ (27,760 cps) was found.

To study the reproducibility of the method, 10 line scans (each 300 μm) were performed at different locations of synthetically prepared laboratory brain standard containing 5 μg g^{-1} Cu. Measured ion intensities of $^{63}Cu^+$ averaged for each of the lines are presented in Fig. 4. The R.S.D. of the measurements was determined to be 6.7%, which proves adequate reproducibility of the method as well as homogeneity of prepared matrix-matched laboratory standards.

3.3. Two-dimensional mapping of brain tissue sections

To define the area of the tumor (indicated by white arrows), we performed autoradiography with the specific pBR receptor ligand [^{3}H]Pk11195. In addition, A_1AR autoradiography was used to define the tumor invasion zone (indicated by red arrows) [6]. Both pBR and A_1AR autoradiographs of GBM-III are shown in Fig. 5a and b, respectively. The sections containing the glioblastoma as well as control brain sections were systematically screened (line by line) with LA-ICP-MS. The measured ion intensities were used to produce two-dimensional images of the element distributions in the analyzed area. In Fig. 5c and d the distributions of Cu and Zn measured on adjacent slices of human brain sample GBM-III are presented. The intensity distributions of Cu and Zn show a similar localization, with maximum ion intensities in the same region (marked by a dotted line), which histologically corresponded to areas of intratumoral hemorrhage. The tumor invasion zone with high A_1AR as well as the cellular tumor mass region with high pBR, can be clearly detected by the LA-ICP-MS measurements. Both elements are

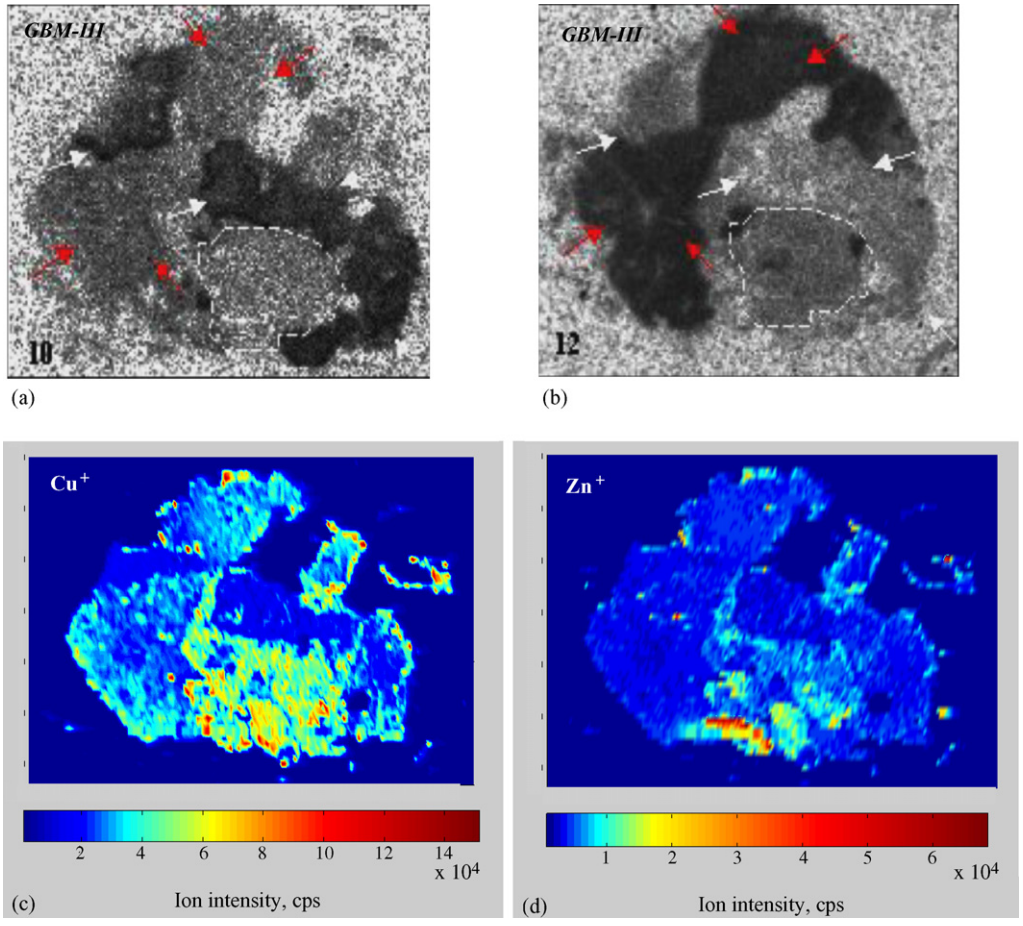

Fig. 5. Two-dimensional distribution of (a) peripheral benzodiazepine receptor (pBR) and (b) A_1 adenosine receptor ligand (A_1A) as well as LA-ICP-MS imaging of (c) Cu and (d) Zn in the glioblastoma sample GBM-III.

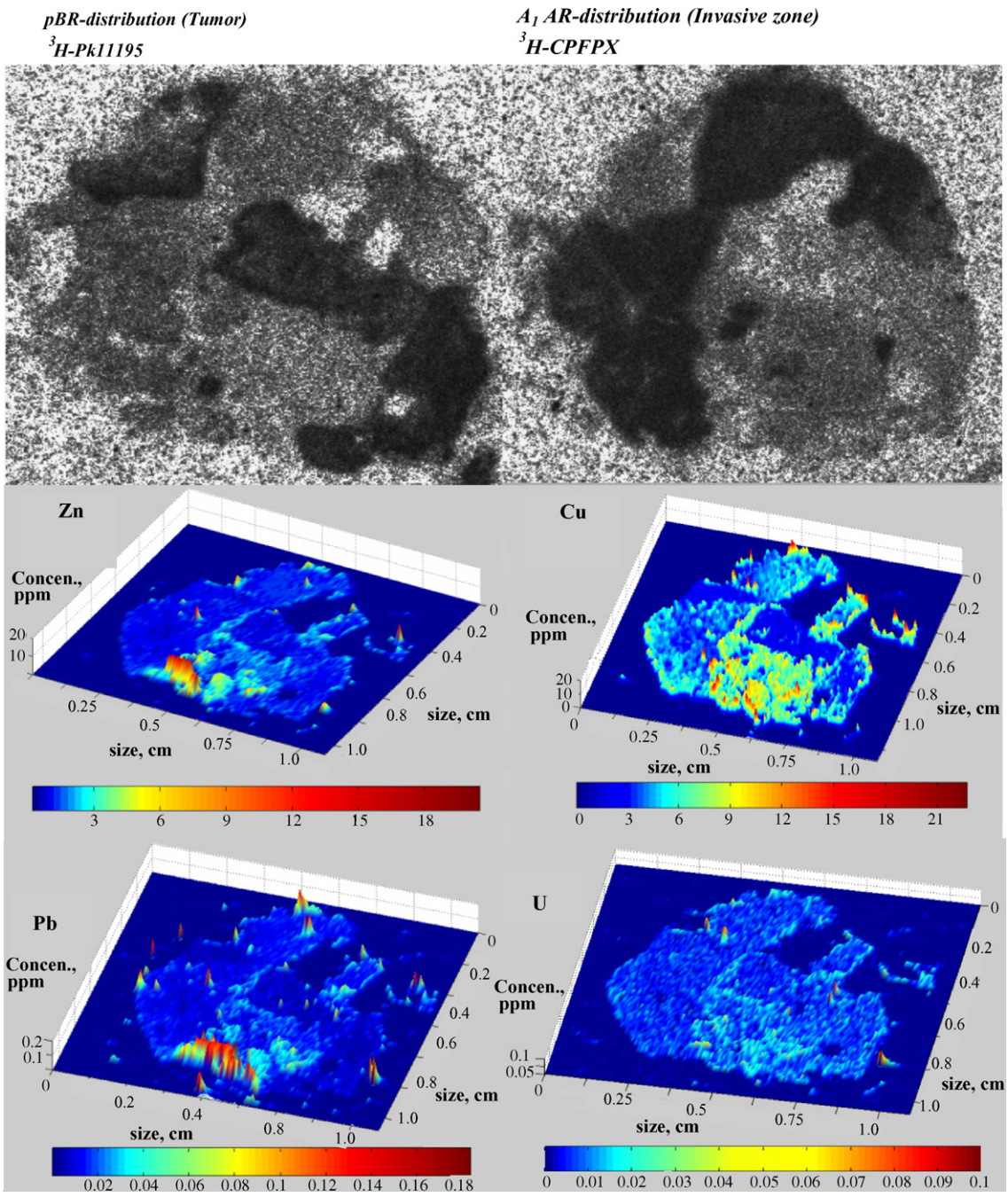

Fig. 6. Lateral distributions of Cu, Zn Pb and U in thin sections of the glioblastoma human brain sample GBM-III measured by developed LA-ICP-MS procedure.

completely lacking within the tumor. Zn is missing in the reactive zone, while little amounts of Cu are found in these areas.

3.4. Quantification of analytical data measured by LA-ICP-MS

Distribution profiles of Cu, Zn, Pb and U in thin sections of human brain tissues measured by LA-ICP-MS were quantified using synthetically prepared matrix matched laboratory standards doped with defined amounts of the elements of interest (1, 5 and 10 $\mu g\,g^{-1}$ of Cu and Zn; 0.01, 0.05 and 0.1 $\mu g\,g^{-1}$ of Pb and U). The correlation coefficients determined for calibration curves were depended on the element analyzed and were in the range of $R^2 = 0.9821$–0.9962.

In Fig. 6 quantitative distributions of Cu, Zn and U in the analyzed sample GBM-III, are presented. In addition to Cu, Zn and U, the Pb concentration was monitored. The results for both analyzed samples show a good correlation with respect to the results determined by receptor autoradiographic methods as well as to the profile of scanned tissues for all elements measured. For sample GBM-III the concentrations for Cu and Zn were determined to be about 10–15 $\mu g\,g^{-1}$, whereas Pb and U were

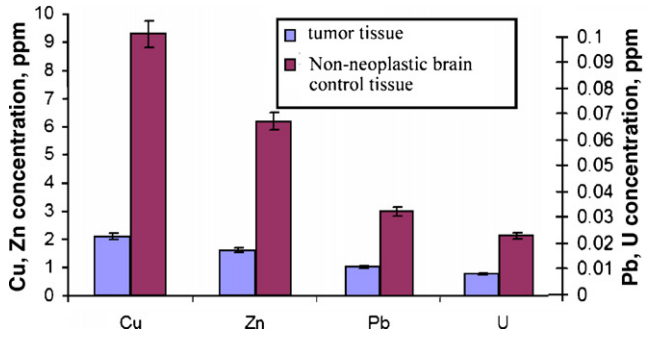

Fig. 7. Comparative measurements of Cu, Zn, Pb and U concentration in sections through the human brain tumor sample GBM-III and the control brain.

in the range of 0.05–0.1 $\mu g\,g^{-1}$, whereas in other glioblastoma multiforme resections, the concentrations for all elements were up to one order of magnitude lower, although the intensity of standards were similar in both measurements.

By comparison with the autoradiographic data, the tumor mass regions are more clearly detected by LA-ICP-MS measurements in sample GBM-III than in other samples measured. It was found by LA-ICP-MS, that the concentrations of all measured elements were lower in glioblastoma multiforme compared to the control brain, whereby the differences in cell density were taken into account. Fig. 7 compares the averages of element concentrations measured in the tumor and control brain regions of the sections of human brain sample GBM-III. For the Cu, Zn, Pb and U concentrations in the glioblastoma and adjacent brain tissues varied by factor 4.4, 3.8, 2.9, 2.7, respectively.

Future systematic studies on larger numbers of human tumor tissues will focus on fundamental processes such as the influence of essential and toxic elements on the development of tumors. A significant improvement of the spatial resolution (less than 1 μm) in LA-ICP-MS for quantitative micro local analysis in biological tissues will be possible by application of near field effect in LA-ICP-MS [30].

4. Conclusions

LA-ICP-MS represents a powerful analytical tool permitting quantitative two-dimensional screening (mapping) of Cu, Zn, Pb and U in thin sections of biological tissues. Using this procedure section of human brain tissue and tumors (thickness 20 μm) were analyzed. LA-ICP-MS measurements on synthetically prepared brain tissue standards proved the adequate reproducibility of the method, which, generally, exhibits relatively good precision and accuracy. The detection limits obtained for Cu and Zn were in the sub-$\mu g\,g^{-1}$ range, while LODs in the low-ng g^{-1} concentration level were determined for Pb and U.

The LA-ICP-MS screening procedure was successfully applied to produce 2D images of element distributions in 20 μm thin sections of human brain tissues and glioblastoma tumors. The images of the spatial distribution of Cu, Zn, Pb and U were compared with the results of other techniques, i.e., conventional histology and receptor autoradiographic methods, which allow the precise localization of the elements in the tumor and the tumor invasion zone. Tumor mass region and tumor invasion zones, respectively, are clearly detected by LA-ICP-MS measurements. These results provide novel information on the distribution of elements in human brain tumors, and may also be an important tool for analyzing brain tumor metabolism and pathogenesis.

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