[Frontiers in Bioscience, 5, a10-17, May 1, 2000]

UV-OZONE ASHING OF CELLS AND TISSUES FOR SPATIALLY RESOLVED TRACE ELEMENT ANALYSIS

B. Gilbert [1], L. Perfetti [1], R. Hansen [2], D. Mercanti [3], M. T. Ciotti [3], P. Casalbore [3], R. Andres [4], P. Perfetti [5], G. Margaritondo [1] and Gelsomina De Stasio [5,6]

[1] Institut de Physique Appliquée, Ecole Polytechnique Fédérale, PH-Ecublens, CH-1015 Lausanne, Switzerland, [2] Synchrotron Radiation Center, University of Wisconsin - Madison, Stoughton WI 53589, USA, [3] Istituto di Neurobiologia del Consiglio Nazionale delle Ricerche, Viale Marx 15, 00137 Roma, Italy, [4] Paul Scherrer Institut, CH-5232 Villigen PSI, Switzerland, [5] Istituto di Struttura della Materia del CNR, Via Fosso del Cavaliere, Roma, Italy, [6] University of Wisconsin - Madison, Department of Physics, Madison WI 53706, USA

TABLE OF CONTENTS

1. ABSTRACT

UV/ozone ashing of thin tissue sections and cell cultures is a simple technique to enhance relative elemental concentrations, while maintaining their spatial location at the sub-micron level. This approach may enhance the capability of spatially resolved analysis techniques to detect the distribution of trace elements in biological matrices. We present results from light microscopy and x-ray spectromicroscopy studies of tissues and cells demonstrating that the micro-structure is very well conserved. We show the signal enhancement resulting from the removal of carbon, which allows otherwise undetectable gadolinium to be mapped in cancer tissue for a novel neutron capture therapy.

2. INTRODUCTION

Many areas of research with the MEPHISTO x-ray photoelectron microscope are concerned with the detection of trace elements in biological samples, and subsequent investigations of their microlocalization (1-3). Electrostatic electron optics in MEPHISTO image the low energy electrons emitted by the specimen surface under soft x-ray illumination to acquire photoelectron micrographs and total electron yield x-ray absorption spectra (XAS) on a microscopic scale. XAS give elemental specificity through the energy position of core levels, and additional chemical state information through the lineshape of the spectra acquired by recording intensity variations as a function of photon energy.

Two important constraints apply to the samples analyzed in MEPHISTO. Firstly, samples must be sufficiently thin or conductive to prevent the formation of surface charge as electrons are removed from the sample. Sections of tissue embedded in epoxy, for example, must be below 500 nm to avoid detrimental charging effects. Secondly, the analyte element must be present with a concentration above the detection limit of the technique, measured for a few specific elements (phosphorus and chromium, both at the L-edge) to be on the order of 100 ppm. Despite these constraints, successful experiments have studied unashed cells and tissues (4), although many such samples cannot be imaged. The range of samples that may be analyzed by spectromicroscopy has been considerably expanded, however, by using the technique UV/ozone ashing to remove organic carbon from biological specimens. Removal of carbon (and other volatile species) has the effects of thinning the sample (tissue sections thicker than 5 μm can be imaged without charging, after ashing) and of greatly enhancing the relative concentration of all remaining elements.

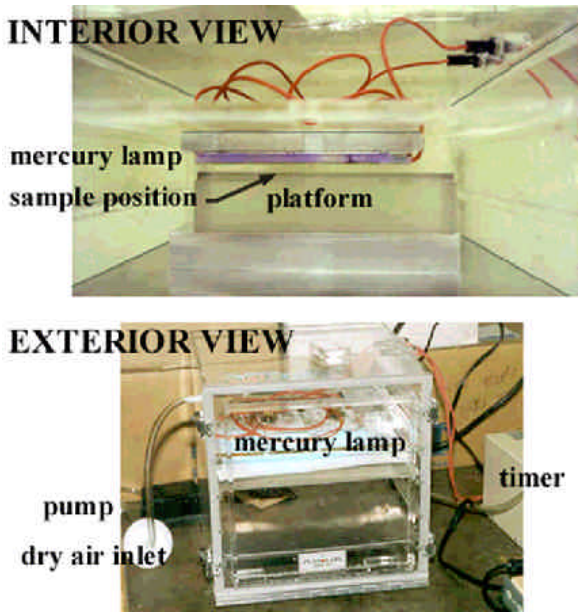

Figure 1. Interior (top) and exterior views of the UV/ozone oven. In the top image the surface being cleaned is the reflective gold surface of a synchrotron x-ray optical component.

We demonstrate here that we do not compromise the lateral resolution of the technique, as no detectable displacement of material takes place during this slow procedure (incineration times greater than 100 hours are common), performed at atmospheric pressure and room temperature (5). A related technique, cold oxygen plasma ashing, was previously used by our and other groups (6-9), which rapidly removed carbon from organic materials, but seemed to introduce an undesirable dislocation of material in some samples. The UV/ozone approach is preferable because, unlike the oxygen plasma environment there are no high energy ions that could displace material by sputtering. Furthermore, the UV/ozone oven is simpler and cheaper to build and operate than a plasma oven. An additional advantage for spectromicroscopy is a consequence of the relative surface sensitivity of the total electron yield technique. The probed depth is limited to approximately 50-100 Å by the escape depth of low energy electrons in materials. In the case of cells, the membrane thickness is approximately 40 Å, and hence there is not a great penetration. Ashing removes this, mostly carbon phospholipid and protein membrane, and enables us to probe well inside the internal regions of a cell. Ashing has clear advantages as a specimen preparation technique for some specific problems in spectromicroscopy, and could be potentially used with any other technique (e.g. Secondary Ion Mass Spectrometry) that seeks to map the distribution of trace elements on an organic matrix.

Finally, UV/ozone ashing may have applications as a precursor step in the digestion of tissues for bulk trace element detection. Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) or Mass Spectrometry (ICP-MS) are examples of sensitive and accurate methods of quantitative elemental analysis, but their use with biological materials first requires tissue digestion and solubilization, typically in nitric acid, accompanied by sonication. The robust organic cell architecture may not be completely dissembled by this process, leaving gross tissue particles that may interfere with accurate analysis. When tissues have first been embedded in a medium, such as paraffin for dissection, tissue digestion is even less effective. Prior sample ashing removes such potential problems, leaving highly soluble inorganic material that we show can substantially improve subsequent quantitative measurements.

3. MATERIALS AND METHODS

3.1. UV/Ozone Oven

The original function of the oven was the cleaning of carbon contamination layers from x-ray optical components (10). Interior and exterior views of the oven are given in Figure 1. It was constructed from components by mounting a low pressure mercury lamp (Jelight Company, Ca., USA) into an acrylic dessicator (Plaslabs, Mi., USA) with an air-tight seal around the door to prevent ozone escaping while the lamp is on. A variable-height platform beneath the lamp can accommodate samples of any dimension. Ambient air is pumped with a 1.2 liter per minute aquarium pump to purge carbon dioxide from the oven and replenish oxygen. Exhaust gas passes through an activated charcoal filter to remove unreacted ozone.

Air that enters the oven passes through two Drierite (W. A. Hammond Drierite Company, Oh., USA) dessicant columns to ensure that samples are ashed in a completely dry environment. Some of the inorganic products of the ashing process are in fact highly hygroscopic salts, and the most serious threat of material dislocation comes from the possibility of water uptake from the air. A modified plastic glove bag (not shown in Figure 1) surrounds the oven door and is purged with dry nitrogen before opening the oven, and ashed samples are stored and transported in miniature dessicators. The low-pressure mercury UV lamp emits two wavelengths of light that are relevant to the ashing procedure (11,12). The light at 1850 Å dissociates molecular oxygen to generate oxygen radicals and ozone that strongly oxidize the sample surface. The emission at 2537 Å photosensitizes carbon at the sample surface, enhancing the reactivity, but also stimulates the photo-dissociation of ozone. The competing effects of the emission bands for ozone creation and destruction, and their different penetration through air result in a strong ozone concentration gradient close to the lamp. Hence the rate of sample ashing varies rapidly with the distance between lamp and sample surface (10). Typical ashing times of tissue sections that we employed ranged between 6 hours (50 nm sections) to 4 days (10 µm sections) at a distance of less than 5 mm from the lamp.

3.2. Total Organic Carbon (TOC) Analysis

Human meningioma tumor tissue was fixed, embedded in paraffin, and four 2.5 µm thick sections were mounted on glass. The paraffin was removed by washing in

xylene, followed by serial immersions in 100%, 99%, 95% and 70% ethanol in water baths and a 100% water bath to rehydrate the tissue and remove the organic solvents. The sections were imaged on a commercial scanner to measure the area of the 2.5 m sections, and hence their volume. One section remained unashed, the others were ashed for 2, 4 and 6 days in the UV/ozone oven They were subsequently digested in 1 ml of 1N analytical grade HCl (aq) with sonication and the volume made up to 10 ml (the minimum sample volume) with ultrapure deionized water. The analysis was made in the Shimadzu TOC-5000 Total Organic Carbon apparatus with independent standards made just prior to analysis and covering the range 5 - 200 mg carbon l^{-1}.

3.3. Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

ICP-AES is a sensitive technique for quantatitive elemental analysis of aqueous solutions that can reach ppb detection levels for some elements (13). The analyte concentration is linearly proportional to the intensity of a specific atomic emission line of the element vaporized in a plasma at 8000 C, and is determined by comparison with concentration standards measured at the same time. The analysis of tissue or cells first requires that the biological material be digested to solubilize the analyte (14,15). This was achieved by sonicating the material in 1N nitric acid (Merck "Suprapur") for 10 minutes, then diluting to the final volume (1.5 ml) and acid concentration (30% w/v). Concentration standards are made at the same acid concentration since solution viscocity can affect the flow rate of liquid through the nebulizer and hence emission intensity measurements of the apparatus. Blank readings are made regularly to check for external sources of the element analyzed, and the emission is recorded from a single concentration standard periodically throughout the measurement to track changes in machine response. The apparatus used was the Perkin Elmer 1000 Spectrometer. The emission wavelengths selected were 3422.4 Å for Gd detection and 3934.7 Å for Ca detection. For Gd detection in cell cultures, the liquid sample volume was 1.5 ml and 9 replicates (repeat emission intensity measurements) were taken and averaged. For Gd detection in individual 2.5 µm tissue sections, the liquid sample volume was 0.5 ml and 5 replicates were averaged. In both cases the maximum photomultiplier tube (PMT) voltage of 850 V was selected. For Ca detection in individal 2.5 µm sections, the liquid sample volume was 0.5 ml, with 5 replicates and a PMT voltage of 500V.

3.4. Spectromicroscopy with MEPHISTO

The MEPHISTO spectromicroscope (1) is an X-ray PhotoElectron Emission Microscope (X-PEEM) that uses an electron optics system (SpectroMicroTech, Orlando, FL, USA) to form a magnified image of the low energy photoelectrons emitted by a specimen under soft x-ray illumination. For this work, MEPHISTO was installed on the Aladdin ring at the Synchrotron Radiation Center. The electron image intensity is amplified by a series of two microchannel plates, and converted into a visible image by a phosphor screen (Galileo, Ca, USA). This image is captured by a video camera (Dage, USA) linked to a Pentium computer for display and data acquisition. The image magnification is continuously variable up to 8,000 times, and the optimum lateral resolution has been measured to be 20 nm (16). The photoelectrons are not energy filtered, so the total photoelectron yield, per unit area per unit time, is recorded as a function of photon energy. Such spectra reflect the x-ray absorption coefficient of the specimen surface and are hence referred to as x-ray absorption spectra. The energy position and lineshape of spectral features provide element identification and chemical state information.

There are several different modes of data acquisition: i) absorption spectra can be acquired directly as intensity variations at the sample surface with photon energy; ii) the distribution of an element or a chemical species can be mapped acquiring three photelectron images, one at and two before an absorption feature, using baseline extrapolation and image ratioing to extract microscopic chemical information while removing topographical effects; and iii) x-ray absorption movies create a stack of photoelectron micrographs scanning photon energy, from which absorption spectra may be subsequently extracted. Distribution mapping and spectrum extraction from x-ray absorption movies are performed with macros specially written for the freely available NIH Image software for Mac. Absorption spectra are plotted in Kaleidagraph for Mac and normalized dividing by reference spectra acquired on a clean Si or Au surface to remove the intensity variations due to the x-ray monochromator. Micrographs and distribution maps are further manipulated in Adobe Photoshop 5.0 to enhance the contrast or add false color.

3.5. Visible Light Microscopy

Optical micrographs were obtained using a Zeiss Axiotech 100 HD microscope connected to a Sony 950 DXC color video camera whose output was captured using a Sony Video Printer, and scanned.

3.6. Sample Preparation

3.6.1. Human glioblastoma tissue

Human glioblastoma tissue from patients administered with BSH prior to surgery, part of European collaboration on Boron Neutron Capture Therapy (BNCT) for cancer, D. Gabel and coworkers, Bremen, Germany (17, and the references therein).

The excised tumor tissue was fixed overnight in a 10% solution of formaldehyde. It was dehydrated by immersion in baths containing increasing concentrations of ethanol (70%, 96% and 99%, 3 exposures for 30 minutes at each concentration) ensuring limited exposure of tissue to ambient air while transferring between baths. The tissue was hardened in toluene for 1 hour and finally embedded in paraffin at 60 C. The tissue block is stored at -20 C. 7-10 µm sections were cut from the paraffin block by microtomy. To acquire thinner sections, a segment of the block was embedded in epoxy and ultramicrotomed (60 - 2000 nm).

3.6.2. Human meningioma tissue

Human meningioma tissue from patients administered gadolinium-based MRI contrast enhancement

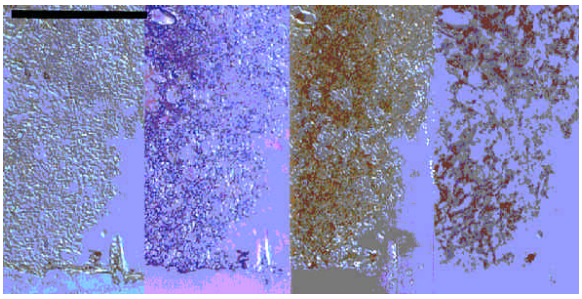

Figure 2. VLM images showing the progress of UV/ozone ashing a 2 µm ultramicrotomed section of human glioblastoma embedded in epoxy. The images were captured in Differential Interference Contrast (DIC) mode, which enhances differences in sample thickness. The ashing times were (from left to right) 0, 6, 18 and 24 hours. Scale bar = 500 µm.

agents for the novel anti-cancer modality gadolinium Neutron Capture Therapy (Gd-NCT), R. Pallini, L. M. Larocca, A. Rinelli, Universita' Cattolica del Sacro Cuore, Rome, Italy (2, and the references therein).

The tissue originated from a human patient undergoing surgery for tumor removal and routinely administered with a gadolinium based contrast enhancement agent for MRI location of the tumor. The tissue was fixed with paraformaldehyde, dehydrated, embedded in paraffin and cut into 2.5 µm thick sections. Adjacent pairs of section were mounted either on glass and stained with ematoxilin and eosin to act as a reference for the VLM, or on clean gold-coated silicon wafer substrates and ashed in the UV/ozone oven for 120 hours for MEHISTO analysis. Two subsequent sections were also taken for tissue digestion and ICP-AES analysis of the average gadolinium concentration.

3.6.3. Cultured human glioblastoma cells

Cultured human glioblastoma cells for in vitro studies of trial compound uptake, D. Mercanti, P. Casalbore, M. T. Ciotti, Istituto di Neurobiologia, CNR, Rome, Italy (18, and the references therein).

The cells originated from a human patient that underwent surgery for tumor debulking and were extracted from the bulk glioblastoma tissue by mechanical and enzymatic dissociation. The cells (mostly glial cells) were then plated and allowed to grow adhering to plastic dishes, cycled several times, and frozen at -80 C for storage. To use the cells in experiments they are warmed to 37 C, plated and cycled again to ensure their viability. We then exposed the cultures to gadopentetic acid for 0-72 hours. Several cultures were prepared for each exposure time, for ICP-AES and MEPHISTO analysis.

4. RESULTS AND DISCUSSION

Figure 2 shows the evolution of sample appearance at the VLM and the obvious loss of material with increasing time of ashing. The sample is a 2 µm section of human glioblastoma tissue embedded in epoxy, and it is clear that while the organic epoxy is completely removed by ashing, an inorganic layer of physiological elements remains.

The initial trial of UV/ozone ashing was performed on a neuron network, and a comprehensive set of absorption spectra of relevant elements was acquired (5). Figure 3 summarizes the dramatic increase in signal observed in MEPHISTO after ashing from sulfur and phosphorus, whose relative concentrations are significantly enhanced by the removal of carbon. The before- and after-ashing spectra have been identically normalized and are presented on the same vertical scale. Although the weakness of the before-ashing spectrum obscures the lineshapes, the energy positions of absorption structures indicate that sulfur and phosphorus are both present in several oxidation states in non-ashed, fixed tissue, but completely oxidized by the ashing process (3).

The strong carbon signal that is detected from tissue before ashing is completely removed by ashing (5). The depth of the specimen probed by the total electron yield technique in MEPHISTO is limited to less than 100 Å, however. An independent Total Organic Carbon (TOC) analysis of subsequent, individual 2.5 µm sections of meningioma tissue was performed after different times of ashing. The paraffin embedding medium had first been removed with xylene as described in the Materials and Methods section. The carbon content normalized to the pre-ashing tissue volume was 190 mg l^{-1} before ashing, and around 30 mg l^{-1} after 2, 4 and 6 days ashing. These measurements indicate that the UV/ozone process might not proceed to completion (complete carbon removal) but stop following the formation of a passivated, carbon-depleted layer. Such layer must be thicker than the MEPHISTO escape depth of 100 Å, since we do not detect a carbon signal.

It is important that ashing does not displace elements (at least not more than the relevant length scale for the experiment) and this is demonstrate by comparisons between ashed and non-ashed tissue, beginning with Figure 4. The comparison of ashed vs non-ashed tissue could not be made with a single tissue section, only with adjacent sections, so some discrepancies are inevitable. The top two images of show close agreement in appearance, however, between fixed tissue on glass at the VLM (top left) and ashed tissue from the same region of an adjacent tissue section imaged in MEPHISTO at 90 eV photon energy (top right). The lower images show sulfur and phosphorus distribution maps (bottom left and right, respectively) imaged at the S and P L-edges which give a very strong signal after ashing. These distribution maps show fine structure of the same or smaller dimensions than the VLM image, and likewise show tissue gaps containing no physiological material.

Gadolinium has potential use in an alternative cancer therapy due to its extremely high capture cross section for thermal neutrons, which otherwise cause very little biological damage traversing tissue (17, and the references therein). The excited Gd nucleus undergoes

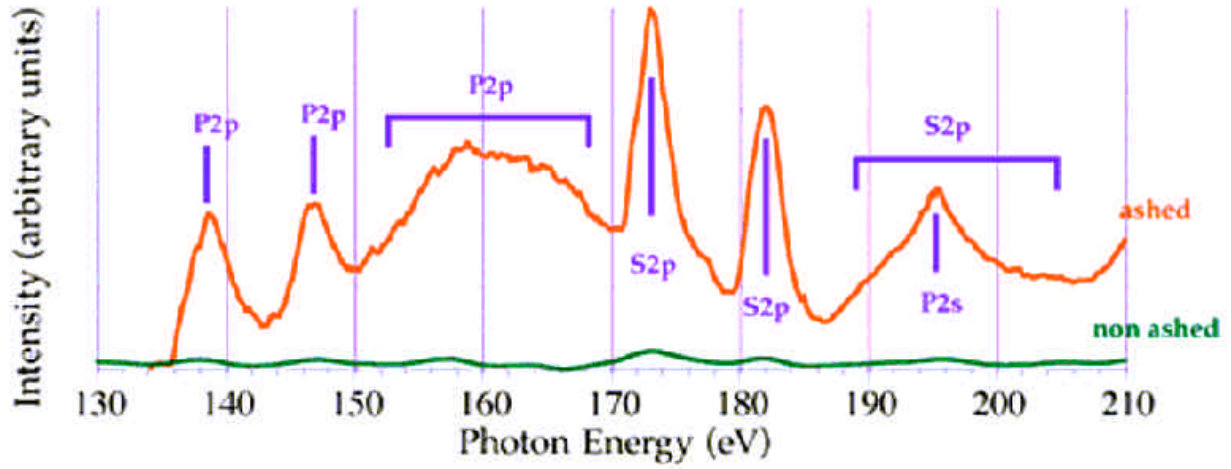

Figure 3. X-ray absorption spectroscopy of ashed and non-ashed human glioblastoma tissue (50 nm, embedded in epoxy) across the sulfur and phosphorus L-edges. Both spectra have been normalized with the same approach so that the peak intensities represent the enhancement of relative concentration of these non-volatile elements. Pure epoxy did not contain detectable levels of either sulfur or phosphorus.

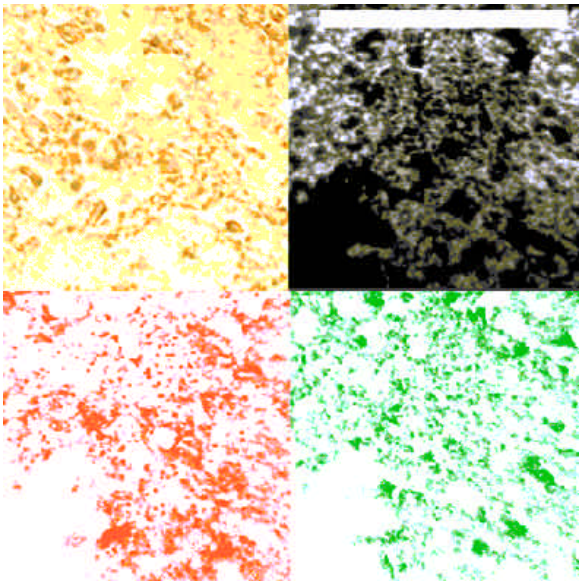

Figure 4. Human glioblastoma tissue. Clockwise from top left: a) VLM image of reference tissue section mounted on a glass slide (no staining). b) MEPHISTO micrograph of adjacent tissue section (2 µm, embedded in paraffin) mounted on a silicon wafer substrate and UV/Ozone ashed for 100 hours. The tissue is imaged at 90eV photon energy, where the tissue appears bright on the substrate. The length of the scale bar is 100 µm. **c)** Phosphorus distribution map of the tissue shown in b), created by a digital ratio of images on and around the P L-edge at 138.5 eV, as described in the Materials and Methods section. d) Sulfur distribution map, acquired at the S L-edge at 173 eV.

internal conversion, releasing energy as high energy electrons and a gamma ray, which may irreparably damage DNA (and cause cell death) if the nuclear reaction takes place in the vicinity of the nucleus. Hence, if Gd can be specifically delivered to the nuclei of tumor cells, and a large tissue area (normal tissue and tumor) is irradiated with thermal neutrons there is a mechanism for tumoricidal reaction. Gadolinium has common use as a contrast enhancement agent for MRI imaging of brain tumors, indicating that there may already exist some specificity of gadolinium for tumor that could be used as the basis of Gd-NCT. It is not known, however, if any gadolinium compounds may enter cell nuclei, and if so, in sufficient quantities to be therapeutically effective.

To address this question we have performed microscopic analysis on sections of meningioma tissue from human patients that underwent surgery. ICP-AES analysis of individual tissue sections did not reveal any gadolinium above the 2 ppm detection limit (after digestion and dilution), and so ashing was essential in order to increase the relative concentration for spectromicroscopy. The first results of these *in vivo* experiments are given in Figure 5. The VLM micrograph (top) shows the same distinctive structure imaged in an adjacent section in MEPHISTO at higher magnification (middle). This structure can be identified as a psamomatous formation, a common feature in meningioma, and was the only place in the tissue where the gadolinium 3d signal was detectable, given on the bottom image. Further experiments are planned to enhance the gadolinium signal sufficiently to address the original problem, and will also require ashing for spatially resolved trace element detection.

A similar experiment was performed in vitro on cultures of human glioblastoma to look at the dynamics of the uptake of gadolinium, and achieve a sufficiently high gadolinium concentration in the cells to study the microlocalization at higher magnification than is currently possible in the real tissue case. The experiment as performed with MEPHISTO is described graphically in

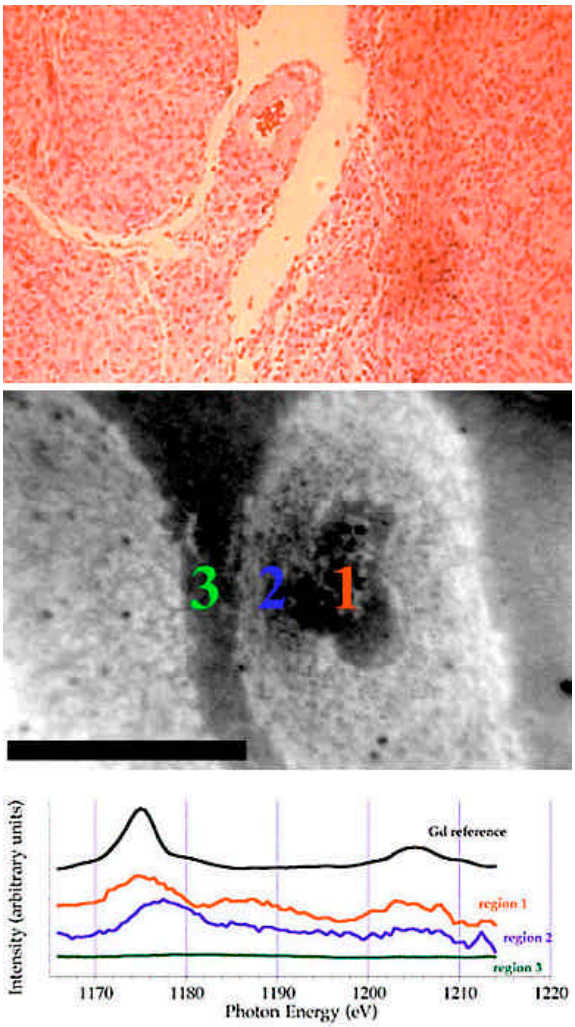

Figure 5. Human meningioma tissue. Top: VLM image of reference tissue section mounted on a glass slide (density stained). The region inside the rectangle is a psamomatous formation, and is shown at higher magnification below. Middle: MEPHISTO micrograph of adjacent tissue section (8 µm, embedded in paraffin) mounted on a gold-coated silicon wafer substrate and ashed for 100 hours. The tissue is imaged at the Gd M-edge at 1170 eV and shows the same psamomatous formation. Scale Bar = 100 µm. Bottom: Gd M-edge X-ray absorption spectrum taken from the psamomatous formation, and from a neighboring tissue area. The spectra originate from the correspondingly labeled regions of the middle image.

Figure 6. The cell cultures are exposed to a solution of the gadolinium compound for a fixed time, and then washed and ashed, to flatten the cells and make the gadolinium spectroscopically accessible. A calcium distribution map (top) enables individual cells to be clearly defined. A gadolinium M-edge movie is then acquired by capturing micrographs at photon energy intervals across this absorption edge (one frame is shown, middle). Gd 3d spectra can be subsequently extracted from carefully-defined areas encompassing either the entire cell or subcellular structures. The gadolinium spectroscopy from many cells showed the expected increase in uptake with time of exposure, and also revealed differences in uptake between individual cells. Further work is also underway to distinguish between gadolinium content in the nucleus and cytoplasm.

The ashing process can be considered as a vertical averaging of spatial information, and this is likely to have been the reason for the low contrast in the Ca distribution maps of ashed cells. To avoid this problem, and have a much higher resolution when investigating the subcellular distribution of elements, we tested the possibility of sectioning cells grown in a culture. Figure 7 shows a 60 nm vertical section (i.e. cut perpendicularly to the substrate) of a glioblastoma cell in a culture, embedded in epoxy, ultramicrotomed and then ashed. In both the oxygen (top) and calcium (bottom) distribution maps, subcellular compartments are visible at quite high contrast. The ashing process was particularly useful for this type of sample because of the nature of the electrostatic electron optics, which require high electric fields for imaging. Before ashing, the cell section is at the very edge of the epoxy section, and this introduces substantial distortions in the local electric field. After ashing, the remaining material is flat enough to allow high magnification imaging.

The data presented here were taken from experiments with specific medical applications in which UV/ozone ashing was an important technique for sample preparation. Ashing enhanced the relative concentration of analytes, and made feasible the challenging problem of determining the microlocalization of trace elements. The limitations of ashing will be twofold. Firstly, the removal of carbon destroys the internal architecture that supports cellular structures, and hence a loss of microstructure is inevitable. Further, as previously mentioned, the residual inorganic compounds (e.g. sulfur and phosphorus calcium salts) may be hygroscopic, and it is therefore essential that biological samples be kept in a dry environment during and after ashing. Even so, it is possible that the inorganic compounds that remain may be hydrated by water present even in fixed tissues, and subsequent crystalization would shift material beyond the original organic boundaries. While further characterization is required at the subcellular level, dislocation of material is not observed on the scale of the images presented (note that Figure 7 contains structures on the order of 1 µm).

The second limitation concerns the extremely oxidizing ozone environment. The MEPHISTO spectromicroscope can obtain chemical state information through the energy position and lineshape of x-ray absorption spectra. Such information may be lost during ashing if the analyte compound may be readily oxidized. Additionally, if the product of oxidization is volatile then the target signal may be lost rather than enhanced by ashing.

Ashing may also be used as an alternative to wet digestion of thin biological samples for quantitative bulk analysis. ICP-AES elemental analysis can be performed on individual sections with a sensitivity reaching a few ppm in

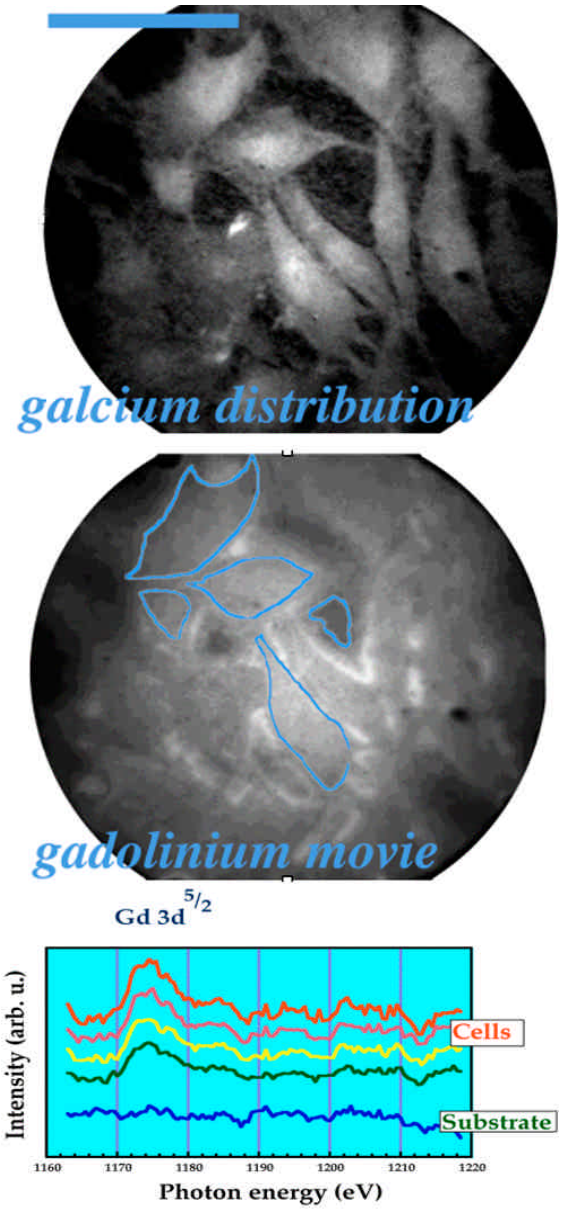

Figure 6. Human glioblastoma cell culture, grown on a gold substrate until confluent, exposed to gadopentetic acid for 72 hours and ashed 100 hours. Top: Calcium distribution map, acquired at the Ca L-edge at 350 eV. The distribution of calcium allows individual cells as well as subcellular structures to be delineated and correlated with further spectroscopic analysis. Scale bar = 100 µm. Middle: One frame of a Gd M-edge movie acquired scanning the photon energy between 1160 - 1220 eV. Gd spectra can be subsequently extracted from carefully-defined specimen areas. Bottom: Normalized Gd spectra acquired from the delineated areas in the middle image, with a high signal from individual cells, and no gadolinium detected from the substrate.

tissue for gadolinium and calcium. In this type of measurement, the minimum liquid sample volume was 0.5 ml (allowing 5 replicates) giving a dilution factor on the order of 10^3, and a corresponding scaling of the detection limits (ppb in solution) with respect to the tissue section volume before digestion. Two digestion techniques were applied to adjacent sections of tumor that were embedded in paraffin. The paraffin embedding medium was removed from one with xylene and rehydrated with successive water/ethanol baths. This sample was placed directly into 5 ml 32% (w/v) nitric acid in an Eppendorf vial and sonicated for 20 minutes. At this point no particulate material could be seen by eye. The second was ashed in the UV/ozone oven for 2 days. The paraffin was not removed by xylene and areas of just paraffin rapidly vanished with ashing. This sample was solubilized by pipetting 5 ml 32% (w/v) nitric acid onto the ashed tissue surface, allowing 2 minutes for the ashed material to dissolve, and then transferring to an Eppendorf vial with the same pipette. The analyte in this trial was physiological calcium. The ICP-AES measurements detected 3 times more calcium detected in the ashed sample than the non-ashed sample. One explanation may be that the extra washing steps involved with paraffin removal extracted free calcium in the first case, but it is also possible that the ashing process liberates calcium bound to proteins, resulting in greater solubilization and a stronger and more accurate reading. Trials of this kind will be repeated with pristine, freeze-fixed samples to further compare ashing as a precursor to tissue digestion.

5. CONCLUSIONS

Distribution mapping of trace elements in biological specimens has been performed in samples as part of ongoing research for a novel cancer therapy. Many of these studies were only possible because the relative analyte concentration was enhanced by prior UV/ozone ashing to remove carbon. The ashing process has been characterized by a number of such experiments and does not show any significant displacement of material due to ashing. The technique may be extended for use with other related techniques such as ion microscopy. Additional advantages specific to the X-PEEM analysis method are that ashed samples are thinner and hence less insulating, and flatter, resulting in undistorted images.

6. ACKNOWLEDGMENTS

We thank our collaborators for supplying the following samples: Human glioblastoma tissue from patients administered with BSH from Michael Neumann, Steffi Steen and Detlef Gabel, Department of Chemistry, Bremen University, Germany. Human meningioma tissue from patients administered gadolinium-based MRI contrast enhancement agents from Luigi Maria Larocca, Alessandro Rinelli and Roberto Pallini, Catholic University of Sacred Heart, A. Gemelli Hospital, Rome, Italy. Cultured human glioblastoma cells for in vitro studies of trial compound uptake from Patrizia Casalbore, Delio Mercanti and M. Teresa Ciotti, Institute of Neurobiology, CNR, Rome, Italy. We gratefully acknowledge the access to the ICP-AES spectrometer granted to us by Dr. Didier Perret of the Department de Chimie of the University of Lausanne, the invaluable help also supplied, and the TOC measurements

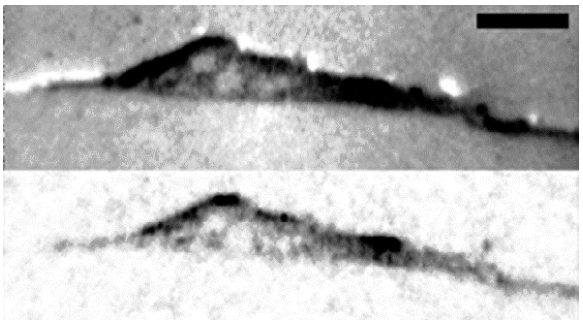

Figure 7. Human glioblastoma cell culture, grown on a gold substrate until confluent, exposed to gadopentetic acid for 24 hours, embedded in LR White, cut with an ultramicrotome (perpendicularly to the substrate) into 60nm thick sections and UV/ozone ashed for 10 hours. Top: Oxygen distribution. Bottom: Calcium distribution. In both images, at least two circular subcellular structures are clearly visible. Scale Bar = 10 µm

performed by Christine Mondi. We also thank the staff of the Synchrotron Radiation Center (a national facility supported by the NSF under Award number DMR-9531009) for their expert assistance.

7. REFERENCES

1. De Stasio G, Capozi M, Lorusso GF, Baudat PA, Droubay TC, Perfetti P, Margaritondo G, Tonner BP: MEPHISTO: a Novel Synchrotron Imaging Photoelectron Microscope. *Rev. Sci. Instrum.* 69:2062-2067 (1998)
2. De Stasio G, Gilbert B, Perfetti P, Margaritondo G, Mercanti D, Ciotti MT, Casalbore P, Larocca LM, Rinelli A, and Pallini R: Frontiers of x-ray spectromicroscopy in biology and medicine: gadolinium in brain cancer. In X-ray and inner shell processes, X99, Aug. 23-27, 1999 Chicago, IL, American Institute of Physics Conference Proceedings Series, in press.
3. Gilbert B, Redondo J, Baudat PA, Lorusso GF, Andres R, Van Meir EG, Hamou MF, Suda T, Mercanti D, Ciotti MT, Droubay TC, Tonner BP, Perfetti P, Margaritondo G, De Stasio G: Spectromicroscopy of Boron for the Optimization of Boron Neutron Capture Therapy (BNCT) for Cancer. *J. Phys. D* 31:2642-2647 (1998)
4. De Stasio G, Dunham D, Tonner BP, Mercanti D, Ciotti MT, Angelini A, Coluzza C, Perfetti P, Margaritondo, G: Aluminium in Cerebellar Neural Cultures. *NeuroReport* 4:1175 (1993)
5. De Stasio G, Gilbert B, Perfetti L, Hansen R, Mercanti D, Ciotti MT, Andres R, White VE, Perfetti P, Margaritondo G: Cell Ashing for Trace Element Analysis: a New Approach Based on UV/Ozone. *Anal. Biochem.* 266:174-180 (1999)
6. Larsson B, Schwitzer PM, Stepanek J, Teichmann S, Weinreich R, Luetolf U, Romer M, Spycher MA. In Hadrontherapy in oncology. Eds: U. Amaldi and B. Larsson. Elsevier, Amsterdam (1994)
7. De Stasio G, Capozi M, Droubay TC, Mercanti D, Ciotti MT, Lorusso GF, Andres R, Suda T, Perfetti P, Tonner BP, Margaritondo G: The Effect of Ashing on Cells: Spectromicroscopy of Physiological Elements. *Anal. Biochem.* 252106-109 (1997)
8. Bellanger JR: Adaptation of a Dry Ashing Procedure for Determination of Selenium by Fluorometry in High-Fat Foods. *J. AOAC Int.* 78:477 (1995)
9. Davicco MJ, Coxam V, Gaumet N, Lebecque P, Barlet JP: Eb-1089, a Calcitriol Analog, Decreases Fetal Calcium Content When Injected into Pregnant Rats. *Exp. Physiol.* 80449 (1995)
10. Hansen RWC, Bissen M, Wallace D, Miller T: Ultraviolet Ozone Cleaning of Carbon-Contaminated Optics. *Appl. Optics* 32:4114-4116 (1993)
11. Hansen RWC, Wolske J, Takacs PZ: UV Ozone Cleaning of a Replica Grating. *Nucl. Instrum. Meth.* A347:254-257 (1994)
12. Hansen RWC, Wolske J, Wallace D, Bissen M: Cleaning of Optical-Surfaces with Photogenerated Reactants. *Nucl. Instrum. Meth.* A347:249-253 (1994)
13. Varma A: Handbook of Inductively Coupled Plasma Atomic Emission Spectroscopy. CRC Press, Boca Raton (1991)
14. Andrasi E, Nadasdi J, Molnar Z, Bezur L, Ernyei L: Determination of Main and Trace Element Contents in Human Brain by NAA and ICP-AES methods. *Biol. Trace Element Res.* 26-27:691-698 (1990)
15. De Stasio G, Perfetti P, Oddo N, Galli P, Mercanti D, Ciotti MT, Koranda SF, Hardcastle S, Tonner BP, Margaritondo G: Metal Uptake in Neurone Cultures: a Systematic Study. *NeuroReport* 3:965-958 (1992)
16. De Stasio G, Perfetti L, Gilbert B, Fauchoux O, Capozi M, Perfetti P, Margaritondo G, Tonner BP: The MEPHISTO Spectromicroscope Reaches 20 nm Lateral Resolution. *Rev. Sci. Instrum.*, 70:1740-1742 (1999)
17. Gilbert B, Perfetti L, Fauchoux O, Redondo J, Baudat PA, Andres R, Neumann M, Steen S, Gabel D, Mercanti D, Ciotti MT, Perfetti P, Margaritondo G, De Stasio G: The Spectromicroscopy of Boron in Human Glioblastomas following Administration of BSH, accepted for publication in *Phys. Rev. E.*
18. De Stasio G, Casalbore P, Gilbert B, Mercanti D, Ciotti MT, Larocca LM, Rinelli A, Perret D, Perfetti P, Margaritondo P, Pallini R: Quantitative Gadolinium Uptake in Human Glioblastoma Cultured Cells for Gadolinium Neutron Capture Therapy (GdNCT). submitted to *Cancer Research.*

Key Words: Ashing, Microchemical Analysis, Trace Element Detection, Spectromicroscopy

Send correspondence to: Dr Ben Gilbert, Institut de Physique Appliquée, Ecole Polytechnique Fédérale, PH-Ecublens, CH-1015 Lausanne, Switzerland, Tel: +41 21 693 54 14, Fax: +41 21 693 46 66, E-mail: bgilbert@dpmail.epfl.ch

This manuscript is available on line at:

http://www.bioscience.org/2000/a/gilbert/fulltext.htm