High-Resolution X-ray Absorption Spectroscopy Studies of Metal Compounds in Neurodegenerative Brain Tissue

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Introduction

Fluorescence mapping and microfocus X-ray absorption spectroscopy are used to detect, locate and identify iron biominerals and other inorganic metal accumulations in neurodegenerative brain tissue at sub-cellular resolution (<5µm). Here, recent progress in developing this technique at the MRCAT beamline is reviewed.

Synchrotron X-rays are used to map tissue sections for metals of interest, and XANES and XAFS are used to characterise anomalous concentrations of the metals *in-situ* so that they can be correlated with tissue structures and disease pathology. Iron anomalies associated with biogenic magnetite, ferritin and haemoglobin were originally located and identified in an avian tissue model with a pixel resolution ~ 5µm [**1**]. Subsequent studies include brain tissue sections from transgenic Huntington's mice, and the first high-resolution mapping and identification of iron biominerals in human Alzheimer's and control autopsy brain tissue [**2**]. Technical developments include use of microfocus diffraction to obtain structural information about biominerals *in-situ*, and depositing sample location grids by lithography for the location of anomalies by conventional microscopy.

Background

The links between neurodegeneration and metal accumulation in the brain are increasingly accepted, but the underlying mechanisms involved are poorly understood. Brain iron accumulation is a feature of most neurodegenerative diseases, including Alzheimer's (AD), Parkinson's (PD), and Huntington's disease (HD) [**3, 4, 5**]. The majority of neurodegenerative diseases lack effective treatment or cure, and in cases involving dementia, such as Alzheimer's disease, diagnosis is usually only confirmed at autopsy. The incidence of iron elevation in regions of neurodegeneration is well documented, but little is know about the chemical state of the additional iron, or the role that it plays. Other metals, including aluminium, copper and zinc, are increasingly associated with aspects of neurodegeneration [**6, 7**].

The form in which metals accumulate in brain tissue is critical in determining whether they can play a significant role in neurodegeneration. For example, iron is normally stored as Fe(III) which is far less reactive, and therefore less toxic, than Fe(II). Factors such as the specific iron compounds present, the valence state, and the precise location with respect to cellular and tissue structures, are all important in determining if such iron accumulations play a significant role in disease progression.

It is known that a variety of iron biominerals are present in brain tissue. These include ferrihydrite-like compounds in the primary iron storage protein, ferritin, haemosiderin deposits, and in some instances magnetite [**8, 9, 10**]. In particular, recent studies have suggested a positive correlation between magnetite concentrations and Alzheimer's disease, both in bulk tissue samples [**11**] and in pathological ferritin extracted from Alzheimer's disease cases [**12**]. From the evidence available, it appears that anomalous iron compounds such as magnetite are present in the human brain as nanoscale deposits, at concentrations of ng – µg/g of tissue. Given that their distribution is as yet unknown, locating such iron oxide accumulations *in-situ* is impractical by conventional microscopy techniques, as in addition to being difficult and time-consuming, these techniques provide very limited information about the nature of the iron biominerals present [**13**]. Detailed *in-situ* studies are necessary if the roles of metal accumulation in disease pathology are to be understood.

We have developed an approach using synchrotron radiation to detect, locate and identify iron compounds and other metals of interest in autopsy brain tissue at sub-cellular resolution. Fluorescence mapping provides a fast and sensitive means of detecting anomalous metal concentrations in a tissue section, and a high-intensity focused beam is used to locate the concentrations to within a few microns. These metal concentrations are then characterised using a combination of X-ray Absorption Near Edge Spectroscopy (XANES) and X-ray Analysis of Fine Structure (XAFS), where they are fitted with combinations of measured and calculated standards respectively.

Methods and Materials

All samples used in this work were obtained and studied in conjunction with the appropriate ethical permissions and procedures. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at University of Florida.

At all stages of tissue preparation it is essential to avoid procedures that may disrupt the nature of the metal compounds present. In these studies, all instruments and containers were acid-cleaned in HCL, and all solutions were placed on strong magnets and filtered in order to minimise metal contamination of samples. All blades used for tissue sectioning were either diamond or Teflon coated.

For HD mice, complete coronal serial sections were cut from mid-brain tissue at 100 - 150µm thick. Sections were flat-embedded for XAS in standard and modified TAAB epoxy resin. Each flat-embedded slide contained sections placed in three segments between sheets of Aclar and Kapton. For the human AD and control tissue samples, 50µm-thick tissue sections were cut from the superior frontal gyrus and processed for XAS in the same way as for the HD mice.

High-resolution fluorescence mapping of metals, XANES and XAFS were performed, where each tissue section was first screened for iron content by comparing the x-ray fluorescence intensity above and below the K-edge for iron at 7112eV. Maps of the tissue sections were obtained by rastering the samples on an x-y stage capable of 100nm positioning resolution, and fluorescence from metals of interest was detected using a 13-channel detector. The initial fluorescence maps were collected at a comparatively low resolution (~100µm), and from these the areas exhibiting anomalous high intensity were scanned at higher resolution to locate anomalies to within a few microns as shown in Fig 1. The microfocused beam was achieved using Kirkpatrick-Baez (KB) mirrors [**1**] to give a well-defined beam and to preserve signal intensity. A CCD detector was used to collect real-time diffraction data.

XANES and XAFS data were collected using the microfocused beam centred at sites of anomalously high fluorescence intensity. XANES spectra were compared with previously obtained spectra from biological and synthetic standards for ferritin, magnetite, and haemoglobin [**1**]. Following background subtraction and energy correction, linear combination fits were performed using IFFEFIT routines after Newville et al. [**15, 2**]. Where the data were of sufficiently high quality, reduced spectra were fitted with calculated XAFS standards.

Results and Discussion

Initial studies have demonstrated that iron compounds can

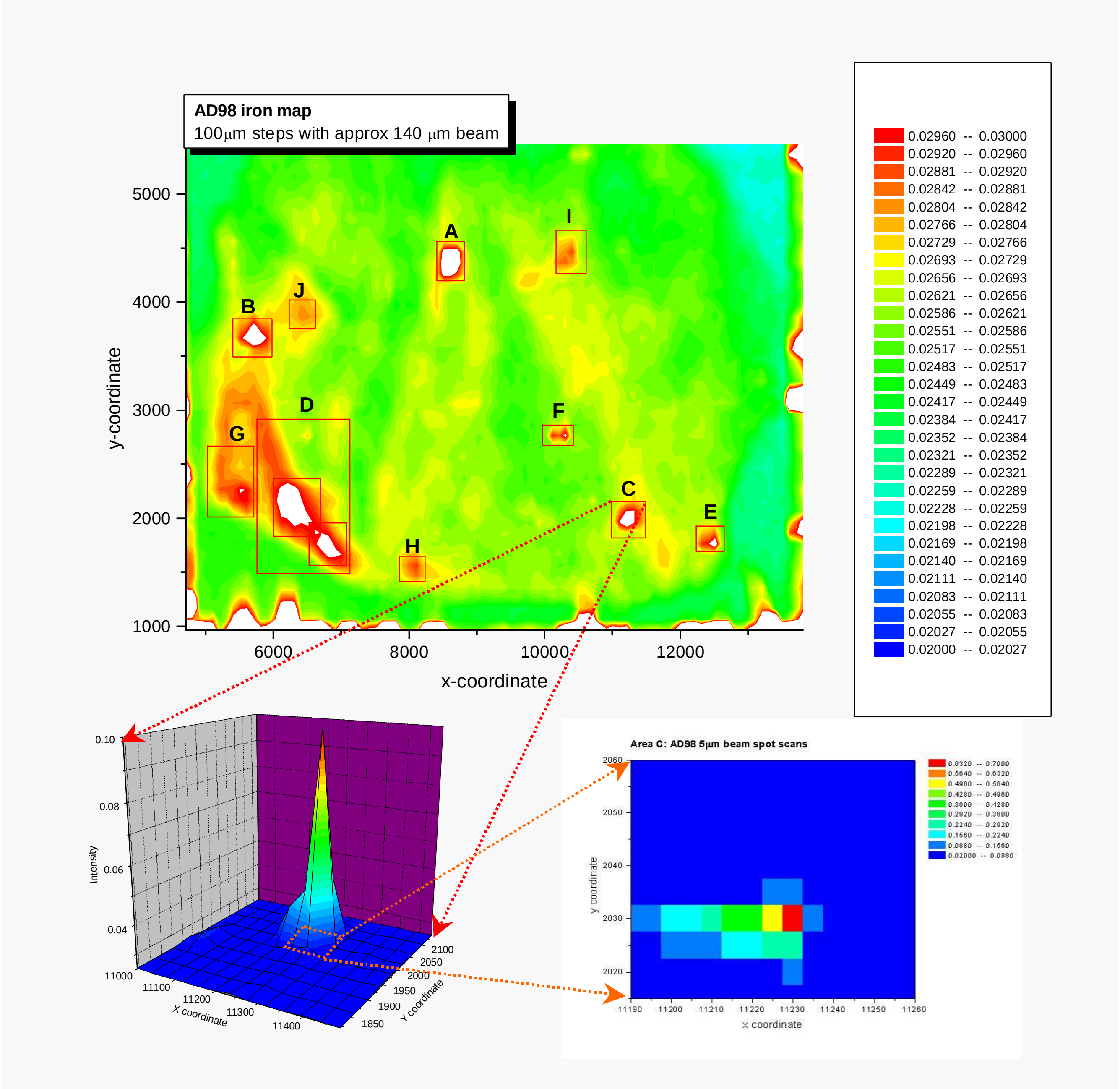

Figure 1: Iron fluorescence intensity measured for an AD tissue section. The main image was mapped at 100µm resolution on MR-CAT. The edge of the sample can be distinguished by the clear reduction in iron intensity. The highest intensity is off-scale (white). The microfocusing process is illustrated for area C, with inset a) showing a wire frame map at 20 µm resolution and b) a pixel map from a 5 µm resolution scan within area C. From b) it is evident that the main iron anomaly is located in a 5µm pixel.

be mapped and characterised in tissue sections using a microfocused beam configuration with <5μm in-plane resolution. The work has resulted in the production of the first large-scale, high-resolution maps of both HD (R6/2) and human AD tissue samples in which iron compounds are characterised at sub-cellular resolution.

The metal fluorescence mapping technique is sensitive enough to resolve 50μm thick tissue sections against background from trace levels throughout the tissue, to resolve structural anatomical details, and to detect anomalous metal concentrations ≤ 5μm diameter when scanning at low resolution with 100-200μm pixels (Fig. 1). This approach enables a comparatively rapid (<24hr) microanalysis of the distribution of high iron concentrations in a tissue area ~ 1cm^2.

The majority of the XAS spectra can be successfully fitted with linear combination fits of XANES standards, and evidence for both physiological ferritin and magnetite has been identified [**1, 2**]. Fits to XAFS data with calculated standards have also been performed, although the form of the ferritin cores provides a particular challenge due to its complexity [**12**], and merits further research in its own right. XAFS from biological samples is often hard to interpret, with challenging signal-to-noise due to the complex nature of the sample material. However, microfocusing at a beamline optimised for XAFS enables spectra of sufficiently high quality to be obtained from iron biominerals in tissue sections.

In the coronal tissue sections, regional variations in iron density allow anatomical structures to be observed, and subsequent histological examinations of the samples are being used to explore correlations between anomalous metal concentrations and disease pathology. To support the subsequent correlation of images, a lithography grid deposition protocol is under development for sample slides. Anomalies in tissue sections can then be located using numbered grids visible in X-ray transmission maps and optical microscopy.

We are extending the technique to other elements of interest such as Zn, Mn, and Cu, and are developing software for real-time analysis that will increase efficiency by reducing the volume of data required for each sample. Microfocus diffraction data obtained with the CCD detector should provide direct structural information to support the XAS data, and the principle has been demonstrated with the observation of diffuse rings consistent with ferrihydrite lattice spacing at the site of an anomaly rich in ferritin.

Conclusions

The combined techniques provide a breakthrough in the study of both intra- and extra-cellular iron compounds and related metals in tissue. High-resolution iron mapping using microfocused x-ray beams has the potential for direct application to investigations of the location and structural form of metal compounds associated with human neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases – a problem which has vexed researchers for 50 years. The information to be gained from this approach has implications for future diagnosis and treatment of neurodegeneration, as well as for our understanding of the mechanisms involved.

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