

High Resolution Secondary Ion Mass Spectrometry Analysis of Trace Elements in Cereal Grain and Roots

Katie L. Moore
Mansfield College



A thesis submitted for the degree of Doctor of Philosophy in the
University of Oxford

Supervised by Professor C. R. M. Grovenor

Hilary Term 2011

ABSTRACT

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This thesis presents information on the subcellular localisation of two important trace elements, selenium and arsenic, in wheat, rice and rice roots for what is believed to be the first time. The general aim of this work was to illustrate the potential of using physical science techniques to solve biological problems. High resolution secondary ion mass spectrometry was undertaken using the CAMECA NanoSIMS50 with a sensitivity down to ppm concentrations and a lateral resolution of less than 100 nm. Selenium in wheat grain was found to be distributed across both the bran layer and the endosperm region with Se-rich hotspots found in the aleurone cells and a higher intensity of Se in the subaleurone region. Arsenic in rice grain was found in two key regions. In grains with high As and high dimethylarsinic acid (DMA) content, As was predominantly localised to the subaleurone region yet in lower concentration, hydroponically grown As(III)-treated grains the As was only localised to the aleurone layer near the ovular vascular trace (OVT). A combined NanoSIMS and S-XRF experiment revealed As hotspots near the OVT. A combination of high pressure freezing, high resolution secondary ion mass spectrometry and TEM was used to localise As in the roots of rice plants revealing a contrasting subcellular distribution of As and Si in the roots even though arsenite and silicic acid are transported across the plasma membranes by the same transporters. Fe plaque forms only on the root epidermis and was shown to be a strong sink for As. Colocalisation of S with As in the vacuoles of the endodermis, pericycle and xylem parenchyma supports the notion that As is stored as arsenite-phytochelatins complexes in the roots while Si is localised in the endodermis cell walls and is not strongly affected by the Lsi2 mutation.

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PREFACE

The work reported in this thesis was carried out by the author in the Department of Materials, University of Oxford, between October 2007 and March 2011, under the supervision of Prof. C.R.M. Grovenor.

No part of this thesis has been previously submitted for a degree at this or any other university. The work of other authors has been freely drawn upon and is duly acknowledged in the text. A list of references has been given at the end of the thesis.

Some of the work described in this thesis has been published in the following journals and has been presented at conferences listed below:

Published work

‘NanoSIMS Analysis of Arsenic and Selenium in Cereal Grain’

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Katie L. Moore, Markus Schröder, Chris R. M. Grovenor

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NMAET IV, National Physical Laboratory, Teddington, UK, Nov. 2010, ‘NanoSIMS Analysis of Trace Element Distributions in Biological Materials’

IOM³ Young Person’s World Lecture Competition, Kuala Lumpur, Malaysia, Sept. 2010

‘NanoSIMS Analysis of Arsenic and Selenium in Cereal Grains’

British Mass Spectrometry Society Conference, Cardiff, UK, Sept 2010, ‘NanoSIMS analysis of arsenic and selenium in roots and cereal grain’

Rothamsted Research day, Harpenden, UK, Jul, 2010, NanoSIMS analysis of arsenic and selenium in roots and cereal grains

Université Joseph Fourier, Grenoble, France, June 2010, Invited talk ‘High resolution localisation of trace elements in biological samples with NanoSIMS’

IOM³ Young Person's Lecture Competition, London, Apr. 2010 'NanoSIMS Analysis of Arsenic and Selenium in Cereal Grains'

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2009 Oxford Biomedical Imaging Festival, Oxford, UK, Sept 2009: Poster Presentation 'NanoSIMS Analysis of Trace Elements in Cereal Grain'

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COST Meeting, Lillehammer, Norway, Sept. 2008: Oral Presentation 'NanoSIMS Analysis of Trace Elements in Cereal Grain'

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1. INTRODUCTION

Trace elements are an important part of our diet, both beneficially and detrimentally. Understanding where they are located in food crops and how they are taken up by roots will help improve diets and avoid exposure to potentially poisonous contamination. Selenium deficiency is a major problem in the UK with the majority of the population only consuming about half of their recommended daily amount. Adding a Se fertiliser to the soil can markedly increase Se concentration in wheat, but it is important to know where the Se is localised within wheat grain to understand how it might be separated by milling. Conversely the irrigation of rice paddy fields in areas such as Bangladesh with As contaminated groundwater has resulted in rice containing significant amounts of this toxic and carcinogenic element. Information concerning the location of the As in rice grain will help to plan strategies to minimise the amount of As consumed while localisation of As in the roots of rice plants will help to understand uptake of this element.

This interdisciplinary project was undertaken in the Department of Materials, University of Oxford in collaboration with Dr Fangjie Zhao, Dr Malcolm Hawkesford, Professor Steve McGrath and Professor Peter Shewry of Rothamsted Research, Harpenden UK, Dr Enzo Lombi, University of South Australia and Professor Chris Hawes and Dr Barry Martin of Oxford Brookes University, UK. These collaborations provided samples, the key biological questions to be answered and helped with interpretation of results (Rothamsted), experience with elemental mapping of trace elements and assistance with synchrotron experiments (Enzo Lombi) and sample preparation and TEM experience (Barry Martin and Chris Hawes).

The aim of this project was to show the potential of using high resolution secondary ion mass spectrometry analysis to investigate the subcellular localisation of important trace elements in cereal plants. SIMS analysis of plant material has been limited even though the potential of the technique has been realised for over 25 years. There are very few applications using SIMS to

localise trace elements with the majority of the work focusing on species present at fairly high concentrations. Although some elemental mapping of Se and As in cereal grains and roots had already been undertaken at the start of this project, these studies were limited in terms of the spatial resolution available.

Chapter 2 gives a detailed description of secondary ion mass spectrometry and presents some recent examples of relevant biological SIMS analysis mainly using the NanoSIMS. A description of the NanoSIMS instrument is given in Chapter 2 while the rest of the experimental techniques used in this project and tuning of the NanoSIMS are presented in Chapter 3. Experiments on the localisation of Se in wheat grain, both mature and immature, are presented in Chapter 4 showing some of the artefacts associated with imaging mature cereal grains and describing new features such as Se hotspots in the aleurone layer which have not been previously observed.

Determining the distribution of As in rice grain proved to be much more difficult than the Se in wheat experiment due to the lower concentration and yield of As. Chapter 5 presents the first subcellular study of the localisation of As in rice grain and also shows the advantage of using combined synchrotron X-ray fluorescence and NanoSIMS analysis.

Chapter 6 concentrates on the localisation of As in rice roots. Understanding the mechanism of uptake of As in the roots is critical to understanding and subsequently minimising As accumulation in rice grain. This chapter reveals many novel features regarding localisation of both As and Si in rice roots showing that these elements follow different distribution patterns despite being transported across the plasma membranes by the same transporters.

Finally, a summary of the important findings and conclusions are presented in Chapter 7 along with suggestions for further work.

2. LITERATURE REVIEW¹

Secondary ion mass spectrometry (SIMS) is the analysis of charged particles emitted by bombardment of a surface with energetic primary ions. Analysis of the mass-to-charge ratio of these particles enables detailed analysis of the surface chemistry. SIMS is a major technique for investigating a wide range of materials due to its combination of very high chemical sensitivity, excellent mass resolution, surface sensitivity, lateral and depth resolution not matched by any other technique. SIMS is being used for the analysis of an increasingly wide range of materials from semiconductors and metals to polymers and biological materials.

2.1. FUNDAMENTALS

Secondary Ion Mass Spectroscopy (SIMS) is the process whereby the surface to be analysed is bombarded by an energetic primary beam of ions causing particles to be emitted from the surface. These emitted (secondary) particles will be a combination of electrons, neutral species, atoms, molecules or atomic and cluster ions (Benninghoven *et al.*, 1987; Vickerman *et al.*, 1989; Wilson *et al.*, 1989). Unless post ionisation processes are used, it is the secondary ions which are detected and analysed to give information about the chemical composition of the sample. The electrons can also be detected and can give an indication of the surface topography and sample structure. SIMS is able to detect all elements (and isotopes) from hydrogen to uranium with parts per billion sensitivity for some elements as well as being able to detect and distinguish between molecular ions (Wilson *et al.*, 1989). High lateral resolution can be obtained (down to 50 nm for some instruments) and it can be applied to any type of material that is stable under vacuum. It is this unique combination of characteristics which makes SIMS a very powerful technique in the physical and life sciences.

¹ A substantial part of this chapter will be published as a book chapter in February 2012: Katie L. Moore, Markus Schröder, Chris R. M. Grovenor 'Imaging Secondary Ion Mass Spectrometry' in Handbook of Nanoscopy, 2 vol., van Tendeloo, van Dyke, Pennycook (Eds.) WILEY-VCH, Weinheim, Germany

2.1.1. PHYSICAL PRINCIPLES

2.1.1.1. The Sputtering Process

A schematic of the sputtering process is shown in Figure 2.1.

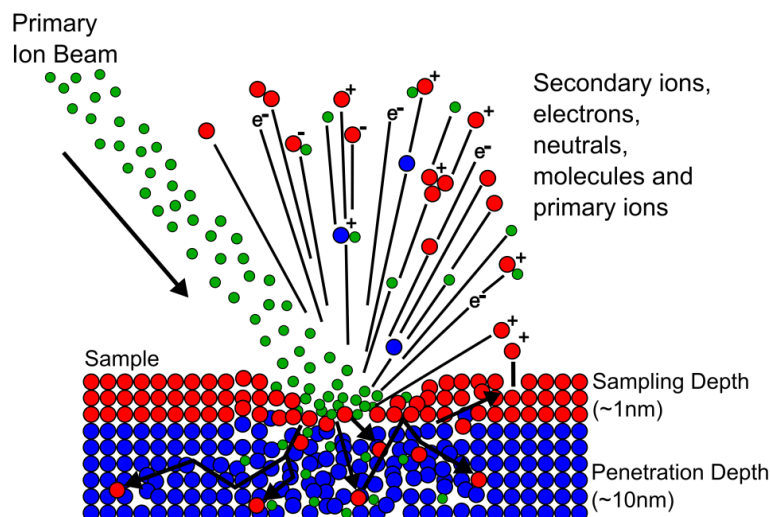


Figure 2.1: A schematic diagram showing the principles of SIMS analysis. Note that the primary ion beam is not drawn to scale and should be several hundred times larger. The collision processes occurring when energetic particles hit a surface and the accompanying surface mixing are also illustrated. Adapted from (Evans Analytical Group, accessed Oct 2010c).

The bombardment of a surface by energetic ions or atoms to cause erosion is known as sputtering (Vickerman *et al.*, 1989). When a high energy particle hits the surface its energy is transferred to the sample by a series of inelastic collisions. In order to achieve desorption of particles from the sample surface the momentum transfer perpendicular to the surface must exceed the binding energy of the particles (0.5 eV to up to 30 eV depending on the surface work function). Sometimes particles are desorbed from the surface by the direct momentum transfer from the primary ion but these “direct-knock-on” processes are rare. Normally the primary particle penetrates up to 20 nm into the sample and loses its energy by a series of collisions (the linear collision cascade model (Sigmund, 1969)) and only momentum transfers by secondary recoils return to the surface. The diameter of the affected area is defined as the area disturbed not

only by the primary ion impact but the resurfacing collision cascade, and can reach diameters of 5-10 nm (CAMECA, accessed Oct 2010b). Only ions from the immediate surface are sputtered from the sample (Guerquin-Kern *et al.*, 2005) hence SIMS is a very surface selective technique. The collision process also results in mixing of the atoms in the near surface region of the solid to a depth of approximately 10 nm (Benninghoven *et al.*, 1987) and roughening of the surface. Benninghoven gave a definition for the so called “static limit” for SIMS analysis (Benninghoven, 1971). If the probability of an incident ion hitting an area twice during one measurement is below 1%, one can assume static conditions and every impact yields signal from undisturbed surface areas (Figure 2.2). Static SIMS uses a primary ion beam with a very low current density (below 10^{12} ions/cm⁻²) to remain within the static limit and this is the key principal of Time of Flight SIMS (ToF-SIMS).

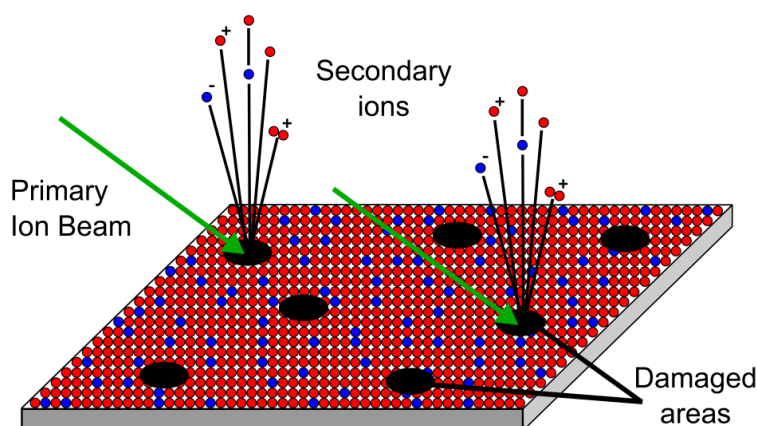


Figure 2.2: Schematic diagram showing the damaged areas after ion impacts during static SIMS analysis. Adapted from (CAMECA, accessed Oct 2010b)

Dynamic SIMS uses a much higher primary ion current density (above 10^{12} ions/cm⁻²) (CAMECA, accessed Oct 2010a) far exceeding the static limit, resulting in fast erosion of the surface and formation of a crater. Static SIMS can provide molecular information whereas dynamic SIMS provides mostly elemental and isotopic information because the rate of damage to the sample surface is much higher resulting in the breaking of most chemical bonds. Near-

surface analysis can still be undertaken, but the faster erosion rate allows bulk analysis of the sample including chemical depth profiling and two and three dimensional imaging.

2.1.2. TECHNICAL DETAILS

In order to extract quantitative chemical information from SIMS analysis it is important to understand how the interaction of the primary ion beam with the specific sample type affects the yield of secondary ions.

2.1.2.1. Yield

The sputter yield is defined as the number of particles removed per primary particle impact and normally lies between 0.1 and 10 (Vickerman *et al.*, 1989) and is given by Equation 2.1:

$$Y = \frac{\text{Number of sputtered atoms}}{\text{Number of primary ions}} = \frac{Sd\rho q}{I_p t} \quad \text{Equation 2.1}$$

where S is the analysed area, d the crater depth, ρ the atomic density, q the charge, I_p the primary current and t the analysis time.

The primary ion beam energy, the mass of the incident ions and angle of incidence all affect the sputter yield, and the yield is also dependent on the physical state of the sample. The crystallinity of the sample surface can have a direct effect on the sputter yield as certain crystallographic planes sputter faster than others (Vickerman *et al.*, 1989). Surface topography also has a large effect on the average sputtering yield and this is particularly evident when the roughness is of a similar scale as the dimensions of the damage cascade, which results in much higher yields in comparison to a flat surface (Littmark & Hofer, 1978). Holes or pores in the sample can reduce the sputter yield by trapping sputtered material.

In addition, not all elements sputter at the same rate so in multi-element samples sputtering results in the preferential loss of one component and the surface becomes enriched in the element

with the lower elemental yield (Werner & Warmoltz, 1976; Vickerman *et al.*, 1989; Albers *et al.*, 1994; Philipp, 2008). The very important influence of yield exerted by the material being analysed, the so called matrix effect, will be described below.

2.1.2.1.1. *Ionisation Efficiency*

The SIMS ionisation efficiency is defined as the fraction of sputtered atoms which become ionised (Evans Analytical Group, accessed Oct 2010c). During the sputtering process very few of the species emitted are in fact spontaneously ionised, for most materials the fraction of charged secondary particles is approximately 1%, so the majority of emitted species are uncharged and cannot be directly analysed.

Secondary ion yields depend on many factors, the most influential being properties of the element itself; the ionisation potential for positive ions and the electron affinity for negative ions. The choice of primary beam can also affect the ion efficiency. An oxygen beam increases the positive ion yield whereas a caesium ion beam increases the negative ion yield. Oxygen bombardment creates oxygen-metal bonds in an oxygen-rich surface zone. When these bonds break during ionisation, the oxygen becomes preferentially negatively charged due to its high electron affinity and its high ionisation potential prevents it from becoming positively charged. This enhances the probability of the local formation of M^+ ions. Caesium implantation on the other hand, decreases the surface work function which means that more secondary electrons are excited over the surface potential barrier hence there are more electrons available leading to an increased probability of negative ion formation (Evans Analytical Group, accessed Oct 2010c). For elements with a high ionisation yield, using a caesium beam may increase the average number of secondary ions emitted per primary ion to 20% (Evans Analytical Group, accessed Oct 2010c). The optimum choice of primary ion species is dependent on the position in the periodic table of the element to be analysed, as shown in Figure 2.3 for caesium and oxygen primary beams.

sputtered ions can escape. Monte Carlo simulation of a collision cascade from a single primary ion indicates the ultimate resolution is about 2nm at this 1 nm sampling depth (Wilson *et al.*, 1989). Of course, to obtain a measurable secondary ion signal a primary ion beam containing very many ions must be used. In practice the spatial resolution is determined by the primary beam diameter. With a 50 nm beam the resolution cannot be better than 50 nm and the pixel size on any image should be matched as closely as possible to the beam size to minimise any further degradation of resolution. The best lateral resolution can be achieved with gallium liquid metal ion sources (Wilson *et al.*, 1989) as they can be focused to smaller diameters than other sources, although there is still a trade off between current and diameter (Levi-Setti *et al.*, 1991).

2.1.2.2.2. Mass Resolution

Mass resolution is just as important as the lateral resolution as it is this which determines the selectivity of the instrument between ions of very similar masses.

Mass resolution, R , is defined in Equation 2.2, where M_1 is the mass of the ion of interest and M_2 is the mass of an adjacent interference peak (Downard, 2004).

$$R = \frac{M_1}{M_1 - M_2} = \frac{M}{\Delta M} \quad \text{Equation 2.2}$$

Some elements and molecular fragments have nominally the same mass, for example $^{79}\text{Br}^-$ and $^{31}\text{P}^{16}\text{O}_3^-$ and $^{40}\text{Ca}^{16}\text{O}^-$ and $^{56}\text{Fe}^-$. Fortunately the small deviation in the atomic masses from integer values, the so called mass deficits, means that with a high mass resolution mass spectrometer most ions can be separated. However, even with many technological advances in SIMS instrumentation, one of the main problems associated with SIMS analysis of biological materials is the difficulty of distinguishing with confidence between elemental and molecular species with nominally the same mass. Great care must be taken to ensure that the correct species is identified or the results are meaningless. At lower (<15 amu) and higher (>100 amu) masses

there are relatively few mass interferences, but almost any combination of carbon, hydrogen, oxygen, phosphorus and nitrogen is possible as a molecular fragment from biological samples. Figure 2.4 shows the spectrum around mass 26 from a coral sample spiked with ^{13}C . The $^{12}\text{C}^{14}\text{N}^-$ and $^{12}\text{C}^{13}\text{C}^1\text{H}^-$ peaks are clearly resolved but the $^{13}\text{C}_2^-$ is not, requiring a mass resolution of ~ 7200 to separate it from the $^{12}\text{C}^{14}\text{N}^-$ peak (Clode *et al.*, 2007). To avoid any possible interference from the small $^{13}\text{C}_2^-$ peak the detector should be positioned at the arrow marked 'a'.

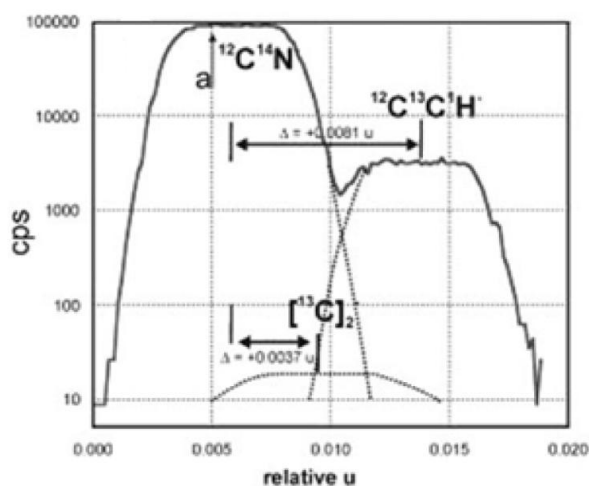


Figure 2.4: A high resolution mass spectra around mass 26 from a coral sample spiked with ^{13}C . The arrow marked 'a' indicates where the detector should be positioned to avoid any interference from the $^{13}\text{C}_2^-$ peak (Clode *et al.*, 2007). Reprinted with permission from John Wiley and Sons.

2.1.2.2.3. Depth Resolution

The final measure of resolution is important when creating a depth profile through a material. The depth resolution is determined by the thickness of the sampling depth from which the secondary ions originate in a single measurement (Liebl, 1975) which is generally about 1 nm (Evans Analytical Group, accessed Oct 2010c). This resolution is degraded by sputtering effects such as atomic mixing, which implants surface atoms some 10 nm into the material, and ion induced surface topography which usually becomes more severe with depth (van der Heide *et al.*, 2003).

2.1.2.3. Sensitivity, Transmission, Mass Resolution and Detection Limits

In ion microscopy, the three key experimental parameters in an analysis are *sensitivity*, *transmission* and *mass resolution*, and the microscopist would generally like, and to some extent needs, all of these to be maximised. Unfortunately these three parameters are related and maximising one will inevitably result in compromising another. The sensitivity of a SIMS instrument is determined by the ionisation efficiency of the element to be analysed and the optical transmission of the ion optics. The first has been seen to be related to ionisation potential or electron affinity as well as chemical environment and the choice of primary beam (Guerquin-Kern *et al.*, 2005). The transmission of the system is given as the ratio of the number of ions formed at the sample surface to the number of ions actually detected, i.e. it is a measure of the number of ions lost along the path to the detectors.

To achieve high mass resolution in a magnetic sector SIMS instrument slits are used to cut off the edges of the secondary beam of ions to produce narrow peaks, as shown in Figure 2.4. This obviously lowers the transmission of the microscope and hence the sensitivity is reduced. Identification of peaks is usually performed under high resolution conditions to avoid mass interferences and the slits are then opened for analysis, reducing the mass resolution but increasing the sensitivity. In a SIMS instrument which uses lenses and apertures to shape the primary beam, such as the NanoSIMS there is another constraint. To achieve a high lateral resolution the primary beam needs to be made as small as possible, which in turn reduces the current in the beam and the sensitivity of the microscope, as fewer secondary ions are generated.

The useful yield, τ , is defined as the ratio of Number of detected ions/Number of sputtered ions and is important as it includes both transmission and ionisation characteristics. Assuming a Poisson distribution for the secondary ions, at least N ($10^4/A\%$) ions must be detected to measure

the secondary ion intensity with an accuracy of A%. The minimum detectable concentration or detection limit, [M], is therefore given by Equation 2.3:

$$[M] = \frac{N}{\rho\tau\nu} \quad \text{Equation 2.3}$$

where ν is the sputtered microvolume (μm^3) and ρ is the density of the sputtered material (atoms μm^{-3}).

2.1.2.4. Steady State

In dynamic SIMS the secondary ion signals from a sample under primary ion bombardment vary in a complex and independent way at the start of the analysis before reaching steady state. This initial region is known as the transient (Wittmaack, 1996). Any analysis performed in this transient region will not be quantitative and comparisons between different samples or analysis on different days becomes impossible. Figure 2.5 shows this effect in a biological specimen, and we can see in that in this case a Cs^+ dose of 10^{17} atoms cm^{-2} is needed to achieve steady state (Guerquin-Kern *et al.*, 2005). In some cases it may not be possible to achieve this steady state at all, for example if the sample is very thin or so sensitive to the ion beam that it may be completely sputtered away before this criterion has been reached. Alternatively the structure and chemistry of the sample may change significantly with depth and all the close-to-surface information would be lost before quantitative analysis can be performed. In static SIMS the dose is far too low to reach steady state so the analysis is often considered to be semi-quantitative. As SIMS is a destructive method it is not possible to improve the accuracy of the measurement simply by analysing the same area again.

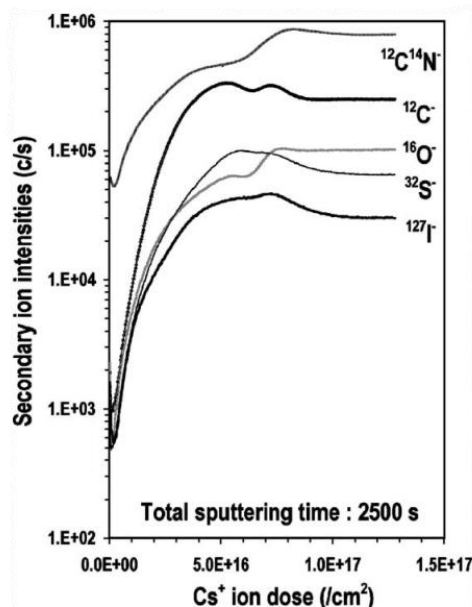


Figure 2.5: Complex ion signal variation from a thyroid follicle as a function of the amount of Cs⁺ implanted before reaching steady state (Guerquin-Kern *et al.*, 2005). Reprinted from *Biochimica et Biophysica Acta*, 1724 (3), Jean-Luc Guerquin-Kern, Ting-Di Wu, Carmen Quintana, Alain Croisy, ‘Progress in analytical imaging of the cell by dynamic secondary ion mass spectrometry (SIMS microscopy)’ 228-238, 2005, with permission from Elsevier.

2.1.2.5. Quantification – Relative Sensitivity Factors

One of the major problems with SIMS is that quantification of the sample composition is very difficult because each element and molecular species has a different ion yield. With careful calibration, each ion of interest can have a measured relative sensitivity factor, and this can be used to give an indication of their suitability for SIMS analysis.

Relative sensitivity factor (RSF) tables have been established for most elements in silicon (Wilson *et al.*, 1989; Evans Analytical Group, accessed Oct 2010c) driven by the semiconductor industry, but RSF tables are now starting to be made for other matrices, including GaN (Evans Analytical Group, accessed Oct 2010a). However, there is a real need to establish these tables for other materials because without this quantification is almost impossible. There has been for instance very little work on RSF values for elements in biological matrices (Ramseyer & Morrison, 1983). The Si tables are still useful in planning experiments because variations of 6 orders of magnitude have been measured for different elements. For instance, under O⁻

bombardment the RSF of Na⁺ ions in Si is 100 times larger than for Si⁺ ions but the value for Br⁺ is 1000x lower (Benninghoven *et al.*, 1987; Wilson *et al.*, 1989). The concentration C_E of an element of interest can be calculated using Equation 2.4:

$$C_E = RSF \cdot \frac{I_E}{I_M} \quad \text{Equation 2.4}$$

where I_E is the detected ion count from the element of interest and I_M is the detected ion count from the matrix element (Wilson *et al.*, 1989). Without an accurate knowledge of these RSF values it is obvious that no reliable information on the relative concentration of these 2 elements in silicon could be obtained. It is useful in SIMS analysis of other materials to use these RSF values in silicon to give at least a rough indication of the likely ion yield.

2.1.2.6. Artefacts in SIMS

As with any technique, there are some important artefacts which have to be borne in mind while conducting SIMS analysis. Some of the effects which can affect secondary ion signals are: phase boundaries; implantation; topography; crater edges; holes; halo effects; diffusion; surface and atomic mixing; the matrix effect; preferential sputtering; induced roughening; redeposition of sputtered material and ion channelling (Levi-Setti, 1988). Some of these effects are controlled by the design of the instrumentation and some are intrinsic to the sputtering process. Some can be minimised or eliminated by careful specimen preparation or instrument and experimental design but others always need to be considered. This is quite a lengthy list of artefacts and, considering the problems associated with quantification and interpretation, one might wonder why SIMS is so popular as an analysis technique. Nevertheless the combination of sensitivity, mass resolution and lateral resolution outweighs these problems in many situations. Some of these artefacts are discussed in the following sections.

2.1.2.6.1. Matrix Effects

In general we expect the ion yield and secondary ion intensity to be closely related to the concentration of the element in the sample, but the chemistry and crystallography of the surrounding matrix can also have a pronounced effect. This is known as the matrix effect and is the general term used to describe differences in sensitivity for a given element in samples of different composition, structure or crystallographic alignment (Wilson *et al.*, 1989). These differences result from changes in the ionisation efficiency and/or the sputtering yield, and the effect is generally further complicated by the use of reactive primary ions. The matrix effect makes it more difficult to measure the concentration of the bulk element than a trace element (Vickerman & Gilmore, 2009) and quantification at internal interfaces is very challenging (Wilson *et al.*, 1989). A well known example of this is given by Wilson *et al.* (1989). The yield of ions of many elements changes across a Si/SiO₂ interface due to the yield enhancement caused by the presence of oxygen. Some differences are shown in Table 2.1.

Element	Relative yield enhancement in SiO ₂
B	6
P	60 – 70
As	20
F	2

Table 2.1: Yield changes at a Si/SiO₂ interface under O₂ primary ion bombardment (Wilson *et al.*, 1989).

Detecting cluster ions containing the primary beam element like MCs⁺ (where M is the atom of interest and Cs⁺ the bombarding species) considerably reduces the variations of intensities due to differences in the sputtering yield, but does not avoid matrix effects altogether (Wilson *et al.*, 1989; Gnaser, 1996; Philipp, 2008). This method allows measurement of the matrix composition but it is often necessary to make a standard sample of known concentration which is similar to the material to be analysed to improve the quantification (Vickerman & Gilmore, 2009). Biological materials are particularly prone to this problem because there tends to be a large

variation in the density in different parts of biological materials which makes the matrix effect very complicated (Burns, 1988). For example the cell membrane is structurally quite different to the nucleus.

2.1.2.6.2. *Surface Topography*

It is very important that the sample surface is flat in order to get reliable SIMS information, as any topography can either enhance or suppress the secondary ion emission. Like secondary electron emission in a scanning electron microscope, the yield of ions at a step edge is often higher and holes can trap sputtered species leading to a lower yield (Levi-Setti, 1988). Topography, if present initially, cannot be removed by sputtering (Wilson *et al.*, 1989). Flat samples also allow optimum transmission to be maintained through the optical path (Guerquin-Kern *et al.*, 2005).

Topography also has a serious effect on depth profiling experiments as the primary ion beam, in particular reactive ions, causes rippling of the crater bottom (van der Heide *et al.*, 2003). As a general rule the surface topography always roughens with increasing ion dose. As the surface does not remain flat during the profile the depth resolution is degraded as not all of the ions are coming from the same vertical position in the sample (Benninghoven *et al.*, 1987).

2.1.2.7. *Sample Charging*

SIMS uses a primary beam of charged particles and for insulating samples this results in a build up of charge on the surface. If in dynamic SIMS a positive primary ion beam is used to produce negative secondary ions, the main effect of positive surface charging is the inhibition or total suppression of negative ions (Vickerman *et al.*, 1989). To minimise charging, insulating specimens are usually coated with a few nanometres of a metal, such as gold or platinum

(Wilson *et al.*, 1989), and it is necessary to sputter this away before analysis can be started. For some very insulating samples a metallic coating is insufficient, or in the case of static SIMS where the top monolayer only is investigated, would prevent analysis of the surface of interest. In these cases an electron flood gun may be used. The bombardment of the surface by low energy electrons is usually sufficient to compensate for the charge build-up (Migeon *et al.*, 1990).

2.2. THE CAMECA NANO SIMS 50

The CAMECA NanoSIMS 50 is an example of a modern dynamic SIMS instrument optimised for high lateral resolution analysis while maintaining high mass resolution ($M/\Delta M = 5,000$) and high sensitivity (ppb for some elements) (Slodzian *et al.*, 1992). For instance a mass resolution of 3500 is achievable while still maintaining 100% transmission (CAMECA, accessed June 2007).

In order to achieve these operating characteristics, the NanoSIMS has several unique features such as coaxial optics and a normal incidence primary ion beam. Working in the ion microprobe mode, the primary ion beam is rastered across the surface and secondary ions are detected as a function of the beam position on the sample, resulting in high lateral resolution and collection efficiency. Figure 2.6 shows schematically the ion optics for the NanoSIMS 50.

The use of reactive primary ions, in this case caesium or oxygen, increases the secondary ion yield of negative and positive ions respectively. If a liquid metal gallium source is used (to achieve a resolution of <50 nm) the elemental signals are 100-1000x lower (CAMECA, accessed June 2007) which would make trace element analysis almost impossible.

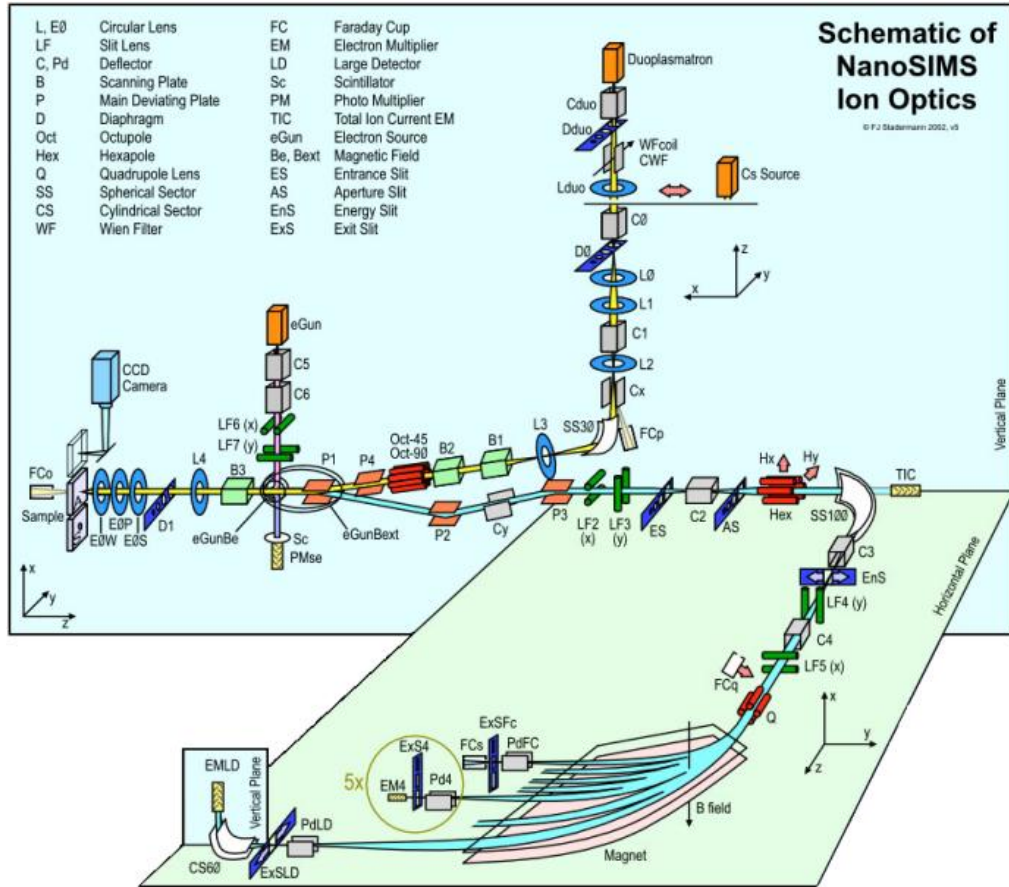


Figure 2.6: Schematic diagram of the NanoSIMS 50 showing the ion optics (Stadermann, accessed Oct 2010). NanoSIMS schematic courtesy of F. J. Stadermann.

The CAMECA NanoSIMS 50 utilises a novel probe forming system which solves many of the problems that were previously encountered in the design of dynamic SIMS instruments. The main conflict in the design of conventional ion optics is that the objective lens of the primary ion column must be as close as possible to the sample to produce a small and intense beam but the extraction optics must also be placed very close to the sample to collect as many secondary ions as possible. The primary and secondary optics both have an irreducible physical size so in conventional optics neither are in their optimum positions. The NanoSIMS solves this problem by simultaneously focusing the primary ion beam and collecting most of the secondary ions through co-linear optics as illustrated in Figure 2.7 (Slodzian *et al.*, 1992). The co-axial optics has several advantages including a shorter working distance, smaller probe size, higher collection efficiency (improved transmission) and minimisation of shadowing effects because of the normal incidence on the sample (CAMECA, accessed June 2007).

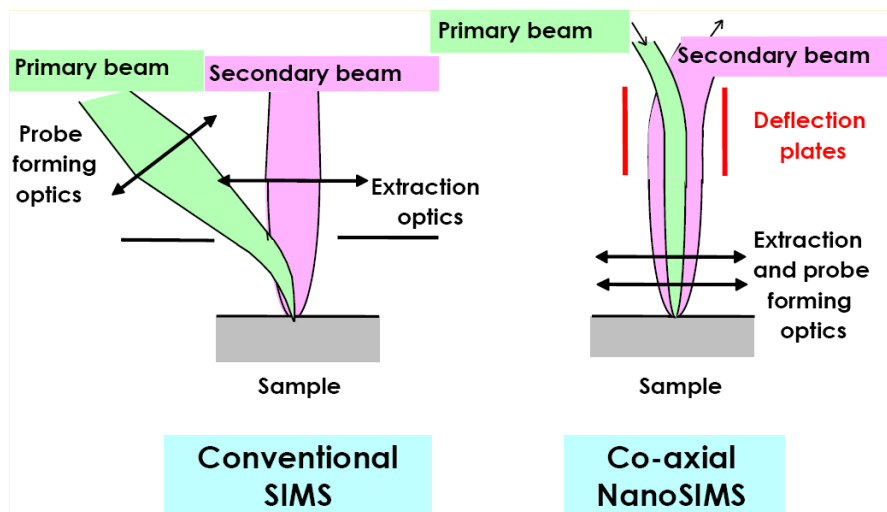


Figure 2.7: Schematic diagram showing the conventional and NanoSIMS probe forming systems (CAMECA, accessed June 2007). Reproduced with permission from CAMECA.

The main disadvantages with this technique are that the primary and secondary ions must be of opposite polarity and equal energy. This constraint means that minimisation of matrix effects using the MCs^+ technique for instance is not possible, and oxygen flooding cannot be used (CAMECA, accessed June 2007). In common with most optical designs the smaller the primary ion beam diaphragm (to improve lateral resolution), the lower the sensitivity, transmission and mass resolution.

The NanoSIMS uses a mass analyser in a Mauttach-Herzog configuration (Benninghoven *et al.*, 1987) with a 90° spherical electrostatic sector and an asymmetric magnet, shown schematically in Figure 2.8. This type of mass spectrometer has reasonable transmission, unrivalled mass resolution ($M/\Delta M > 10^4$) high energy acceptance and preserves the lateral resolution which allows high mass resolution analysis with a small spot size (Vickerman *et al.*, 1989).

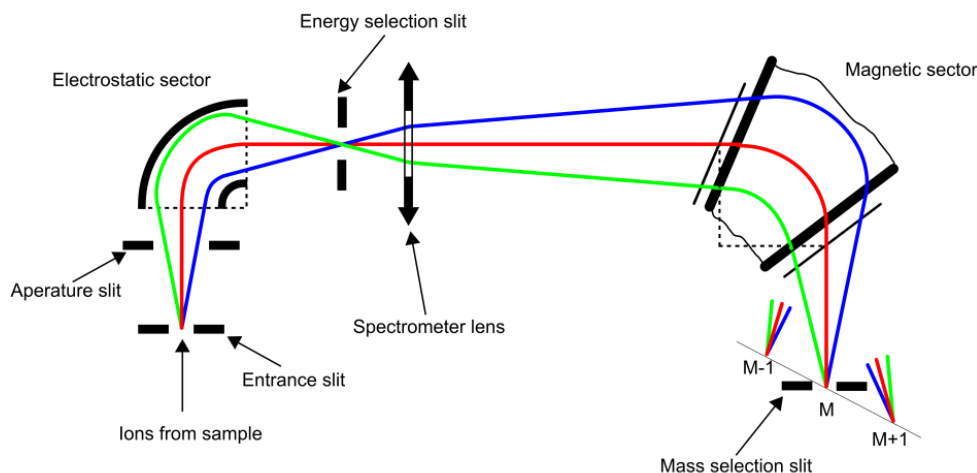


Figure 2.8: Schematic diagram of a double focusing mass spectrometer showing the electrostatic and magnetic sectors. Redrawn from (Migeon, 2008).

Ions sputtered from the surface of a sample have an inherent kinetic energy spread. If these were to enter a magnetic field directly the mass resolution of the spectrometer would be degraded because magnetic fields do not just focus charged particles according to their mass-to-charge ratio but also by their velocity. To counteract this problem an electrostatic sector and a slit are placed before the magnet to create an energy spread before entering the magnetic field. The electrostatic sector is mass independent as indicated by Equation 2.5:

$$r = \frac{2V}{E} \quad \text{Equation 2.5}$$

where r is the radius of the ion, V the applied voltage and E the field (Migeon, 2008).

When the ions enter the magnetic field they are separated according to their mass-to-charge ratio according to Equation 2.6, where r is the radius of the ion, M is the mass, E_k the kinetic energy of the ion, q the charge and B the magnetic field (Hoffmann & Stroobant, 2007). The faster ions are deflected less by the magnetic field but the initial energy spread created by the electrostatic sector means that the energy dependence of Equation 2.6 is compensated.

$$r = \frac{\sqrt{2ME_k}}{qB} \quad \text{Equation 2.6}$$

After mass selection, the secondary ions are detected using electron multipliers which can accurately measure low intensity ion signals. Another advantage of the NanoSIMS design over previous magnetic sector SIMS instruments is the ability to collect several ion signals at the same time, five ions for the NanoSIMS 50 and seven for the NanoSIMS 50L (Slodzian *et al.*, 1992). This multicollection capability allows a more accurate reconstruction of the sample chemistry as each ion image is originating from exactly the same volume which is not the case for images from mass spectrometers which have to acquire images sequentially. This ensures reliable isotopic measurements and image registration.

2.2.1. COMPARISON OF SIMS WITH OTHER TECHNIQUES

Secondary ion mass spectrometry falls somewhere in between the best imaging and elemental detection techniques. Although the lateral resolution of SIMS is not as good as energy dispersive x-ray spectroscopy and electron energy loss spectroscopy in a transmission electron microscope (Newbury, 1986; Benninghoven *et al.*, 1987), these techniques can neither distinguish between isotopes of the same element prohibiting the use of isotopic spiking experiments (Guerquin-Kern *et al.*, 2005) nor detect molecular species. In contrast SIMS does not have the sensitivity of some chemical analysis techniques, but these generally derive their sensitivity from sampling a very large volume which means there is no opportunity to localise elements since spot sizes can be as large as 5-15 mm (Evans Analytical Group, accessed Oct 2010b). Synchrotron based X-Ray techniques, such as X-Ray fluorescence, have an incident beam size approaching the lateral resolution of SIMS analysis but the spatial resolution is significantly lower due to the large interaction volume of the X-Rays. However, synchrotron mapping can be over a very large scale, requires minimal sample preparation as the samples can be analysed in air (Lombi *et al.*, 2011)

and the sensitivity is better than that of SIMS, approaching the ppb range (Lombi *et al.*, 2009). Although other techniques have individual characteristics which are better than SIMS it is the combination of characteristics listed previously which makes SIMS a major competitor.

2.3. BIOLOGICAL SIMS

SIMS has its roots in the semiconductor industry and its development was driven by the need to accurately measure low concentrations of dopants in semiconductors (Wilson *et al.*, 1989). However, it has become widely used for element localisation in geochemistry, metallurgy and electronics (Fragu *et al.*, 1992). The SIMS community has for a long time been dominated by chemists and physicists who have mainly focused on improving the instrumentation with little interaction with the biological community (Heeren *et al.*, 2006). SIMS is essentially a physical science technique, so why is it increasingly being used to study biological specimens? The main reasons are its high sensitivity, detection of all elements and isotopes, molecular imaging combined with a reasonable lateral resolution and the potential for 3D imaging (Burns, 1982; Chandra *et al.*, 2000).

The earliest experiments using SIMS to image biological specimens were performed by Galle, who studied sodium and potassium ions in renal tissue and human blood (Galle, 1970). The first SIMS instruments were, however, not suitable for biological secondary ion mass spectrometry. The primary ion beam was large and intense, with a diameter of 25-250 μm , an energy of 10 keV and a beam current of 10 to 100 nA (Guerquin-Kern *et al.*, 2005). These conditions are highly destructive for most biological samples which tended to be completely vaporised after one analysis. Trace element analysis, which requires long acquisition times, was almost impossible. Other problems included poor mass resolution ($M/\Delta M \sim 300$) and a lateral resolution of around 1 μm (Guerquin-Kern *et al.*, 2005). Both of these values were insufficient for biological analysis, for example it was not possible to separate $^{12}\text{C}^{14}\text{N}^-$ ($m=26.045$) and $^{12}\text{C}_2^1\text{H}_2^-$ ($m=26.024$) or $^{31}\text{P}^{16}\text{O}_3^-$ and $^{89}\text{Br}^-$ or to obtain much intercellular detail in images.

The ability of SIMS to map molecular ions was quickly found to be particularly useful for biological materials. Perhaps the most useful ion is CN^- which is a characteristic fragment of amino acids and therefore allows mapping of protein distributions (Levi-Setti, 1988). It is also widely used to show the general morphology of biological samples (Grignon *et al.*, 1992) since membranes also have a high yield of CN^- , as shown in later examples.

SIMS analysis of biological materials generally falls into two classes. The first is elemental mapping which exploits the sensitivity of SIMS combined with its high mass resolution and lateral resolution. Mony and Larras-Regard used SIMS to investigate the distribution of selenium in mouse kidney after diet supplementation with different chemical forms of selenium (Mony & Larras-Regard, 2000). In this study they found that the selenium was mainly localised in the cells of the proximal tubules and the basal lamina around these cells indicating that these are important in controlling the bioavailability of selenium and preventing it reaching toxic levels. The authors noted that the selenium was not always localised with sulphur indicating a more complex metabolism than a simple substitution for sulphur. The lateral resolution in this study was limited to about $0.2\text{ }\mu\text{m}$ so cellular features are not visible. Figure 2.9 shows NanoSIMS images of a section from a rat kidney after ingestion of a selenium-containing drug allowing direct imaging of the drug and resolution of intracellular features which was not possible in the study by Mony and Larras-Regard (2000).

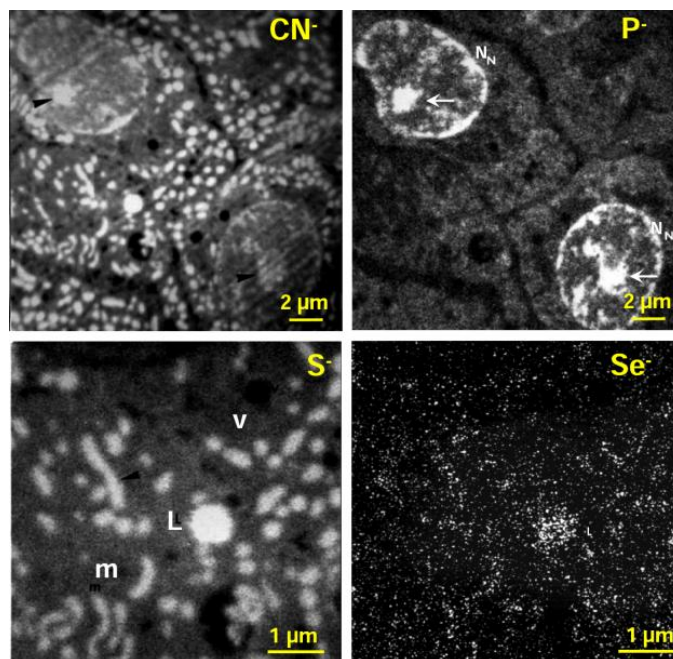


Figure 2.9: NanoSIMS images of a rat kidney after ingestion of a Se-containing drug, bottom images show the lysosome region at higher magnification (mitochondria (M), lysosome (L), nucleus (N)) (CAMECA, accessed Apr 2008). Reproduced with permission from CAMECA, sample from M-C Mony and E. Larras-Regard.

The second main use of SIMS is in ratio or spiking measurements where stable isotopes (Clode *et al.*, 2007; Clode *et al.*, 2009) or “exotic” atoms (Guerquin-Kern *et al.*, 2005; Lechene *et al.*, 2006; Behrens *et al.*, 2008) are incorporated as labels into cells or tissue samples as a means to understand transport and sequestration mechanisms, and uses the high mass resolution and spatial resolution of SIMS. Labels such as ^{13}C or ^{15}N are treated by cells in essentially the same way as the more naturally occurring isotopes (Clode *et al.*, 2007) but are easily distinguished by SIMS (Figure 2.4). This technique is more commonly used in dynamic SIMS instruments because there are fewer mass interferences and, unlike ToF-SIMS instruments, they are not able to map for the larger fragments which could provide direct information about the position of specific molecules. There are several elements which have isotopes with a low natural abundance that can be used for spiking experiments, many of which are relevant to biological materials, including ^2H , ^{13}C , ^{15}N , ^{18}O , ^{34}S , ^{41}K , and ^{44}Ca .

In Figure 2.10 ^{15}N has been used to monitor protein renewal in the mouse cochlea using ^{15}N -leucine and to demonstrate quantitative analysis of isotopes in subcellular compartments. The

mouse cochlea is a highly organised tissue with several different cell types. Located in a subcellular structure of this tissue is the stereocilium, the mechanosensing organelle of hair cells. As nitrogen is not easily ionised in SIMS analysis it must be measured as the CN^- ion (Clode *et al.*, 2009). Initial experiments on untreated control samples proved that the NanoSIMS could be used to measure a $^{15}\text{N}/^{14}\text{N}$ ratio of 0.368% in the cochlea very similar the natural isotope ratio of 0.3673% even though the structures are only 250 nm across (Lechene *et al.*, 2006). Figure 2.10 shows a NanoSIMS image of the stereocilia, labelled Sb1, from a mouse which was fed on a ^{15}N -leucine diet for 9 days. The $^{12}\text{C}^-$, $^{13}\text{C}^-$, $^{12}\text{C}^{14}\text{N}^-$ and $^{12}\text{C}^{15}\text{N}^-$ images were acquired simultaneously, and the $^{12}\text{C}^{15}\text{N}^- / ^{12}\text{C}^{14}\text{N}^-$ and $^{13}\text{C}^- / ^{12}\text{C}^-$ ratio images were obtained by dividing the images pixel-by-pixel after analysis. There is strong contrast in the $^{12}\text{C}^{15}\text{N}^- / ^{12}\text{C}^{14}\text{N}^-$ ratio image because ^{15}N has been incorporated into the proteins, but the $^{13}\text{C}^- / ^{12}\text{C}^-$ ratio image has no contrast because no additional ^{13}C was added. A hue saturation intensity (HSI) image can be used to illustrate the ^{15}N incorporation into the tissue, which can then be expressed as a percentage of protein renewal, with values ranging from 0% (blue) to 60% (pink) as shown in Figure 2.10 (g).

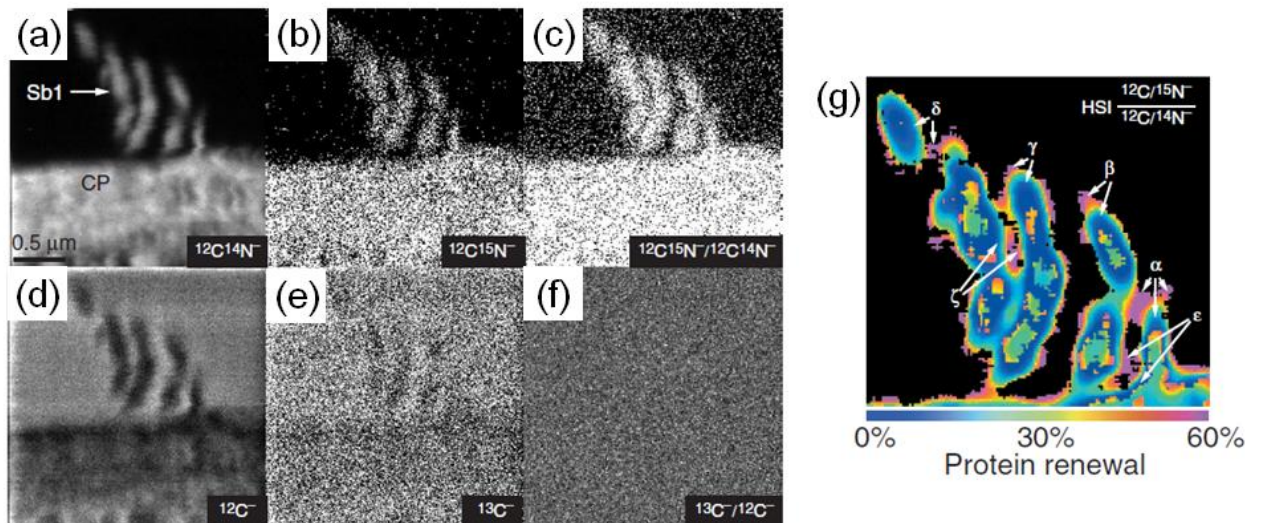


Figure 2.10: NanoSIMS images of stereocilia from a mouse cochlea fed with ^{15}N -Leucine for 9 days (Lechene *et al.*, 2006). (a) $^{12}\text{C}^{14}\text{N}^-$ (b) $^{12}\text{C}^{15}\text{N}^-$ (c) $^{12}\text{C}^{15}\text{N}^- / ^{12}\text{C}^{14}\text{N}^-$ ratio image (d) $^{12}\text{C}^-$ (e) $^{13}\text{C}^-$ (f) $^{13}\text{C}^- / ^{12}\text{C}^-$ ratio image (g) HSI image of the $^{12}\text{C}^{15}\text{N}^- / ^{12}\text{C}^{14}\text{N}^-$ ratio derived from (a) and (b). The colours correspond to the excess ^{15}N derived from the measured $^{12}\text{C}^{15}\text{N}^- / ^{12}\text{C}^{14}\text{N}^-$ isotope ratio, expressed as a percentage of the ^{15}N excess in the feed, which is a measure of protein renewal. The HSI image is $3 \times 3 \mu\text{m}$. With permission from BioMed Central Ltd.

In the second example, ^{13}C ions have been mapped to understand the uptake and metabolism of carbon in coral. This animal was chosen because it is thought that symbiotic algae photosynthetically fix inorganic carbon from seawater and subsequently translocate this to the coral host. In order to understand coral reef formation and the growth and storage of these fixed carbon compounds it is important to understand the role of the individual cell types (Clode *et al.*, 2007). Colonies of coral were incubated in seawater artificially enhanced with $\text{NaH}^{13}\text{CO}_3$ before fixing, decalcification, embedding and sectioning. The $^{13}\text{C}^-$ signal was readily detected with the NanoSIMS and the sub-cellular resolution enabled the ^{13}C distribution to be clearly visualised within the algal symbionts, as can be seen in Figure 2.11. The authors demonstrate that it is necessary to have sufficient mass resolution to be able to distinguish $^{13}\text{C}^-$ from the isobaric interference of $^{12}\text{C}^1\text{H}^-$ ($M/\Delta M > 1660$), and that the position of the detector needs to be carefully chosen to avoid the low mass tail of the $^{12}\text{C}^1\text{H}^-$ as shown in Figure 2.11B. Taking a ratio of the $^{13}\text{C}^-$ image with the $^{12}\text{C}^-$ image, as shown in Figure 2.11A d) minimises the effects of topography and matrix effects which could alter the ion yield of the C^- ions. The $^{13}\text{C}/^{12}\text{C}$ image in Figure 2.11 shows regions of significant ^{13}C accumulation in a ring-like structure in the algal symbiont.

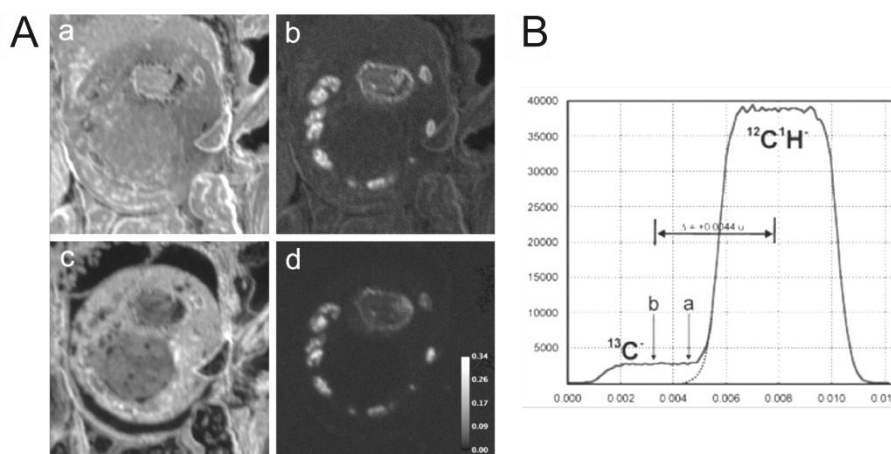


Figure 2.11: A) High resolution NanoSIMS images of a single symbiotic alga in a ^{13}C -labeled coral *Galaxea fascicularis* (Clode *et al.*, 2007): (a) $^{12}\text{C}^-$; (b) $^{13}\text{C}^-$; (c) $^{12}\text{C}^{14}\text{N}^-$; (d) $^{13}\text{C}/^{12}\text{C}^-$. FOV = 10 μm . Scale on (d) indicates $^{13}\text{C}/^{12}\text{C}$ ratio values with a minimum of 0 and a maximum of 0.34. B) Mass scan around mass 13 showing the interference of the $^{12}\text{C}^1\text{H}^-$ peak on the $^{13}\text{C}^-$ peak, positioning of the detector at point b avoids interference from the low mass tail of the $^{12}\text{C}^1\text{H}^-$ peak which would occur at point a. Reprinted with permission from John Wiley and Sons.

2.3.1. SAMPLE PREPARATION OF BIOLOGICAL MATERIALS FOR SIMS ANALYSIS

SIMS analysis needs to be performed under ultra high vacuum conditions ($<10^{-7}$ Pa) to prevent scattering of the primary and secondary ions by residual gas molecules and analysis of contaminants adsorbed onto the surface (Vickerman *et al.*, 1989; Downard, 2004). Biological materials inherently consist of a large fraction of water (about 80% (Cooke & Kuntz, 1974; Mentre, 1992)) and this must be removed before analysis can be undertaken. It is this process which is the most difficult and causes the most problems in the study of samples from the life sciences (Chandra & Morrison, 1995). Guerquin *et al.* (2005) state that “An ideal sample preparation should preserve both the structural and chemical integrity of the living cell. However, this requirement must be adapted to the instrument resolution and appropriate to the specificity of the analysis under investigation”.

There is a considerable literature on these sample preparation issues and all the authors stress the care that must be taken to achieve a final specimen with chemistry representative of the living sample.

2.3.1.1. Chemical Fixation

This type of relatively simple preparation is suitable for situations where the analyte of interest is tightly bound to a cellular structure, for example to a membrane, organelle or DNA. This process involves immersing the tissue into a fixative such as, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, dehydration in a series of increasing ethanol solutions and finally resin embedding (Hayat, 2000). Although this method is suitable for structural analysis by electron microscopy, and is one of the simplest methods of preparing biological specimens, it causes complete redistribution of diffusible ions such as Na^+ and other small molecules, and is therefore an inappropriate method if the *in vivo* distribution of these ions is to be studied with SIMS (Stika *et al.*, 1980; Chandra & Morrison, 1992; Mentre, 1992).

An illustration of chemical fixation is shown in the following example. The most common method currently used to locate markers and labels in cells is to use an optical technique such as fluorescence microscopy. This technique although powerful and very widely used suffers from several problems (Lau *et al.*, 2010). It requires the use of bulky dyes which can modify the behaviour of the molecule of interest in the cell (Guerquin-Kern *et al.*, 2005), is diffraction-limited to 200-700 nm and the molecule of interest may not be naturally fluorescent. Imaging SIMS is increasingly finding use in the study of the metabolisms of cells as well as in research on targeted drug delivery and the localisation of metals, as the technique and instruments improve. By using an element or isotope which is not naturally found in biological samples, small molecules can be labelled without affecting the shape, binding potential or lipophilicity, and the chemical localisation of many molecules or non-fluorescent compounds can be directly explored. Lau *et al.* (2010) recently presented results comparing on the same cell the distribution of 5-bromo-2-deoxyuridine (BrdU) in HeLa cells mapped with conventional fluorescent immunochemistry and NanoSIMS analysis of the $^{79}\text{Br}^-$ and $^{80}\text{Br}^-$ ions (Lau *et al.*, 2010). A typical result is shown in Figure 2.12 and the distribution of BrdU from the two techniques is identical. They subsequently demonstrated the imaging of the distribution of a Mo-containing drug, ATN-224, a therapeutic copper chelator for which there is no fluorescent marker. In this case NanoSIMS imaging not only provided a means of locating the drug in the cell, but when combined with organelle specific fluorescent staining the drug could be located to individual lysosomes.

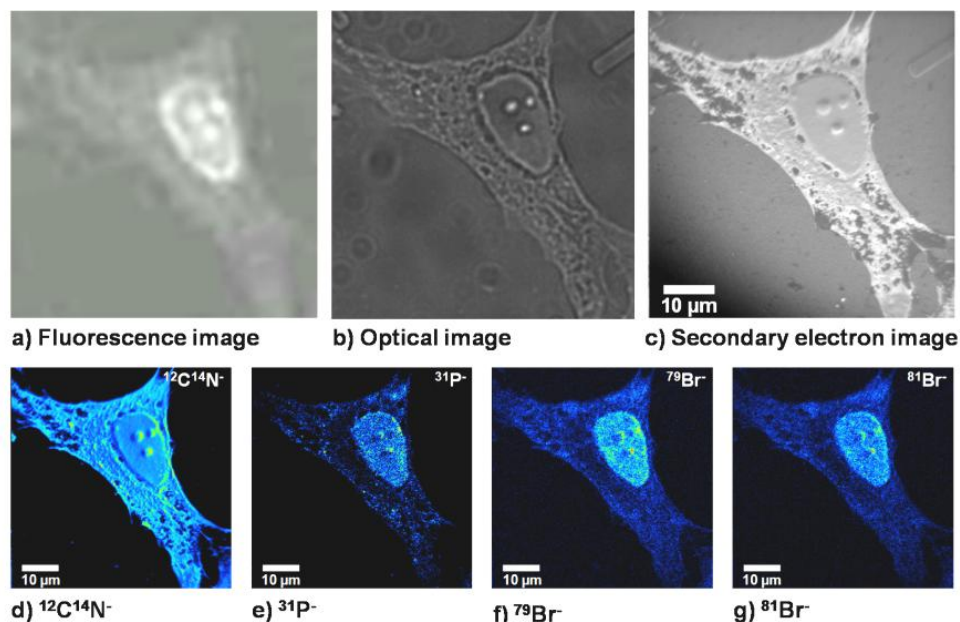


Figure 2.12: Images of the same HeLa cell taken with a) fluorescent, b) optical and c)-g) NanoSIMS microscopes. The position of the nucleoli is identical in all the images (Lau *et al.*, 2010). Reprinted with permission from John Wiley and Sons.

2.3.1.2. Cryofixation

Cryofixation is the main alternative to chemical fixation (Levi-Setti, 1988; Dahl & Staehelin, 1989). This route attempts to immobilise all the molecules and ions in their *in vivo* state by freezing the sample so fast that ice forms in the amorphous state and takes up the same volume as the liquid water it replaces (Steinbrecht & Zierold, 1987; Studer *et al.*, 2001). Growth of ice crystals must be avoided as they are normally acicular and will damage the cells (McDonald & Auer, 2006). Having made this choice of sample preparation there are various routes that can be taken (Giddings & Staehelin, 1980; Livesey & Linner, 1987; Kellenberger, 1991; Chandra & Morrison, 1992; Hajibagheri & Flowers, 1993; Huang *et al.*, 1994).

If the SIMS instrument has a cryostage then it is possible to analyse the sample in the hydrated state. This has the advantage that complex sample preparation methods do not need to be used and the redistribution of diffusible ions should be avoided, but the ion yields from a frozen sample can be difficult to quantify, water is preferentially removed along the z-axis and matrix effects can be severe (Chandra *et al.*, 1986; Derue *et al.*, 2006b; Chandra, 2008). Figure 2.13

shows cryo-ToF-SIMS images of the metaxylem from a cross section of a bean stem indicating how this technique can be used to monitor the uptake of nutrients into plant specimens (Metzner *et al.*, 2008). This image was taken with a spatial resolution of $\sim 1\ \mu\text{m}$ allowing the distribution of the elements of interest to be mapped at a subcellular level. For instance, it is possible to see the dendritic ice crystals within each of the cells showing that the sample is not entirely vitrified. The water distribution in the sample is shown in Figure 2.13A by the $^1\text{H}_3^{16}\text{O}^+$ image which is the most abundant water-related ion species in this case (Metzner *et al.*, 2008). Figure 2.13E clearly shows that the tracer for K transport is predominantly localised in the xylem vessels with some spreading to neighbouring tissues. In contrast the Na is localised in the xylem parenchyma, the cells which surround the xylem. This is consistent with what is understood about nutrient transport properties in these cells, and show the potential of cryo-ToF-SIMS in detecting multiple tracers at high resolution and sensitivity for studying these mechanisms in great detail.

Figure 2.13 has been removed due to Copyright restrictions.
The original image can be found in the following reference:

Figure 5 from Metzner R, Schneider HU, Breuer U, Schroeder WH. 2008. Imaging nutrient distributions in plant tissue using time-of-flight secondary ion mass spectrometry and scanning electron microscopy. *Plant Physiology* 147: 1774-1787.

Figure 2.13: Cryo-TOF-SIMS images of the surface of a bean stem cross section showing the metaxylem. The images show the maps of $^1\text{H}_3^{16}\text{O}^+$, $^{23}\text{Na}^+$, $^{39}\text{K}^+$, and $^{41}\text{K}^+$. E shows the ratio of $^{41}\text{K}^+$ to $(^{39}\text{K}^+ + ^{41}\text{K}^+)$ in percent. The field of view is $195 \times 195\ \mu\text{m}$ (Metzner *et al.*, 2008).

For microscopes which do not have a cryostage the best preparation route is to fix and dehydrate the sample at low temperature to avoid redistribution artefacts, and then infiltrate with resin.

Embedded samples can then be sectioned using a microtome and deposited on a silicon wafer for analysis. As an example of the preservation of the structure of delicate (highly vacuolated) plant samples by high pressure freezing, Figure 2.14 shows elemental maps from the nickel hyperaccumulator *Alyssum Lesbiacum* at the subcellular scale (Smart *et al.*, 2010). Ultra-rapid cryofixation is generally accepted to be the preferred method for preserving the most challenging biological material in the *in vivo* state (Steinbrecht & Zierold, 1987; Chandra *et al.*, 2000; Smart *et al.*, 2010; Sparks *et al.*, 2010), and so high-pressure freezing (HPF) followed by freeze substitution with acetone and resin embedding was chosen for this sample. Samples were sectioned with a microtome to a thickness of 0.5 μm and deposited onto Si wafers. The images in Figure 2.14 show a cross-section of a leaf with a stomata complex, epidermal cells and palisade mesophyll cells. The left hand set of images (Figure 2.14A) were taken with the Cs^+ beam and the right (Figure 2.14B) with the O^- beam. The Cs^+ beam gives a much higher resolution, better than 80 nm in the CN^- image, allowing features such as the cell cytoplasm and starch grains within a single chloroplast in one of the two stomatal guard cells to be clearly resolved. The nickel distribution (dry shoot Ni content of $7.87 \pm 1.89 \text{ g/kg}$) is uniform across the central vacuole of the epidermal cells. Analysis of the electropositive ions, however, shows that the *in vivo* distribution of the highly diffusible ions such as $^{23}\text{Na}^+$, $^{24}\text{Mg}^+$, $^{39}\text{K}^+$ and $^{40}\text{Ca}^+$ have been altered significantly during the sample preparation. It is now well established that the Na/K and K/Ca ratios give an important calibration of the quality of the sample preparation and less rigorous sample preparation will result in the complete redistribution of these elements (Chandra & Morrison, 1995; Derue *et al.*, 2006a; Smart *et al.*, 2010). For example the K/Ca ratio in the epidermal cell vacuoles is much lower than would be expected for living cells so even with the use of advanced sample preparation techniques achieving excellent morphological preservation it is hard to avoid redistribution of the fastest diffusing ions.

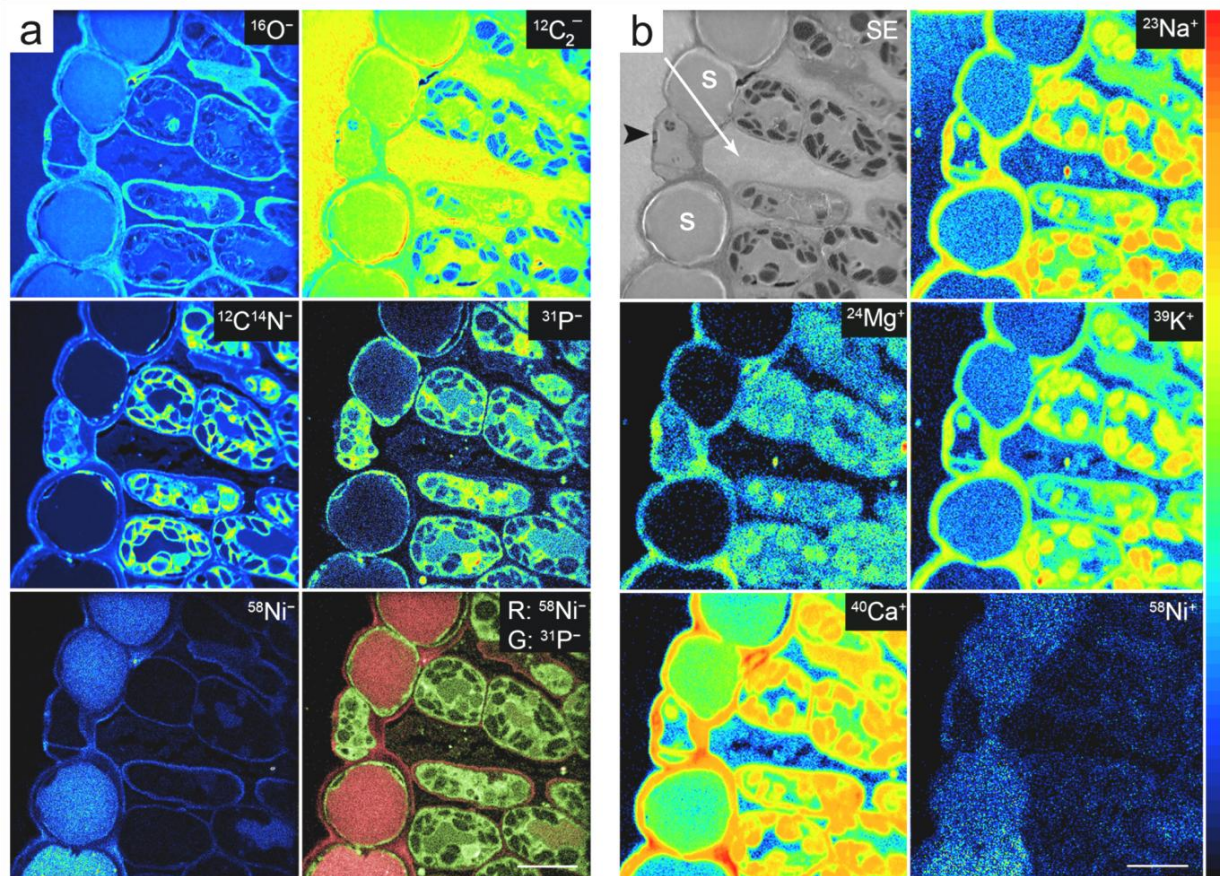


Figure 2.14: NanoSIMS analysis of a cross-section of a nickel rich *Alyssum Lesbiacum* leaf prepared by high pressure freezing and freeze substitution (Smart *et al.*, 2010). A) NanoSIMS maps from a peripheral region of the leaf cross-section including a stomatal complex obtained using the Cs^+ primary ion beam showing $^{16}\text{O}^-$, $^{12}\text{C}_2^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{31}\text{P}^-$ and $^{58}\text{Ni}^-$ ion maps and a colour overlay for Ni and P. B) NanoSIMS maps obtained using the O^- primary ion beam for the distribution of $^{23}\text{Na}^+$, $^{24}\text{Mg}^+$, $^{39}\text{K}^+$, $^{40}\text{Ca}^+$ and $^{58}\text{Ni}^+$ signals from the same area shown in A). The secondary electron (SE) image was acquired with the Cs^+ beam and shows the morphology of the sample. Scale bar = 10 μm . Reprinted with permission from John Wiley and Sons.

2.4. CONCLUSIONS

SIMS instrumentation has been improved in the past 15 years to the point where high resolution imaging of elemental and molecular species can be reliably carried out even on dilute species. The range of sample types that can be analysed has also been expanded, so that imaging SIMS is now a general purpose analytical technique covering the whole range of physical science and biological materials.

3. EXPERIMENTAL TECHNIQUES

This chapter gives an overview of the sample preparation and analytical techniques used to undertake the work reported in this thesis. A more detailed description of the specific sample preparation protocols relevant to the different materials investigated is given at the start of each chapter, and where different NanoSIMS tunings were used, this is indicated in the text.

3.1. *SAMPLE PREPARATION*

Sample preparation for the mature cereal grains was relatively simple and could be carried out at the Department of Materials without the need for specialist equipment. Where more advanced sample preparation was required for the immature wheat grains and rice roots the facilities in the Plant Sciences Department of Oxford Brookes University were used under the guidance of Dr. Barry Martin and Professor Chris Hawes.

3.1.1. HIGH PRESSURE FREEZING

A BAL-TEC HPM 010 high pressure freezer (HPF) (BAL-TEC AG) was used to rapidly freeze samples of immature wheat grain and rice roots. The machine is shown in Figure 3.1; the sample holder, loaded into position, is shown on the left and the bath of liquid nitrogen, for manipulating and storing samples post freezing, is shown in the centre. Plant material must be sectioned to fit inside the planchettes, which are shown schematically in Figure 3.2. A maximum thickness of 0.6 mm is possible, although thinner sections will freeze better, with a maximum diameter of 2 mm. Samples were sectioned under MES buffer (2-(N-morpholino)ethanesulfonic acid) to prevent the samples drying out. The sectioned material was coated with hexadecene, a non-penetrating cryoprotectant, to fill any airspaces around the section before a second planchette was placed on top and the planchettes were clamped together in the sample holder. Once the sample holder was inserted into the machine the planchettes were exposed to a pressure of 210

MPa while pressurised jets of liquid nitrogen at $-196\text{ }^{\circ}\text{C}$ impinged on the sample for a duration of 30 ms at which point the temperature of the sample reached $-50\text{ }^{\circ}\text{C}$ and was completely frozen. After freezing, planchettes were immediately removed from the sample holder and placed into liquid nitrogen where they were split apart and the half containing the sample was placed into a cryotube and stored for further processing.

High pressure freezing has a relatively slow cooling rate in comparison to some other freezing techniques, however it is able to freeze much thicker samples with nearly complete vitrification because the high pressure suppresses the formation of ice crystallisation and growth therefore minimising ice crystal damage (Moor & Riehle, 1987; Hayat, 2000).



Figure 3.1: The BAL-TEC HPM 010 high pressure freezing machine

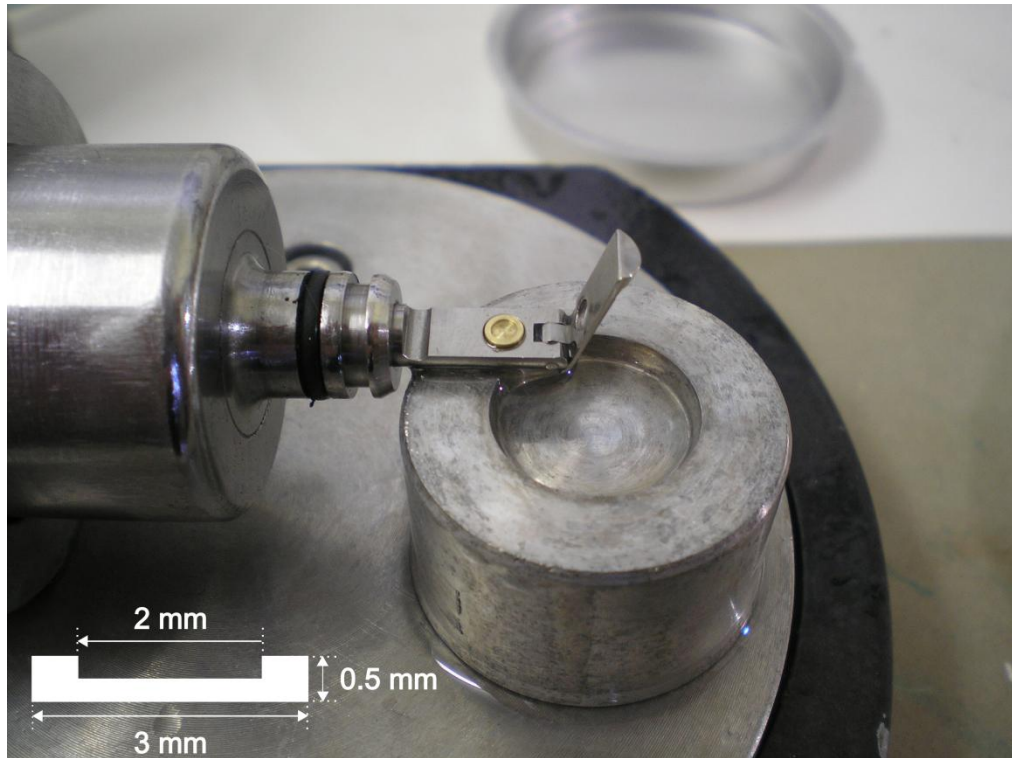


Figure 3.2: A planchette loaded into the open HPF sample holder. The end is closed to ensure a tight seal between the planchettes and is loaded into the machine as shown in Figure 3.1. The insert shows a cross section of the planchette inserted into the holder.

3.1.2. FREEZE SUBSTITUTION

Freeze substitution involves replacing water in the sample with an organic solvent, in this case acetone, at a temperature below which secondary ice crystals can form, that is, below about -70°C (Morphew, 2002; McDonald, 2007). Once substitution is complete, samples can be warmed to room temperature without fear of recrystallisation or ultrastructural changes as water is now absent. Osmium tetroxide was added to the freeze substitution system not only to provide contrast in the TEM but also to act as a fixative by permanently cross-linking lipids and proteins (Lewis & Knight, 1992). By adding the fixative at low temperature it becomes distributed throughout the cell prior to the system reaching the temperature at which fixation can take place, minimising the need for diffusion across large distances which is required during chemical fixation (McDonald, 2007).

The following freeze substitution protocol is derived from protocols for the preparation of plant, animal and insect material in the literature (Steinbrecht & Zierold, 1987; Studer *et al.*, 2001) and has been developed for plant material over many years by Dr. Barry Martin (Plant Sciences Department at Oxford Brookes University). Freeze substitution was carried out using the Reichert AFS (Leica) freeze substitution system which was pre-cooled to -160 °C. Planchettes were loaded into microporous pots and placed on top of acetone slush in universal aluminium containers in the freeze substitution system along with an equal number of aluminium containers containing 2 % osmium tetroxide in acetone (HPLC Grade). The freeze substitution system was programmed to maintain -160 °C for 30 minutes before heating the sample at a rate of 15 °C/h to -85 °C where the temperature was maintained for 24 h at which point the acetone melts and the specimen microporous pots were transferred into the thawed osmium tetroxide solution. The freeze substitution machine was then programmed to -85 °C for 26 h, heating at a rate of 2 °C/h, 8 hours at -60 °C, heating at a rate of 2 °C/h, 24 h at -30°C heating at 1 °C/h and finally held at -20 °C for 24 hours. At this point samples were removed from the freeze substitution system and transferred to a fridge at 4 °C for 24 h.

3.1.3. RESIN EMBEDDING

All subsequent stages were carried out at room temperature. Samples were washed in acetone three times, each time for 20 minutes, after which they were carefully removed from the microporous pots and the individual samples were removed from the planchettes. The sections were embedded gradually in TAAB Low Viscosity Resin (Medium) using a graded resin/acetone (v/v) series: 10, 25, 50, 75 and 100% with each step lasting 2 hours apart from the 100 % resin step where the samples remained in resin overnight. Over the next 4 days the samples were kept in 100 % TAAB Low Viscosity Resin (Medium) and the resin was changed every morning and afternoon. Individual sections were placed into embedding moulds filled with resin and polymerised at 70 °C for 24 hours.

3.1.4. MICROTOMY

Resin blocks were shaped with a razor blade to make block faces trapezoidal. A Reichert Ultracut E Ultramicrotome with a diamond knife was used to create 1 μm thick sections for NanoSIMS analysis. Sections were picked up with a hair and deposited onto a droplet of water onto the NanoSIMS substrates. Sections were stretched flat on a hotplate and were examined with a light microscope to assess the structural preservation of the sections as shown in Figure 3.3.

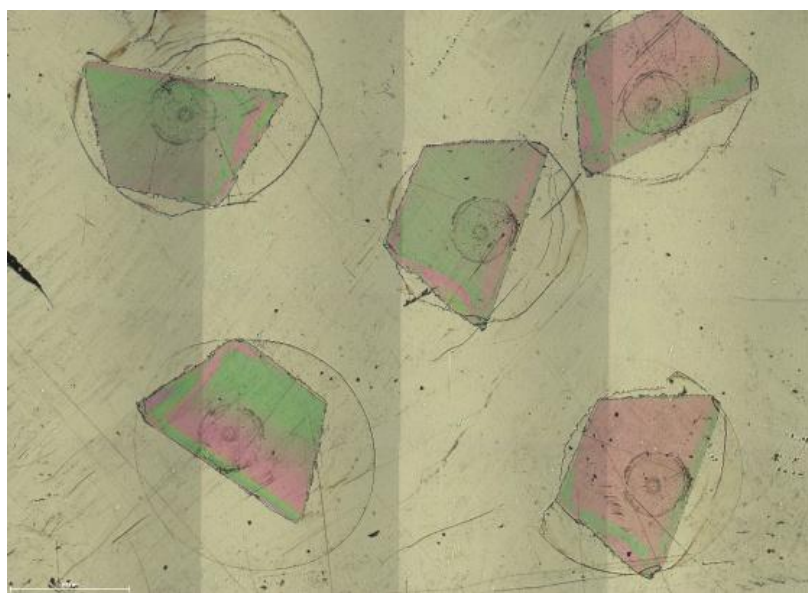


Figure 3.3: Light micrograph image showing stretched microtome sections on a Pt coated plastic substrate

Ultrathin TEM sections were cut at approximately 80 nm (silver coloured sections) and collected directly onto formvar coated copper grids.

3.1.5. STAINING SAMPLES

To assess the quality of structural preservation of the resin embedded samples, initial microtomed sections were examined using transmission light microscopy. To enhance contrast, sections were stretched flat on an optical slide and stained using a droplet of 1% toluidine blue in 1 % sodium borate solution for 30 s on a hot plate. The stain was washed off with distilled water, the slide dried and then examined with the optical microscope. If the structural preservation was

high, i.e. the section was complete and there were few tears in the plant material, further sections were cut and deposited onto the NanoSIMS substrates and left unstained.

TEM samples were stained with uranyl acetate for 5 minutes in the dark followed by a 5 minute stain with lead citrate. Samples were washed after each stain by dipping them rapidly into ultra-pure water several times and left to dry before inserting them into the TEM.

3.1.6. PLATINUM SPUTTER COATING

Just prior to loading samples into the NanoSIMS they were all coated with Pt using a Cressington 208HR high resolution sputter coater to a thickness of 10 nm, monitored with a thickness controller. For the mature cereal grains the coater stage was tilted and rotated during coating to ensure an even coating of Pt across the slightly topographic surfaces. For the flatter microtomed sections the coater was not tilted, only rotated.

3.2. *LIGHT MICROSCOPY*

An Axioskop 2 MAT light microscope with an AxioCam digital camera (Carl Zeiss MicroImaging GmbH) was used in reflectance mode to assess the quality of sample preservation and to map the samples prior to loading into the NanoSIMS so that features of interest could be easily correlated and identified using the NanoSIMS digital camera (CCD).

3.3. *SCANNING ELECTRON MICROSCOPY AND ENERGY DISPERSIVE X-RAY SPECTROSCOPY*

Three scanning electron microscopes (SEM) were used for this work, a JEOL JSM-6300 and a JEOL 840A microscope both with tungsten filaments and a JEOL 840F field emission instrument. Samples for X-ray microanalysis were coated with carbon using a Cressington 208C carbon coater for a duration of 12 s. Energy dispersive X-ray microanalysis (EDX) was carried

out on both the JEOL 840A and JEOL JSM-6300 using Oxford Instruments INCA software. Mapping of the samples required long acquisition times, several hours, in order to detect the low sulphur concentrations. As and Se in the samples used for this study were below the detection limits of this technique.

3.4. *NANO SIMS*

High resolution SIMS analysis was undertaken using the CAMECA NanoSIMS 50 with most of the work in this thesis being undertaken on this instrument situated in the Department of Materials, Oxford.



Figure 3.4: The Oxford CAMECA NanoSIMS50 with the key features labelled

A detailed description of the operation of the NanoSIMS is given in the literature review. It should be stated that during the course of this work two important changes were made to the NanoSIMS instrumentation which affected the imaging quality and transmission of the

instrumentation. In November 2009 a new lens block which holds the three immersion lenses for focusing the primary ions and extracting the secondary ions was replaced and the widths of the ES slits were narrowed. All the work on the rice roots was undertaken after the slits were changed therefore the results were taken with a comparable transmission through the machine which is important for quantification.

3.4.1. ION SOURCES

Due to the coaxial optics used in the NanoSIMS, it is possible only to detect secondary ions of opposite polarity to the primary ions. This means that the NanoSIMS requires two separate ion sources, a Cs^+ source to generate negative ions and a duoplasmatron source to generate positive ions. For the majority of the work presented in this thesis the Cs^+ source was used as the ions of interest, Se and As, have the highest yield when emitted as negative ions. Figure 3.5 shows a schematic of a Cs^+ thermal ionisation source.

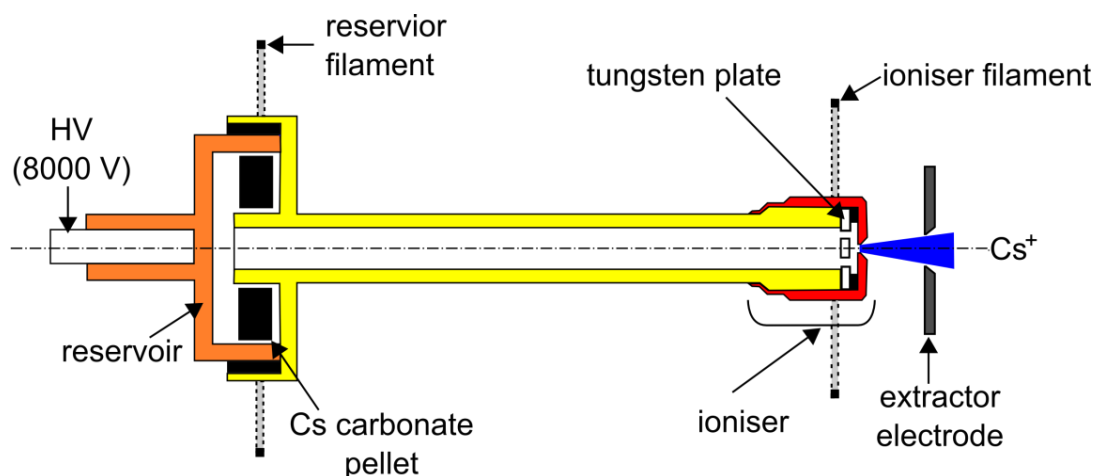


Figure 3.5: A schematic diagram of a Cs^+ thermal ionisation source. Redrawn from (CAMECA, accessed Feb 2011)

Caesium vapour is generated from a caesium carbonate pellet (Cs_2CO_3) which is heated via a filament to 400 °C. A tungsten plate is enclosed in the ioniser head which is heated to 1100 °C and when the vapour comes into contact with this the Cs ionises to Cs^+ ions (CAMECA, accessed Feb 2011). When an electric field is applied to the tungsten plate the ions are extracted

and can be used as primary ions. The reservoir and ioniser are heated separately by electron bombardment via annular filaments. An extraction electrode placed in front of the ioniser generates an electric field extracting and accelerating the Cs^+ ions (CAMECA, accessed Feb 2011).

A limited amount of data was acquired using the duoplasmatron. Oxygen gas is introduced into a hollow cathode and an arc between the hollow cathode and the anode initiates a plasma. A conical intermediate electrode then maintains the discharge and the magnetic coil focuses the plasma onto the axis. The duoplasmatron generates both positive and negative ions (O_2^+ , O^+ , O_2^-) but on the NanoSIMS only the O^- ions are selected. This is achieved using a combination of a positive voltage on the extraction electrode and because the O^- ions are only situated towards the edge of the plasma the axis of discharge must be displaced relative to the extraction hole. A Wien filter is then used to scan across the mass range to more accurately select the O^- ions and generate a smaller spot size (CAMECA, accessed Feb 2011). A schematic diagram of the duoplasmatron is shown in Figure 3.6.

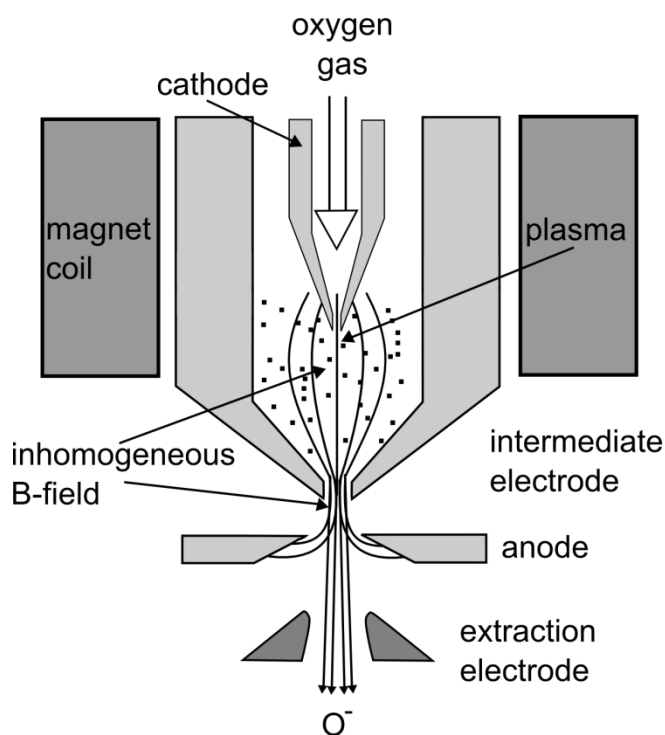


Figure 3.6: A schematic diagram of a duoplasmatron. Adapted from (Evans Analytical Group, accessed Feb 2011)

3.4.2. TUNING THE INSTRUMENT

Full tuning of the instrument was carried out periodically over the course of this work which involved tuning the secondary ion optics to maximise transmission and mass resolving power. This tuning was undertaken on a silicon square patterned mesa which also allowed the focusing and astigmatism to be checked.

At the start of each session the magnet was set to the highest mass of interest and allowed to stabilise before the start of analysis. The source was heated and the current was maximised by changing the voltages on the C0x, C0y, C1x and C1y deflector plates. The current was measured for each of the values of the aperture D1. Typical values and ratios for the different aperture sizes are shown in Table 3.1. The positions of the trolleys were then tuned carefully using high mass resolution (HMR) scans on appropriate standards.

Aperture position	D1 diameter (μm)	Cs ⁺ current (pA)	Duoplasmatron current (pA)
D1 = 0	NA	106	709
D1 = 1	750	12	87
D1 = 2	300	2.5	18
D1 = 3	250	1	7
D1 = 4	200	0.6	—

Table 3.1: Table showing the sizes of the D1 apertures and the typical associated currents

After tuning the instrument on the standards, an area of interest was selected using the CCD camera and the Z value was noted as the point when the sample was in focus in the CCD. With the D1 aperture fully removed, Cs⁺ ions were implanted into the area for the amount of time calculated to implant the required dose to bring the sample to steady state as described in the experimental chapters. The D1 aperture was then reinserted, usually to position 2, and the secondary ion optics were tuned again on the sample. The beam was defocused to avoid creating

a regular array of holes into the sample during tuning and the raster area was reduced to avoid artefacts from the crater edge. HMR scans from the sample were checked against the HMR scans from the standards, the Cy and P2/P3 deflector plates were checked and adjusted if necessary to improve transmission, and the value of EOS immersion lens was checked though usually not changed from its default value. The image was refocused and the astigmatism adjusted to allow the image to be acquired. Depth profiles, as a series of images, were usually performed overnight. For the low concentrations of Se and As present in these samples dwell times were usually around 60 ms taking approximately 1 hour for a 256 x 256 pixel image. For the resin embedded roots, a typical crater depth after cleaning and a 1 hour image was 100 nm with approximately 4-5 nm removed in a 1 hour image.

3.4.3. IMAGE ANALYSIS

Image analysis (line scans, measuring signal intensities, drift correction, summing, colour merging and ratioing of images) was undertaken using ImageJ (Rasband, 1997-2009) using the NRIMS Analysis Module (Harvard).

3.5. *ELECTRON PROBE MICRO-ANALYSIS*

Electron Probe Micro-Analysis (EPMA) was undertaken by Chris Salter in the Department of Materials, University of Oxford, using a JEOL 8800R. EPMA is able to make quantitative elemental analysis of samples with an approximate interaction volume of a few cubic microns and a sensitivity down to 100 ppm (0.01%) (Goodhew *et al.*, 2000). Electrons interacting with the sample generate characteristic X-rays which are detected using wavelength dispersive X-ray (WDX) spectrometers. X-rays from a particular element are selected using a crystal of known interplanar spacing. Only X-rays satisfying Bragg's law will be diffracted and be counted with the detector. Different elements can be detected by changing the position or type of crystal. The

detector and crystal must move together to remain on the Rowland circle so that the X-ray take-off angle remains constant to ensure that elements are counted efficiently (Goodhew *et al.*, 2000). The JEOL 8800R has four WDX detectors therefore it was often necessary to reset the crystals and perform a second run to map all the elements of interest. The signal intensities are corrected for matrix effects using atomic number (Z), absorption (A) and fluorescence (F) corrections to display the results as a concentration (Goodhew *et al.*, 2000).

3.6. *FOCUSED ION BEAM*

Focused ion beam (FIB) microscopes operate in a similar way to SEMs, however instead of using electrons a focused beam of ions is used. The machine can be operated in low current mode to generate ion-induced secondary electron images or in high current mode specific areas of the sample can be milled away to create micron-sized structures, for example site specific foils for the TEM (Giannuzzi & Stevie, 2005). In this work a FEI FIB 200 was used to simulate the effect of the NanoSIMS ion beam interaction with the sample. A gallium (Ga) liquid metal ion gun, operated at 30 kV was used at 1 nA for milling and 300 pA for imaging.

3.7. *TRANSMISSION ELECTRON MICROSCOPY*

Transmission electron microscopy (TEM) is routinely used in both materials science and biology. It uses a highly focused beam of electrons which are transmitted through a very thin sample. The image is formed from the interaction of these electrons with atoms in the sample. In biological TEM it is necessary to stain the samples with heavy ions such as osmium, lead or uranium to improve contrast and selectively highlight structural features, for example membranes or nuclear material (Hayat, 2000). A Hitachi 7650 TEM operating at 120 keV was used in the Plant Sciences Department at Oxford Brookes University to assess the level of structural preservation after HPF and to correlate elemental signals observed in the NanoSIMS with structural features. Images were acquired using an AMC bottom mounted digital camera.

3.8. *INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY*

Inductively coupled plasma mass spectrometry (ICP-MS) was undertaken at Rothamsted Research using an Agilent ICP-MS 7500ce spectrometer following acid ($\text{HNO}_3\text{:HClO}_4$ 85:15) digestion of 0.25 g of sample diluted to 20 ml. ICP-MS is a highly sensitive technique which allows quantitative measurement of very low concentrations. The sample to be analysed is converted into a liquid, generally using acid digestion, and then pumped into a nebuliser where it is mixed with argon gas to form an aerosol. The sample then enters the plasma where it is decomposed and ionised. The ionised plasma then enters the quadrupole where ions are separated based on their mass-to-charge ratio before entering a detector. The number of ions detected for a given element is directly proportional to the concentration of that element in the sample. The Se and As detection limits for the Agilent ICP-MS spectrometer used for this work are around 0.1 ppb in solution equating to around 8 ppb ($\mu\text{g/kg}$ dry weight) (FJ Zhao, personal communication).

3.9. *SYNCHROTRON ANALYSIS*

A synchrotron is a very large scale microscope in which many different experiments can be undertaken. In a synchrotron electrons are accelerated to very high speeds first via a linear accelerator, then a booster ring before they are injected into a large storage ring. Once in the storage ring, large magnets bend the electrons around the ring and as electrons pass through a magnet they lose energy in the form of light. Third generation synchrotrons, including the APS, and DIAMOND synchrotrons used in this work, use insertion devices which are placed into the straight sections of the beam causing the electrons to follow a wiggling path producing a very intense and tuneable light which is directed out of the storage ring towards a beamline where it can be used for experiments. A schematic diagram is shown in Figure 3.7.

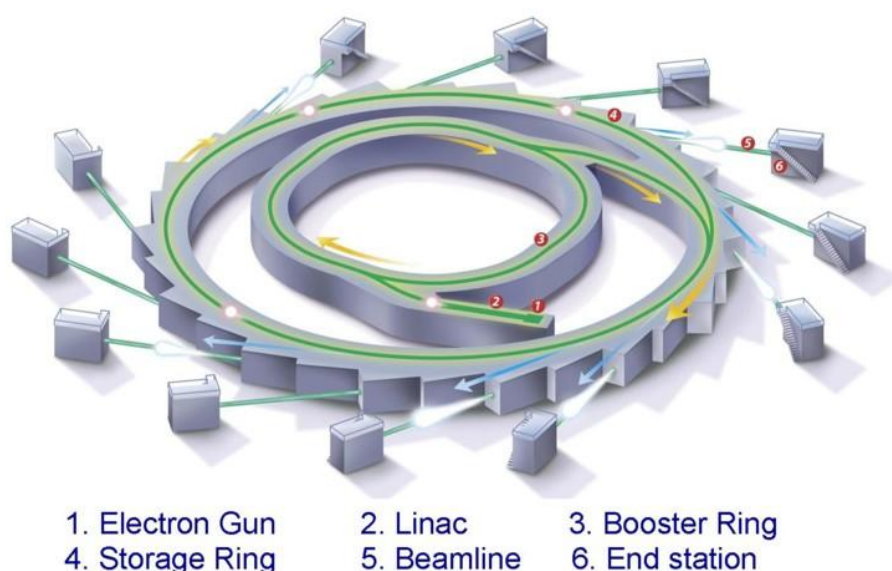


Figure 3.7: A schematic diagram showing the key components of a synchrotron (Australian Synchrotron, accessed Feb 2011). Image courtesy of the Australian Synchrotron.

Synchrotron X-Ray Fluorescence (S-XRF) mapping requires a tightly focussed X-ray beam. The sample is placed on a stage in front of the beam (which remains stationary) and the stage is rastered in front of the beam to build up a chemical image of the sample. The technique is very similar to that of energy dispersive X-ray microscopy but uses X-rays to cause X-ray fluorescence instead of electrons. Characteristic X-rays are detected using a multi-element energy dispersive X-ray detector so that the entire spectrum can be acquired simultaneously. S-XRF has the advantage of very good sensitivity (approaching the ppb range), reasonable lateral resolution (a few microns) and the only major sample preparation requirement is that of a thin sample as the high energy X-rays generated by a synchrotron are very penetrating and a thick sample causes a degradation in lateral resolution. The major drawback is that it is difficult to detect light elements ($Z < 15$) in low concentrations in biological samples (Lombi *et al.*, 2011) which makes it difficult to locate trace metal distributions to structural features which could be indicated by mapping these lighter elements. Experimental conditions relating to each experiment are given in the text. To excite As X-rays and other elements of interest an incident X-ray energy of 13 keV was used at the APS and 12.4 keV at DIAMOND.

4. SELENIUM IN WHEAT GRAIN²

4.1. INTRODUCTION

The structure of wheat is shown schematically in Figure 4.1. The majority (70-80%) of the wheat grain is comprised of the endosperm (Mills *et al.*, 2005) which is rich in starch grains embedded in protein, both of which are needed for germination of the grains (Belderok, 2000). The fused pericarp and seed coat surround the endosperm. In the centre of the grain the cells are rounded, only in the outermost layer of the endosperm, the aleurone layer, do the cells have a different morphology. The aleurone layer is a single layer of cubic cells containing spherical phytate granules rich in proteins, vitamins, minerals and enzymes needed for the germination process (Belderok, 2000).

Figure 4.1 has been removed due to Copyright restrictions. The original image can be found in the following reference:

Grundas ST, Benjamin C 2003. WHEAT | Grain structure of wheat and wheat-based products. In: B. Caballero, L. C. Trugo, P. M. Finglas eds. *Encyclopedia of Food Sciences and Nutrition*. Oxford: Academic Press, 6137-6146.

Figure 4.1: Schematic diagram showing the internal structure of a wheat grain. (a) longitudinal section; (b) cross section; E, epidermis; H, hypodermis; I, inner pericarp; C, cross cells; TC, tube cells; T, testa; N, nucellar layer; A, aleurone layer; EN, endosperm; PE, peripheral cells; PR, prismatic cells of endosperm; SE, scutella epithelium; S, scutellum; EM, embryo (Grundas & Benjamin, 2003).

² I have published a substantial part of this chapter in the following reference and some of the text and images have been used verbatim in this chapter: **Moore KL**, Schroder M, Lombi E, Zhao FJ, McGrath SP, Hawkesford MJ, Shewry PR, Grovenor CRM. 2010. NanoSIMS analysis of arsenic and selenium in cereal grain. *New Phytologist* **185**: 434-445.

When wheat grains are milled, fracture occurs underneath the aleurone cells and the outer layers, known as the bran, are separated from the endosperm. The bran therefore consists of the fused pericarp, the seed coat and the aleurone layer (Belderok, 2000). In general the bran is removed in the refining process and therefore the wheat loses some of its nutritional value and some beneficial trace elements.

It has been known since the 1950s that selenium (Se) is an important trace element for animals (Schwarz & Foltz, 1957). Selenium is a vitally important part of the Se-containing enzyme glutathione peroxidase which prevents tissue degeneration by acting as an antioxidant (Fordyce *et al.*, 2000; Hartikainen, 2005). On the other hand it is also well known that Se is toxic to humans at high doses, and the range between deficient and toxic levels is small; <17 µg/g per day and >900 µg/g per day respectively (Fordyce *et al.*, 2000; Hawkesford & Zhao, 2007). The recommended intake of Se in the UK is 60 µg/d for females and 75 µg/d for males (Broadley *et al.*, 2006).

Selenium deficiency in humans has been linked to many different physiological disorders. In some parts of China, the Se intakes are extremely low and this has been linked to an endemic degenerative heart disease known as Keshan disease and also to Kashin-Beck disease, an osteoarthritis disorder (Hartikainen, 2005). A low Se intake has also been linked to cardiovascular disease, pancreatitis, asthma and inflammatory response syndrome (Rayman, 2000) and there is evidence to suggest that low Se can impact on the function of the immune system, response to viral infection, male and female fertility and thyroid function (Broadley *et al.*, 2006). Clinical trials have also indicated that Se in quantities greater than those required to maximise the expression of the selenoenzymes can act as an anticarcinogen (Combs, 2001).

It has been estimated that between 0.5 and 1 billion people worldwide may be deficient in Se and this includes populations in developed countries (Combs, 2001). In 1974 the intake of Se per

person in the UK was $>60 \mu\text{g/d}$ (Broadley *et al.*, 2006); however by 1995 this had reduced to 29–39 $\mu\text{g/d}$ (MAFF, 1997) and in 1999 the intake was estimated as 35 $\mu\text{g/d}$ (Rayman, 2002) which is significantly below the reference intake. This decrease is primarily from a reduction in the amount of wheat imported from North America which has a higher Se content than European wheat (Hawkesford & Zhao, 2007). There has also been a fall in the consumption of cereal products, in particular bread which is still one of the main sources of Se in our diet along with meats and fish (Hawkesford & Zhao, 2007).

Agronomically biofortifying foods with Se is considered to be more effective than direct Se supplementation. This is because the Se is assimilated into the plant in organic forms which are more bioavailable to humans, and there is less chance of consuming excessive amounts of Se than from Se supplements (Hartikainen, 2005; Hawkesford & Zhao, 2007). Selenium in wheat grain has high bioavailability, 83%, compared to 57% for tuna and exceeds 50% for the inorganic selenate and selenite in supplements (Thomson, 2004).

UK soils contain little Se and hence home grown wheat grain is low in Se content. To increase the amount of Se consumed via home grown wheat a Se-containing fertiliser could be applied to the soil. Sodium selenate, Na_2SeO_4 , is more widely used than selenite as both pot and field studies have shown that the former results in a much higher Se concentration in the plant (Hawkesford & Zhao, 2007). The best example of using Se-fertilisers to biofortify food crops comes from Finland where the application of 10 mg Se/kg to multi-element fertilisers has resulted in an increase in the average Se intake from 25 $\mu\text{g/day}$ to 110 $\mu\text{g/day}$ with the Se concentration in spring wheat increasing from $< 10 \mu\text{g/kg}$ to 250 $\mu\text{g/kg}$. It is generally accepted that Se is taken up by plants from the soil predominantly as selenate via the sulphate transporters in the plasma membranes of the roots (Broadley *et al.*, 2006; Hawkesford & Zhao, 2007). However, the shared transporters mean that Se biofortification and sulphate fertilisation are linked and an increase in the uptake of one is likely to result in a decrease in the other.

There is a limited amount of published research concerned with the localisation of Se in cereal grains. Flour fractions containing aleurone cells were shown to contain only slightly higher Se concentrations than flours that did not (Eurola *et al.*, 1990). The embryo was shown to have only 1.5 times higher Se content than the endosperm (including the aleurone) and that the Se was quite evenly distributed throughout the whole of the grain in comparison to other mineral nutrients (e.g. zinc (Zn), iron (Fe) and potassium (K)) which were concentrated in the aleurone (Lyons *et al.*, 2005a). These results are similar to those found using S-XRF to map the Se distribution in rice grains where an enrichment of Se was found in the embryo with a concentration gradient decreasing from the outer parts of the grain to the centre (Williams *et al.*, 2009).

4.2. PROJECT AIMS

It is important to know precisely where the Se is situated in the grain as the milling process separates the bran layer and embryo from the rest of the grain. The bran layer and embryo are much less frequently used in foods so if the Se is predominantly localised in these tissues there will be less benefit from the Se biofortification of the wheat grain. Previous research on the localisation of Se in wheat did not have the necessary resolution to distinguish whether the Se was localised in the aleurone layer or in the regions just beneath. The aim of this project was therefore to investigate the potential of SIMS analysis as a method of localising a trace element within wheat grain and to establish where the Se was localised at high resolution. Particular focus was placed on the aleurone, endosperm and embryo regions and to establish which elements were co-localised with Se with the aim of understanding the mechanisms of transport.

4.3. SAMPLE PREPARATION

4.3.1. SAMPLE GROWTH

Wheat (*Triticum aestivum* L. cv. Hereward) grain samples were obtained from a field experiment conducted on the University of Nottingham (UK) experimental farm in 2007. Selenium fertilizer was applied to the crop at the stem extension stage in the form of sodium selenate (400 g Se ha⁻¹). Other inputs were the same as in normal agronomic practice. The wheat was harvested at maturity. The concentration of Se in the wheat was measured using inductively-coupled plasma mass-spectroscopy (ICP-MS) (Agilent LC1100 series and Agilent ICP-MS 7500ce; Agilent Technologies, Santa Clara, CA, USA) after an acid digestion (HNO₃ - H₂O₂) in a microwave digester, and was found to be 16.7 mg kg⁻¹ Se. This is significantly higher than most UK wheat which has a range of 0.006 – 0.86 mg kg⁻¹ with a mean of 0.032 mg kg⁻¹ Se (Adams *et al.*, 2002). The wheat plants showed no obvious signs of suffering from Se toxicity even with this high concentration.

4.3.2. MATURE WHEAT GRAINS

It was important to find a suitable method for preparing the mature wheat grains prior to analysis in the NanoSIMS. As the wheat grains were naturally desiccated sample preparation was relatively simple, however a method needed to be established to obtain a flat surface.

4.3.2.1. Fracture

In order to make a preliminary investigation on the structure of the wheat grain, samples were initially just fractured in half by hand. While this allowed some examination of the internal structure the surface was very rough, as shown in Figure 4.2 below, and this prevented good structural and compositional analysis. With this method the elongated starchy endosperm cells are clearly visible and there is some evidence of starch grains within them. In the majority of cases the only part that remains of the aleurone layers is the cell walls, the phytate granules are

either not exposed or they have fallen out. Only the aleurone cell in the bottom right of the image contains phytate granules. Both the fused pericarp and the seed coat are intact on the outer edge of the grain however this surface was too rough to perform EDX analysis and is therefore not a suitable preparation method for the NanoSIMS.

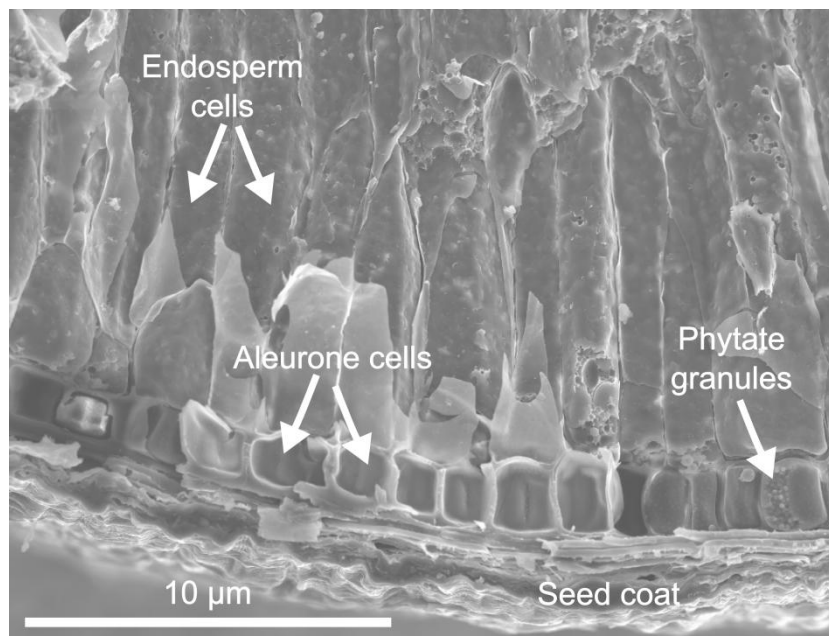


Figure 4.2: SEM image of the edge of a wheat grain showing the surface after fracturing by hand. The endosperm cells are clearly visible however the phytate granules in the aleurone cells are not exposed or have fallen out.

4.3.2.2. Resin Embedding and Microtome Sectioning

Microtomy is widely used to create a flat surface for analysis so one grain of wheat was prepared to allow sectioning. The sample was placed into an Eppendorf tube in LR White for 1 week to allow resin impregnation into the grain. The orientation of the wheat grain in the Eppendorf tube is shown in Figure 4.3.

The resin was cured at 50 °C in an oven overnight after which the cured resin block was removed from the tube. The tip of the resin containing the end of the wheat grain was shaped with a razor blade into a pyramid for microtome sectioning. A LKB Ultratome Nova (Ultratome Nova, Bromma, Sweden) microtome with a glass knife was used to section the wheat grain.

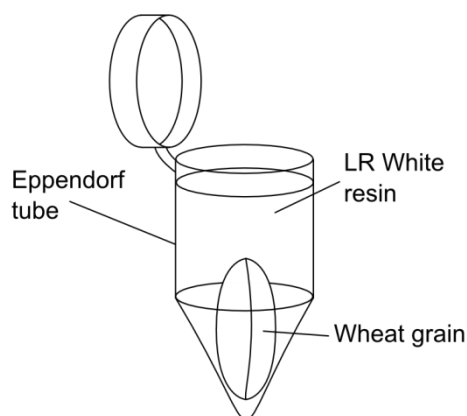


Figure 4.3: Schematic diagram showing the mounting of the wheat grains in the Eppendorf tube

Several problems were encountered with this technique. It was quite difficult to shape the point so that the region of interest, in this case the outer layers of the grain including the aleurone, were situated towards the tip of the pyramid which would subsequently be sectioned. There was no impregnation of the resin into the grain as the outer layers of the grain are resilient to impregnation. Although the grains are desiccated there is enough residual water remaining in the grain to prevent impregnation of the hydrophobic LR White resin. This caused the wheat grain to absorb water and become soggy when it came into contact with the water in the microtome trough and cut sections tended to stick back onto the freshly sectioned surface. When the sections were floated onto water the starch grains tended to break away from each other causing both elemental and cellular redistribution. Figure 4.4 is an optical micrograph of the best, unbroken microtome section that was obtained from the mature grain sectioned using this method. It was not possible to identify the type of cells or where they came from within the grain and the only elements detected with EDX analysis were carbon and oxygen hence it was decided that this method was not suitable for preparing mature wheat grains.

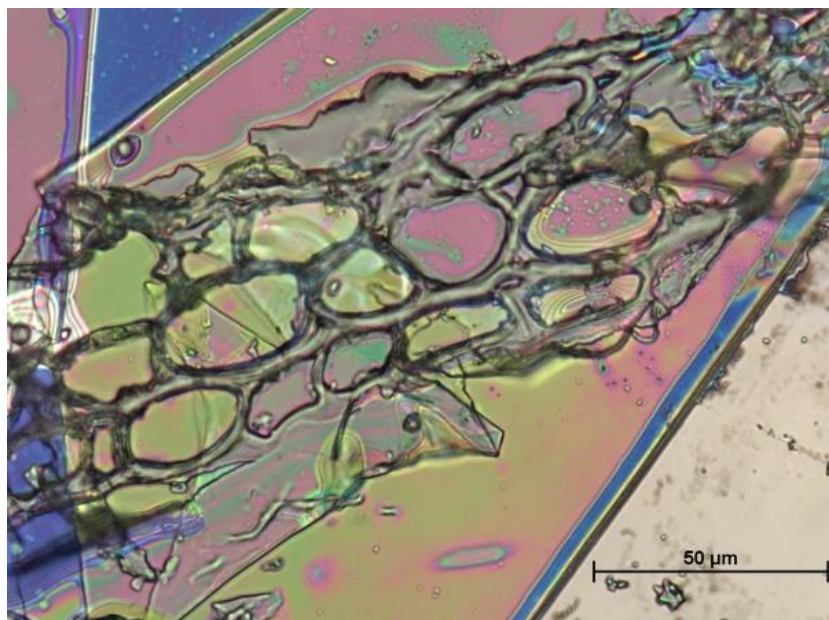


Figure 4.4: Optical image of a microtomed section of a mature wheat grain

4.3.2.3. Lathe Sectioning

Microtome sectioning involves moving a sharp edge over the sample fairly quickly to create the section, hence it was decided that a lathe may generate a sufficiently flat surface for NanoSIMS analysis. A sample of wheat was mounted into a 10 mm brass ring using LR White to hold the sample in place. The resin was polymerised by placing the NanoSIMS ring onto a glass slide onto a hotplate while holding the grain in place with a pair of tweezers. Half of the grain was left protruding from the ring and the majority of the protruding part was sectioned off using a razor blade. A lathe facing tool was then brought across the surface of the ring very quickly to remove the remaining part of the grain. This method created a macroscopically flat surface; however when the grain was examined by SEM it was found that there was a lot of mechanical damage and redistribution of material which would cause problems with relating the structure to the chemical distribution. Figure 4.5 shows the damage caused by the lathe, the phytate granules have been redistributed across the surface, the aleurone cells have been badly damaged and the lathe has caused fracture around the starch grains rather than sectioning through them.

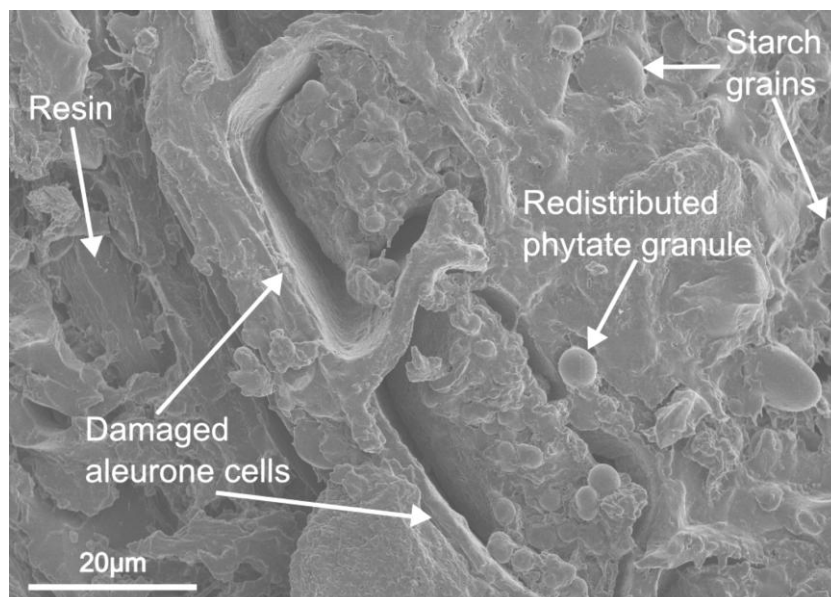


Figure 4.5: SEM image showing the damage caused by sectioning the wheat grain with a lathe facing tool. Significant redistribution and fracture has occurred making it difficult to determine the actual structure of the sample.

4.3.2.4. Razor Blade Sectioning

The flattest surfaces were achieved by hand sectioning with a sharp razor blade. A wheat sample was mounted into a NanoSIMS ring using LR White resin to hold the sample in place and half of the grain was left protruding in the same way as described previously. A razor blade cleaned with acetone was used to section the grain. This was usually performed in two steps; first the sample was cut close to the resin surface and then cut level with the top of the ring in the final cut by running a new razor blade along the top of the ring. The quality of preservation that can be achieved using this method can be seen in Figure 4.6. It was not possible to achieve this level of preservation over the whole surface of the sample as the grain tended to fracture about half way across the grain but there is sufficient area to be analysed with SEM, EDX and NanoSIMS.

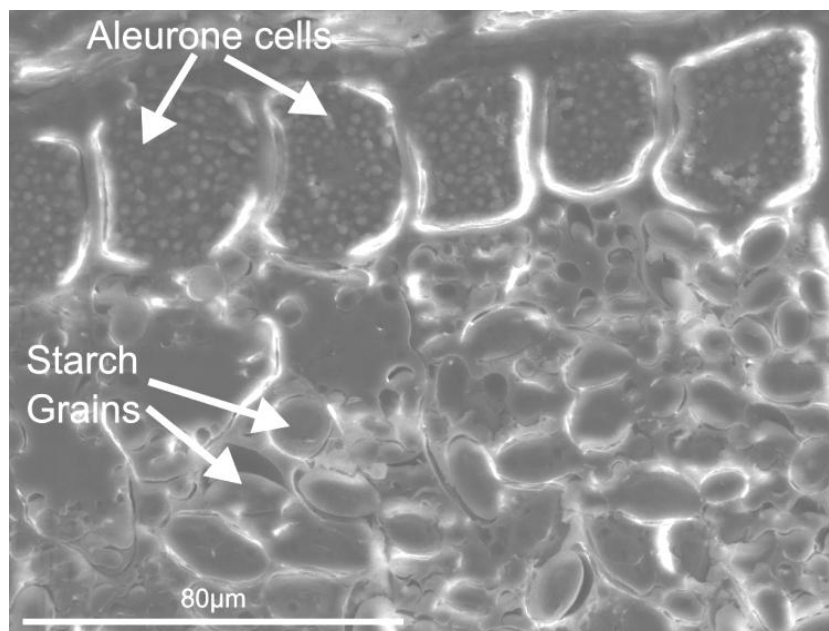


Figure 4.6: SEM image of the outside edge of a wheat grain showing the high level of preservation achievable using a razor blade

As this simple method achieved the best preservation of the wheat grains it was decided that this would be used to prepare the mature wheat grain samples for the NanoSIMS.

4.3.3. IMMATURE WHEAT GRAIN SAMPLES

In order to investigate how the Se distribution varies with development of the wheat grain, and to investigate the Se distribution in the embryo, immature samples were also prepared. Grains were taken from the plant at 14 and 21 days post anthesis (DPA) which corresponds to the start and middle of grain filling. Individual ears were tagged on the day of flowering to allow the timing of grain filling to be monitored as shown in Figure 4.7 a). Two different concentrations of Se were applied to the plants, 5 ppm and 10 ppm as sodium selenate in compost, the actual average concentration of Se in the grain was however quite high at 29 ppm and 52 ppm respectively which explains why some of the wheat plants showed signs of Se toxicity in the form of bleached leaves. Within the ear the grains are attached to the stalk in groups of three, only the outer two grains were selected as the middle grain tends to be rather small. The open seed coat

was peeled back and the grain was then gently pulled off the stalk taking care not to squash the grain in the process. Grains were only taken from a region half way down the ear, as shown in Figure 4.7 b). Different ears were used for the HPF and chemical fixation as it was not logistically possible to move the plants between Rothamsted Research and Oxford Brookes continuously, but the same plant was used for both the 14 DPA and 21 DPA sample preparation in both cases. Grains were only removed from the ear just prior to preparation to prevent them drying out.

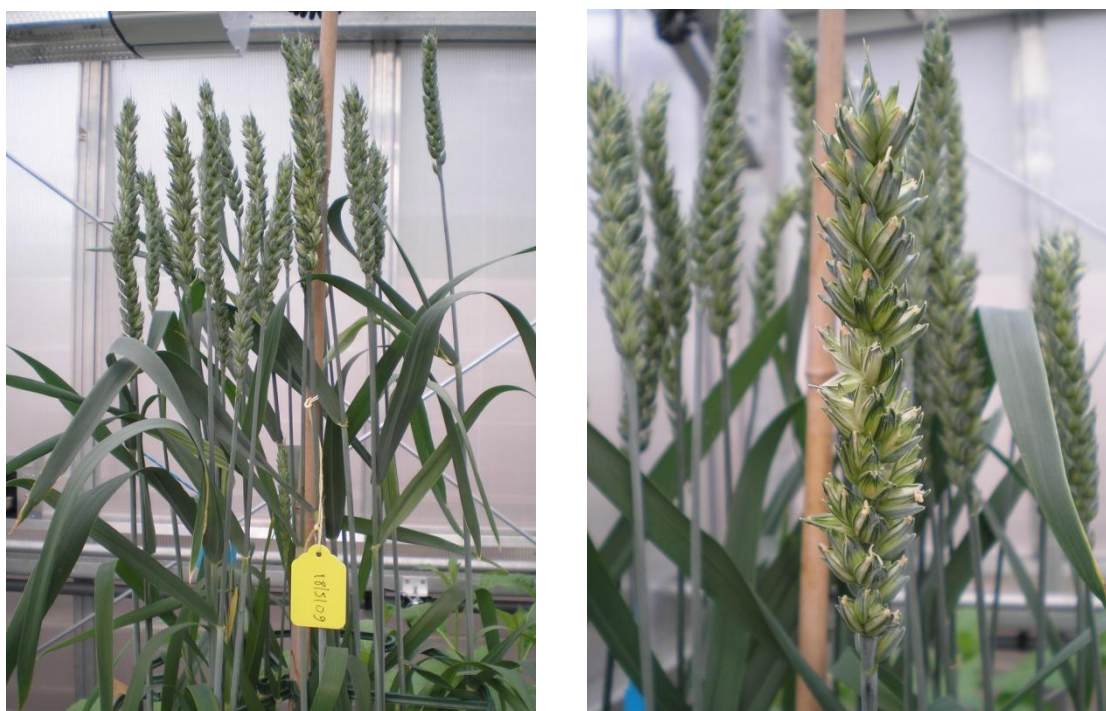


Figure 4.7: Immature wheat grain plants at 14 days post anthesis a) the tagged ear which flowered on 18/05/09 b) The analysed ear with grains removed from the middle for sample preparation

4.3.3.1. Chemical Fixation

Chemical fixation was carried out at Rothamsted Research with the help of Dr Cristina Gritsh. Fresh buffer (0.1 M sodium cacodylate, pH 7.4) and fixative (3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) were prepared and brought up to room temperature before use. A drop of fixative was placed onto a piece of clean dental wax and the wheat grains were cut under this drop of fixative. Sections of wheat with a maximum thickness of 1 mm were cut with a razor blade from the embryo and endosperm regions both longitudinally and transversely.

Care was taken when cutting the sections using a sliding motion with the razor blade to avoid squashing the grain and new razor blades were used frequently to avoid using a blunt blade. Once sectioned the pieces of wheat were placed into a vial containing sufficient volume of fixative to cover the sections and the vial was placed into a rotator at room temperature for 3-5 hours. Sections were washed 3 to 4 times in 0.1 M sodium cacodylate buffer for 20-30 minutes for each change ensuring that the samples are not exposed to air at any time. The samples were then dehydrated in a graded ethanol series at room temperature as follows: 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% with each step lasting 30 minutes with the exception of the 70% step where the sample was left at 4°C overnight and the 100% step which was carried out with dry ethanol and was repeated 3 times for 20 minutes each. Infiltration was carried out with increasing concentrations of LR White resin (medium grade) as follows:

100% Ethanol: LR White 4:1 1hr

100% Ethanol: LR White 3:2 1hr

100% Ethanol: LR White 2:3 1hr

100% Ethanol: LR White 1:4 1hr

100% LR White, 3 changes of 1hr, then two changes of resin a day for four days.

Samples were placed in a rotator at RT during the day and in the fridge at night. To embed the samples flat bottomed capsules were filled with resin. One section was then placed in the bottom of each and orientated appropriately (Figure 4.8). The lid was closed and the resin was polymerised in an oven purged with nitrogen gas to remove the oxygen which can prevent polymerisation of the LR White resin. Polymerisation was carried out at 55 °C for 16h before the samples were left to cool down in a fume cupboard.

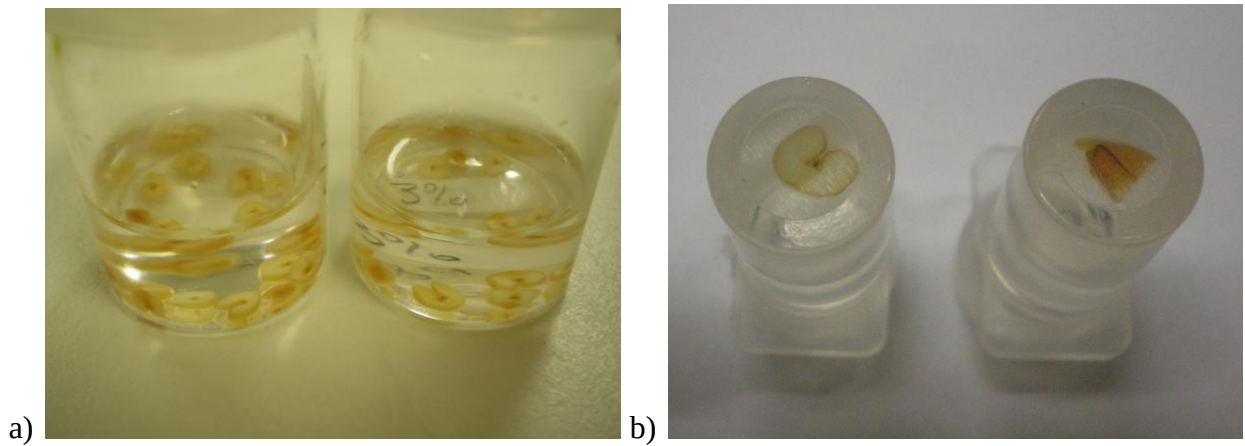


Figure 4.8: a) Sections of immature wheat grain in 100% LR White resin prior to mounting samples individually in capsules for polymerisation. b) A transverse and longitudinal section in capsules after polymerisation

4.3.3.2. High Pressure Freezing and Embedding

Wheat grains were sectioned under MES buffer instead of the chemical fixative used above, again taking care not to squash the grain. The sections for the HPF needed to be considerably thinner ($<600\ \mu\text{m}$) than those cut for the chemical fixation which proved to be significantly more challenging, especially when trying to cut the embryo region because the embryo tended to pop out from the grain as it was sectioned. Once a section of the appropriate thickness had been created a histology punch was used to create a portion of the sample which was of the right diameter to fit into the 3 mm planchettes. At 21 DPA the wheat grains were noticeably bigger and it was not possible to fit as much of a section into the histology punch. The sections were coated with a drop of hexadecene, a cryoprotectant. Another planchette was placed on top and immediately frozen in a BAL-TEC HPM 010 high-pressure freezer (BAL-TEC AG) freezing the sample at a pressure of 210 MPa at $-196\ ^\circ\text{C}$ for a duration of 30 ms. The subsequent freeze substitution and embedding were carried out using the protocols outlined in the experimental methods chapter.

4.3.3.3. Microtomy

For both methods of preparation the resin block faces were cut with a razor blade to make them trapezoidal. A diamond knife on a Reichert Ultracut E Ultramicrotome was used to create 1 μm thick sections for NanoSIMS analysis. Sections were picked up with a hair and deposited onto a droplet of water on a Si wafer. Sections were stretched flat on a hotplate and were examined with a light microscope to assess the structural preservation of the sections. To prevent charging during NanoSIMS analysis the samples and Si substrates were coated with 10 nm of platinum before loading into the NanoSIMS.

4.4. RESULTS AND DISCUSSION

4.4.1. SEM AND EDX

Energy dispersive X-Ray spectroscopy was used to map the major elements in the aleurone cells so that suitable elements for analysis in the NanoSIMS could be identified. The corresponding elemental maps for the SEM image in Figure 4.6 are presented in Figure 4.9. The sample was carbon coated to prevent charging and the EDX maps were acquired at voltage of 15 kV with a three hour acquisition time. The phytate granules contained within the aleurone cells are predominantly composed of phytic acid (structure shown in Figure 4.10). Phytic acid is known to have a high affinity for several cations including Fe, Zn, Mn, Ni, Cd, Cu, Mg, Ca, Na and K (Persson *et al.*, 2009) and the EDX maps are consistent with this showing that Mg, P and K are strongly localised within the aleurone cells. The areas within the aleurone cells where these elements are not detected are likely to be the location of aleurone nuclei. The concentration of sulphur was quite low so it was difficult to detect with EDX. The long acquisition time made it possible to see some localisation in the sub-aleurone region as indicated in Figure 4.9 c); however the drift in the SEM means that the maps are smeared from the top left towards bottom right of the images. The sub-aleurone region is known to be particularly protein-rich in

comparison to the endosperm region further into the grain so this result was as expected (Tosi *et al.*, 2009). It can be seen in Figure 4.6 that the regions of S localisation correspond to regions without any starch grains and are hence likely to be protein-rich regions.

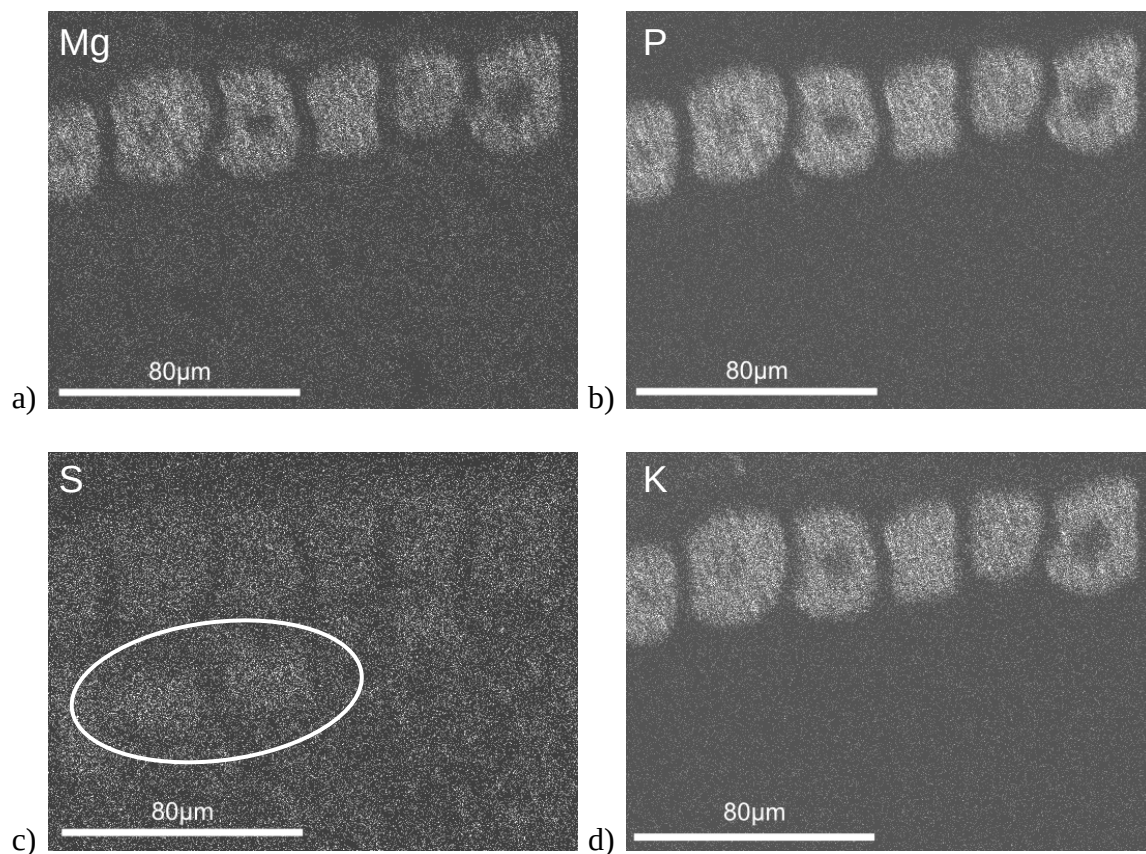


Figure 4.9: EDX maps of the area shown in Figure 4.6 showing a) Mg, b) P, c) S, d) K. The highlighted area on the sulphur image indicates the location of the sub-aleurone protein with a weak enhancement of S indicating a protein-rich region

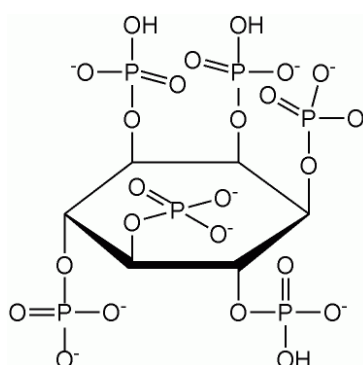


Figure 4.10: Molecular structure of phytic acid

4.4.2. NANOSIMS OF MATURE WHEAT GRAINS

4.4.2.1. Detector Positioning (HMR)

As the bulk concentration of Se in the samples was low it was necessary to tune the detector on a standard to ensure correct positioning of the detector for Se. Sodium selenate, dissolved in water and re-crystallised onto a resin surface, was used to tune the spectrometer. A high resolution mass spectrum (HMR) was taken around mass 80 for both the sodium selenate standard and the wheat grain sample with the peaks around this mass shown in Figure 4.11. On the Se standard there is one very large and intense peak which can be assumed to be $^{80}\text{Se}^-$. The wheat grain sample shows two obvious peaks. The left hand one lines up exactly with the Se peak from the standard hence the detector can be positioned with confidence at the vertical line indicated on the graph. It is not clear what the right hand peak could be, possible ions with the correct mass are C_6H_8 or SO_3 but these seem unlikely. This method of tuning on a Se standard before tuning on the sample was undertaken every morning before analysis.

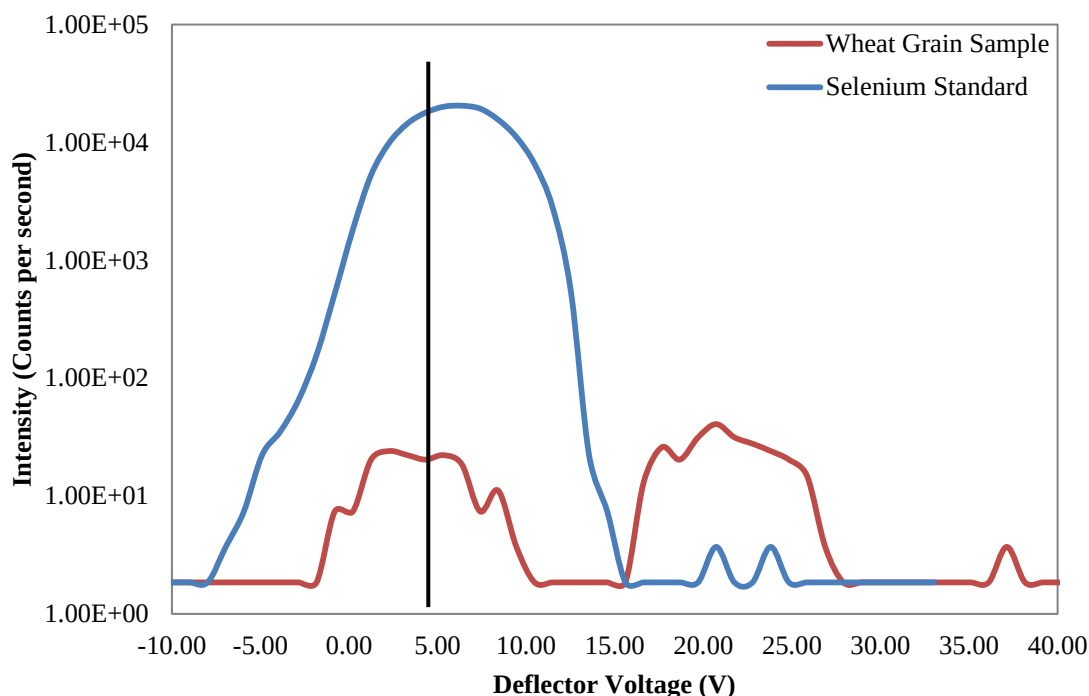


Figure 4.11: High resolution mass spectra around mass 80 from the sodium selenate standard and the wheat grain sample. HMR scans are exported as deflector voltages.

The molecular structure of phytic acid, as shown in Figure 4.10, indicates that every phosphorous atom is bonded to 4 oxygen atoms. This meant that mapping of $^{31}\text{P}^{16}\text{O}^-$ ions would indicate the position of the phytate granules. In fact, mapping $^{31}\text{P}^-$ and $^{32}\text{S}^-$ with detector 5 tuned to Se was only possible if the deflector voltages in front of the detectors were changed as the detectors could not be positioned close enough together. Surface contamination on a GaP standard was used to accurately position the detector for $^{31}\text{P}^{16}\text{O}^-$.

4.4.2.2. NanoSIMS Analysis

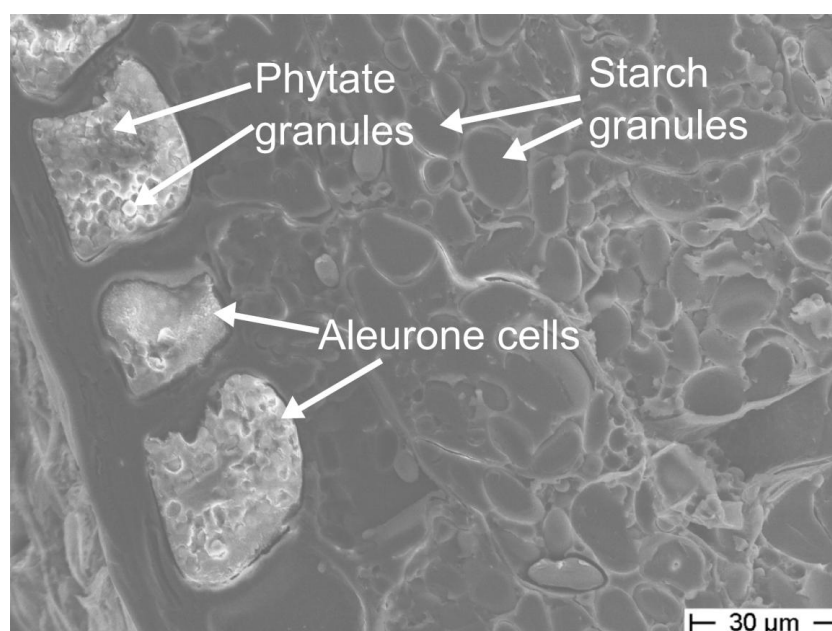


Figure 4.12: Scanning electron micrograph of the outer edge of a mature wheat grain showing the aleurone layer (left) and the starchy endosperm (right). This area was subsequently analysed in the NanoSIMS. Reprinted with permission from John Wiley and Sons.

Figure 4.12 shows a scanning electron micrograph of an area of a wheat grain selected for NanoSIMS analysis. It shows the outer part of the endosperm, with a single layer of thick-walled aleurone cells surrounded by the outer layers of the grain (testa and pericarp) and enclosing the starchy endosperm cells. The aleurone cells are clearly well-preserved, with phytate granules being easily visible. The starchy endosperm cells show sectioned starch granules rather than rounded grains which have been fractured around the edge as in Figure 4.5. The surface, although not completely flat, is not so rough that reliable NanoSIMS analysis is prevented.

Figure 4.13 shows NanoSIMS ion maps of the area shown in Figure 4.12 taken with a dwell time of 60 ms per pixel and a current of 2 pA. Significant differences were observed between the individual elemental maps showing that the influence of topography does not dominate the local ion yield. This indicates that the images reveal genuine variation in composition. The $^{12}\text{C}^{14}\text{N}^-$ signal intensity in Figure 4.13 is much stronger in the regions around the starch granules which is consistent with the granules being surrounded with a protein-rich matrix. The NanoSIMS images also show that there is a greater concentration of protein in the sub-aleurone region, which is consistent with our knowledge of protein distribution in wheat grain (Tosi *et al.*, 2009), and also corresponds with an increase in Se concentration in this region. The oxygen map shows a more even distribution, apart from in the aleurone cells, where intense regions of signal are observed. These regions correspond with high intensity in the $^{31}\text{P}^{16}\text{O}^-$ map and show the location of the phytate granules. Although a bulk concentration of 16.7 mg kg^{-1} Se is very high for wheat grain (normal values lie within the range $0.01 - 2 \text{ mg kg}^{-1}$ Se (Hawkesford & Zhao, 2007)), it still presents a serious analytical challenge for techniques with high spatial resolution. At this concentration, however, the $^{80}\text{Se}^-$ signal is readily detected by the NanoSIMS and is relatively uniformly distributed across the protein matrix of the aleurone cells but more concentrated in the region surrounding the starch granules in the starchy endosperm cells. These results are consistent with those of (Euroola *et al.*, 1990), who showed that flour fractions containing the aleurone cells had only slightly higher Se concentrations than flours that did not. The Se distribution is very similar to that of sulphur (see also (Feeney *et al.*, 2003)), which is not unexpected as these elements share the same mechanisms of uptake and transport (Hawkesford & Zhao, 2007), with about 80% of the Se in the wheat grain being present in proteins as selenomethionine or selenocysteine (which replace methionine and cysteine, respectively) for similarly Se-enriched grains (Hart *et al.*, 2011).

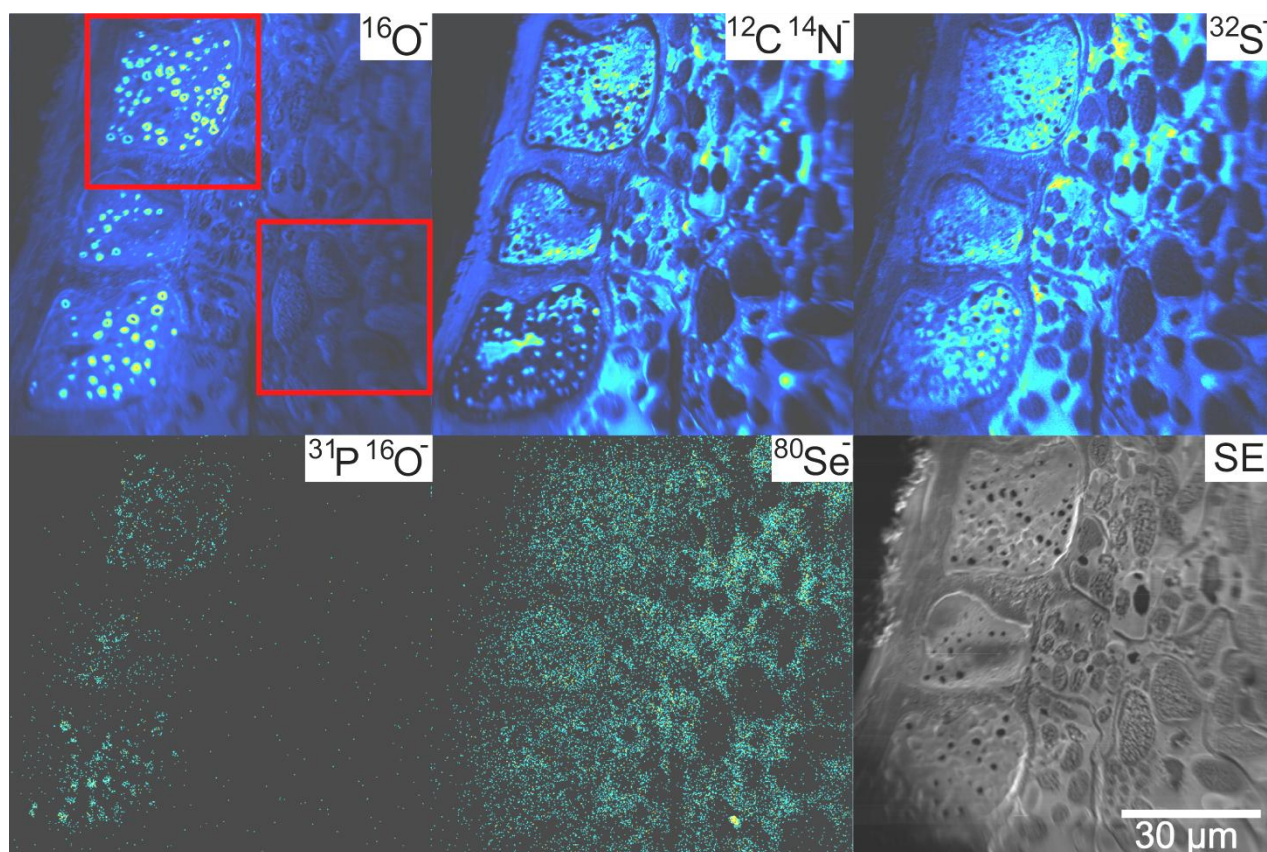


Figure 4.13: NanoSIMS maps of $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{31}\text{P}^{16}\text{O}^-$, $^{80}\text{Se}^-$ and the secondary electron image from the outer part of the wheat grain showing three aleurone cells and part of the starchy endosperm region. Reprinted with permission from John Wiley and Sons.

Higher magnification images of the aleurone cell and starchy endosperm cells from the areas indicated by the boxes in Figure 4.13 are shown in Figure 4.14 a) and b), respectively. These images were taken under the same analytical conditions so the Se ion counts can be quantitatively compared. The line scan of Figure 4.14 c) indicates that the concentration of Se is greater inside the aleurone cell than in the surrounding region but that the distribution within the cell is relatively uniform. Figure 4.14 b) and d) show that Se is concentrated in the protein region surrounding the starch granules in the starchy endosperm cells, with little signal detected inside the starch granules. Figure 4.14 b) and d) also show that Se very closely follows the distribution of S.

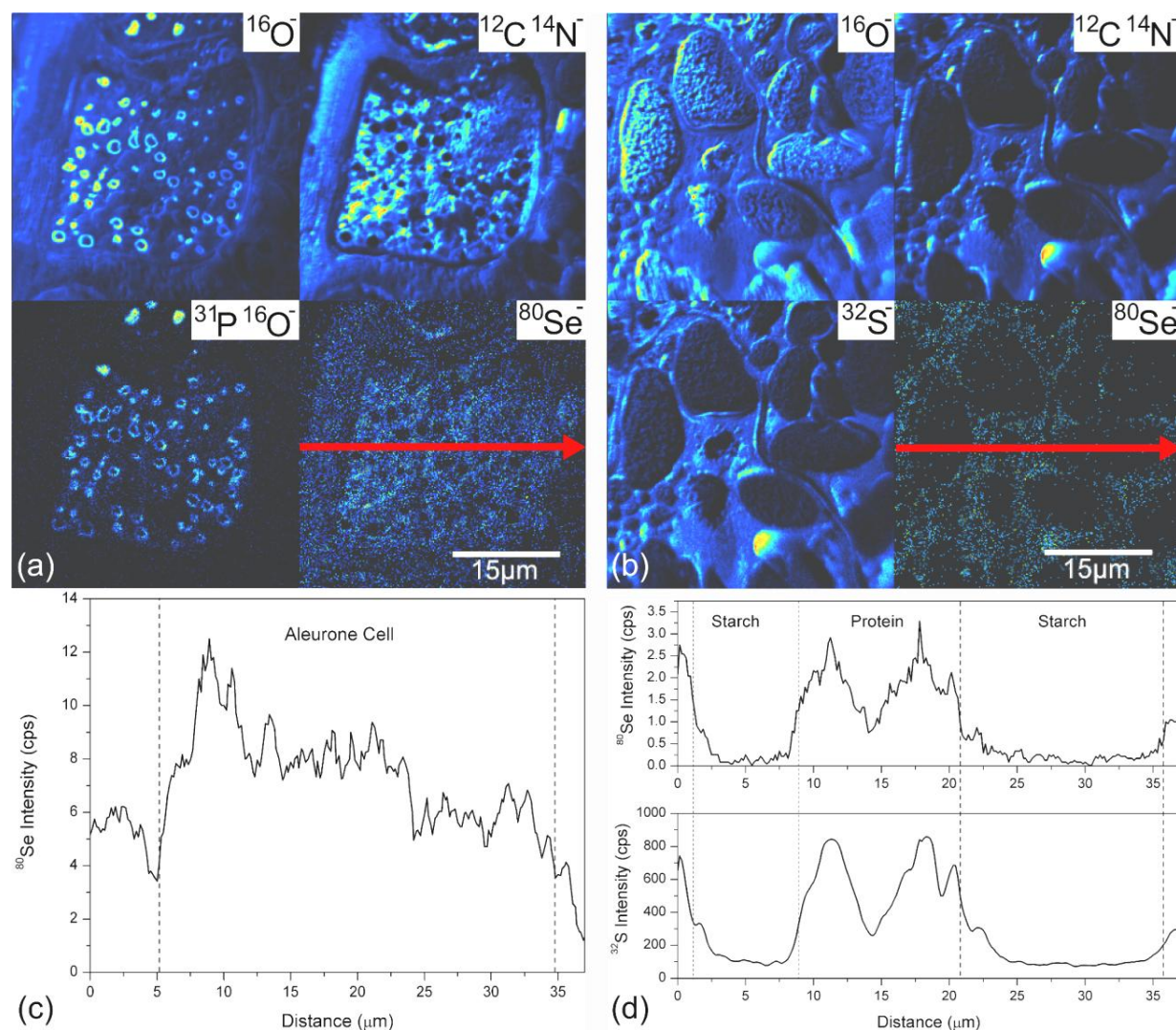


Figure 4.14: Higher magnification NanoSIMS ion maps of (a) the wheat aleurone cell indicated in Figure 4.13 showing $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{31}\text{P}^{16}\text{O}^-$ and $^{80}\text{Se}^-$ signals (b) the wheat starchy endosperm region indicated in Figure 4.13 showing $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$ and $^{80}\text{Se}^-$ signals. (c) Line scan for $^{80}\text{Se}^-$ across the wheat aleurone cell as indicated by the arrow in (a), with the intensity in counts per second (cps). (d) Line scan for $^{80}\text{Se}^-$ and $^{32}\text{S}^-$ ions across the starchy endosperm region as indicated by the arrow in (b), with the intensity in counts per second (cps). The line scans were averaged over 400 pixels to improve the counting statistics using a box with a width of 10 pixels (parallel to the line) and a height of 40 pixels (normal to the line) and stepped along the image in 1.5 μm intervals. Reprinted with permission from John Wiley and Sons.

The Se distribution in the starchy endosperm region is more defined in Figure 4.15. This image was acquired from a different grain to that of Figure 4.13 so it is possible that this grain had a higher Se concentration. As was shown in Figure 4.14, there is a strong relationship between the Se and sulphur distributions. In this image it is clear that the $^{31}\text{P}^{16}\text{O}^-$ signal was more concentrated inside the starch grains and is presumably associated with phospholipids present

within the starch granules. The characteristic ‘porous’ structure observed inside all of the starch grains, and shown most clearly in the $^{31}\text{P}^{16}\text{O}^-$ image of Figure 4.15 will be discussed in more detail in section 4.4.4.1.

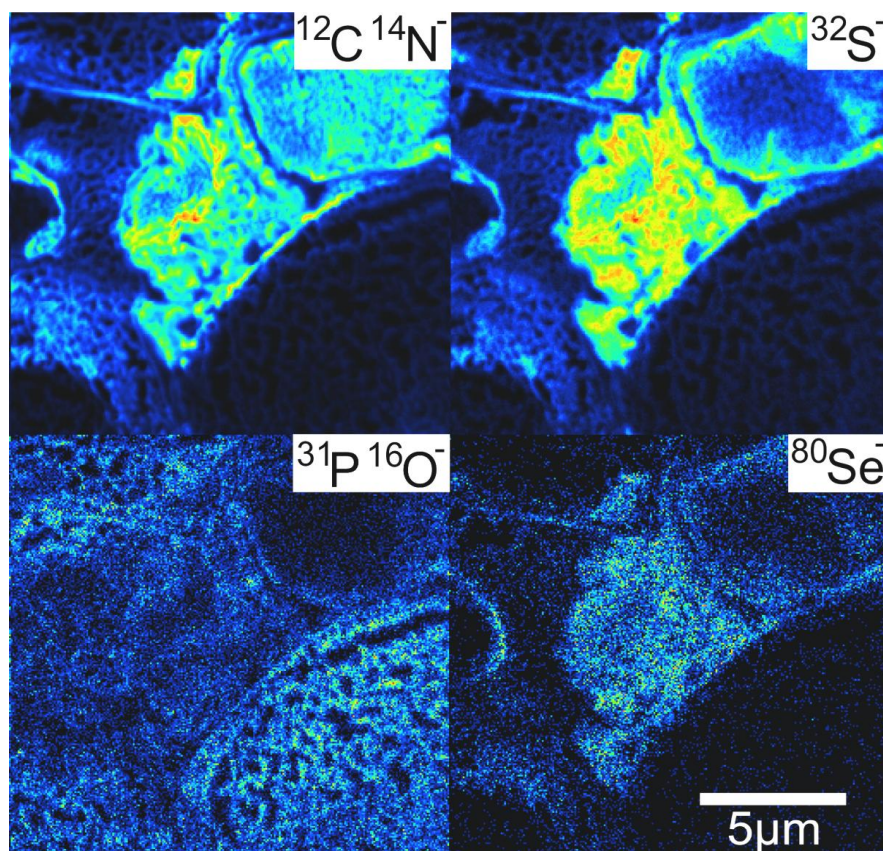


Figure 4.15: High resolution NanoSIMS maps of $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{31}\text{P}^{16}\text{O}^-$, $^{80}\text{Se}^-$ from the starchy endosperm cell region of mature wheat grain. Reprinted with permission from John Wiley and Sons.

4.4.2.2.1. *Se-rich Hotspots*

Further imaging of the aleurone cells from the same grain as that analysed in Figure 4.13 revealed the presence of Se-rich hotspots, which are shown in Figure 4.16 and Figure 4.17. The oval feature in the centre of the aleurone cell in Figure 4.16 which is devoid of phytate granules is likely to be the cell nucleus. Higher resolution imaging, Figure 4.18 and Figure 4.19, showed that these hotspots are approximately 1-2 microns in size and appear to be situated on the outside of the phytate granules and are located across the whole of the cell. Surprisingly the same hotspots are not seen in the sulphur maps, with less S detected in these areas in comparison to the surrounding areas. In the other regions of the cell the distribution of the Se follows quite closely that of S.

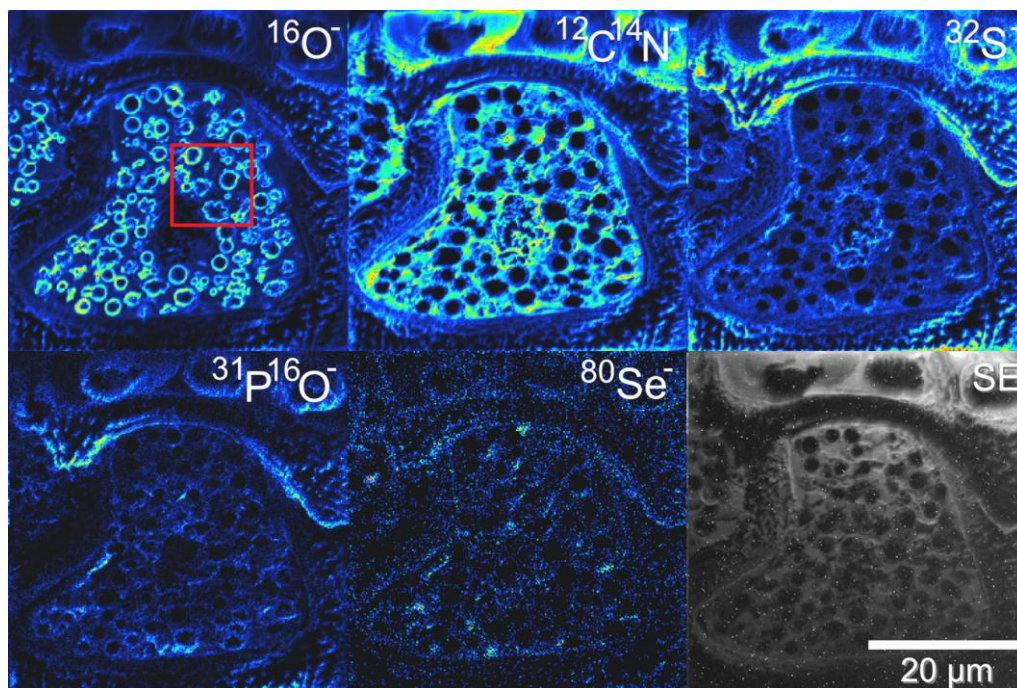


Figure 4.16: NanoSIMS maps of $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{31}\text{P}^{16}\text{O}^-$, $^{80}\text{Se}^-$ and the secondary electron image of a wheat aleurone cell. The phytate granules are clearly visible in the $^{16}\text{O}^-$ image as characteristic rings and hotspots are just visible in the Se image. It is likely that the feature in the centre of the image is the cell nucleus. The secondary electron signal is rather poor from this area and this image is presented on a log scale to allow the features to be observed more clearly. The red box on the $^{16}\text{O}^-$ image shows the position of the higher resolution image presented in Figure 4.18.

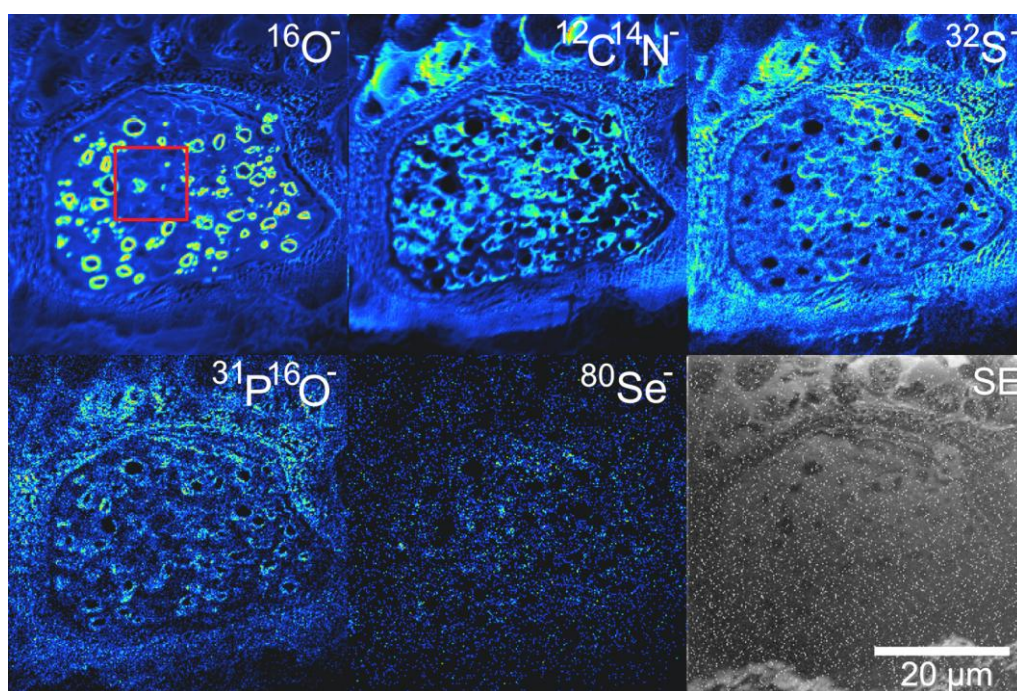


Figure 4.17: NanoSIMS maps of $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{31}\text{P}^{16}\text{O}^-$, $^{80}\text{Se}^-$ and the secondary electron image of a wheat aleurone cell almost adjacent to the cell in Figure 4.16. The phytate granules are clearly visible in the $^{16}\text{O}^-$ image as characteristic rings and hotspots are just visible in the centre of the Se image. The secondary electron signal is rather poor from this area and this image is presented on a log scale to allow the features to be observed more clearly. The red box on the $^{16}\text{O}^-$ image shows the position of the higher resolution image presented in Figure 4.19.

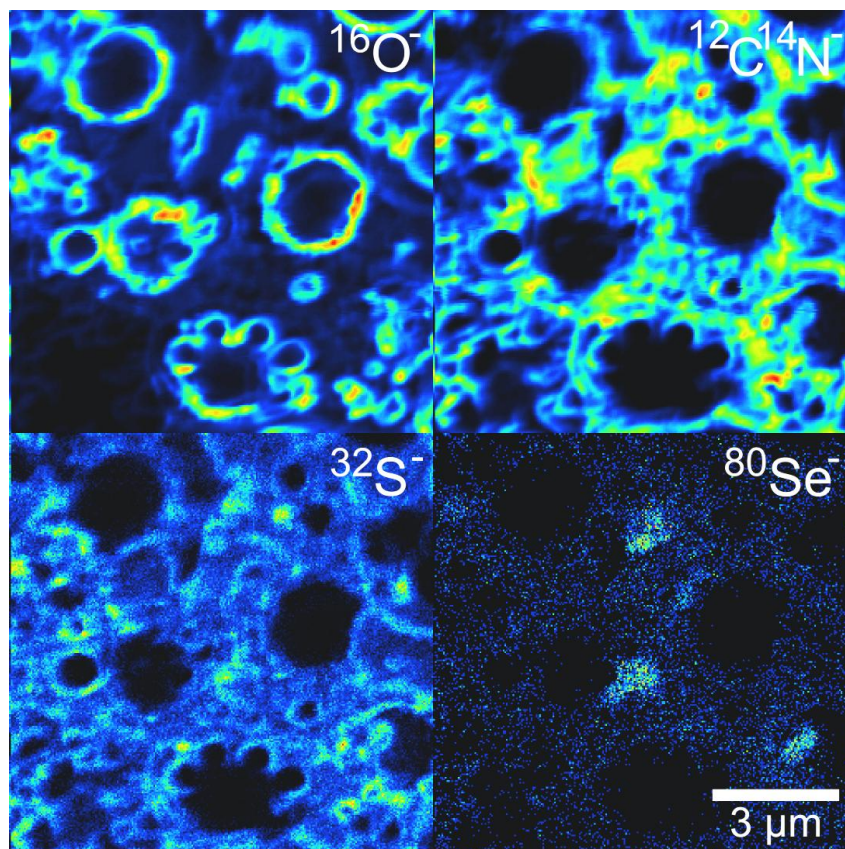


Figure 4.18: Higher resolution NanoSIMS maps of $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, and $^{80}\text{Se}^-$ of the area marked by the red box in Figure 4.16. The Se-rich hotspots seem to be on the outside of the phytate granules.

Both the colour merge image in Figure 4.19, and the line scans shown in Figure 4.20 and Figure 4.21 clearly show that there is no apparent association of the Se in the hotspots with sulphur. In Figure 4.20 it is especially clear that there is a reduction in the sulphur intensity through the hotspots. There is not enough signal from the background Se to clearly show in the line scans that the Se does follow the sulphur in the cell matrix, this is shown best in Figure 4.19 although some of this signal variation may be due to topography.

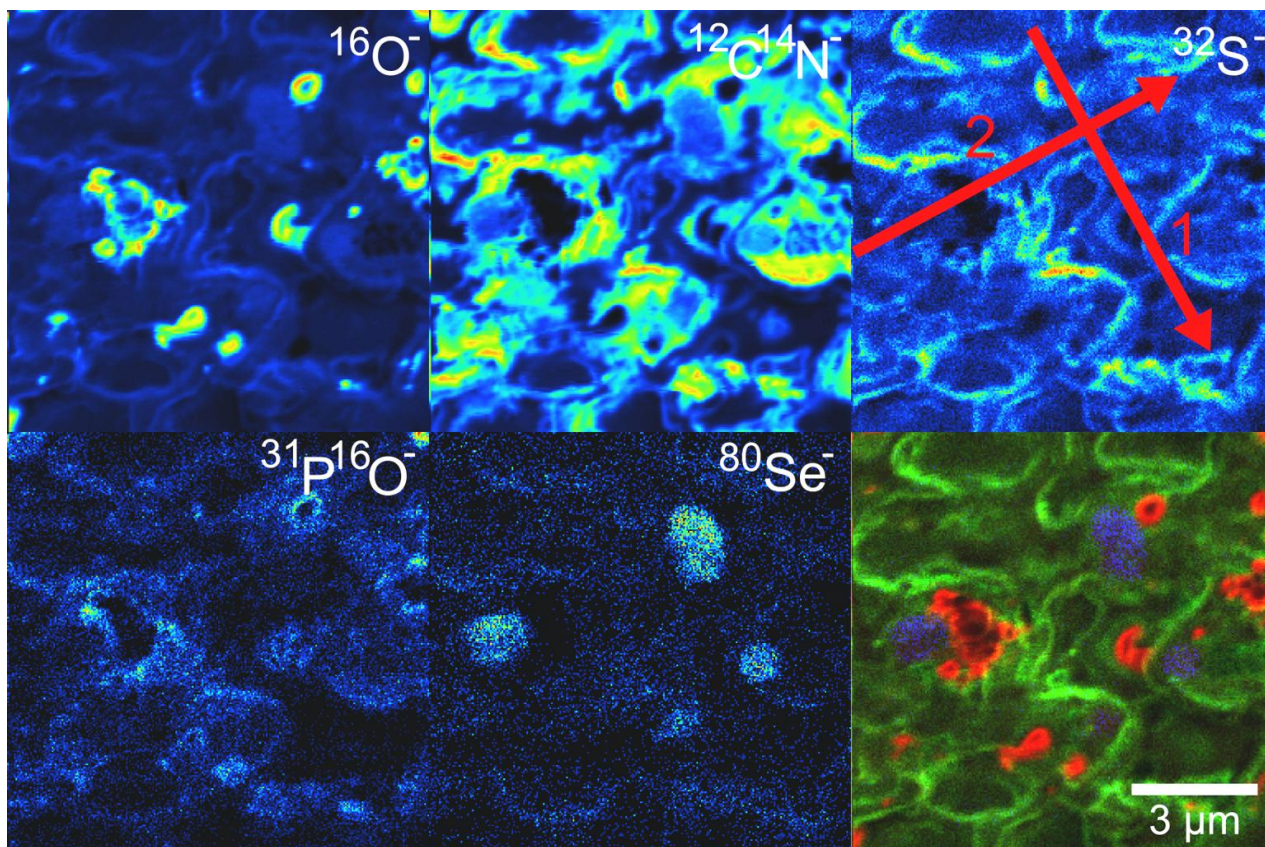


Figure 4.19: Higher resolution NanoSIMS maps of $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{31}\text{P}^{16}\text{O}^-$, $^{80}\text{Se}^-$ and a colour merge of the area marked by the red box in Figure 4.17. These Se-rich hotspots also seem to be on the outside of the phytate granules and there is no apparent association of the Se in these hotspots with the sulphur. The arrows mark the position and direction of the line scans. Colour merge: red = $^{16}\text{O}^-$, green = $^{32}\text{S}^-$, and blue = $^{80}\text{Se}^-$. This image clearly shows that there is no co-localisation of the Se in the hotspots with the sulphur. Reprinted with permission from John Wiley and Sons.

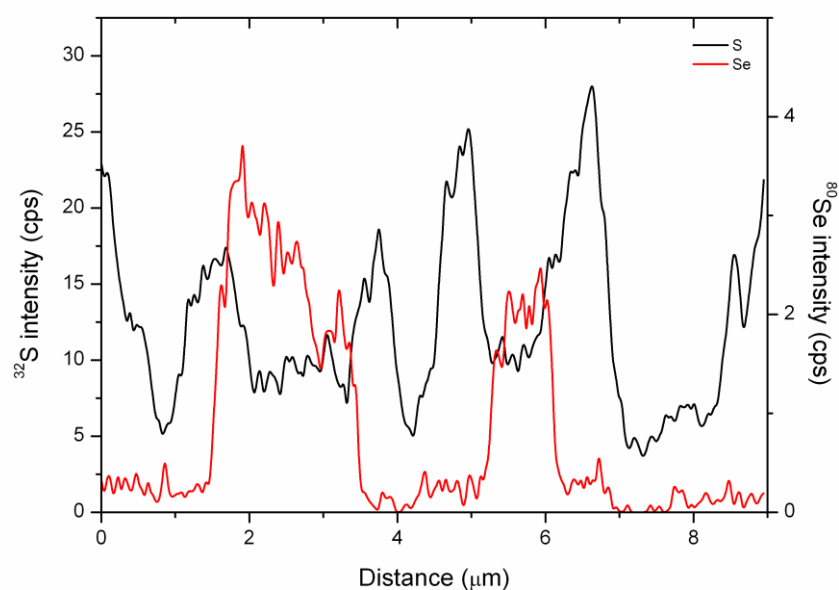


Figure 4.20: Line scan for $^{32}\text{S}^-$ and $^{80}\text{Se}^-$ across two hotspots as indicated by the arrow marked 1 in Figure 4.19, with the intensity in counts per second (cps).

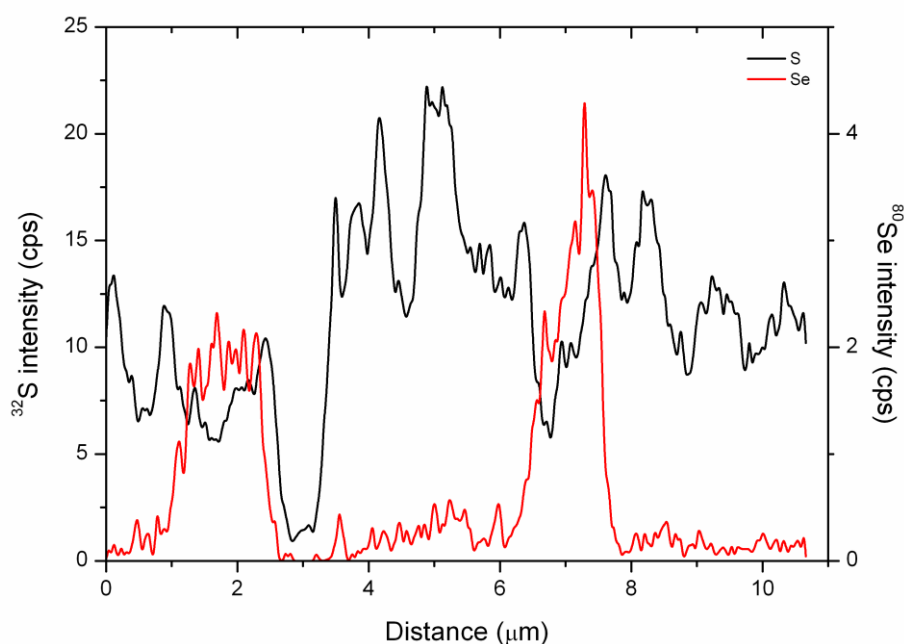


Figure 4.21: Line scan for $^{32}\text{S}^-$ and $^{80}\text{Se}^-$ across two hotspots as indicated by the arrow marked 2 in Figure 4.19, with the intensity in counts per second (cps).

Wheat is relatively tolerant to high concentrations of Se (Lyons *et al.*, 2005b) and it is possible that the Se-enriched wheat grain stores Se in these localised regions, which may be derived from vacuoles, to prevent high Se concentrations having toxic effects more widely within the grain. This is the first time that localisation of Se to this type of feature has been observed in wheat grain and further work is needed to confirm the identity of the compartments and determine the processes that lead to this apparent sequestration. It would be interesting to investigate whether Se hotspots also occur in grain containing lower levels of Se, although analytically this will be difficult as the Se concentration will be approaching the detection limit even of the NanoSIMS. Analysis of similarly Se-fertilised grain by HPLC-ICP-MS showed little difference in the form of Se between the bran and white flour fractions; both containing selenomethionine as the major Se compound (Hart *et al.*, 2011). It is therefore not clear what the likely form of Se is within these Se hotspots.

The embryo was a region of particular interest for analysis with the NanoSIMS as Eurola *et al.* (1990) had indicated that the embryo should contain approximately 1.5 times more Se than the endosperm. Very little is known about the embryo and where the Se is localised in this region. Unfortunately it was not possible to analyse the embryo in the mature wheat grain samples with the NanoSIMS because no signal could be obtained from this region due to sample charging. This was presumably due to a difference in composition, possibly the high oil content of the embryo, which inhibited secondary ion formation.

4.4.3. EPMA

Electron microprobe analysis was performed on another wheat grain prepared with a razor blade in the same way as described previously. The same grains that were used for NanoSIMS analysis could not be used for this technique as they had been coated with a 10 nm coating of Pt. The M_{α} Pt X-ray has an energy of 2.0485 keV which sits too close to the P K_{α} X-ray line, with an energy of 2.0134 keV, to separate out the peaks to allow accurate mapping of the phosphorous (which the NanoSIMS has indicated is an important element). Also the NanoSIMS primary ion beam introduced surface roughening, hence it was necessary to perform EPMA analysis on a new sample.

The purpose of performing EPMA analysis on these samples was to see if the micron-sized hotspots could be detected with this technique as well. This would therefore eliminate the possibility of these being artefacts from the imaging technique. The sensitivity of the microprobe is about 100 ppm, therefore it was unlikely that with an average concentration in the grain of 16.7 ppm that the microprobe would be able to map the Se distribution, but the concentration in these Se rich hotspots should be much higher, hence if they are present they could be observed.

The backscattered electron image in Figure 4.22 shows the structure of the analysed area of the wheat grain. At the bottom of the image three aleurone cells can be seen and inside each of these

the phytate granules show up as very bright circles. There appears to have been some shrinkage of the middle aleurone cell away from the cell wall, however the inside of the cell appears to have remained intact. At the top of the image the starchy endosperm region has been mapped and individual oval shaped starch grains can be observed in the endosperm cells. In Figure 4.23 a slightly larger area than that of Figure 4.22 has been mapped showing the same three aleurone cells but also some of the outer layers. There does appear to be a gap between the layers at the bottom of the image where there are no elemental signals however this has not affected the area of interest.

Consistent with the EDX results in Figure 4.9, potassium, magnesium, oxygen and phosphorous, as shown in Figure 4.22 and Figure 4.23, are strongly located in the phytate granules. In these EPMA maps it is possible to see the individual phytate granules in the chemical maps, hence it is possible to clearly see the association of these elements to these regions. There is an area in the centre of the left-hand aleurone cell where no phytate granules are observed and it is possible that this is where the nucleus was located. Phosphorous usually indicates metabolic activity, however the two regions of medium phosphorus intensity in the sub-aleurone region are probably not nuclei because the aleurone cells are the only live endosperm tissue at maturity (Khan & Shewry, 2009).

The sulphur image is very similar to that observed in the NanoSIMS images. Sulphur is detected both inside the aleurone cells and also in the protein regions in between the starch grains. Similar to the NanoSIMS images shown in Figure 4.16 and Figure 4.17 there is a slightly higher concentration of S around the aleurone cells towards the outside of the grain. It was expected that the Se map would be very similar to the sulphur map however it is clear that the Se distribution is very homogeneous and the only differences in the distribution are observed where there is topography. There are two possible reasons for this distribution. Either the position of the detector was not positioned correctly for Se or, more likely, the Se distribution is too low to be

detected by EPMA and this is just background signal. Most of the points in this image have at most 1-2 counts and due to the homogeneity of the image, background is the most likely explanation. It would appear that there are no Se hotspots present in these cells. Biological materials differ greatly so it is possible that the grain analysed in the NanoSIMS had a much higher Se concentration than the grain analysed here, hence no hotspots are observed in Figure 4.23.

The microprobe did allow imaging of elements such as potassium, calcium and magnesium which cannot be imaged with the caesium ion beam in the NanoSIMS. The calcium map showed the presence of intense regions in the sub-aleurone region and there is also a very definite line of calcium in the pericarp. The concentration of iron in the wheat grain is very low and close to the sensitivity limit for the microprobe, however it is possible to see that more iron is located inside the aleurone cells than in any other part of the imaged area. It is likely that most of the iron signal outside of the aleurone areas is background noise because the large gap between the outer layers, shown most clearly in the Se image, is not visible in the iron image. Although the maximum counts in the Fe image are similar to those of the Se image these are much more localised therefore are more likely to be real variations in composition. The silicon map is rather homogeneous apart from the bottom left hand corner. This high silicon concentration region is likely to be from the pericarp or testa of the wheat grain. High silicon intensity is expected in the husk as it forms a protective layer for the grain.

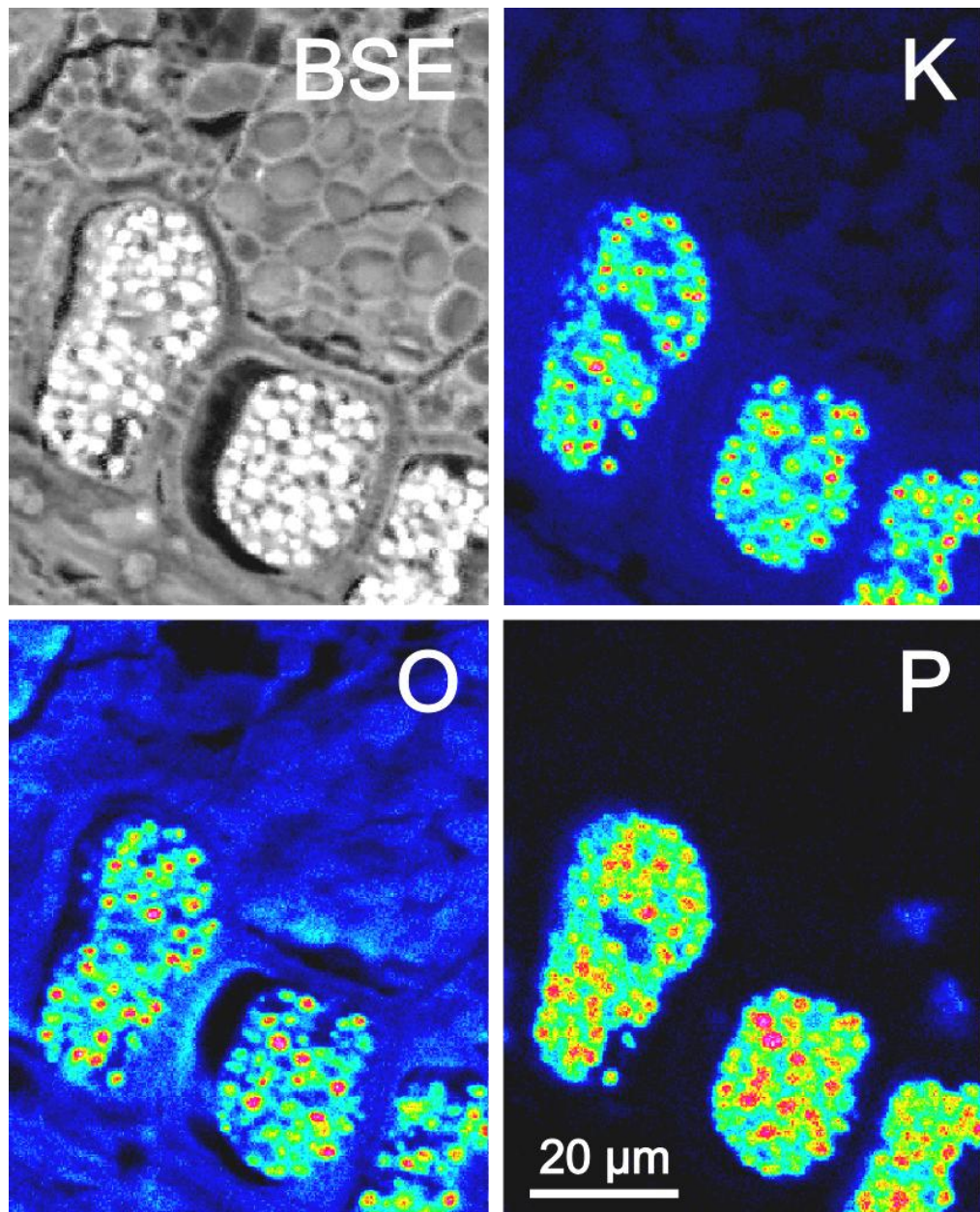


Figure 4.22: EPMA maps showing the backscattered electron image (BSE), K, O and P from the edge of a mature wheat grain sectioned with a razor blade. Three aleurone cells are observed at the bottom of the image and the starchy region is at the top.

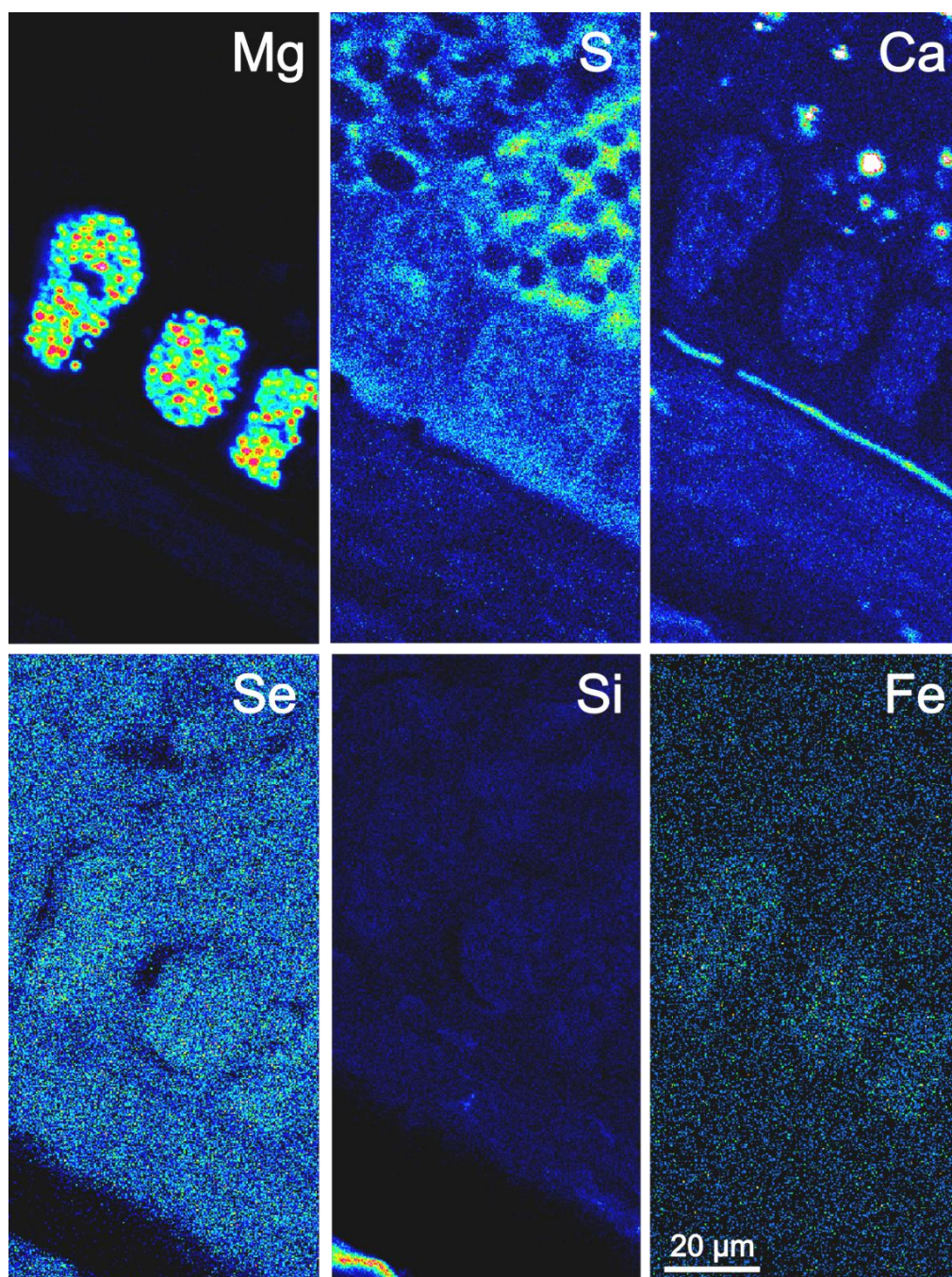


Figure 4.23: EPMA maps showing the Mg, S, Ca, Se, Si and Fe signals. This area is a slightly expanded area from that shown in Figure 4.22.

4.4.4. FOCUSED ION BEAM IMAGING

Cleaning and imaging of the grain with an intense primary ion beam resulted in artefacts being introduced into the NanoSIMS images making interpretation of the results more difficult. In order to study these, a focused ion beam microscope (FIB) was used to simulate the effect of the NanoSIMS primary ion beam on the wheat grain. In the NanoSIMS it is necessary to first

remove the 10 nm platinum layer from the grain and implant caesium before analysis so it is not possible to observe directly how the surface topography develops with sputtering. The FIB is capable of much higher beam currents (for these experiments 300 pA) than the NanoSIMS (15-25 pA) and only 2 nm of platinum was necessary to prevent charging so this allowed the evolution of the structures to be monitored much more quickly. The FIB scans the primary ion beam over the sample surface in a raster in the same way as the NanoSIMS and forms the image with the ion-induced secondary electrons.

4.4.4.1. Starch

The first artefact appears on cleaning and imaging of the starchy endosperm region of the endosperm. Within all of the starch grains examined in the NanoSIMS a porous structure is observed yet the surrounding protein region is unaffected. This structure can be seen in the NanoSIMS secondary electron image of Figure 4.24. This porous structure was not observed in any of the starch grains examined before SIMS analysis by optical or SEM microscopy. Two possible causes are proposed for why this structure appears. Either the ion beam reveals morphology inherent within the starch grain or the ion beam is preferentially removing either the amylopectin or amylose polysaccharide components of the starch.

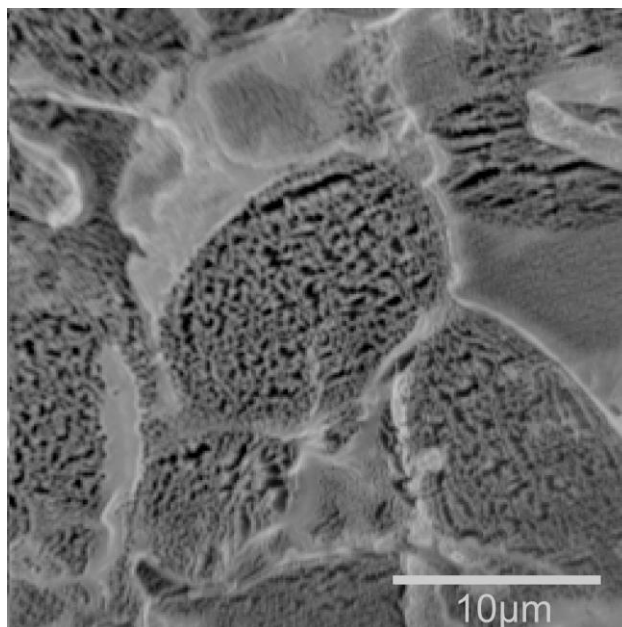


Figure 4.24: NanoSIMS secondary electron image showing a porous structure inside the starch grains after exposure to the Cs^+ primary ion beam

Successive FIB images were taken of the starchy region of razor blade sectioned wheat grain, removing small quantities of material each time. Initial experiments were performed on normal wheat grain and the same structure was found to be created very quickly by the ion beam. In order to establish if the structure was inherent to the starch grains or if one of the starch components were preferentially removed, wheat grains with 100% amylopectin (waxy) starch were obtained from Rothamsted Research and the same experiment was performed.

FIB images of the 100% amylopectin starch before and after imaging with the ion beam are shown in Figure 4.25. When the resulting image (Figure 4.25 b)) is compared with the NanoSIMS secondary electron image of Figure 4.24 it can be seen that the structures are identical. As the same structure develops in the 100% amylopectin starch grains it can be concluded that this structure is not caused by preferential removal of either amylose or amylopectin and hence the ion beams in both the FIB and the NanoSIMS must be revealing a structure which is inherent within the starch grains. Continued imaging of starch grains with an electron beam in an SEM does not cause the same structure. Although this artefact causes

problems with understanding the element localisation within the starch grains it appears not to affect the protein region in the same way. As the majority of the Se is located within this protein region the Se localisation can still be investigated with confidence. It is not clear why this structure appears with ion beam imaging. Previous work investigating the internal structure of starch grain in wheat (Yuryev *et al.*, 2004), Arabidopsis (Zeeman *et al.*, 2002) and potato tubers (Pilling & Smith, 2003) have all indicated that there are semi-crystalline growth rings within the starch grain which consist of amylopectin clusters and are surrounded by amorphous regions. It is possible that the ion beam breaks apart these rings leading to the observed structure.

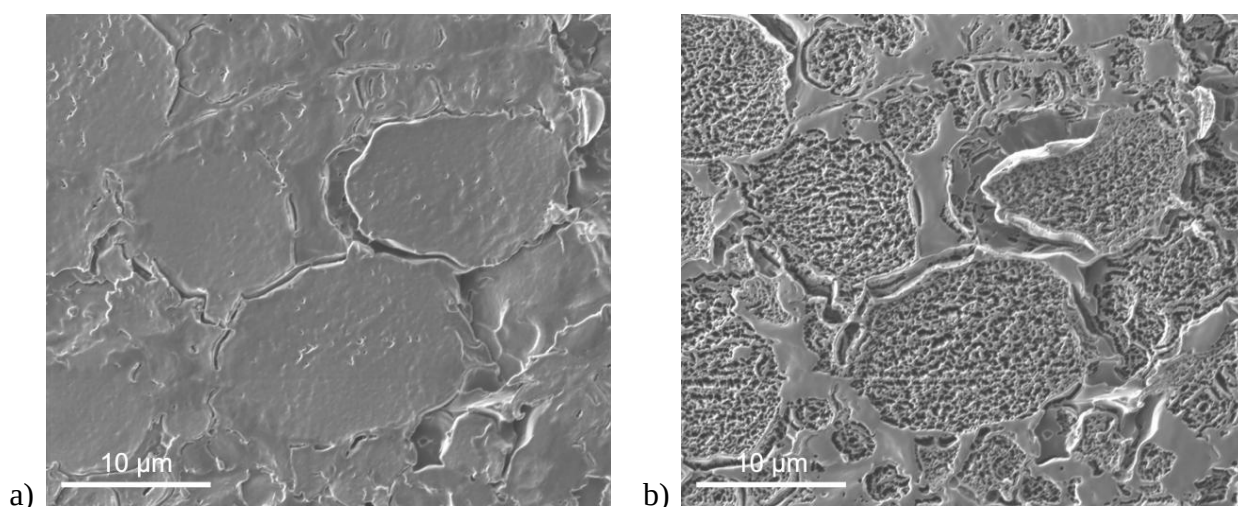


Figure 4.25: Focused ion beam secondary electron images of a wheat grain containing 100% amylopectin starch showing a) razor blade sectioned starch before FIB imaging b) porous structure after FIB imaging which is identical to the structure which is revealed in the NanoSIMS

4.4.4.2. Phytate Granules

The other observed artefact affected the phytate granules within the aleurone cells. In all the NanoSIMS images of the aleurone cells the phytate granules appear as a ring of high intensity in the O^- and PO^- images accompanied by a circular region with no intensity in the CN^- and Se^- images. It was expected that the phytate granules would appear in the O^- and PO^- images as filled circles as the chemical structure of phytic acid (Figure 4.10) indicates that there is a high concentration of P and O, and EDX and EPMA have also shown this to be the case. This is not the first time this feature has been seen in SIMS analysis of wheat grain. Heard *et al.* used SIMS to look at the aleurone layer of wheat grain and accounted for this effect as local charging within

the phytate resulting in the suppression of the secondary ion signals (Heard *et al.*, 2002). The characteristic effect of charging, streaks running across the image as the sample discharges, were not seen in any of the NanoSIMS images therefore this may not be the full explanation.

The initial concern was that these phytate granules were being preferentially sputtered away leaving a hole from which no signal could be detected. To investigate if this was the case a razor blade sectioned wheat grain sample was placed into the FIB and, as with the starch, successive images were taken to see how this effect develops over time. The phytate granules are not dark in the first FIB image (Figure 4.26 a)) because the 2 nm platinum coating had not been removed. With continued imaging (Figure 4.26 b)) it became apparent that the inside of the phytate granules became dark in the same way as is observed in the NanoSIMS. The FIB also indicated the presence of a membrane covering the phytate granules. The phytate granule at the top of Figure 4.26 a) has not been cross sectioned with the razor blade and before FIB imaging it appears spherical as expected. With continued imaging there is a ‘peeling back’ of what would appear to be a membrane. In Figure 4.26 b) three regions of this outer membrane remain and the underlying matrix is exposed in between which also has a low electron yield.

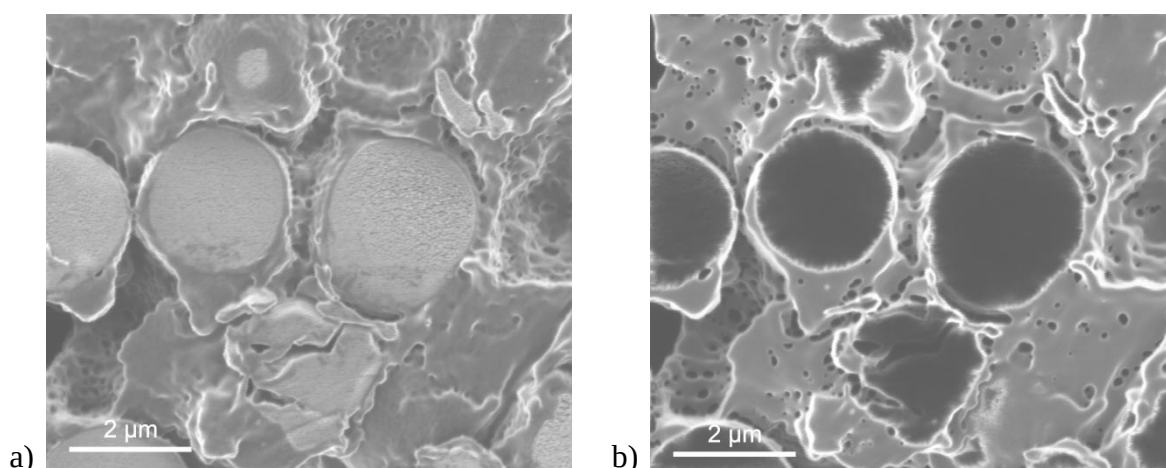


Figure 4.26: Focused ion beam secondary electron image of wheat phytate granules a) before FIB imaging b) after FIB imaging. Note the phytate granule in the middle at the top of the image, there is a gradual ‘peeling back’ of a surrounding membrane which reveals the underlying membrane which has a low electron yield.

This successive FIB imaging did not fully answer the question of whether these areas were being preferentially sputtered away as there was not enough secondary electron yield coming from these areas to see if any material remained. A second FIB experiment was performed which utilised the higher primary ion beam currents available in the FIB to remove a large part of material. A well preserved aleurone cell was selected and half of the cell was placed out of the field of view of the primary ion beam to protect it from damage. A trench was milled across the other half of the cell using a 1 nA beam until it was approximately 5 μm deep. The protected half of the cell was then moved into the field of view and imaged using the ion beam until the dark regions in the phytate granules were exposed. Tilting the whole grain by 30 degrees allowed examination of the phytate granules from the side to see if material had been preferentially removed. The trench can be seen in Figure 4.27 a) and in the higher magnification FIB image of the edge of this trench (Figure 4.27 b)) is it immediately apparent that the phytate granules have not been sputtered away preferentially. There is also no evidence of charging within the phytate granules.

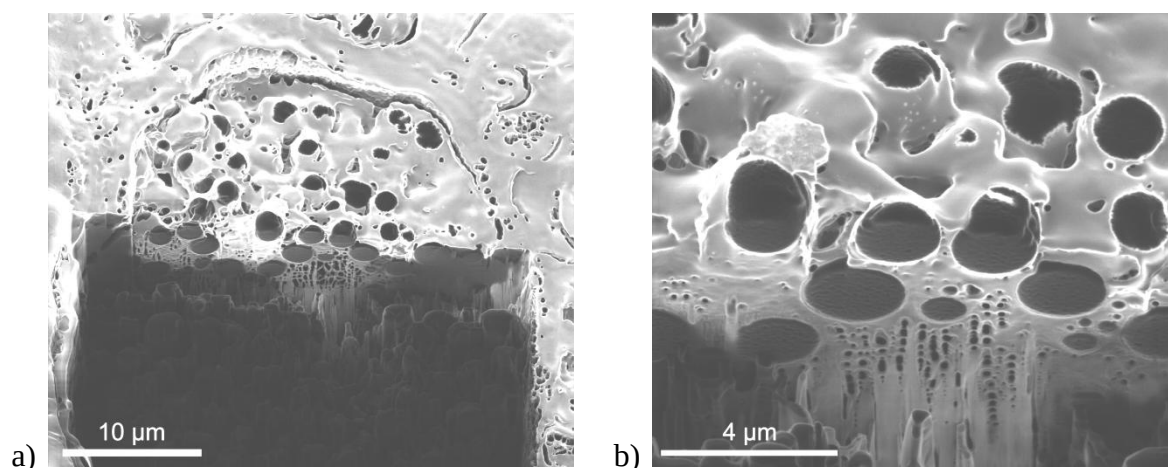


Figure 4.27: Focused ion beam image of a) the milled trench b) higher magnification image showing that the phytate granules are still filled and are not preferentially sputtered away

This leads to the assumption that the reason these granules are dark is because the yield of secondary electrons from these regions is very low. This is possibly due to the well-known matrix effect (Benninghoven *et al.*, 1987; Wilson *et al.*, 1989) and hence the yield of secondary

ions is also suppressed, and no signal is seen in the NanoSIMS. The yields of $^{16}\text{O}^-$ and $^{31}\text{P}^{16}\text{O}^-$ secondary ions are very low from the bulk of the phytate granules even though the local concentrations of these elements are high. The yields of these same ions adjacent to the surrounding material (matrix) are much higher and this leads to the ring structure. The main problem associated with this artefact is that it is not possible to detect any elemental signal from within the phytate granules therefore it is not possible to determine the Se distribution inside them. The assumption can, however, be made that there is more Se in the surrounding matrix than inside them because there is no ring structure, consistent with the $^{12}\text{C}^{14}\text{N}^-$ and $^{32}\text{S}^-$ images.

4.4.5. NANOSIMS OF IMMATURE WHEAT GRAINS

4.4.5.1. Optical Imaging

Before NanoSIMS analysis was undertaken maps were made of the sample on the Si wafer so that the NanoSIMS analysis could be accurately positioned, an example of this is shown in Figure 4.28 for the chemically fixed, 14 DPA sample from a plant treated with 10 ppm sodium selenate, which was used for the majority of the analysis presented in the following NanoSIMS section. In this transverse section through the wheat grain it is possible to see the embryo, the starchy endosperm, the crease and the nucellar projection. The aleurone layer is well preserved around the edge of the wheat grain however some of the outer layers, specifically the epidermis and the hypodermis have pulled away from the rest of the pericarp. This damage was caused during the sectioning of the wheat grain as these outer layers are very difficult to preserve due to their fragility.

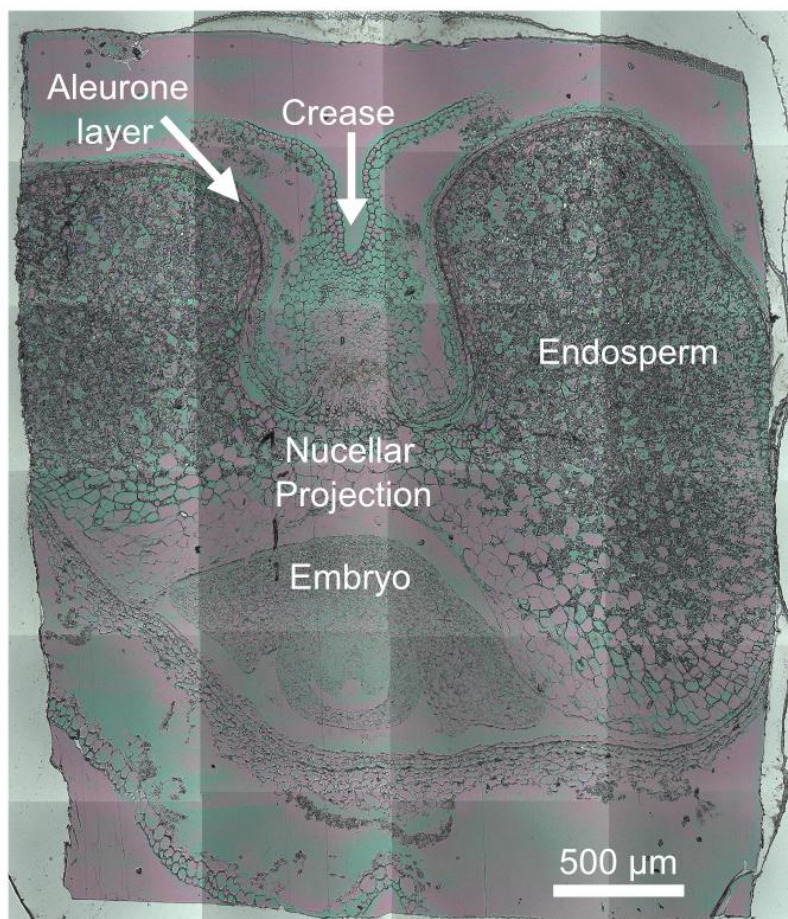


Figure 4.28: Optical map of microtomed wheat grain, 14 DPA, 10ppm sodium selenate, chemically fixed deposited onto an Si wafer.

It is clear from the secondary electron images in Figures 4.29, 4.30 and 4.31 that the same structure appears in the starch grains as was observed in the mature wheat grains in both the NanoSIMS and the FIB. It would therefore appear that neither the sample preparation nor the embedding procedure controls the sputtering behaviour of the starch grains. Protein bodies are visible both at 14 DPA (Figure 4.29) and at 21DPA (Figure 4.30) which is consistent with the images of wheat grain development that have been published previously (Tosi *et al.*, 2009), however in the 21 DPA sample the image of the central endosperm apparently shows some protein matrix surrounding the starch grains with a lower concentration in addition to the protein bodies observed in the adjacent cells. In one analysed region of a 14 DPA HPF sample the protein bodies had already broken down and instead a protein matrix is observed between the starch grains (Figure 4.31). This observation is consistent with that of Tosi *et al.* (2009) who

showed that at 14 DPA some of the endosperm cells have a coherent protein matrix whereas others still contain protein bodies. In all three figures it is clear to see that the selenium is incorporated into the protein which is consistent with that shown previously for the mature wheat grains. The selenium distribution of Figure 4.31 is very similar to that of Figure 4.14 b), again indicating that the sample preparation had very little effect on the Se distribution. In the top left hand corner of Figure 4.29 some very fine lines can be observed which are most likely to be starch membranes which have broken and are no longer attached to the starch grain. A similar intact starch membrane can be observed surrounding the starch grain at the bottom of the image. These membranes cannot be seen in Figures 4.30 and 4.31 because the surrounding protein matrix overwhelms the signal. Endosperm cell walls are clearly shown at the top and bottom of Figure 4.30 and across the middle of Figure 4.29.

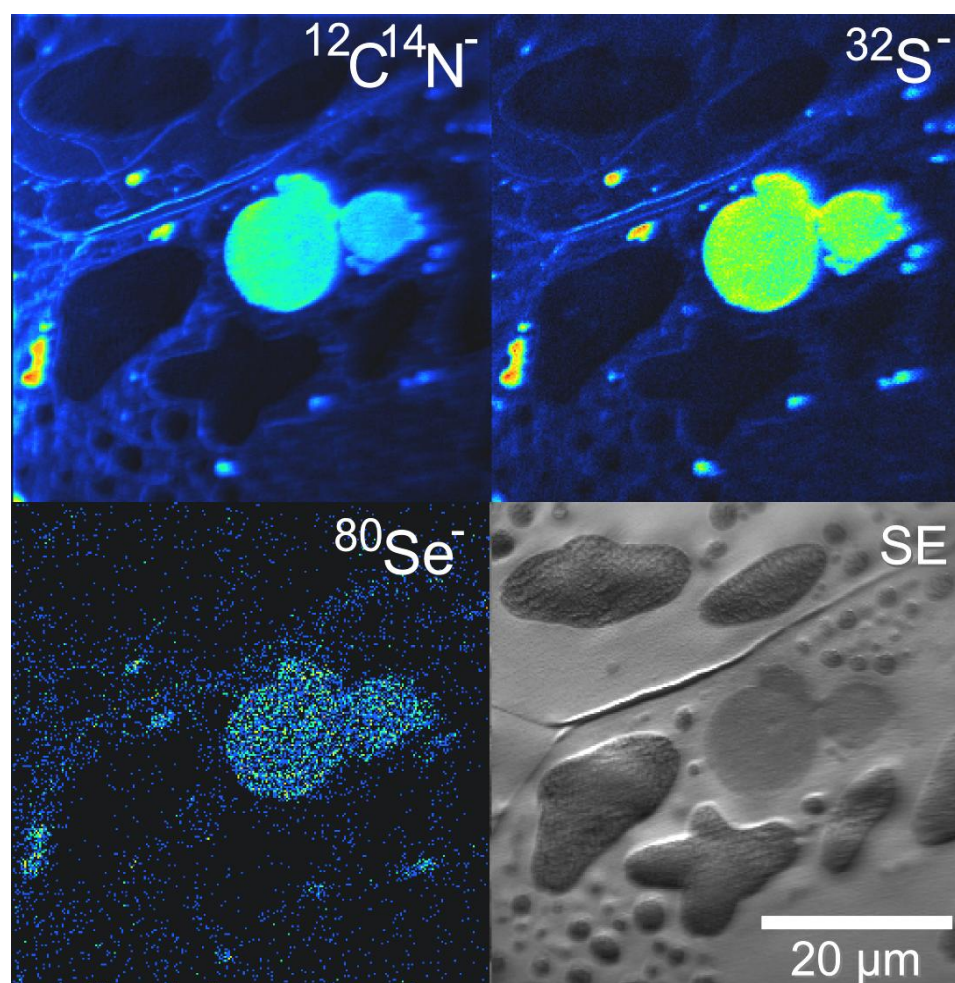


Figure 4.29: Chemically fixed wheat grain sampled at 14 dpa and treated with 10 ppm sodium selenate. Image is taken from the starchy endosperm region showing individual starch grains and protein bodies containing strong $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$ and $^{80}\text{Se}^-$ signals.

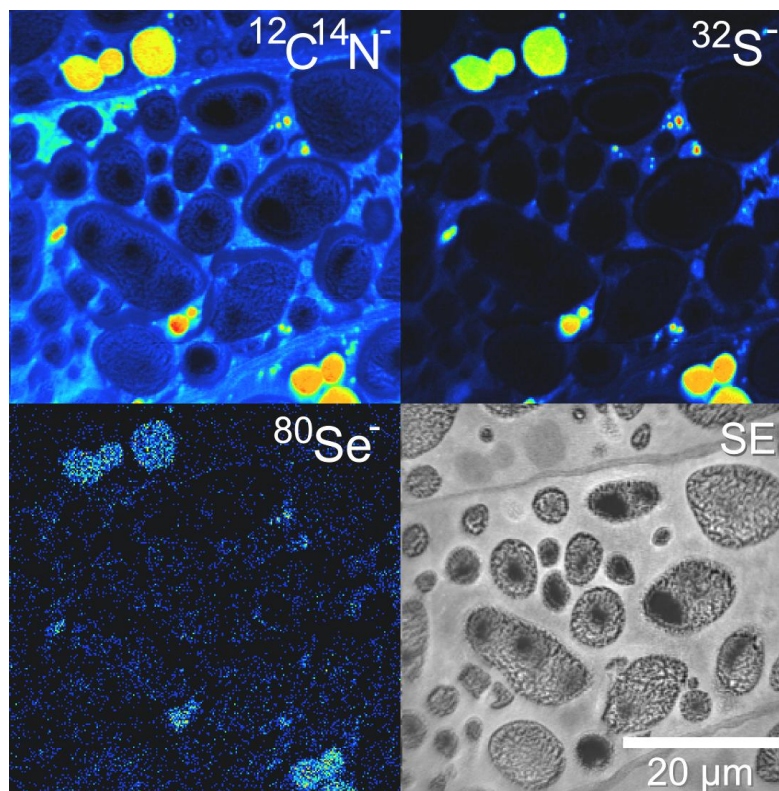


Figure 4.30: High pressure frozen wheat grain sampled at 21 DPA and treated with 5 ppm sodium selenate. Image is taken from the starchy endosperm region showing individual starch grains and protein bodies containing strong $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$ and $^{80}\text{Se}^-$ signals.

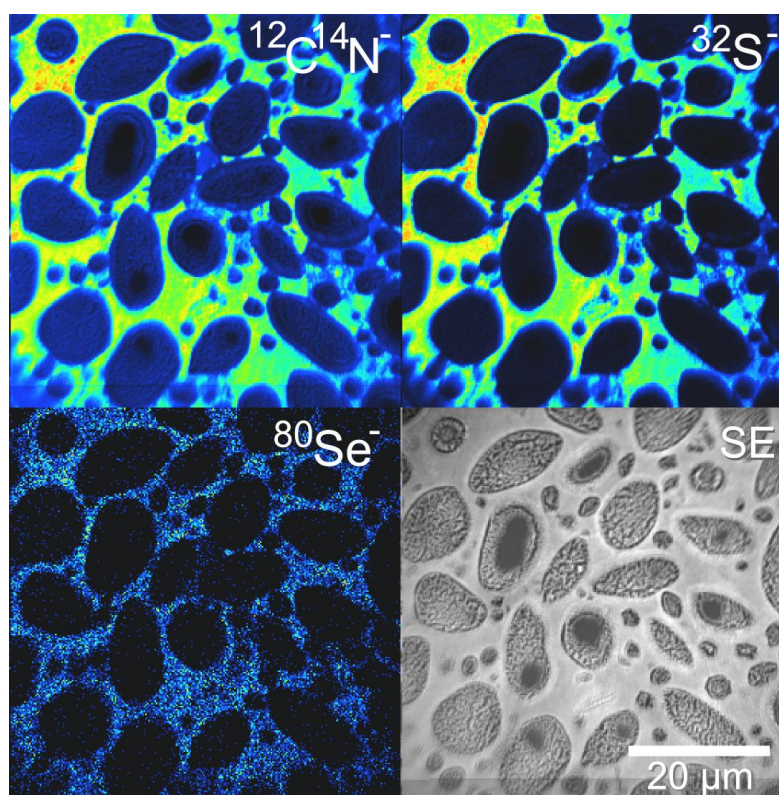


Figure 4.31: High pressure frozen wheat grain sampled at 14 dpa and treated with 5 ppm sodium selenate. Image is taken from the starchy endosperm region showing individual starch grains surrounded by a Se containing protein matrix (the protein bodies have naturally broken down)

4.4.5.2. Aleurone Cells

It was expected that Se hotspots would be observed in the aleurone layer near the phytate granules similar to those shown in Figure 4.19. Figure 4.32 shows part of an aleurone cell. To the right of the image is the nucleus, containing three nucleoli, which is surrounded by phytate granules clearly indicated in the $^{31}\text{P}^{16}\text{O}^-$ image. The HPF process has preserved the subcellular structure of the cell allowing the fine detail within the nucleus to be resolved which was not possible in the mature wheat grain (c.f. Figure 4.16). Both $^{32}\text{S}^-$ and $^{80}\text{Se}^-$ are co-localised with $^{12}\text{C}^{14}\text{N}^-$ within the nucleoli but not within the rest of the nucleus. Similarly to the results from the mature wheat grains there are Se hotspots which are close to the phytate granules but Se is not localised inside the granules. In contrast, however, these hotspots show co-localisation with $^{32}\text{S}^-$ which was not seen previously in the mature wheat grains (Figure 4.19 and Figure 4.20). Similar results are also observed with the chemically fixed samples (Figure 4.33). It would appear that there are many types of granule contained within the aleurone cell which have different behaviours with regard to the Se and S distributions. It is not clear how the wheat grain is able to partition the Se into these small hotspots, especially when considering that some are not colocalised with $^{32}\text{S}^-$ or $^{12}\text{C}^{14}\text{N}^-$.

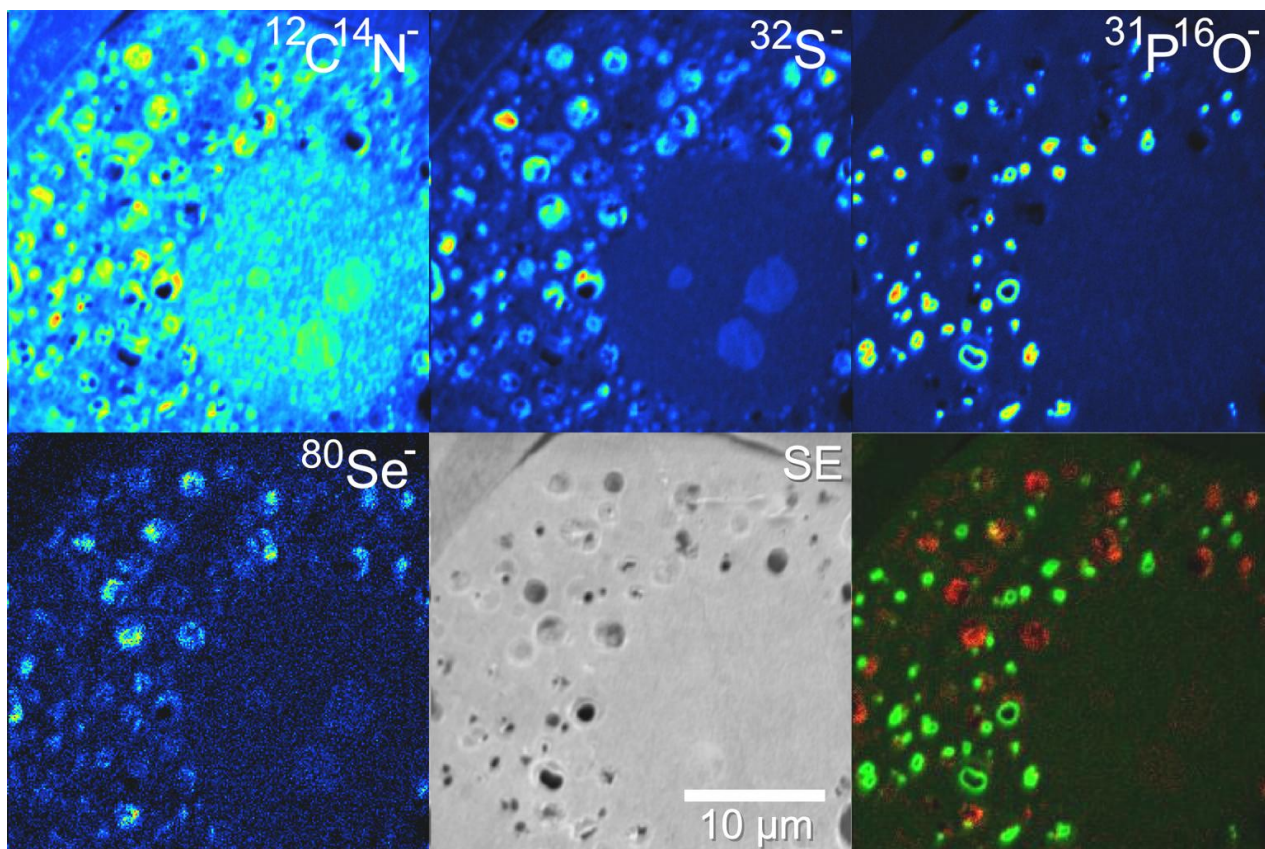


Figure 4.32: High pressure frozen wheat grain sampled at 14 DPA and treated with 5 ppm sodium selenate. The image shows part of an aleurone cell including three nucleoli contained within the nucleus which is surrounded by phytate granules. The colour merge image shows the relative locations of the $^{80}\text{Se}^-$ (red) and the $^{31}\text{P}^{16}\text{O}^-$ (green).

For the mature wheat grains, with a concentration of 16.7 ppm Se, only a few hotspots were observed whereas for the immature wheat grains with a significantly higher Se concentration more Se hotspots were observed within the cells, again suggesting that a high Se concentration causes the formation of these hotspots, possibly to prevent wider Se toxicity.

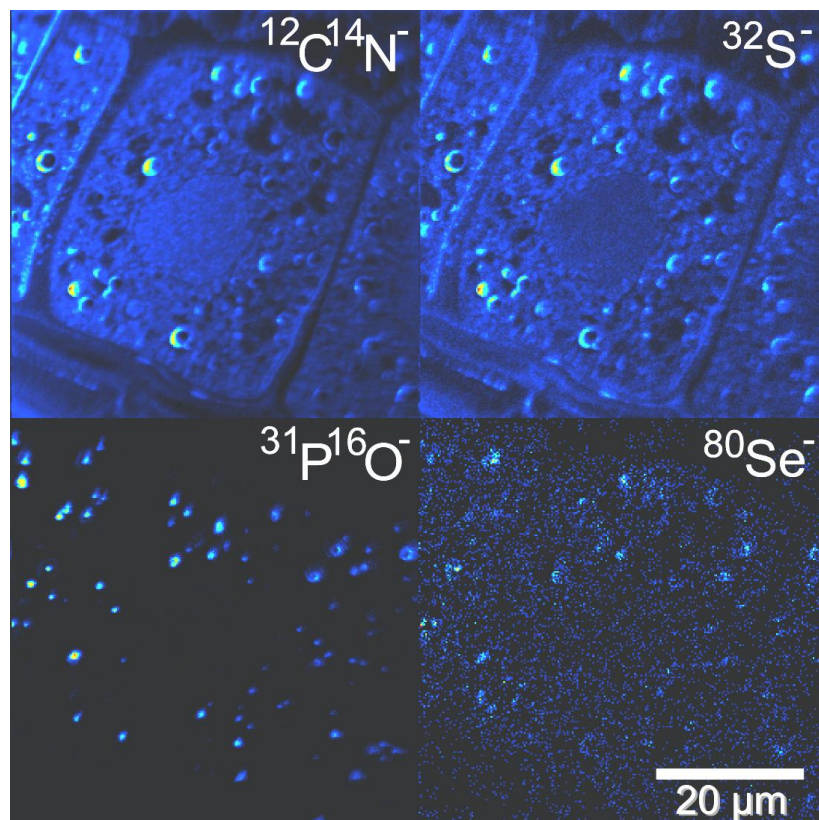


Figure 4.33: Chemically fixed wheat grain sampled at 14 DPA and treated with 10 ppm sodium selenate. The image shows an aleurone cell with the nucleus which is surrounded by phytate granules.

SEM of a chemically fixed section adjacent to that shown in Figure 4.32 provides an explanation for the differences in shapes between the granules observed in the NanoSIMS images of the aleurone cells prepared by chemical fixation and to some extent HPF. There is clearly some damage to the sections although it is not clear at which point during sample preparation this damage was introduced. Some of the phytate (or other granules) seem to be well preserved while others show tears along their outer edge and some seem to be missing their content altogether. The SEM images also show that there is a similar level of damage to the starch grains. These tears would lead to topography effects in the NanoSIMS image and may explain the high signal intensities observed for a few of the phytate granules in the CN and S images of Figure 4.33. It is likely that the tearing occurred during the microtomy, however poor impregnation of the resin may have caused this to be a particularly weak point in the sample.

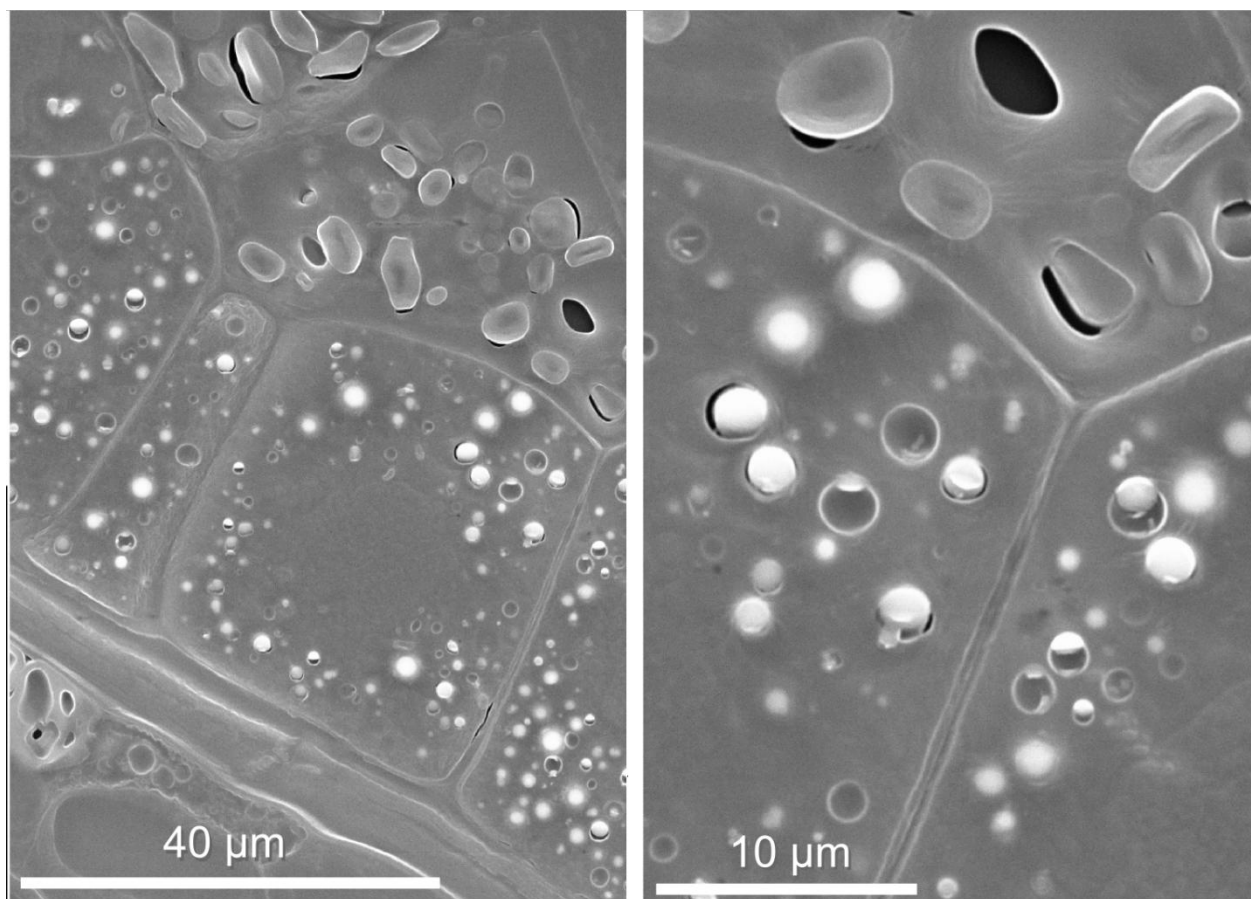


Figure 4.34: SEM images of an adjacent section to that shown in Figure 4.33 showing damage to the phytate and starch granules that has occurred during the sample preparation.

4.4.5.3. Embryo

One of the main purposes of undertaking this analysis was to be able to analyse the embryo region of the wheat grain as this could not be analysed in the mature wheat grain. Eurola *et al.* (1990) reported that the embryo contains 1.5 times higher Se concentration than the endosperm which includes the aleurone so it was expected that this would be a region of higher Se concentration. In all the NanoSIMS images of the embryo in both the chemically fixed and HPF samples, the selenium concentration was not noticeably higher. The selenium distribution tended to be rather uniform across the imaged area, as shown in the NanoSIMS images of Figures 4.35 and 4.36 with only the nucleoli showing an increase in Se concentration similarly to the nucleoli of the aleurone cells. Note; in order to form this Se image, 9 consecutive images needed to be summed together.

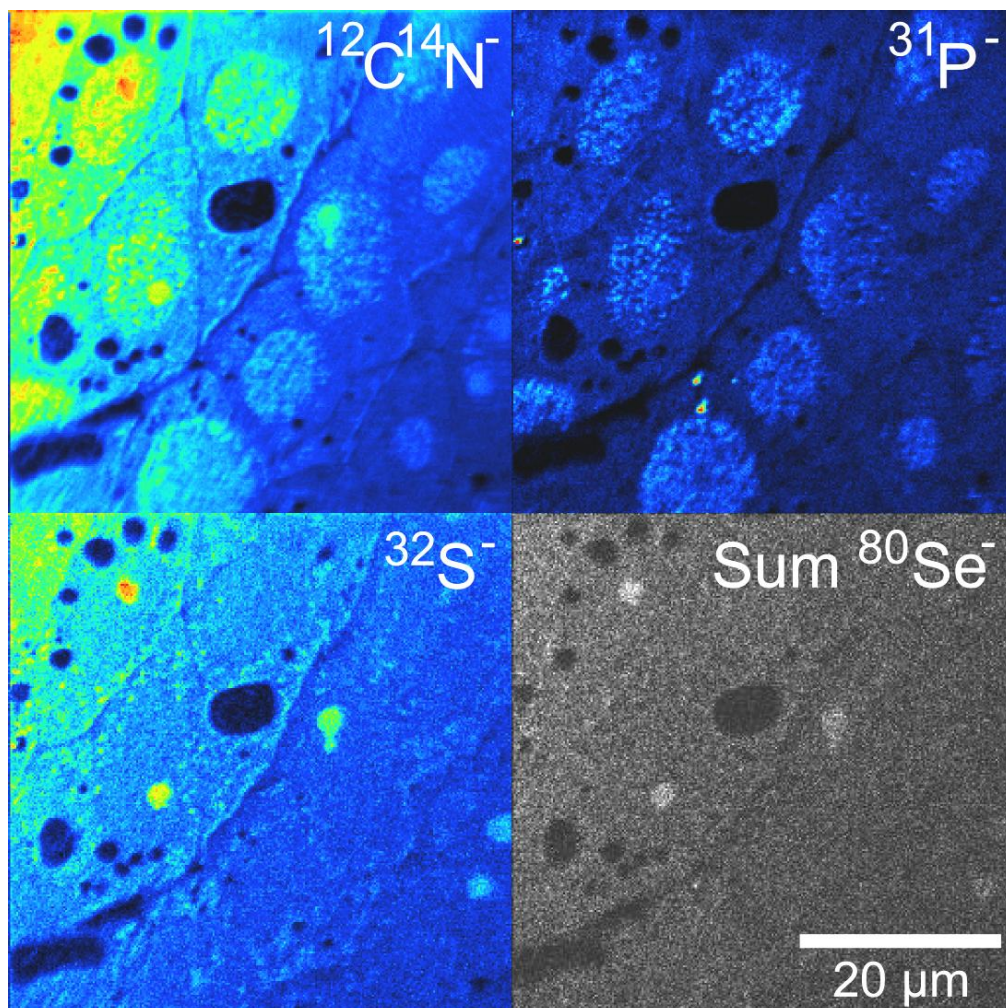


Figure 4.35: Chemically fixed wheat grain sampled at 14 DPA and treated with 10 ppm sodium selenate. The image shows part of the embryo region near the radicle. The $^{12}\text{C}^{14}\text{N}^-$ and $^{31}\text{P}^-$ images reveal the presence of many nuclei. The Se image appears to be very uniform apart from the location of the nucleoli and is the sum of 9 separate images.

NanoSIMS images of the tips of the seminal roots were obtained and are shown in Figure 4.36 and the position of the acquisition is indicated on the optical image of the same area. The embryo contains large numbers of cell nuclei, required for growth and cell division, shown clearly in the $^{12}\text{C}^{14}\text{N}^-$ image. In the tips it was not necessary to sum together several images to be able to resolve the Se distribution, however the Se concentration was still rather low and hence the enrichment of Se in the nucleoli could not be observed. Although little information can be obtained from these images with regard to the Se distribution it is clear to see that very little Se has been redistributed into the resin during infiltration as the airspace adjacent to the tips does not contain Se.

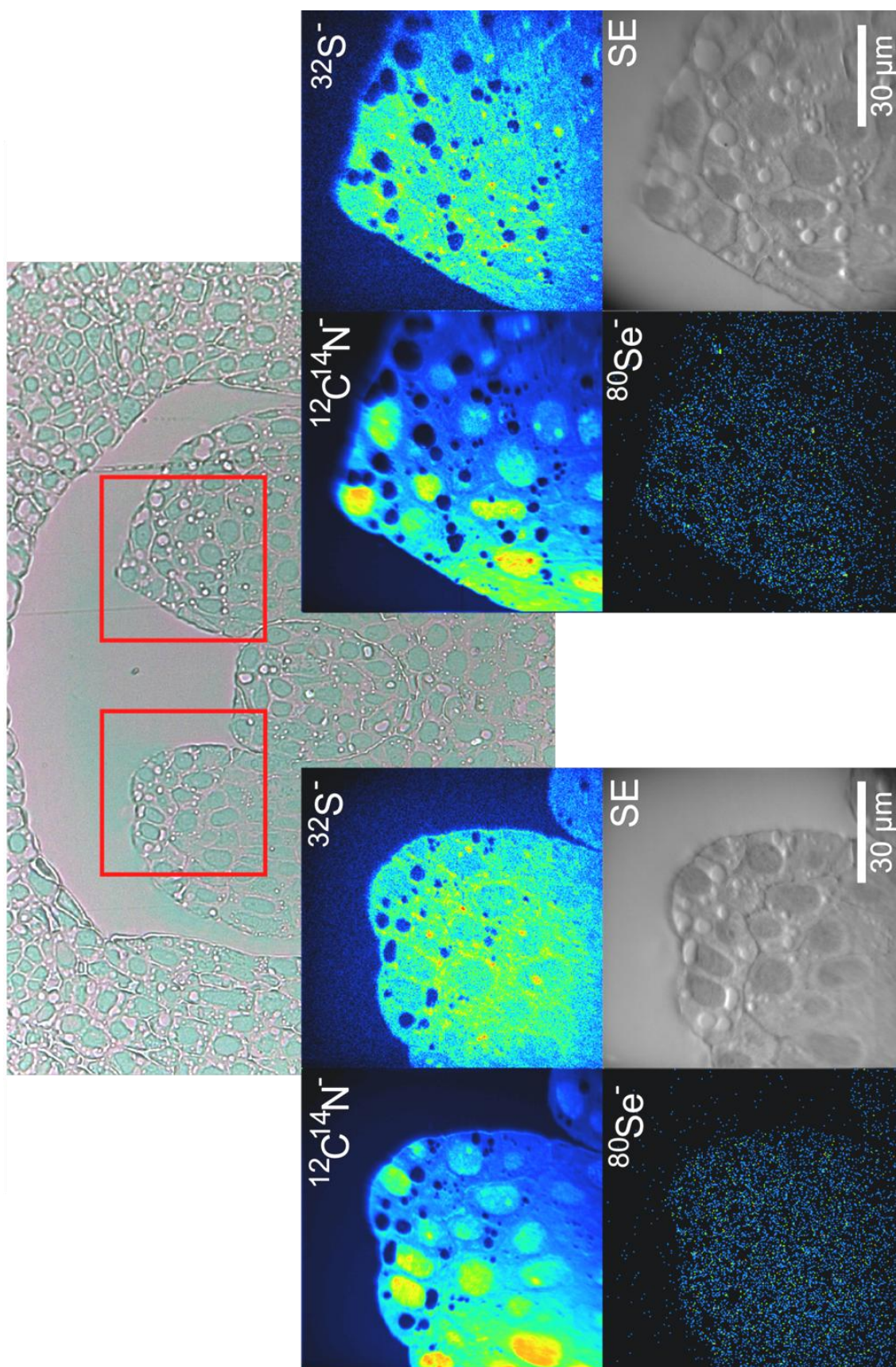


Figure 4.36: Chemically fixed wheat grain sampled at 14 DPA and treated with 10 ppm sodium selenate. Optical image shows the location of the NanoSIMS analysis on the tips of the lateral roots. Nuclei are clearly visible in the $^{12}\text{C}^{14}\text{N}^-$ and secondary electron images (SE). Selenium appears to be uniformly distributed throughout the embryo and very little seems to have been redistributed as the resin contains almost no Se.

4.4.5.4. Nucellar Projection

The nucellar projection was another part of the embryo region which could not be analysed in the mature wheat grain. Most of this region degenerates during development (Khan & Shewry, 2009) however this region was also too close to the embryo and hence the same charging problems prevented analysis. It is widely believed that the breakdown of the nucellar projection cells results in nutrients being released into the developing endosperm and embryo (Khan & Shewry, 2009). This region could therefore be an important point within the developing wheat grain where a high Se concentration can be localised. The Se in this region appeared to be only accumulated within the protein-rich regions and not within any of the vacuoles although it would appear that there is some Se localised to nuclei clearly indicated by the $^{31}\text{P}^-$ image.

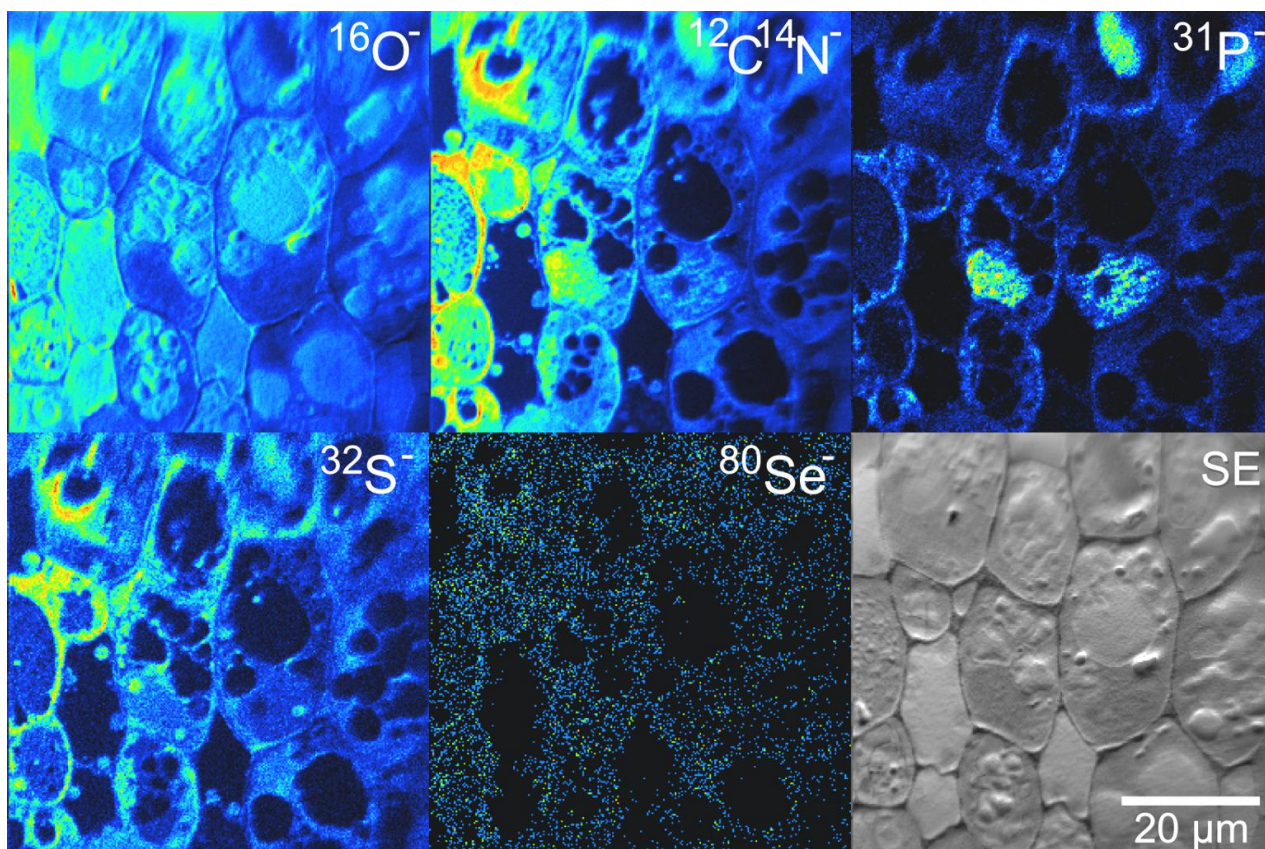


Figure 4.37: NanoSIMS image of the nucellar projection from a chemically fixed wheat grain sampled at 14 DPA and treated with 10 ppm sodium selenate. Selenium is co-localised with the protein rich regions but is not localised within the vacuoles. Three nuclei are visible in the $^{31}\text{P}^-$ image.

4.4.5.5. Quantification of Se

In order to be able to quantitatively compare the Se concentration in different regions of the wheat grain ratios of Se/CN and Se/S were calculated for each area. It was expected that the embryo and the aleurone layer would show a higher concentration of Se based on previous results (Euroola *et al.*, 1990; Lyons *et al.*, 2005a). Figure 4.38 shows that for the embryo there is an approximately linear relationship between the Se/CN and Se/S ratios so where Se is enriched relative to S it is also enriched relative to protein. This indicates a possible substitution of Se for S in proteins which was not necessarily expected in this nuclei-rich region. The data for the protein bodies seems to be following a similar trend although with only two points it is not possible to draw a firm conclusion; however this is exactly what would be expected for the protein bodies. For the aleurone cells the Se/CN ratio for 4 points is relatively constant whereas the Se/S ratio changes quite significantly (different Se enrichment). This would indicate that the Se localised within these cells is independent of the protein fraction, i.e. Se is not incorporated into the protein. This conclusion agrees with the NanoSIMS images shown previously in which Se hotspots are observed in the aleurone cells. With only one point for the nucellar projection it is difficult to draw any conclusions about the way the Se is incorporated, but the Se/CN and Se/S ratios are both quite high leading to the assumption that there is quite a high Se concentration in this region.

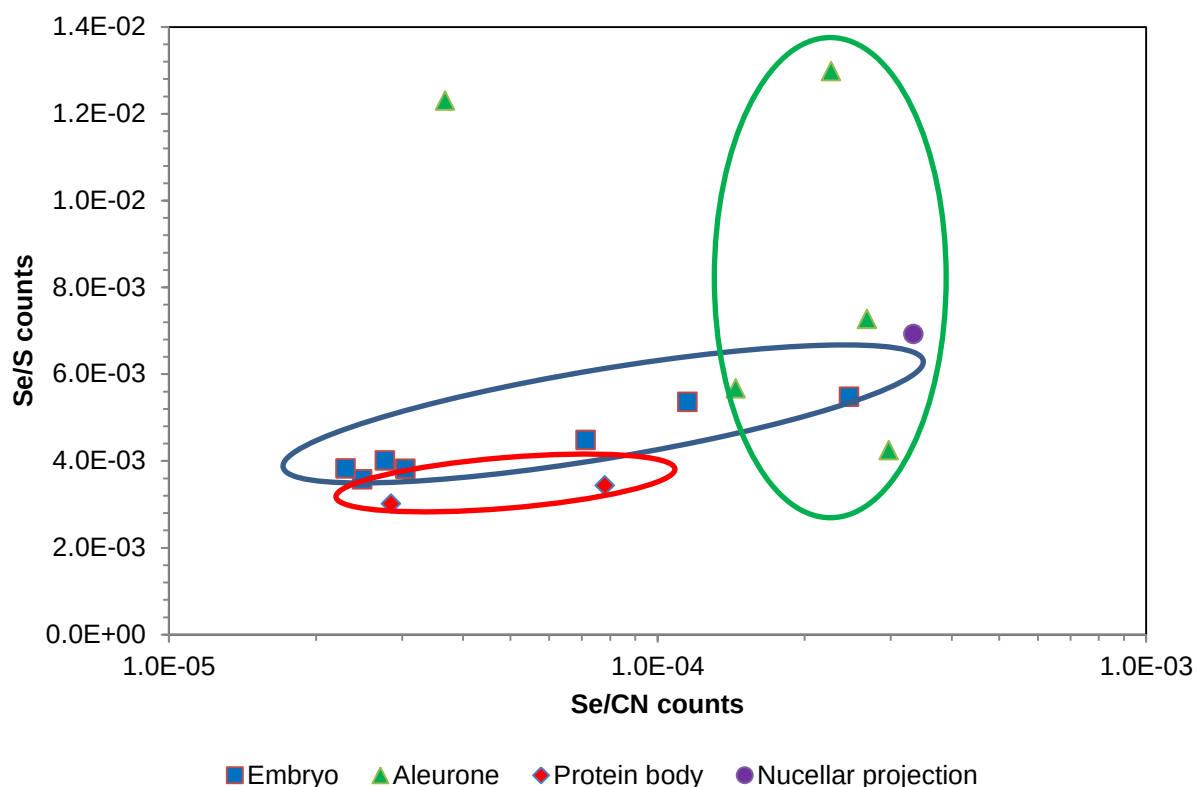


Figure 4.38: Graph showing the relationship of Se to CN and S. The embryo and protein bodies seem to show that Se is substituted for S in the proteins, the aleurone Se appears to be independent of the protein fraction.

4.4.5.6. Iron Localisation

Iron (Fe) deficiency is one of the most common nutritional disorders in the world, affecting an estimated 2 billion people (WHO, 2002; Zimmermann & Hurrell, 2007), and is particularly prevalent in developing countries where cereals form a major part of the diet (WHO, 2002). Iron deficiency can affect resistance to infection, cognitive development and pregnancy (Mayer *et al.*, 2008). Cereal grains are important components of strategies to alleviate micronutrient deficiencies as they are the primary staple food for the human diet (Brinch-Pedersen *et al.*, 2007). Although Fe is present in cereal grains, its bioavailability is generally very low (Zimmermann & Hurrell, 2007). The amount of phytic acid, the main storage form for P in cereals, is believed to control the bioavailability of Fe since phytic acid strongly chelates metal ions including Fe (Ockenden *et al.*, 2004) forming insoluble complexes which cannot be digested or absorbed by humans due to the lack of intestinal phytase enzymes (Abebe *et al.*, 2007;

Persson *et al.*, 2009). Phytic acid is mainly present in phytate granules inside the aleurone cells of cereal grains and Fe also mainly accumulates in this tissue (Mazzolini *et al.*, 1985; Ockenden *et al.*, 2004), accounting for 70% of the total iron in mature barley grains (Brinch-Pedersen *et al.*, 2007). However, the bulk analysis techniques used to determine the localisation of Fe have limited resolution and give no indication of the subcellular Fe distribution.

NanoSIMS imaging of aleurone cells from a high pressure frozen sample revealed $^{56}\text{Fe}^{16}\text{O}^-$ highly localised to the phytate granules, in particular those with a high $^{31}\text{P}^{16}\text{O}^-$ concentration as shown in Figure 4.39. Careful tuning of the $^{56}\text{Fe}^{16}\text{O}^-$ signal was required due to a peak overlap of the $^{56}\text{Fe}^{16}\text{O}^-$ signal with the $^{28}\text{Si}_2^{16}\text{O}^-$ signal otherwise any holes in the section through to the underlying Si wafer gave very large numbers of counts and an inaccurate apparent $^{56}\text{Fe}^{16}\text{O}^-$ distribution.

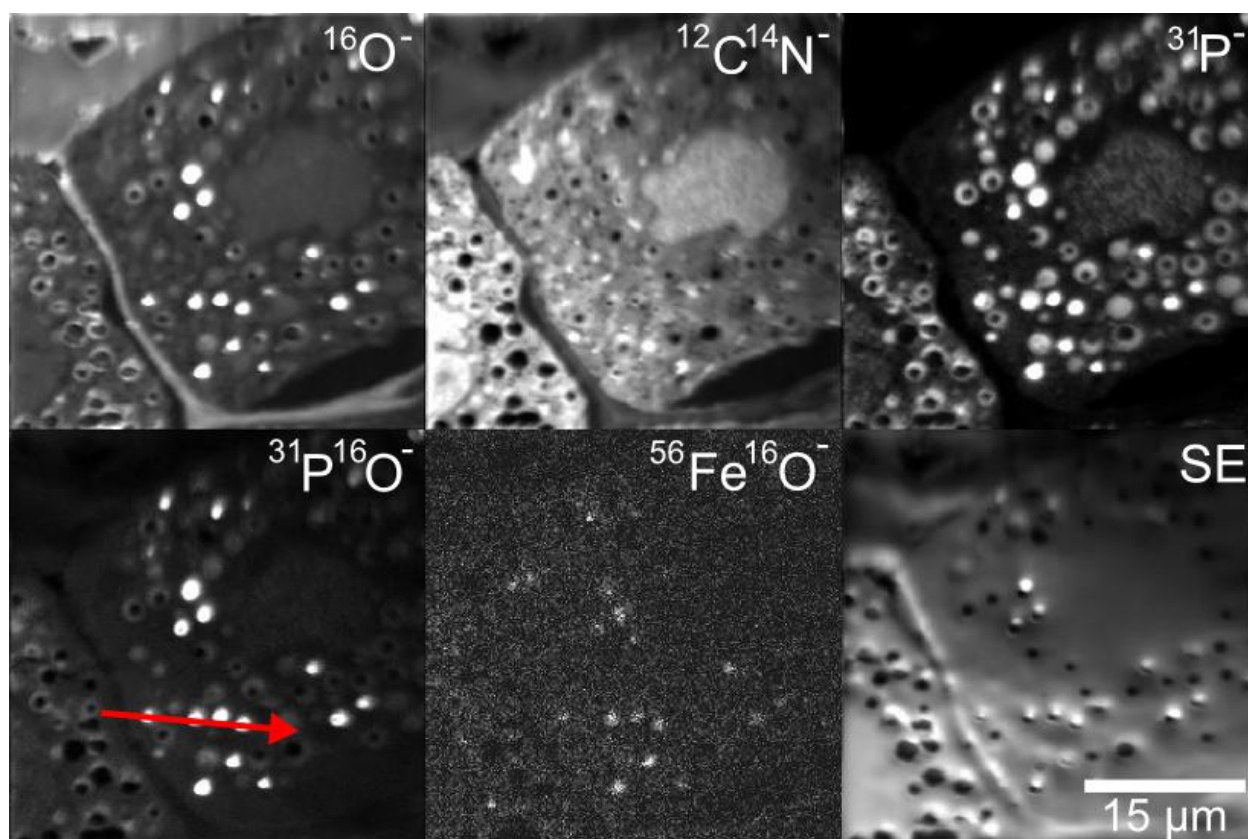


Figure 4.39: NanoSIMS images summed from 8 separate images of an aleurone cell of an immature wheat grain prepared with HPF. The $^{56}\text{Fe}^{16}\text{O}^-$ is tightly localised to the phytate granules as indicated by the $^{31}\text{P}^{16}\text{O}^-$ image.

Taking a line scan across the phytate granules shows the colocalisation of the $^{56}\text{Fe}^{16}\text{O}^-$ with both $^{31}\text{P}^-$ and $^{31}\text{P}^{16}\text{O}^-$. The line scan across the $^{31}\text{P}^{16}\text{O}^-$ image indicated by the red arrow is shown in Figure 4.40.

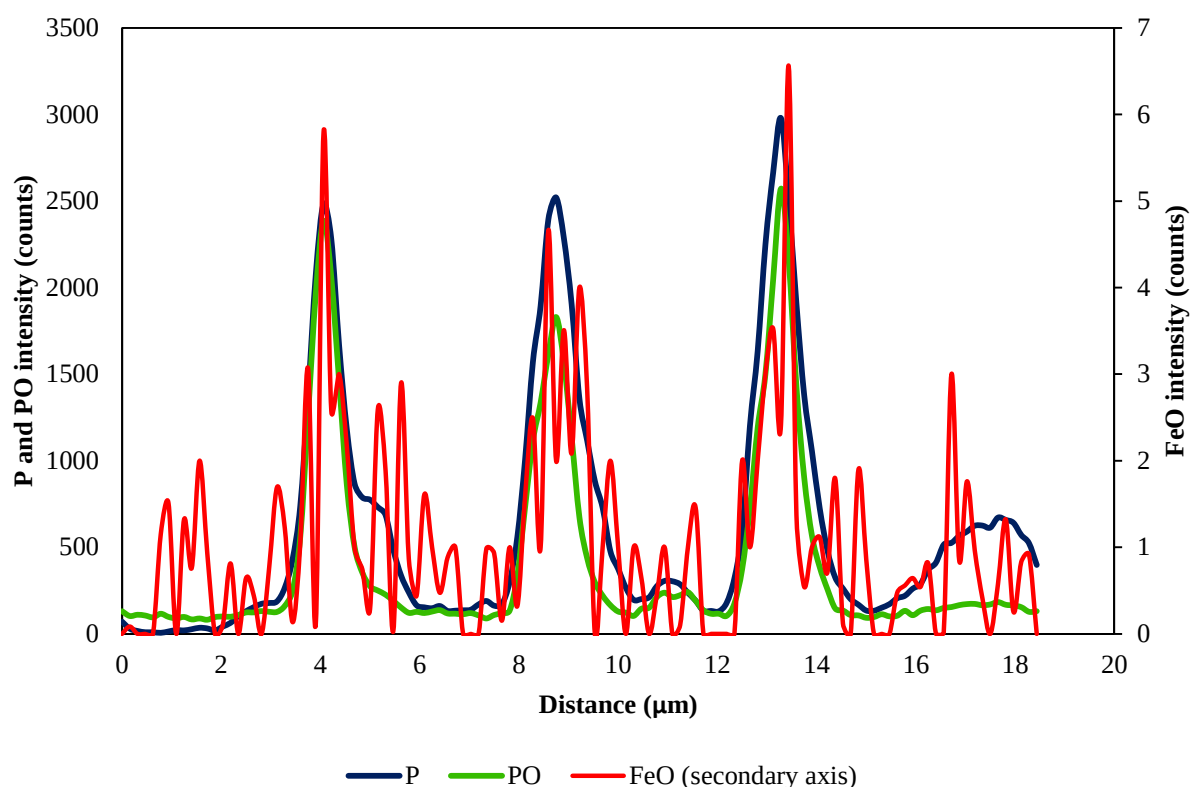


Figure 4.40: Line scan across the phytate granules in Figure 4.39, as indicated by the red arrow, The $^{56}\text{Fe}^{16}\text{O}^-$ is exactly correlated with $^{31}\text{P}^-$ and $^{31}\text{P}^{16}\text{O}^-$.

Imaging of the endosperm region, approximately 100 μm into the endosperm from the aleurone, revealed that some Fe in wheat was not colocalised with phytate (Figure 4.41). This Fe was uniformly distributed in the cytoplasm yet noticeably absent from the starch grains themselves and from the protein bodies. Although this iron corresponds to only a fraction of that present in the whole grain it may be more bioavailable as it is not present as phytate.

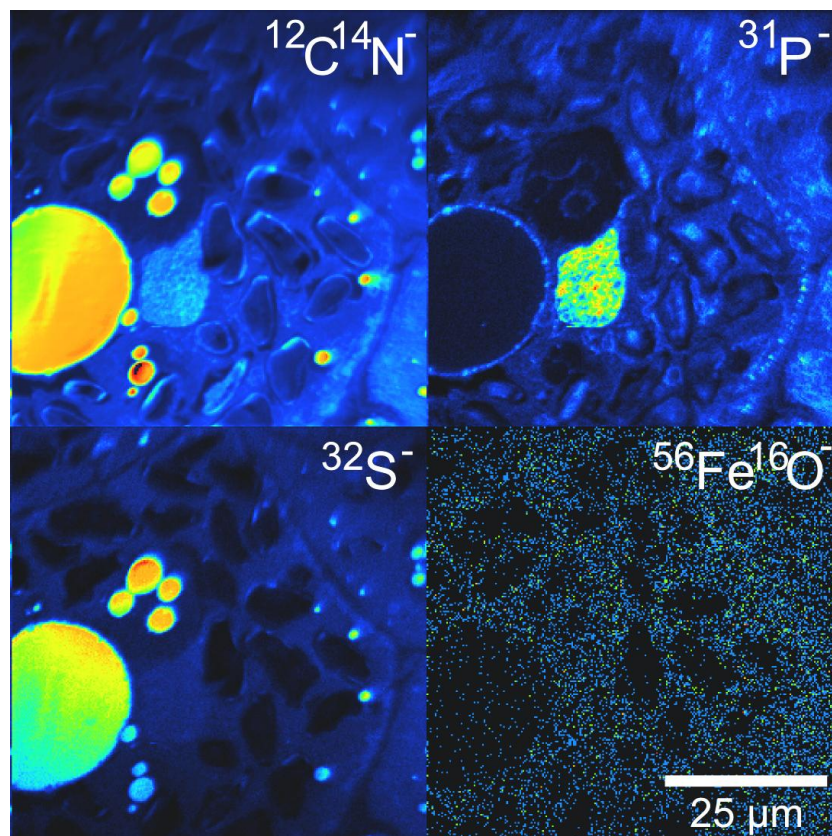


Figure 4.41: NanoSIMS image of the endosperm region of an immature wheat grain prepared with HPF showing $^{56}\text{Fe}^{16}\text{O}^-$ localised to the cytoplasm surrounding the starch grains.

4.4.6. ASSESSMENT OF SAMPLE PREPARATION

Sample preparation for the immature wheat grains was more complicated than that for the mature wheat grains, however it would appear that both the chemical fixation and high pressure freezing methods are capable of preserving the distribution of Se well with no obvious redistribution effects. Structurally there appeared to be little difference between the level of preservation between the two techniques. As Se was expected to be incorporated into proteins as Se-containing amino acids in the wheat grains, chemical fixation is actually a valid sample preparation technique in this case as the element should not be washed out during the dehydration steps. Na/K ratios, used to give an indication of sample preservation, are discussed in Chapter 6. For ease of preparation the mature wheat grains are able to provide most of the information required and there is also less chance of introducing sample preparation artefacts however there are still problems associated with charging of the embryo region and beam/imaging artefacts from the starch and phytate granules.

5. ARSENIC IN MATURE RICE GRAINS³

5.1. INTRODUCTION

Rice is a staple crop which is the main food for about 50% of the world's population (The Rice Association, accessed Mar 2008) making up more than one fifth of the calories consumed worldwide by humans (Smith, 1995). The majority of these calories come from complex carbohydrates or starch (Lotus Foods, accessed Mar 2008).

The structure of a rice grain is shown in Figure 5.1. When rice arrives from the paddy field the outer husks are first milled using a rice huller to produce brown rice. This outer husk which forms a protective coating to the grain has to be removed because it is indigestible, and removing it has no effect on the nutritional value of the remaining rice.

Figure 5.1 has been removed due to Copyright restrictions. The original image can be found in the following reference:

Juliano BO 1993. Rice in human nutrition. International Rice Research Institute: Food and Agriculture Organisation of the United Nations.

Or directly from this link:

<http://www.fao.org/docrep/t0567e/T0567E01.GIF>

Figure 5.1: Schematic diagram showing the structure of a rice grain (Juliano, 1993)

³ I have published a substantial part of this chapter in the following reference and some of the text and images have been used verbatim in this chapter: **Moore KL**, Schroder M, Lombi E, Zhao FJ, McGrath SP, Hawkesford MJ, Shewry PR, Grovenor CRM. 2010. NanoSIMS analysis of arsenic and selenium in cereal grain. *New Phytologist* **185**: 434-445.

Dehusked, unpolished rice grains are covered by the nutrient-rich bran and aleurone layers. These outer layers are darker in colour and are rich in nutrients such as vitamins, minerals and dietary fibres (Matsuo & Hoshikawa, 1993). Polishing of the resulting brown rice produces white rice, and in this process the outer bran layers and the embryo are removed. The milling of rice aims to improve the appearance and taste of cooked rice and increase its digestibility (Matsuo & Hoshikawa, 1993).

Arsenic (As) is known to be a toxic element and is a group 1 human carcinogen (International Agency for Research on Cancer, 2004). It has been shown to affect fertility, cause birth defects, impair the development of children (Williams *et al.*, 2007) as well as cause skin lesions and many types of cancer, including skin and internal cancers (Smith *et al.*, 2000). In some areas of South-East Asia the mortality rate from drinking stagnant surface water contaminated with microorganisms was extremely high, hence a program of digging tube wells into aquifer sediments was initiated to provide ‘safe’ drinking water (Smith *et al.*, 2000). At the time when these wells were dug, As in water was not recognised as being a problem and hence tests on the groundwater did not include this element (Smith *et al.*, 2000). Tube wells in Bangladesh have been in use since the 1940’s however since the 1970’s and 80’s an ever increasing number of these tube wells have been dug with a corresponding increase in the number of individuals drinking from them. The problems associated with drinking As contaminated water only really came to light in the late 80’s and was confirmed in 1993 (Smith *et al.*, 2000). In Bangladesh, one of the worst hit As contaminated areas, nearly half of the tube wells have a concentration higher than $10\text{ }\mu\text{g L}^{-1}$, the maximum recommended level set by World Health Organisation for As in drinking water (World Health Organization, 1996), however in some cases the concentration can approach 2 mg L^{-1} (Meharg & Rahman, 2003). The digging of these tube wells has resulted in what has been described as “the largest mass poisoning of a population in history” (Smith *et al.*, 2000) with up to 77 million people in Bangladesh alone estimated to be exposed to As contaminated water (Argos *et al.*, 2010).

In populations not drinking As-contaminated water, food is the main source of As in the human diet, with fish being the major source, especially in countries such as Japan and the USA. However this As tends to be in less toxic organic forms such as arsenobetaine, monomethylarsonic or dimethylarsenic acids (Schoof *et al.*, 1999). Generally, intake of the more toxic inorganic As from terrestrial foods is low except for regions where rice forms a major part of the diet (Williams *et al.*, 2007). The As-contaminated water is not just used for drinking; it is also widely used for irrigating crops in Bangladesh and West Bengal, India, in particular for irrigation of rice paddies to allow rice to be grown during the dry season (Meharg & Rahman, 2003; Martin *et al.*, 2010). In some regions of Bangladesh with elevated As in the groundwater the soil As level can be as high as 46 - 83 $\mu\text{g g}^{-1}$ compared with a background level of $\sim 5 \mu\text{g g}^{-1}$ (Meharg & Rahman, 2003) and across Bangladesh it is estimated that ~ 1000 tonnes of As is added to the soil each year through irrigation water (Ali *et al.*, 2003). Rice (*Oryza sativa*) is one of the main foods in these As-epidemic areas (Rahman *et al.*, 2008) and in Bangladesh makes up 73% of the calorific intake (Meharg & Rahman, 2003). Rice is particularly efficient in the uptake of As and is a generic problem, however if As is building up in paddy field soils the problem is worsened and rice becomes a major source of As in the diet. Even if safe drinking water could be introduced into Bangladesh it is unlikely that the solution will be able to provide enough water for irrigation or may be too costly, hence strategies to reduce the As concentration in rice still need to be investigated (Meharg & Rahman, 2003).

One key research area to help mitigate this problem is to understand how and where As is trafficked and sequestered into rice grains so that strategies can be developed to reduce the As content of rice grown on contaminated soils.

5.1.1. PREVIOUS WORK ON AS LOCALISATION IN RICE GRAIN

The first studies regarding the localisation of As in rice grain were limited in terms of the techniques that were available. Initially the As concentration in different parts of the rice grains was quantified by separating the grain using milling and polishing. Using this method the As concentration could be measured in the husk, bran and endosperm using Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) methods (Ren *et al.*, 2006; Rahman *et al.*, 2007; Sun *et al.*, 2008). However this separation method can never be very accurate in distinguishing between these individual layers. Sun *et al.* (2008) polished rice grain until 7% of its mass had been removed, this value previously calculated as the percentage of the grain that constitutes the bran (Franz & Sampson, 2006). However, it is difficult to control the polishing process so that the entirety of the bran layer is removed and not any of the regions just beneath. As these milling data are averaged over several grains and there is a lot of inhomogeneity between the shapes and sizes of rice grain, this method can never be very accurate. This technique can however be used to indicate differences between the separate regions of the grain with the general trend or As concentrations; endosperm < whole grain < husk < bran (Ren *et al.*, 2006; Rahman *et al.*, 2007; Sun *et al.*, 2008). This method is still commonly used to test differences between different cultivars because it is a very simple and quick way of getting an indication of the concentration of elements in different regions of the grain, and indicates where the focus should lie for analysis by techniques with improved spatial resolution.

The first study showing the spatial localisation of As in rice grain used two different methods, laser ablation ICP-MS and synchrotron X-ray fluorescence (S-XRF) (Meharg *et al.*, 2008). Laser ablation ICP-MS was carried out with a spot size of 200 µm and the result is shown in Figure 5.2. A fair proportion of a grain was sampled using this technique, however it is difficult to make accurate assumptions from these results because of the size of the spot. The authors state that this map indicates that As is more concentrated in the epidermal layers of the grain (Meharg *et al.*,

2008) but the analysis points are not close enough to the edge of the rice grain to include the aleurone or pericarp layers, although the data do indicate a higher As concentration towards the edge of the grain with low As in the centre.

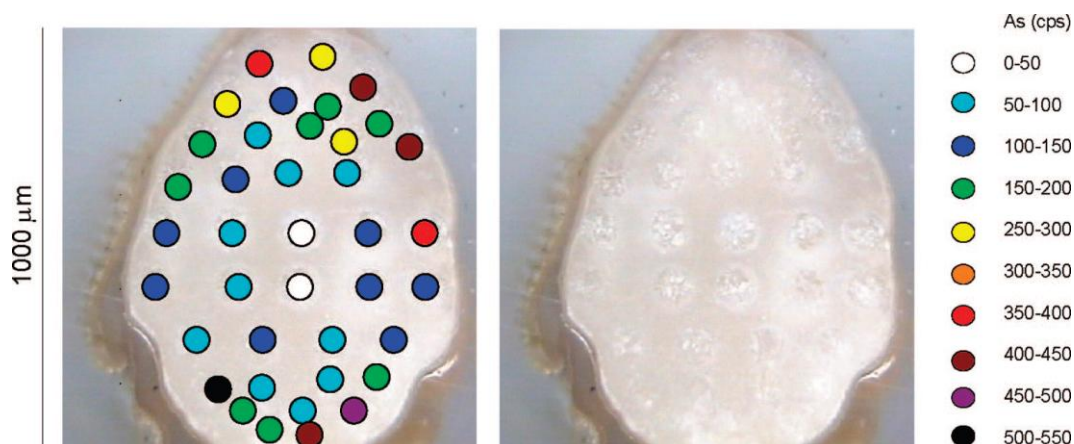


Figure 5.2: Laser ablation ICP-MS analysis of an As containing rice grain. As counts per second (CPS) were recorded at each point of ablation (shown in the right image) and are coloured according to the concentration (left image) (Meharg et al., 2008). Reprinted with permission from Meharg AA, Lombi E, Williams PN, Scheckel KG, Feldmann J, Raab A, Zhu YG, Islam R. 2008. Speciation and localization of arsenic in white and brown rice grains. *Environmental Science & Technology* 42: 1051-1057. Copyright 2008 American Chemical Society.

In the same paper, the authors use S-XRF mapping which has a much higher spatial resolution. They investigated the difference between the As distribution in brown and white rice and included other trace metals such as Fe and Zn. Synchrotron X-ray beams are highly penetrating which makes interpretation of S-XRF maps from thick samples quite difficult as the resulting image is a 2D compression of 3D data. The authors found it difficult to thin section unembedded rice grains because they tended to fracture. Embedding of the rice grains was not considered as it could have changed the speciation of the As for X-ray Absorption Near Edge Spectroscopy (XANES) analysis (a synchrotron technique allowing the speciation to be determined from small targeted regions of the sample rather than bulk analysis with mass spectrometry methods). Although this method is not ideal for ease of interpretation of the data, it allowed mapping and speciation without the risk that sample preparation had introduced artefacts.

The maps of brown rice revealed strong As localisation at the surface of the grain in a region which the authors associate with the surface longitudinal groove or ovular vascular trace (OVT) which runs along the length of the ventral side of the grain (Figure 5.3). Several other elements are also localised to this region including Fe, Zn, Mn and Cu.

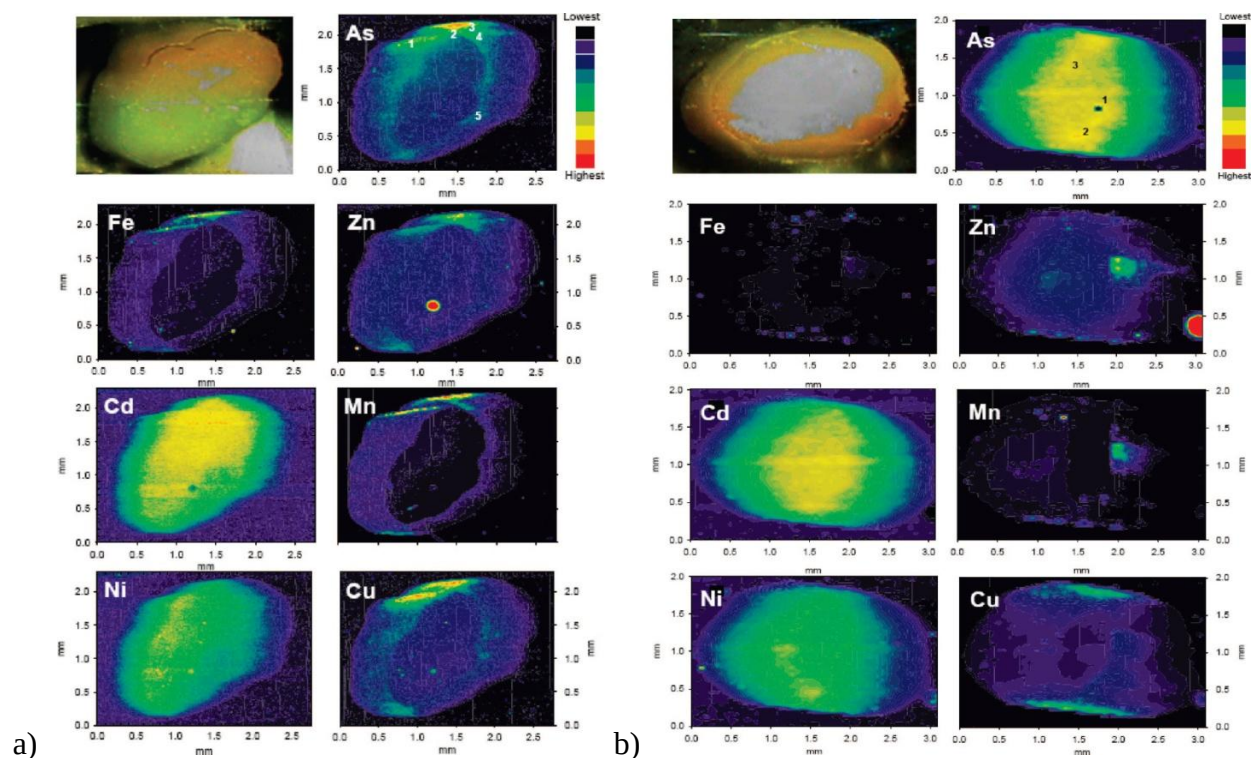


Figure 5.3: S-XRF elemental maps of hand sectioned rice grain a) Bangladesh brown rice and b) U.S. white rice. The colour bar indicates the signal intensity, with black corresponding to the lowest concentration and red the highest. XRF maps were acquired at 15 μm steps. Note the smearing to the right of the image due to the thickness of the sample. The brown rice sample shows a high As concentration in the OVT region which is noticeably missing from the white rice sample (Meharg *et al.*, 2008). Reprinted with permission from Meharg AA, Lombi E, Williams PN, Scheckel KG, Feldmann J, Raab A, Zhu YG, Islam R. 2008. Speciation and localization of arsenic in white and brown rice grains. *Environmental Science & Technology* 42: 1051-1057. Copyright 2008 American Chemical Society.

The authors conclude from their results that the As is preferentially localised to the aleurone layer but it is not clear from these maps that this conclusion can be reached. The synchrotron maps were acquired with quite a low resolution, a beam size of 10 μm and a step size of 15 μm , which means that at most only a couple of points lie in the aleurone layer. While it does appear that there is some slight enhancement around the edge of the grain it is difficult to be sure that this is not an artefact from the thick sample. Nevertheless the hotspot at the top of the image is

very clear and is noticeably absent from the image of the white rice. These data are consistent with the milling data but also shows that the As distribution is not homogenous around the grain and therefore a simple milling process does not give a true representation of the As distribution. The image of the white rice also indicates how other nutrients are affected by the removal of the bran layer, with a noticeable decrease in the nutritional value of the grain especially for Fe, Mn and Zn.

Subsequently a method was developed to allow thin sectioning of mature rice grain without the need to embed the grain in resin and therefore increase the quality of the mapping (Lombi *et al.*, 2009). Samples were first fractured and glued to a metal support then sectioned thinly using a vibrating blade microtome. To stop the section fracturing during cutting, a piece of X-ray transparent Kapton tape was pressed onto the surface of the grain and the cutting knife was run beneath this. This allowed 70 μm thick sections to be created both longitudinally and transversely which were ready for S-XRF analysis. An optical image of one of these sections and the corresponding S-XRF map is shown in Figure 5.4. It is clear that at the top of the grain in a region between the husk and the endosperm corresponding to the position of the OVT there is a distinct As hotspot. This result is consistent with those of Meharg *et al.* (2008), however in this case there is no noticeable enhancement of As in the aleurone layer. The OVT, in conjunction with the chalazal and nucellar tissues, transports nutrients into the endosperm (Krishnan & Dayanandan, 2003), and it is therefore not unexpected that there is a higher As concentration in this region of the grain.

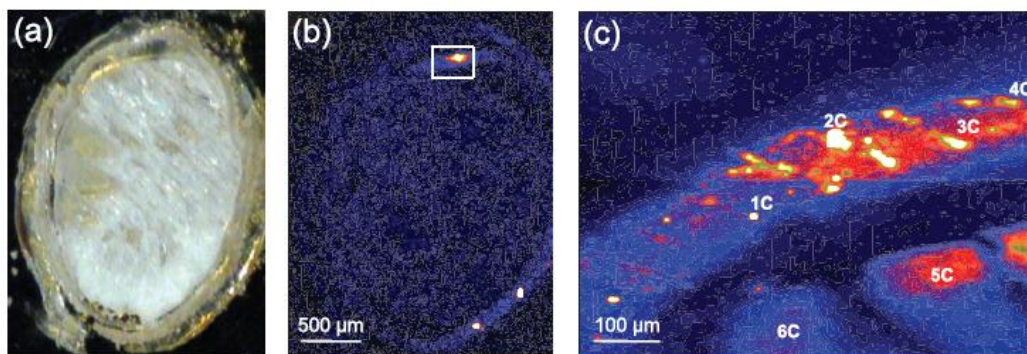


Figure 5.4: a) Optical image of the thin section created with the vibrating microtome blade with the ventral side of the grain at the top of the image. b) Arsenic XRF map of the same sample acquired with 20 μm steps showing a region of high As signal at the top of the sample c) higher resolution map from the region indicated by the white square in b) at the ovular vascular trace (Lombi *et al.*, 2009). Reprinted with permission from John Wiley and Sons.

Although synchrotron experiments are capable of mapping large areas with good resolution and high sensitivity, one of the main problems is that the hard X-rays used do not allow mapping of lighter elements which could be used to infer the mechanisms of uptake and help to locate As within the grain. In the same paper Lombi *et al.* (2009) used a combination of S-XRF, to map the distribution of the heavier trace elements, and μ -Particle Induced X-Ray Emission (μ -PIXE) to map the lighter macronutrients as shown in Figure 5.5. A 2.5 MeV proton beam with a spot size of 5 μm was used to scan the surface of the sample. High concentrations of Cu, Fe, Mn and Zn were observed in the husk, but the distributions of these elements varied considerably in other parts of the rice grain. Zn and Mn were found to be concentrated within the central part of the embryo, however their distributions are slightly different. It would appear that Zn and possibly Cu decrease in concentration from the aleurone inwards towards the centre of the endosperm. Mn and Fe were found predominantly localised to the aleurone region. The sample used for the μ -PIXE analysis did not contain the embryo, so this region cannot be compared. The S distribution is quite striking, with a clear gradient in concentration dropping from the subaleurone to the centre of the endosperm similar to the distribution of the Zn. This S distribution has also been confirmed in another study of rice with PIXE (Lombi *et al.*, 2011). As

expected based on previous results (Ogawa *et al.*, 1979), P and K were highly localised in the aleurone and Si was mainly observed in the husk.

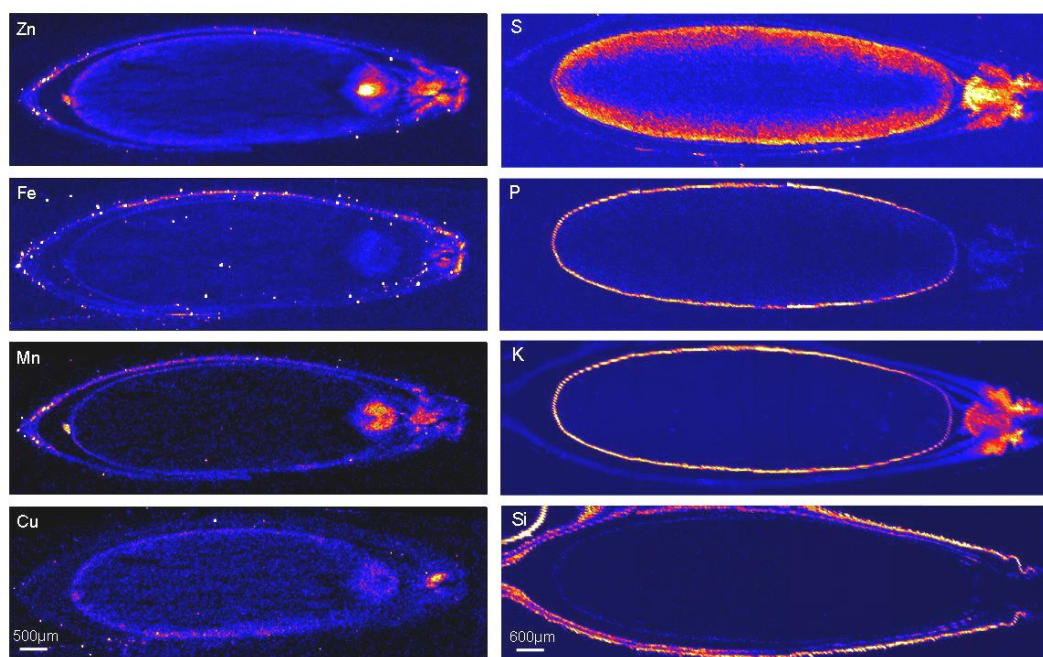


Figure 5.5: Elemental maps of longitudinal sections of rice grains. Zn, Fe, Mn and Cu maps were acquired using S-XRF while S, P, K and Si were collected with PIXE. The step size used was 25 µm in both cases (Lombi *et al.*, 2009). Reprinted with permission from John Wiley and Sons.

In the same paper the authors also show that the embryo contains a higher concentration of As than the endosperm suggesting that As preferentially accumulates in the protein and nutrient rich parts of the grain.

More recently, synchrotron fluorescence microtomography has been shown to be an effective way of looking at whole rice grains without the need to section the sample thinly (Carey *et al.*, 2010). This method reconstructs the elemental distribution from one plane of a 3D sample. The sample is scanned stepwise across the X-ray beam, rotated by 1° and repeated for 0° to 180°. In this study the effect on the resulting As distribution in the grain of supplying developing rice grains with dimethylarsinic acid (DMA) and arsenite was investigated in plants with cut stems. This study also investigated the roles of xylem and phloem transport using, respectively, Sr and Rb. Other researchers have shown that Sr can be used as a tracer for Ca and Rb for K based on

the fact that they have similar chemical properties (Metzner *et al.*, 2008). S-XRF is not able to map lighter elements such as K and Ca effectively but is able to detect these heavier elements more effectively and can therefore be used to give an indication of the location of K and Ca.

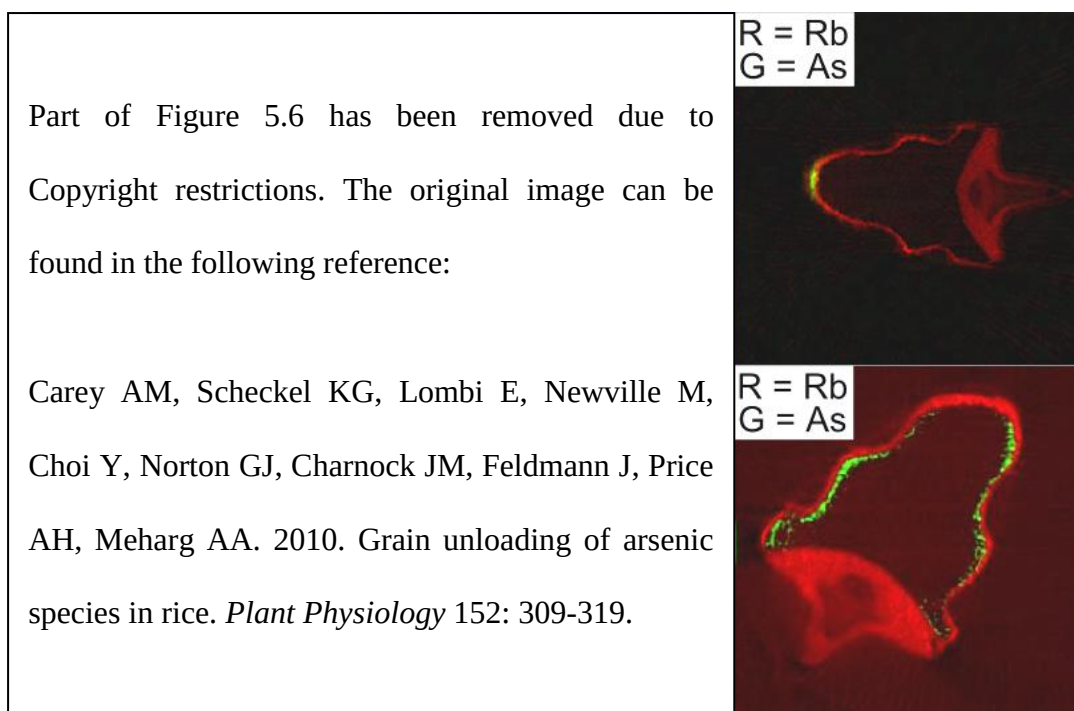


Figure 5.6: Fluorescence microtomography images showing the distributions of As and Rb in immature rice grain for arsenite (top) and DMA (bottom). Colour merge images of Rb (red) and As (green) were created using ImageJ and are not included in the original publication (Carey *et al.*, 2010)

The results of this study are very striking, showing a dramatic difference in the As distribution depending on the arsenic treatment. When the rice plant is exposed to arsenite the As remained predominantly in the OVT region as shown by the top row of images in Figure 5.6 but treating with DMA resulted in a completely different distribution in which the authors state that “As has dispersed into, and throughout, the nucellar epidermis region, with migration into the outer parts of the endosperm evident” (Carey *et al.*, 2010). Assuming the Rb follows the same distribution of K then it can be assumed that this indicates the position of the aleurone layer and the colour merge clearly shows that the As is localised to the OVT region for the arsenite treatment with some overlap with the aleurone as indicated by the Rb. However when the same colour merge is performed for the DMA treated sample it is clear that the As is not localised to the outer bran

layers of the grain and is in fact only localised within the subaleurone and endosperm. While the authors do state that some As is localised within the endosperm it is not clear that they have noticed the extent to which there is very little As in the outer bran layers.

With the data of Carey *et al.* (2010) indicating a difference in the As distribution dependant on the speciation of the As supplied, it is now possible to re-examine the previously published work to see if these distributions are consistent. Although in much of the previously published work the As is not supplied as a well-controlled form, i.e. as DMA or As(III), as in the experiment of Carey *et al.* (2010), other work has indicated that the growth conditions can affect the final speciation in the grain and hence the distribution is also likely to be affected in a similar way. Paddy field grown rice tends to have a much lower total As concentration in the grain than greenhouse experiments (Khan *et al.*, 2010), and a lower total As concentration equates to a high proportion of inorganic As in the grain. With higher total As concentrations a greater proportion of As is in the form of DMA (Meharg *et al.*, 2008; Khan *et al.*, 2010), as is shown graphically in Figure 5.7.

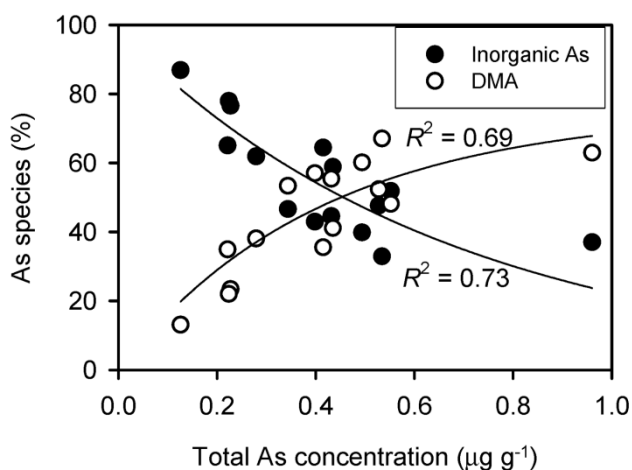


Figure 5.7: Relationship between the percentages of inorganic As or DMA and total grain As concentration from rice grain from a greenhouse experiment and from paddy fields in Bangladesh showing an increase in % DMA with increasing total As concentration (Khan *et al.*, 2010). Reprinted with permission from Khan MA, Stroud JL, Zhu YG, McGrath SP, Zhao FJ. 2010. Arsenic bioavailability to rice is elevated in Bangladeshi paddy soils. *Environmental Science & Technology* 44: 8515-8521. Copyright 2008 American Chemical Society.

In the sample analysed by Meharg *et al.* (2008) μ -XANES revealed a large proportion of the outer layers, in particular the OVT region, to be rich in inorganic As whereas the central regions of the white rice were found to be much higher in DMA. Similarly in the results of Lombi *et al.* (2009) the majority of the As was found using XANES and HPLC-ICP-MS to be inorganic As and only in the endosperm was DMA found to contribute a significant proportion of the total extracted As. In both of these cases the As was localised to the OVT region which is consistent with that observed by Carey *et al.* (2010), and in both cases the samples were obtained from As contaminated sites rather than being greenhouse grown. In another S-XRF study, the distribution of As in rice grain was studied over time to examine how As is transported and unloaded into the grain (Zheng *et al.*, 2010).

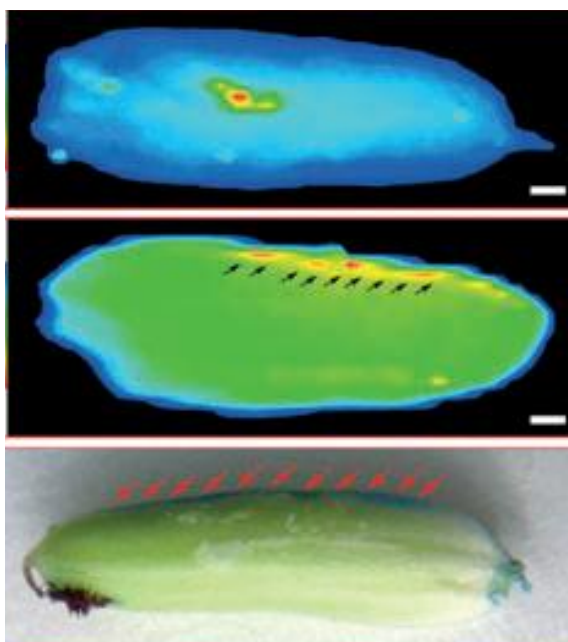


Figure 5.8: S-XRF elemental images showing the As concentration changing with time. The top image is taken from a rice grain at 14 days after flowering (DAF), the middle image a ripened caryopsis at 32 DAF and the bottom image shows an optical image of the same grain at 32 DAF with the OVT indicated by the position of the aniline blue dye. The scale bar is 500 μ m (Zheng *et al.*, 2010). Reprinted with permission from John Wiley and Sons.

This experiment was conducted in a greenhouse although relatively low doses of As were used and the final As concentration in the grain was quite low hence the proportion of inorganic As was again quite high. At 14 days after flowering (DAF) a high As signal was observed in the centre of the grain (top image of Figure 5.8) however when grain filling had finished the As

signal was mainly observed along the OVT as shown in the middle image. The position of the OVT is shown in the bottom image by staining with aniline blue dye. The authors suggest that unloading of inorganic As is associated with carbohydrate accumulation during grain filling as the inorganic As concentration increased with increasing biomass.

All this previously published data indicate that there are likely to be discrepancies in the localisation of As in rice grain depending on the As speciation and that growth conditions need to be carefully considered when analysing the results.

5.2. PROJECT AIMS

Although significantly more spatially resolved studies have been undertaken for As in rice grain, in comparison to Se in wheat grain, the studies are still at relatively low resolution and it is still somewhat difficult to precisely locate the As in the grain and to study in detail the mechanisms of uptake. Previous work had indicated two regions where there is likely to be an accumulation of As which were obvious targets for NanoSIMS analysis, the OVT region and the aleurone/subaleurone layer. The aim of the NanoSIMS experiments was to determine subcellular information regarding the As distribution and link this information with what is already known from S-XRF and milling data.

5.3. SAMPLE PREPARATION

5.3.1. SAMPLE GROWTH

Rice (*Oryza sativa* L. cv. Oochikara) grain samples were obtained from a pot experiment conducted in a glasshouse at Rothamsted Research (Harpenden, UK). The sample growth protocols are outlined elsewhere (Xu *et al.*, 2008), but briefly sodium arsenite (10 mg As kg⁻¹ soil) was added to the soil at the beginning of the experiment and rice was grown under flooded conditions. At maturity, rice grains were collected and dehusked, but not polished, so that the

aleurone layer was intact. The bulk concentration of As in the rice (measured using ICP-MS) was 2.5 mg kg^{-1} , rather similar to the highest concentrations ($1.7\text{-}1.8 \text{ mg kg}^{-1}$) measured in rice grains collected from As-contaminated fields in Bangladesh by Meharg & Rahman (2003) grown in soils with comparable As concentrations to those used in this experiment. These grains contained around 70% DMA.

In an additional experiment rice plants were treated with $10 \text{ }\mu\text{M}$ As(III) or DMA for two weeks during flowering in a hydroponic experiment (with renewal of the nutrient solution once a week) after which the plants were transferred into nutrient solution without As until grain maturity. The bulk concentration of As in these grains, measured with ICP-MS, was 0.83 mg kg^{-1} for the As(III) treatment, 3 times less than the high As grains above, and 1.99 mg kg^{-1} for the DMA treatment. The speciation in the grains was almost entirely inorganic As in the As(III) treatment and almost all DMA in the DMA treatment.

5.3.2. WHOLE GRAIN PREPARATION

Samples were initially prepared in exactly the same way as described for the mature wheat grains. Individual rice grains were mounted in the NanoSIMS rings in LR White resin and then sectioned flat with a razor blade. This method alleviated some of the problems encountered by other authors where fracturing of the rice grain on sectioning was a problem. Although this provided a flat surface for analysis, the first samples prepared with this method could not be analysed in the NanoSIMS as no ion signals could be detected from the sample itself which suggested charging. When the samples were removed from the NanoSIMS it was obvious that they had shrunk in the high vacuum as they still contained a significant amount of residual water. The shrinkage not only broke the conductive platinum coating on the surface of the samples but also caused damage to the outer aleurone cells as these are the mechanically weakest part of the grain. The break in the platinum coating can be seen in Figure 5.9a) from the fact that the rice grain shows much more contrast than the surrounding resin. It would not have been possible to

properly image the aleurone of these grains because the damage to the aleurone cells was too severe (Figure 5.9 b)).

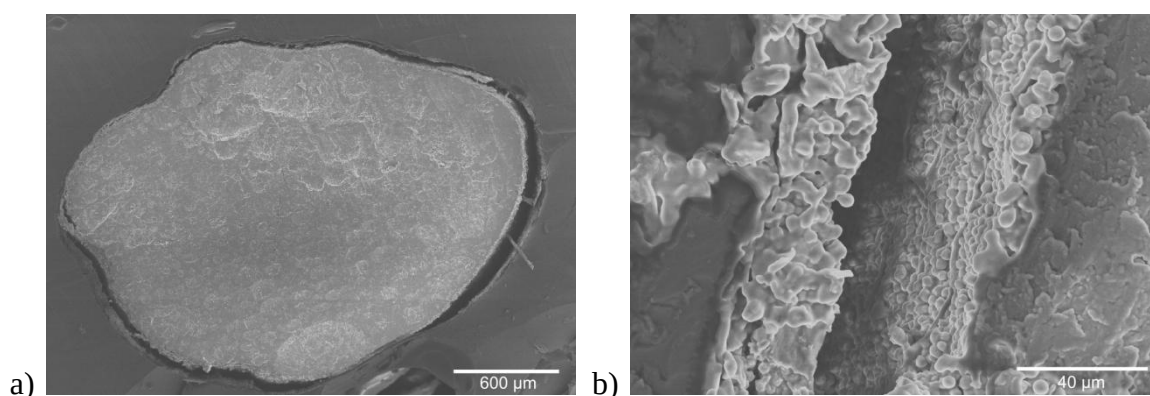


Figure 5.9: SEM images showing the shrinkage of the rice grain after placing in the high vacuum of the NanoSIMS a) The shrinkage of the whole grain b) fracture along the aleurone cells part of which is stuck to the embedding resin (left)

It was clear that rice samples needed to be desiccated before mounting and loading into the vacuum of the NanoSIMS to prevent shrinkage. Unmounted samples were held under a continually pumping vacuum at a pressure of approximately 10^{-2} mbar to reduce the water content. In order to establish how long the samples needed to be kept under vacuum the weight of 5 grains was measured daily until no significant change in weight was observed (Figure 5.10). After approximately one week under vacuum the rice grains showed no further reduction in weight.

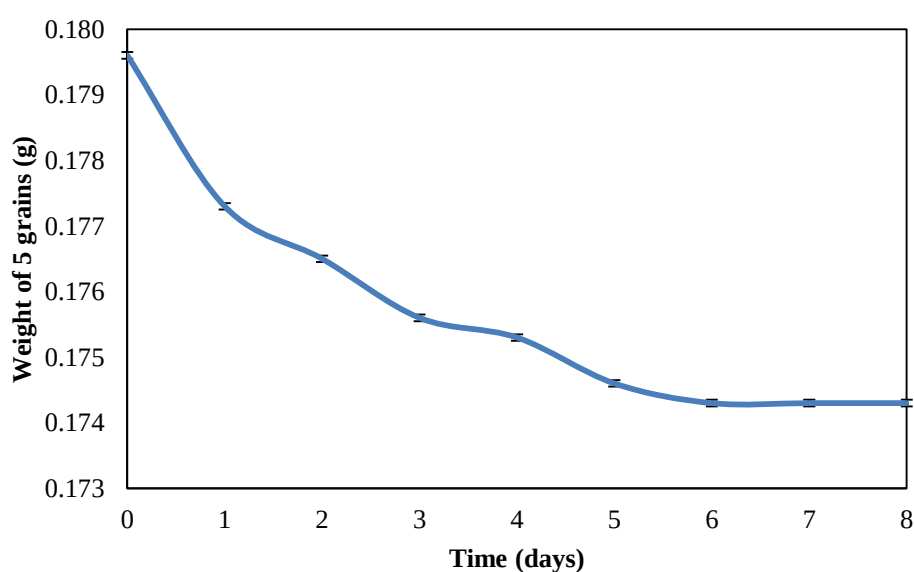


Figure 5.10: Graph showing a decrease in weight over time of 5 rice grains desiccated under a vacuum of 10^{-2} mbar

The rice grains were then removed from vacuum and left at atmospheric pressure and humidity and weighed daily to see how quickly they re-absorbed water (two grains were removed for preparation for the NanoSIMS.) The weight gain is shown in Figure 5.11. This result indicated that the samples needed to be kept under vacuum until they were ready to be prepared and that they should be placed into the NanoSIMS as soon as possible after mounting, sectioning and Pt coating. Pre-desiccation of all subsequent grains meant that analysis could be undertaken without shrinkage.

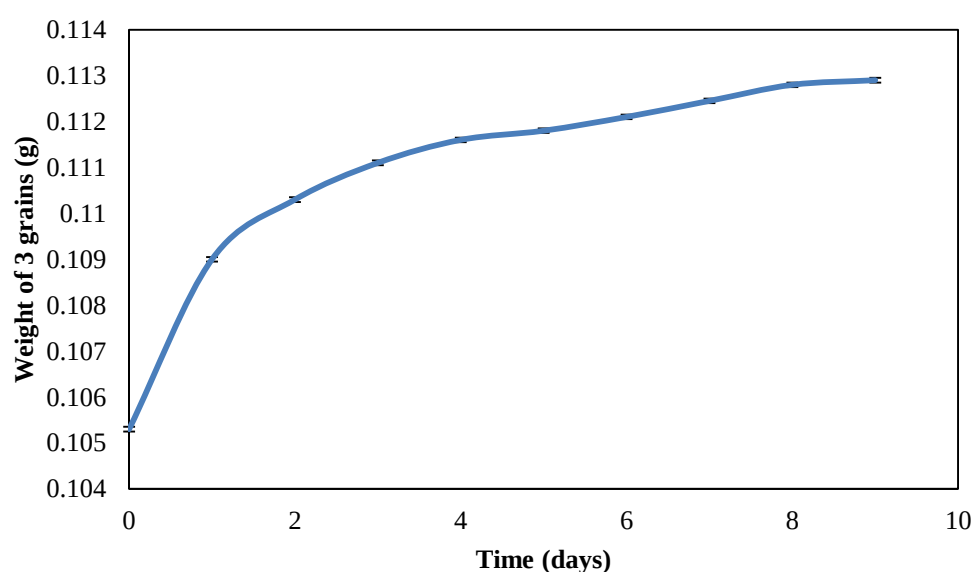


Figure 5.11: Graph showing an increase in weight with time of 3 rice grains after taking them out of vacuum

5.4. RESULTS AND DISCUSSION

5.4.1. SEM AND EDX

Initially to investigate the internal morphology of the samples, the rice grains were just broken in half by hand in the same way as the wheat grain. There are similarities in the way the grains broke, in both cases the elongated endosperm cells can be seen, however in rice the starch grains seem to be much more irregular in size and their shape is much less ovular. These features can be seen in Figure 5.12.

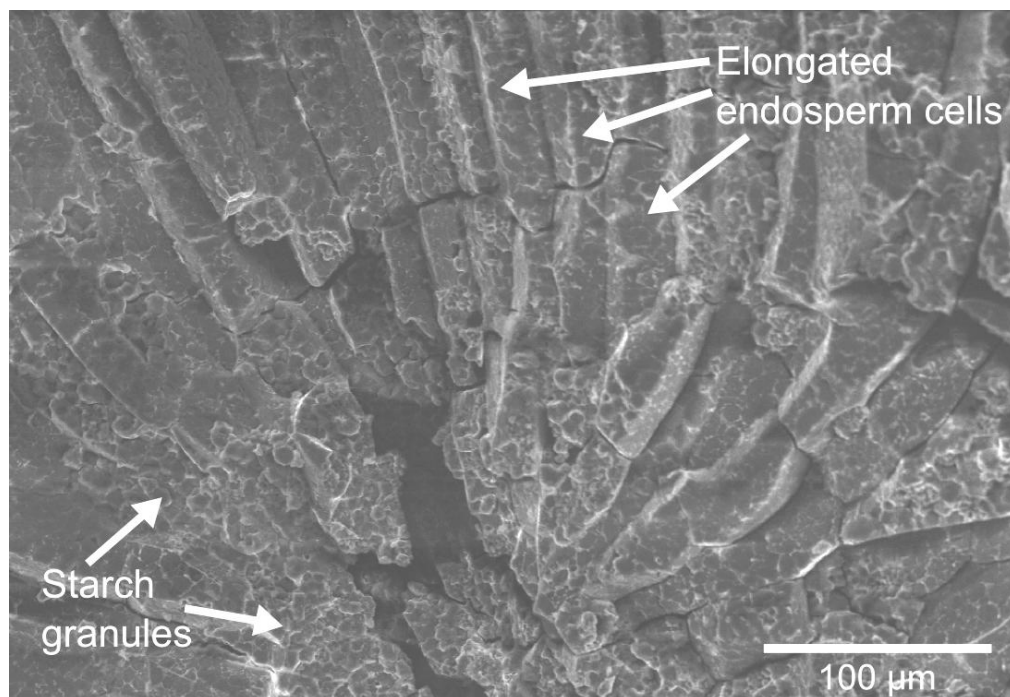


Figure 5.12: SEM image of the centre of a hand fractured rice grain showing the elongated endosperm cells and the irregular starch grains

SEM and EDX of the outside edge of a razor blade sectioned rice grain showed the similarity between the wheat and rice grains (Figure 5.13). Both have a single layer of aleurone cells surrounding the starchy endosperm. In many places along the edge of the rice grain the aleurone layer is less clearly defined than in the wheat and the individual aleurone cells are not as regularly shaped. Whereas in the wheat these cells tend to be very square, in the rice they are much more rounded. It is not possible to clearly see the individual starch grains in the razor blade sectioned rice grain and the rice phytate granules are much larger than those in the wheat and can be individually resolved in the EDX images. The chemical distribution and elements detected in rice are almost identical to that in the wheat, with Mg, P and K localised in the aleurone cells and sulphur more concentrated in the starchy endosperm. These results are consistent with those reported previously (Ogawa *et al.*, 1979). The absence of sulphur signal gives an indication of the location of the starch grains which cannot be seen in the secondary electron image. Although the carbon and oxygen signals are weak in EDX there is enough signal from these samples to clearly see the distribution.

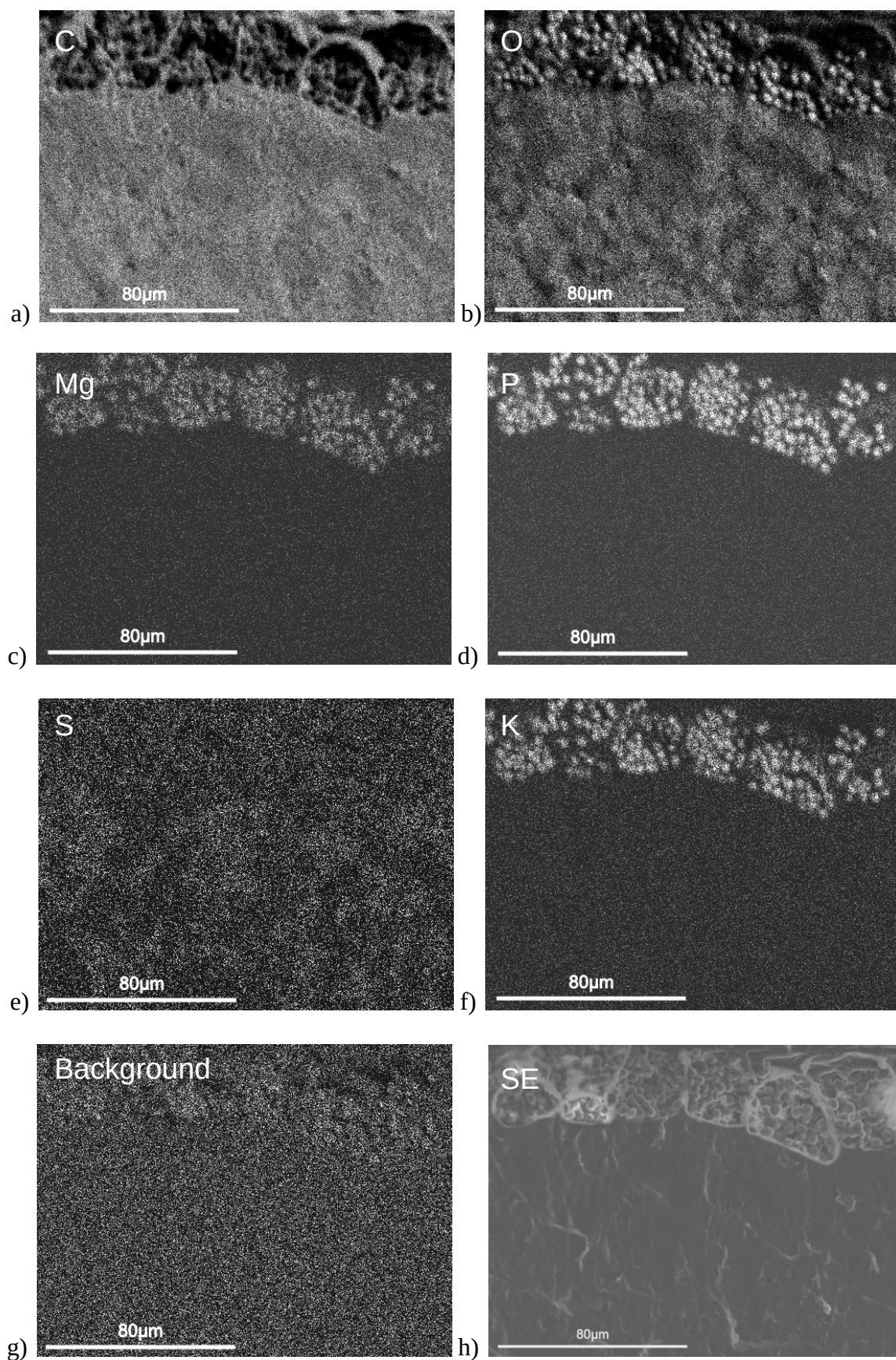


Figure 5.13: EDX maps of the edge of a rice grain showing a) carbon, b) oxygen, c) magnesium, d) phosphorous, e) sulphur, f) potassium, g) background (1.5-1.8 keV), h) secondary electron image.

There is a high oxygen signal detected from the phytate granules and this is accompanied by a reduction in the amount of carbon. The sample is carbon coated so carbon should be expected to be quite uniform across the surface of the sample yet there is a distinct reduction in signal from the phytate granules. This fits in with what is known about the chemical structure of phytic acid (as described in Chapter 4). The background map, acquired from the spectrum between 1.5 and 1.8 keV gives an indication of the topography of the sample and the locations from which the overall signal yield is higher. There is some enhancement in the rather topographic aleurone layer but this is not significant considering the strong position-sensitive signals from all the elements of interest.

5.4.2. NANOSIMS OF AS IN RICE

5.4.2.1. Detector Positioning (HMR)

Similarly to the Se wheat grain samples, the bulk concentration of As in the rice grains was very low and the position of the detector needed to be carefully tuned using a standard. A solid gallium arsenide (GaAs) polished standard was used for this purpose. A HMR scan was taken around mass 75 on the GaAs sample and on the rice grain. A very large, intense signal was observed for the scan on GaAs indicating the position of $^{75}\text{As}^-$ and it is clear that the left hand peak from the rice sample lines up exactly with this peak (Figure 5.14). Hence it is clear that the As can be detected from the rice and that the detector can be accurately positioned in the centre of the left peak from the sample. This tuning was carried out each morning before analysis on a sample was undertaken. Similarly to the mapping of the Se in the wheat grain, the position of the phytate granules was mapped as $^{31}\text{P}^{16}\text{O}^-$.

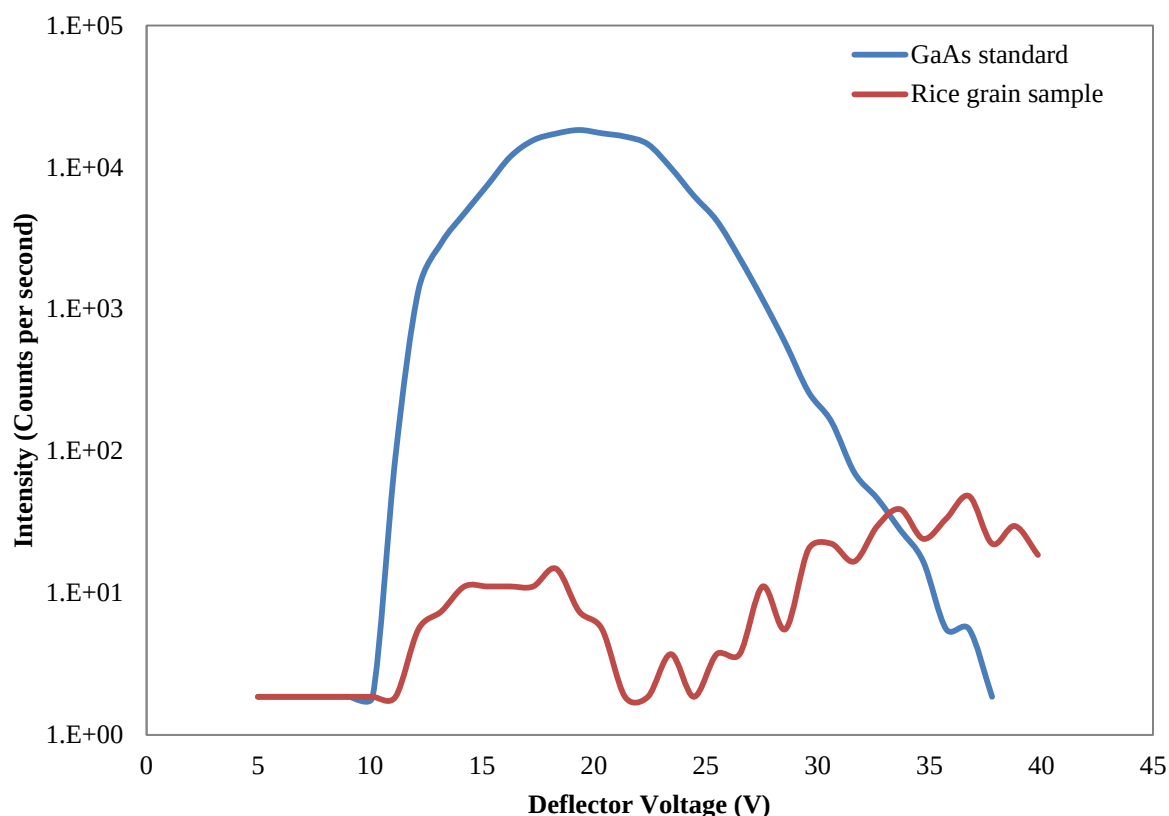


Figure 5.14: High resolution mass spectra around mass 75 from the solid gallium arsenide standard and a typical rice grain sample. HMR scans are exported as deflector voltages. Right hand peak from the sample could be C_6H_3 .

The NanoSIMS experiment on As in rice can be estimated to be about 100 times more challenging than the Se in wheat experiment. Not only is the bulk As concentration about 10 times lower but the RSF value (see Chapter 2) for As is 10 times lower than that of Se if one can use Si RSF values as a benchmark for yield of ion species from biological samples. However if the As is strongly localised as indicated by the S-XRF mapping then the local concentration will be increased, making the mapping of As distributions possible.

5.4.2.2. NanoSIMS Analysis of Soil-Grown High As Rice Grains

Initial analysis was focused on the aleurone layer away from the OVT region. Figures 5.15, 5.16 and 5.17 show results from three separate rice grains with similar results obtained from a further two samples. A dose of $5 \times 10^{16} \text{ Cs}^+ \text{ ions cm}^{-2}$ was implanted into a $100 \mu\text{m}$ area, then each image was acquired with a dwell time of 60 ms and a current of 4 pA. A higher dose was not

used for these samples as this resulted in significant damage to the starch grains and almost complete removal of the protein-rich regions. The three results presented here are almost identical in their elemental distributions and morphology. On the left of each image is part of the endosperm region and on the right two cells from the aleurone layer. Within each of these cells the phytate granules are clearly preserved, and similarly to the results from the wheat grains, they also show the characteristic ring structure in the $^{31}\text{P}^{16}\text{O}^-$ and $^{16}\text{O}^-$ images. In Figure 5.17 half of an aleurone cell remains unsputtered, presumably because it is lower than the rest of the surface, and hence the characteristic phytate granule structure is not observed, whereas in Figure 5.16 it appears that the top aleurone cell has been sectioned through the cell wall between aleurone cells and hence few phytate granules are observed. Also similarly to the wheat grain the subaleurone region is particularly protein rich as indicated by the $^{12}\text{C}^{14}\text{N}^-$ and $^{32}\text{S}^-$ images. The starch grains in rice have a much more circular morphology in cross section in comparison to the oval wheat starch grains, and a characteristic structure appears within the grains with sputtering as was confirmed with the FIB using the same method as outlined in Chapter 4. The starch grains are similarly surrounded by a protein rich matrix with decreasing concentration from the aleurone towards the centre of the grain. The S distribution is corroborated by the results of Lombi *et al.* (2009) who showed using PIXE a S distribution in rice grains that decreased in concentration from the outer parts of the grain to the interior of the endosperm (presented above in Figure 5.5) and confirmed by (Lombi *et al.*, 2011). Arsenic was predominantly localised to this subaleurone protein matrix, with a significantly lower concentration observed in the aleurone layer itself. Essentially no As was localised within the starch grains and any counts that were observed here could be a result of either redistribution from the sectioning or As atoms which were redistributed during the cleaning and implantation. Similarly to the gradient observed for the S and CN, the As concentration decreases from the outside edge to the centre of the grain. Figure 5.19 is taken from the starchy region of the grain 300 μm into the endosperm beneath the aleurone layer. It is clear that the As is still localised to the protein matrix between the starch grains however due to the decrease in the concentration of protein there is a concurrent decrease

in the As concentration. These results do not fit with results reviewed above where it was suggested that “once the As-rich pericarp and aleurone layer are removed, the remaining As is distributed homogeneously in the white rice grain” (Meharg *et al.*, 2008). My results show that the As concentration in the aleurone layer was significantly lower than in the underlying endosperm. Figure 5.18 shows the low As content of the aleurone layer more clearly. When ion signals are detected from the phytate granules, a ring is observed surrounding the edge of the phytate granule as explained in Chapter 4, and shown clearly in Figure 5.18 for the $^{16}\text{O}^-$ and $^{31}\text{P}^{16}\text{O}^-$ images. This ring is not observed in the As image therefore it can be assumed that the As is not associated with phytate unlike other trace metals such as Fe and Zn (Ozturk *et al.*, 2006; Abebe *et al.*, 2007). In Figure 5.17 there is a large hotspot of As in the aleurone layer which was not observed in any other sample or aleurone area. It is not clear what this feature is and hence where this As signal originates from. In the $^{16}\text{O}^-$ and SE images it can be seen that this As hotspot is associated with a triangular feature which appears to be sitting on top of the aleurone cell. Although it is not possible to tell whether this feature has been redistributed during the cutting process or whether it is in fact part of the aleurone cell, it is possible that topography has enhanced the signal at this point.

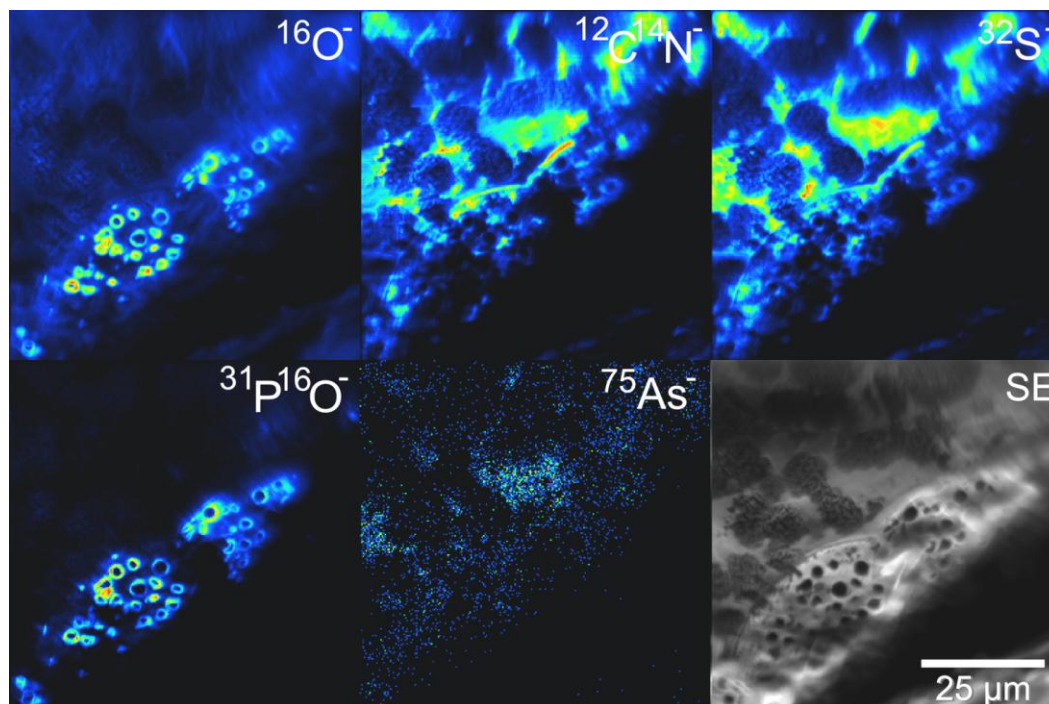


Figure 5.15: NanoSIMS maps of $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{31}\text{P}^{16}\text{O}^-$, $^{75}\text{As}^-$ and the secondary electron image from the outer part of a rice grain showing two aleurone cells and part of the starchy endosperm region. The As is predominantly localised in the subaleurone protein. Parts of this image have been published in the following review paper: Zhao FJ, McGrath SP, Meharg AA. 2010. Arsenic as a food chain contaminant: Mechanisms of plant uptake and metabolism and mitigation strategies. *Annual Review of Plant Biology*, Vol 61: 535-559.

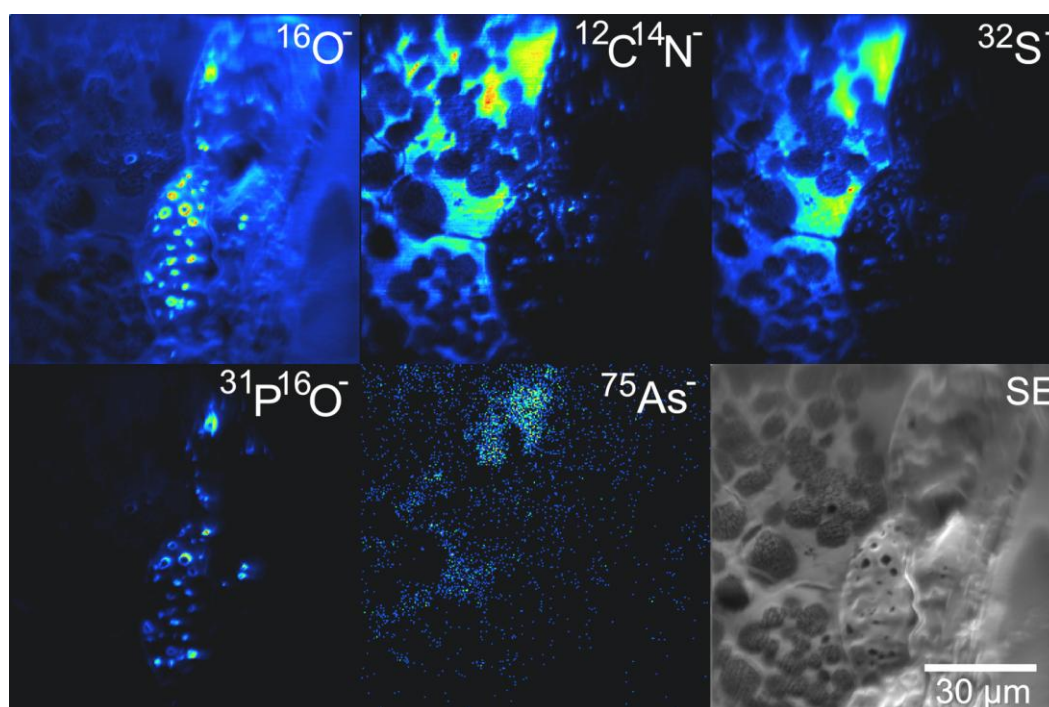


Figure 5.16: NanoSIMS maps of $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{31}\text{P}^{16}\text{O}^-$, $^{75}\text{As}^-$ and the secondary electron image from the outer part of a second rice grain showing two aleurone cells and part of the starchy endosperm region. The As is again predominantly localised in the subaleurone protein. Reprinted with permission from John Wiley and Sons.

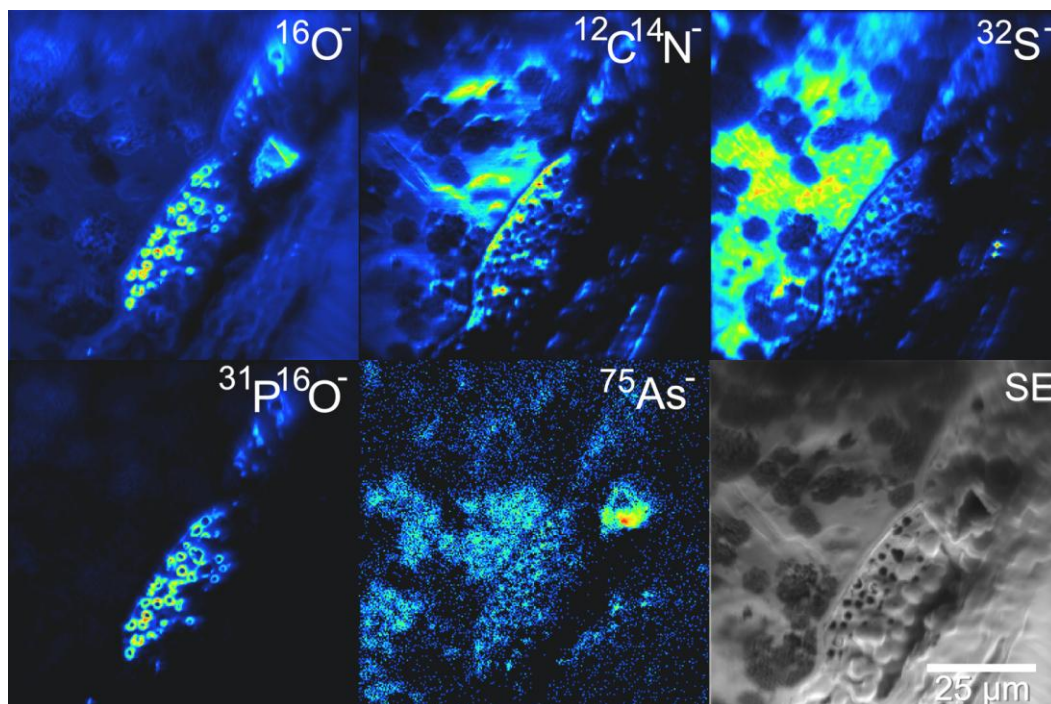


Figure 5.17: NanoSIMS maps of $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{31}\text{P}^{16}\text{O}^-$, $^{75}\text{As}^-$ (log scale) and the secondary electron image from the outer part of a third rice grain showing two aleurone cells and part of the starchy endosperm region. The As is again predominantly localised in the subaleurone protein, but for this sample there is a large As hotspot in the aleurone layer and there appears to be some As association with the phytate granules.

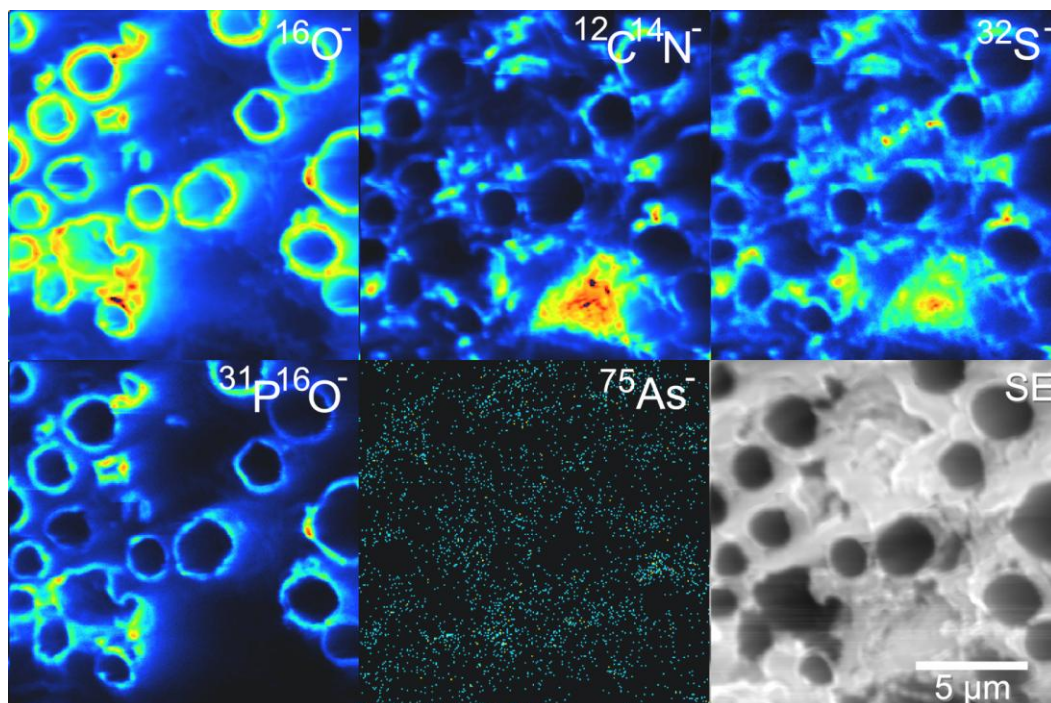


Figure 5.18: Higher resolution NanoSIMS maps of $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{31}\text{P}^{16}\text{O}^-$, $^{75}\text{As}^-$ and the secondary electron image from the centre of a rice aleurone cell showing the characteristic ring phytate structure and very little As localised within the aleurone layer itself. Reprinted with permission from John Wiley and Sons.

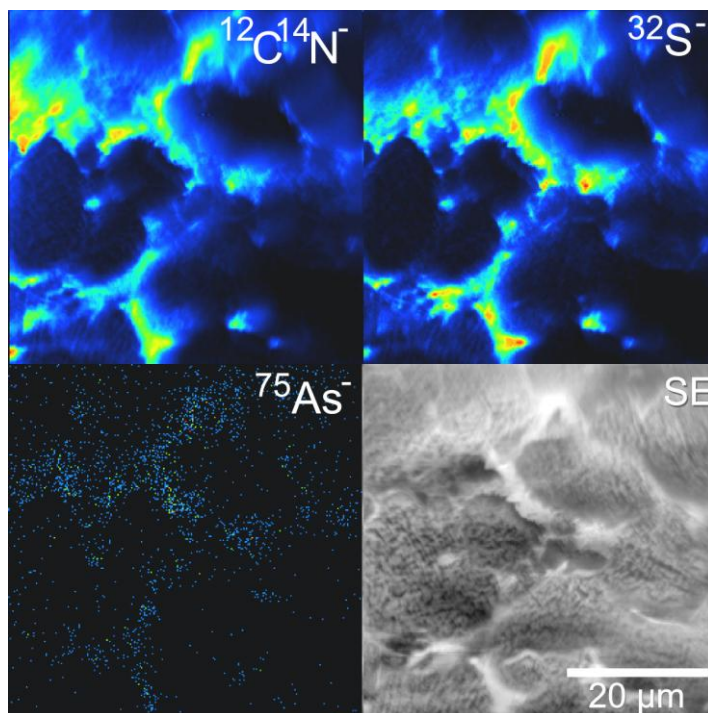


Figure 5.19: Higher resolution NanoSIMS maps of $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{75}\text{As}^-$ and the secondary electron image from the starch endosperm region 300 μm from the aleurone layer showing a much lower concentration of protein and hence As in comparison to the subaleurone region.

Imaging of the OVT region was an obvious target based on the results of Meharg *et al.* (2008) and Lombi *et al.* (2009), and it was expected that there would be a high As concentration region in this part of the grain. Figure 5.20 shows that for these soil-grown rice grains, NanoSIMS analysis detects no dramatic increase in As concentration at this point (see quantification in Figure 5.28). However, As was observed within the multilayered aleurone cells just inside the OVT and nucellar projection in contrast to the images shown above where very little As was observed within the aleurone cells. A schematic diagram showing the position of these multilayered aleurone cells relative to the position of the OVT is shown in Figure 5.21.

Arsenic was not observed outside of these cells, i.e. towards the OVT (bottom of Figure 5.20). The As hotspot localised in the top left of the As image increased in intensity with depth profiling and could be similar to those found in the sample analysed with S-XRF (as discussed below in Section 5.4.3).

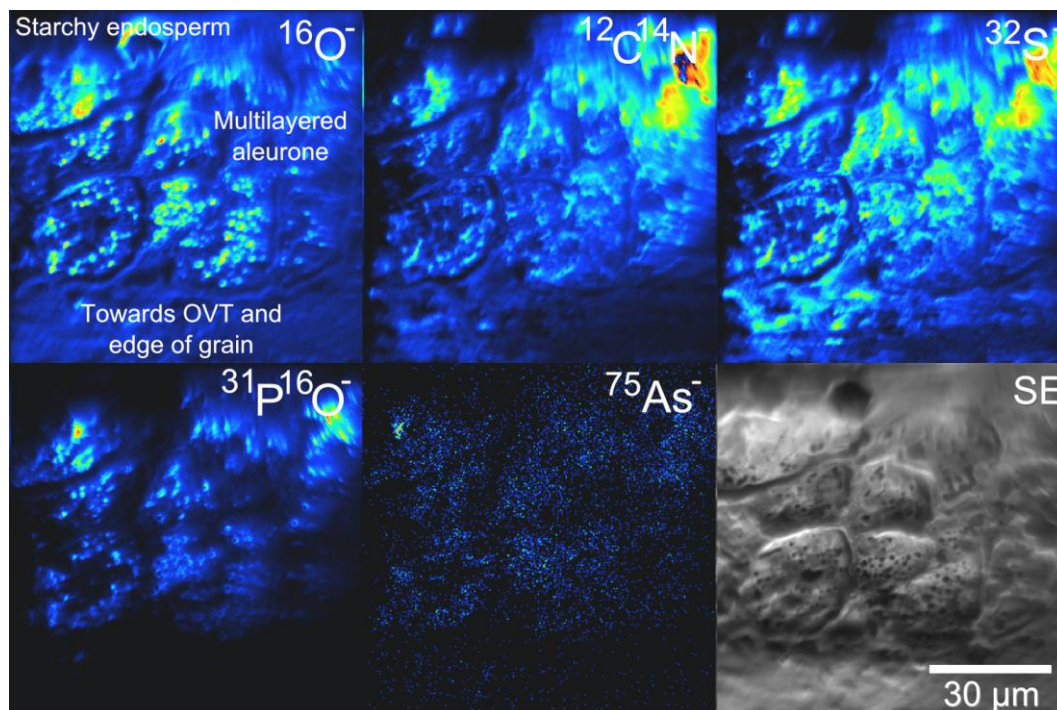


Figure 5.20: NanoSIMS images of $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{31}\text{P}^{16}\text{O}^-$, $^{75}\text{As}^-$ and the secondary electron image from the ovular vascular trace region of a rice grain. The outside edge of the rice grain is at the bottom of the image with the starchy endosperm at the top.

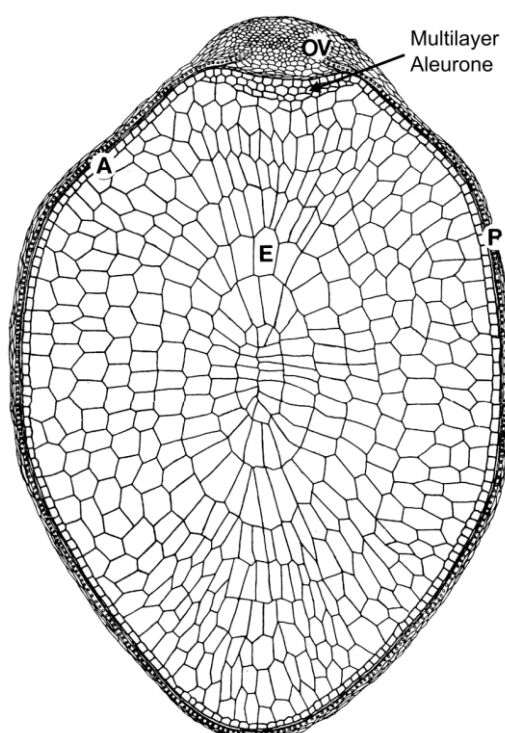


Figure 5.21: Schematic diagram of a transverse section of a mature rice grain. A, Aleurone; E, endosperm; OV, ovular vascular trace; P, pericarp (Krishnan & Dayanandan, 2003). With kind permission from Springer Science+Business Media: Journal of Biosciences, 'Structural and histochemical studies on grain-filling in the caryopsis of rice (*Oryza sativa* L.)' 28, 2003, page 459, S. Krishnan and P. Dayanandan, Figure 4.

In a separate experiment, S-XRF mapping at the DIAMOND synchrotron was used to map the distribution of As in a rice grain treated only with DMA (Figure 5.22). The bulk grain speciation was measured with HPLC-ICP-MS and found to be predominantly DMA, very similar to the soil-grown high As concentration grains analysed with the NanoSIMS above and also with a fairly high bulk As concentration of 1.99 ppm. A vibratome was not available at the synchrotron so the rice was hand sectioned using a razor blade. The OVT region was identified (away from the embryo) and a transverse section was cut to a thickness of $\sim 470\ \mu\text{m}$. This was slightly thicker than the ideal thickness for S-XRF mapping and hence there is some distortion of the image towards the right hand side but not so significant that the images cannot be interpreted. The As image is very similar to that presented by Carey *et al.* (2010) and to the NanoSIMS data presented above. The subaleurone region contains a higher concentration of As which then decreases towards the centre of the grain, also shown in the line scan across the As image (Figure 5.23). The aleurone layer is best indicated by the Fe map as a distinct line around the edge of the rice grain, as shown in Chapter 4 for the wheat grain phytate granules. The Fe intensity decreases towards the bottom right of the image caused by smearing of the signal due to the thickness of the sample. The position of the OVT is towards the top right of the image, at the point where Zn is strongly localised and it can be seen that there is no enhancement of As at this point. Strong Zn localisation in the OVT was also observed by Meharg *et al.* (2008). A colour merge of As and Fe shows the relative locations of these two elements indicating that there is, as indicated by the NanoSIMS data, very little As localised to the aleurone layer in grains containing a high proportion of DMA and this is also confirmed by the line scan shown in Figure 5.23. The Fe hotspots towards the centre of the rice grain (bottom of the image) are likely to be contamination from the razor blade used to cut the rice grain.

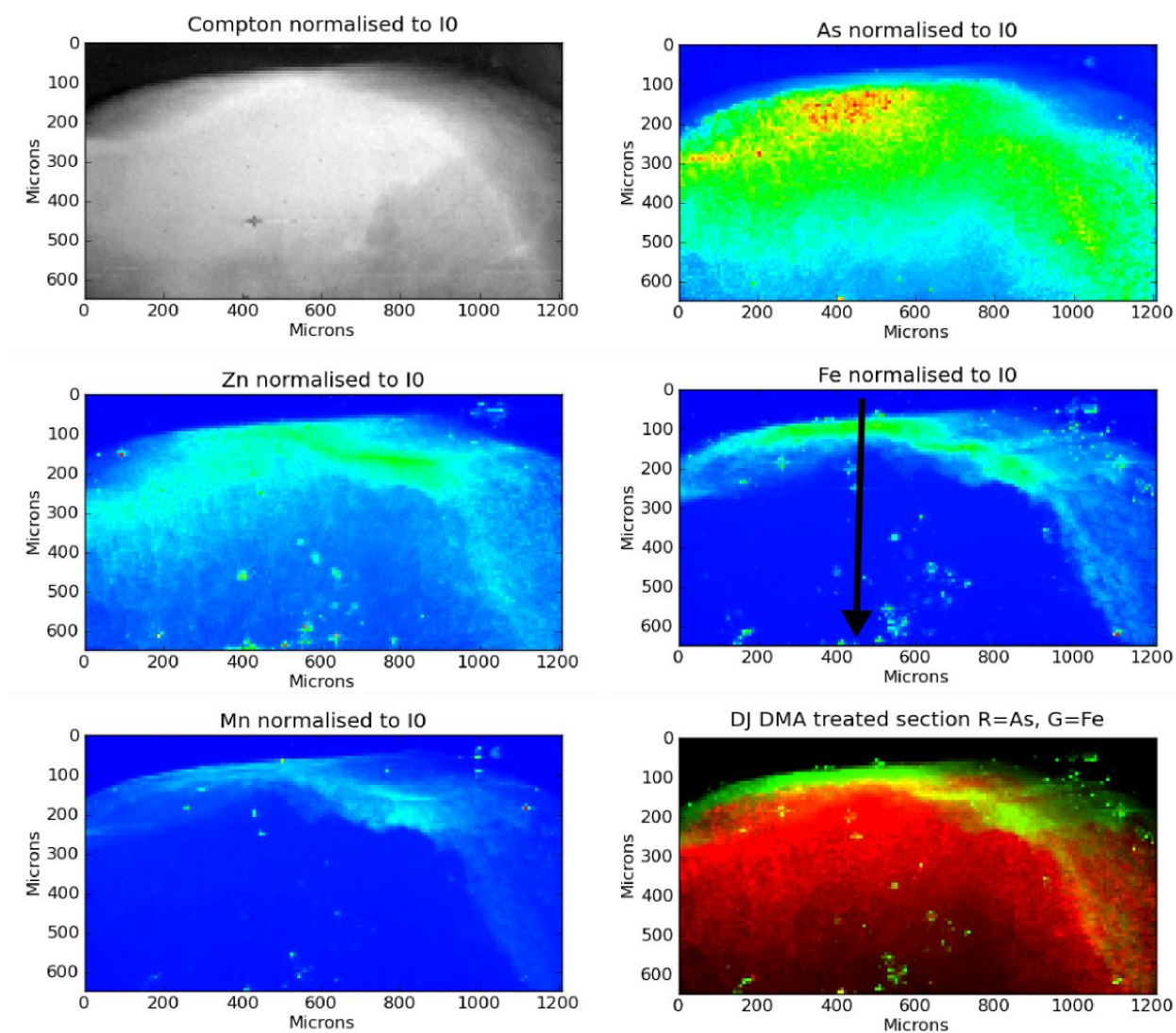


Figure 5.22: S-XRF images of part of a DMA treated rice grain which includes the OVT at the top right of the image. All images are normalised to I_0 , the incoming X-ray flux, to minimise differences in X-ray signal from the sample. The Compton image indicates variations in thickness of the section. The black arrow on the Fe image indicates the position of the line scan presented in Figure 5.23. The beam size was $5\mu\text{m}$ and the step size was $8\mu\text{m}$, 1.5 s count time.

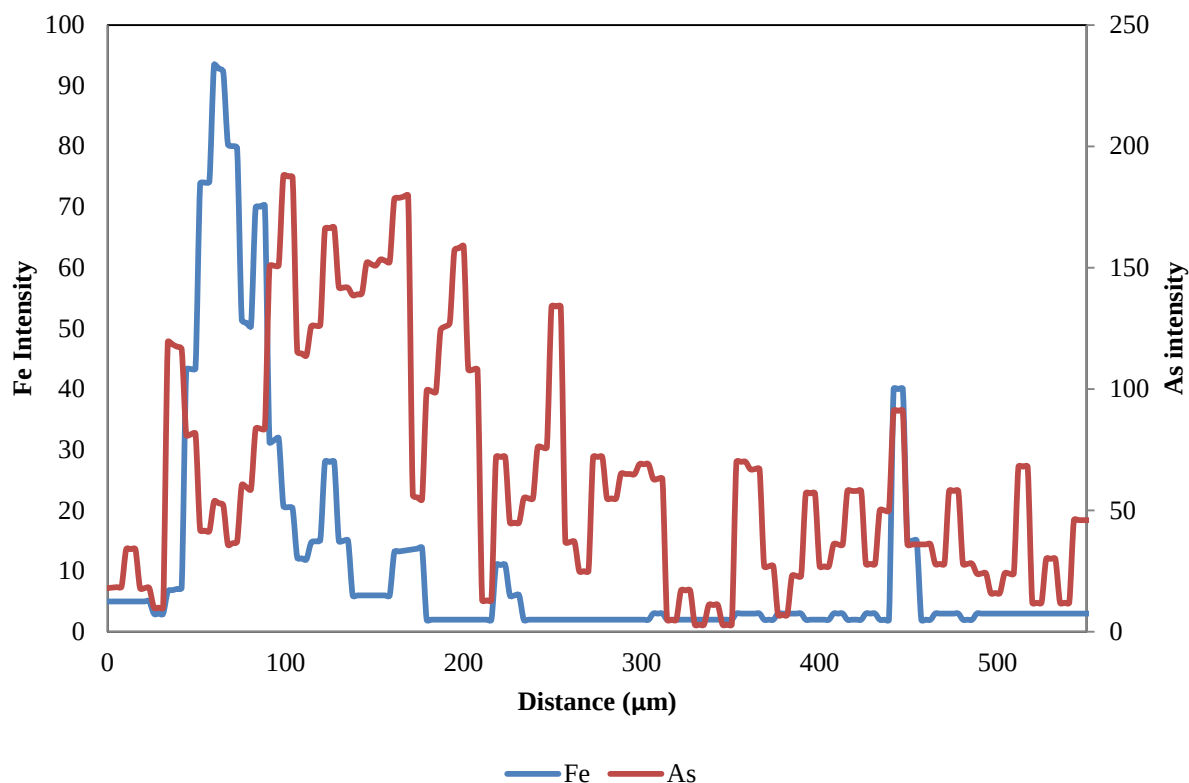


Figure 5.23: Line scan taken across the As and Fe images shown in Figure 5.22 as indicated by the black arrow. The Fe is localised to the aleurone layer with very little As colocalisation. The As signal decreases from the subaleurone region towards the centre of the grain.

5.4.2.3. NanoSIMS Analysis of Hydroponically Grown As(III)-Treated Rice

A further experiment was conducted on hydroponically grown rice grains treated with As(III) with a significantly lower bulk concentration of As in the grain (0.83 ppm, mainly inorganic As). Mapping of the aleurone and subaleurone region in an area away from the OVT revealed a distinctly different As distribution to that of the soil-grown high As concentration and DMA treated grains. Instead of subaleurone localisation of As there was comparatively very little As detected in this region and most of this As was only localised to the aleurone layer (Figure 5.24). The distribution of the As followed that of the $^{31}\text{P}^{16}\text{O}^-$ distribution suggesting that the As was localised to the phytate granules with the same ring like structures observed (as explained previously in Chapter 4).

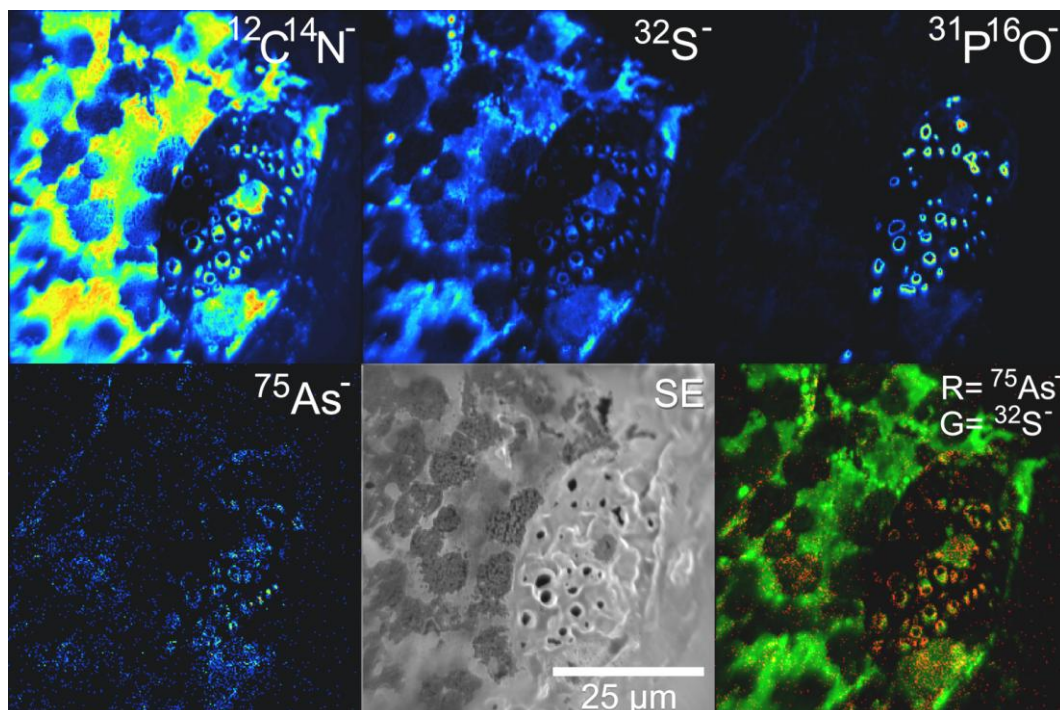


Figure 5.24: NanoSIMS maps of $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{31}\text{P}^{16}\text{O}^-$, $^{75}\text{As}^-$ and the secondary electron image from the outer part of a hydroponically grown As(III)-treated rice grain away from the OVT. The colour merge shows the relative localisation of $^{75}\text{As}^-$ (red) and $^{32}\text{S}^-$ (green) indicating that the As is predominantly localised to the phytate granules in the aleurone rather than to the subaleurone protein.

Imaging of the multilayered aleurone layer near the OVT of this same rice grain indicated that in these hydroponically grown As(III)-treated grains, this was the part of the grain where the majority of the As was localised. This multilayered aleurone region and the area analysed with the NanoSIMS is shown in Figure 5.25. It is quite apparent in the NanoSIMS image that there is strong localisation of As to this particular region of the rice grain, with As colocalised to the phytate granules (Figure 5.26). There is a noticeable absence of As at the bottom of the image where part of the endosperm region has been imaged.

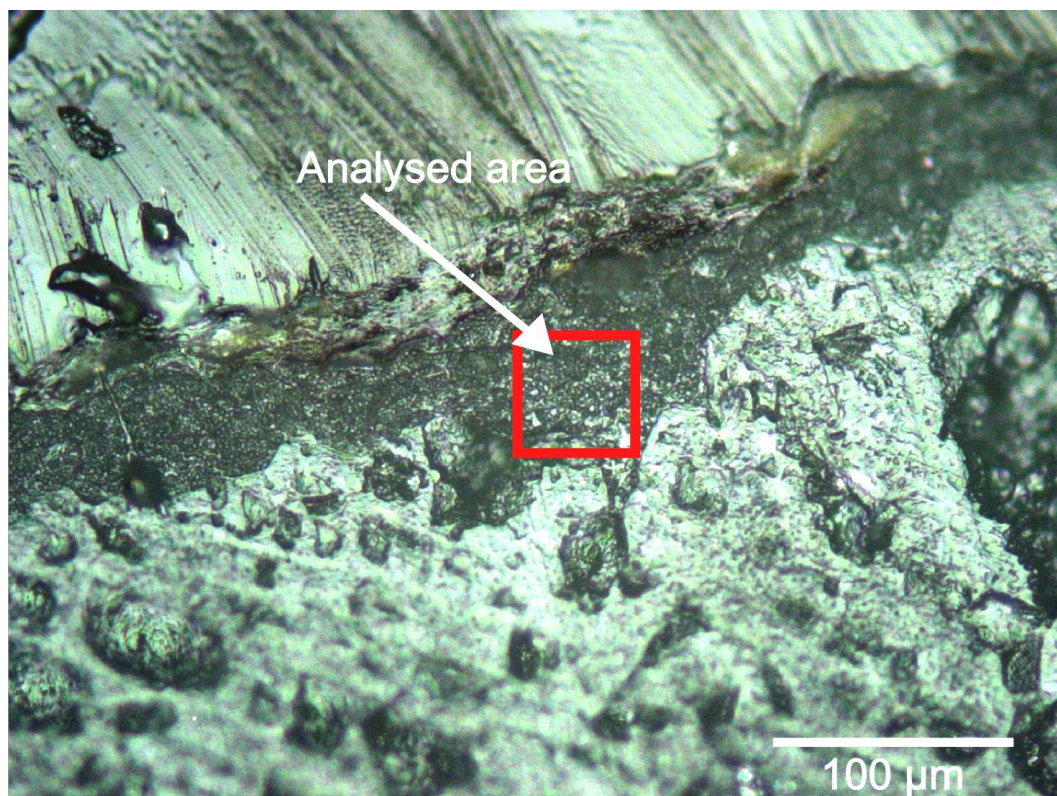


Figure 5.25: Optical image near the OVT region of a hydroponically grown As(III)-treated rice grain showing the multilayered aleurone cells. The red box indicates the position of the NanoSIMS analysis as shown in Figure 5.26.

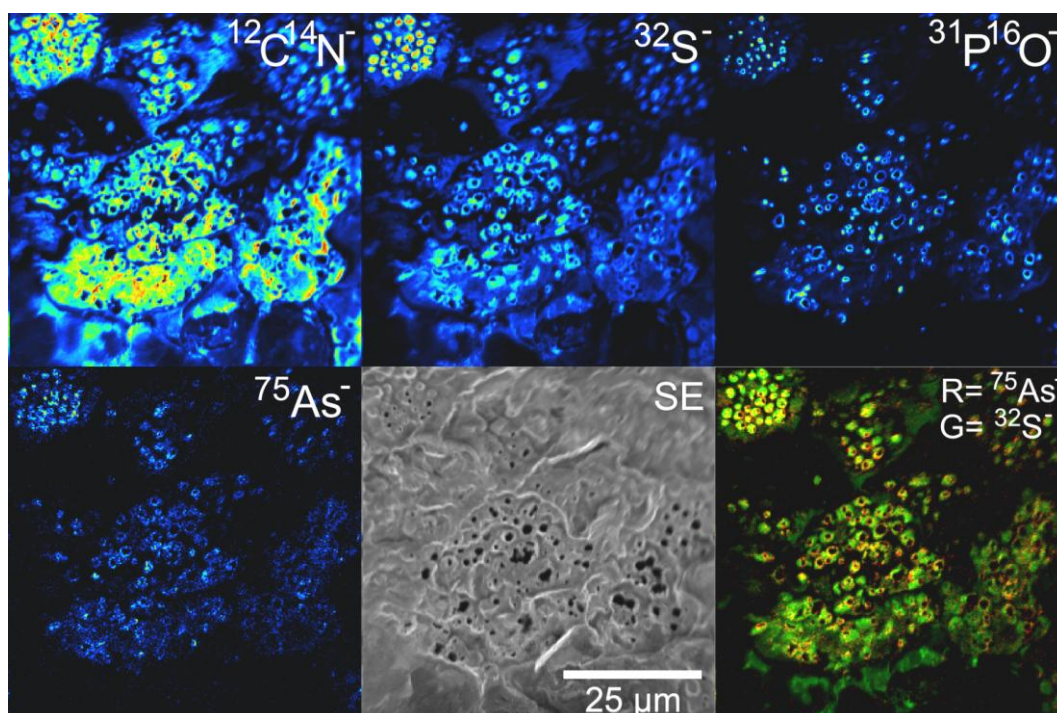


Figure 5.26: NanoSIMS maps of $^{12}\text{C}^{14}\text{N}^-$, $^{32}\text{S}^-$, $^{31}\text{P}^{16}\text{O}^-$, $^{75}\text{As}^-$ and the secondary electron image of the multilayered aleurone cells near the OVT region of a hydroponically grown As(III)-treated rice grain. The colour merge shows the relative localisation of $^{75}\text{As}^-$ (red) and $^{32}\text{S}^-$ (green). As is strongly localised to the aleurone type cells in this region with little As in the endosperm region at the bottom of the image.

S-XRF analysis at DIAMOND of a similarly treated rice grain to that analysed with the NanoSIMS also indicated a similar distribution, with As highly localised apparently to the multilayered aleurone region near the OVT region. The Fe map can again be used to give an indication of the position of the aleurone layer however there is a noticeable reduction in Fe concentration where there is high As and Zn concentration in this multilayered aleurone region as shown in Figure 5.27 suggesting that these cells differ from other aleurone cells.

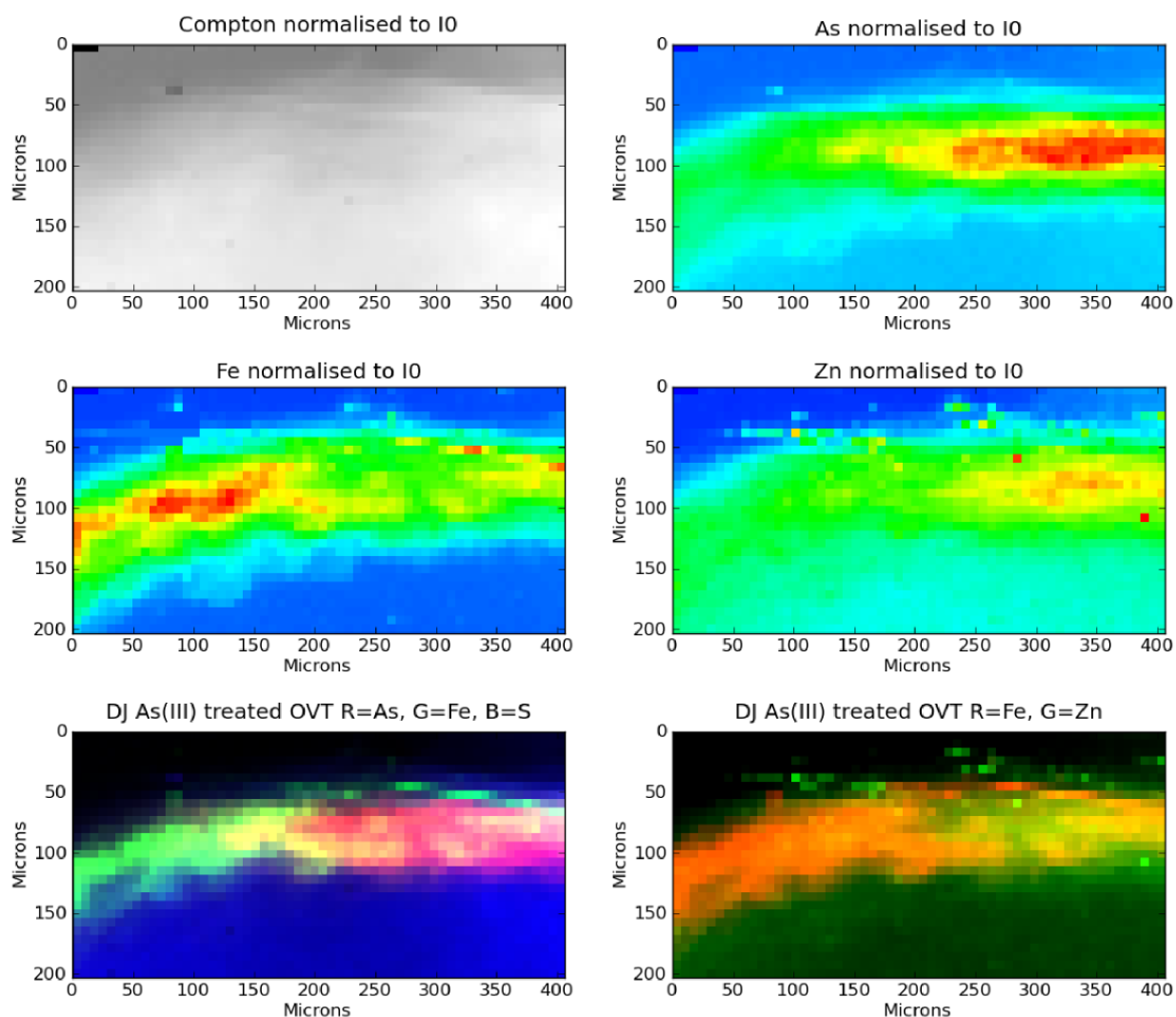


Figure 5.27: S-XRF images of the OVT region of a hydroponically grown As(III)-treated rice grain showing As highly localised to this region. All images are normalised to I0, the incoming X-ray flux, to minimise differences in X-ray signal from the sample. The Compton image indicates variations in thickness of the section. The beam size was 5 μm and the step size was 7 μm , 1.5 s count time. Sample was hand sectioned to a thickness of 0.53 mm.

Comparing the $^{75}\text{As}^-/^{12}\text{C}^{14}\text{N}^-$ ratio of the multilayered aleurone cells near the OVT and the aleurone/subaleurone regions from the soil-grown and hydroponically grown As(III)-treated grains clearly indicates the differences in As distribution between differently treated grains. Figure 5.28 shows that the aleurone cells in the OVT region of the hydroponically grown As(III)-treated grain have a distinctly higher ratio than any other region, with the aleurone/subaleurone region of this same grain exhibiting a very low ratio. For the soil-grown high As concentration grains there is little difference in the ratios between the aleurone cells near the OVT and the aleurone/subaleurone as apparent from the NanoSIMS images.

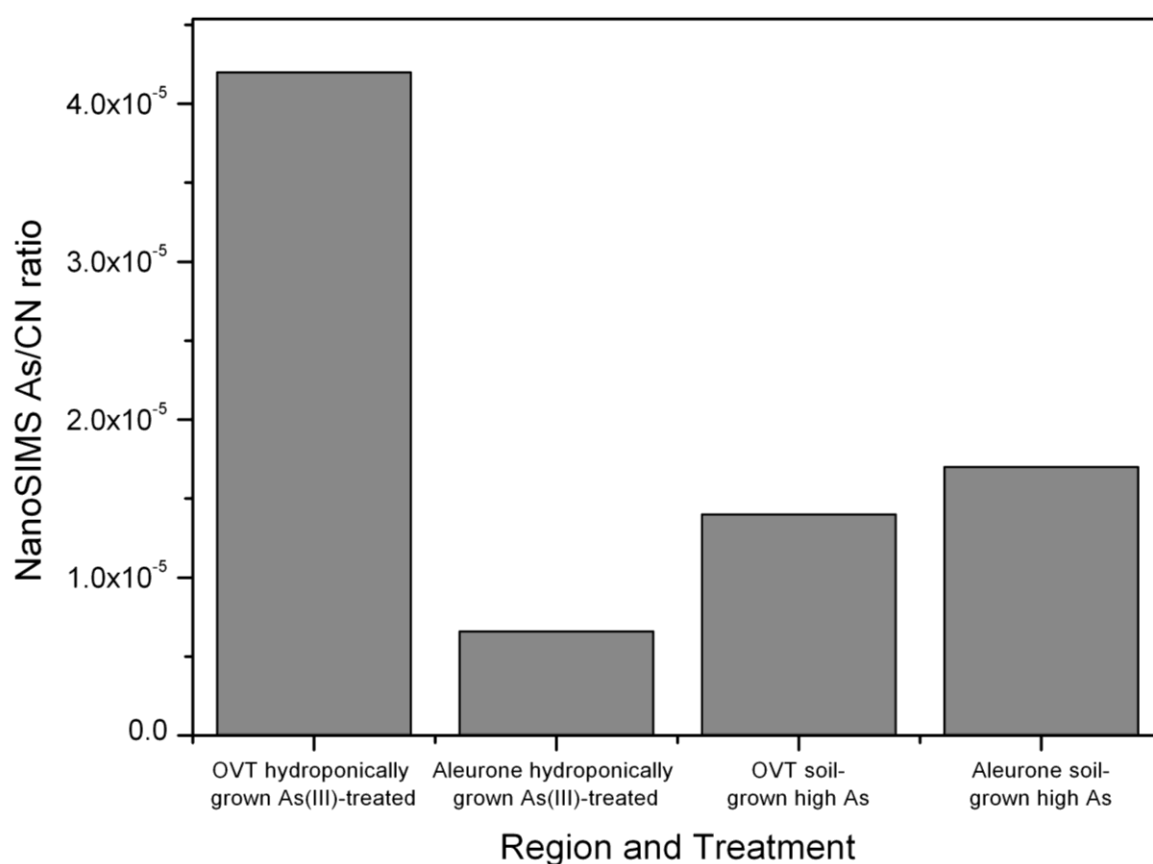


Figure 5.28: NanoSIMS $^{75}\text{As}^-/^{12}\text{C}^{14}\text{N}^-$ ratios of the aleurone cells near the OVT and the aleurone/subaleurone regions from the soil-grown high As concentration grains and the hydroponically grown As(III)-treated grains. This graph indicates that As is strongly localised to the aleurone cells near the OVT in the As(III)-treated grains and more evenly distributed across the OVT and subaleurone regions in grains with a high DMA content.

These data correlate very well with those of Carey *et al.* (2010) who also showed strong As localisation near the OVT for the As(III) treated grains and a much more diffuse localisation for

the DMA treated grains. In this study, however, it has been possible to more precisely target which cell layer the As is localised and show more clearly the distinct differences that can be observed for differently treated or different concentration grains.

5.4.3. S-XRF AND NANOSIMS OF AS IN THE SAME RICE SAMPLE

Although the NanoSIMS is able to map at higher resolution than most analytical instruments it is not able to map very large areas. The maximum area which can be mapped in a single image without distortions is limited to about 90 μm . It is therefore impractical to attempt to map the whole of a rice grain with this technique and there are also problems with re-deposition of sputtered material around analysed areas which would make large scale sequential mapping difficult. In order to compare directly S-XRF and NanoSIMS results it was decided to analyse the same soil-grown high As concentration rice grain with both techniques to ensure that by choosing semi-random areas to analyse across the rice grain, as above, regions with a high As concentration were not missed. While EPMA is able to map larger areas it did not have sufficient sensitivity to map the ppm concentrations of As in these samples.

One rice grain sample was prepared in the same way as described previously for NanoSIMS analysis by mounting in LR White resin and sectioned flat with a razor blade, but not coated with Pt at this stage. Although this sample preparation was not ideal for synchrotron analysis, as explained previously, it was necessary to ensure that the same sample could subsequently be analysed in the NanoSIMS. This sample was sent to Enzo Lombi (Centre for Environmental Risk Assessment and Remediation, University of South Australia) for analysis at the Advanced Photon Source (APS) at Argonne National Laboratory (Argonne, IL, USA). Synchrotron X-Ray Fluorescence was carried out on beamline 20ID. Half of the grain (3 mm x 1.7 mm) was mapped using S-XRF with a step size of 15 μm , the beam focused to approximately 2 μm and with a dwell time of 1 sec/pixel.

The sample is mounted in the synchrotron stage which allows the sample to be raster scanned rather than scanning the beam, allowing larger scale mapping. The incoming X-Ray beam and the detector are both positioned at 45° to the sample surface. The sample set up is shown in Figure 5.29.

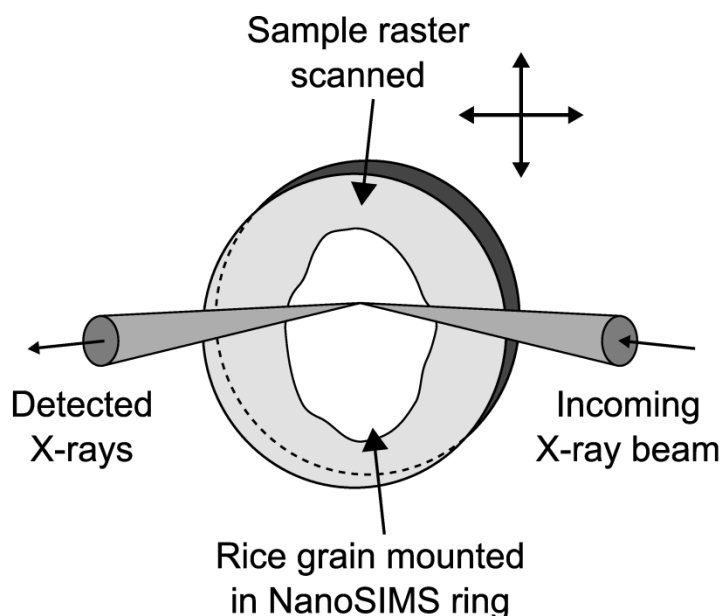


Figure 5.29: Schematic diagram showing the arrangement of the sample and X-ray beams in a S-XRF experiment. The incoming and detected beams are at 90° to each other.

The As S-XRF map obtained from this sample is shown in Figure 5.30. The thickness of the sample, which was necessary to prepare the sample for NanoSIMS analysis, and the detector positioning caused the map to be distorted towards the left hand side. This is because the high energy X-Rays are very penetrating and since the detector is positioned to one side as the sample is scanned, signals from the deeper parts of the sample are not sampled at the same x-position as the points normal to it on the surface. Even with this distortion it is clear to see that at the top of the map, in an area near the OVT, there is a region of high As intensity and hence the purpose of combining these techniques, for what is believed to be the first time, has been achieved with the synchrotron mapping identifying areas of high As to be subsequently targeted with the NanoSIMS.

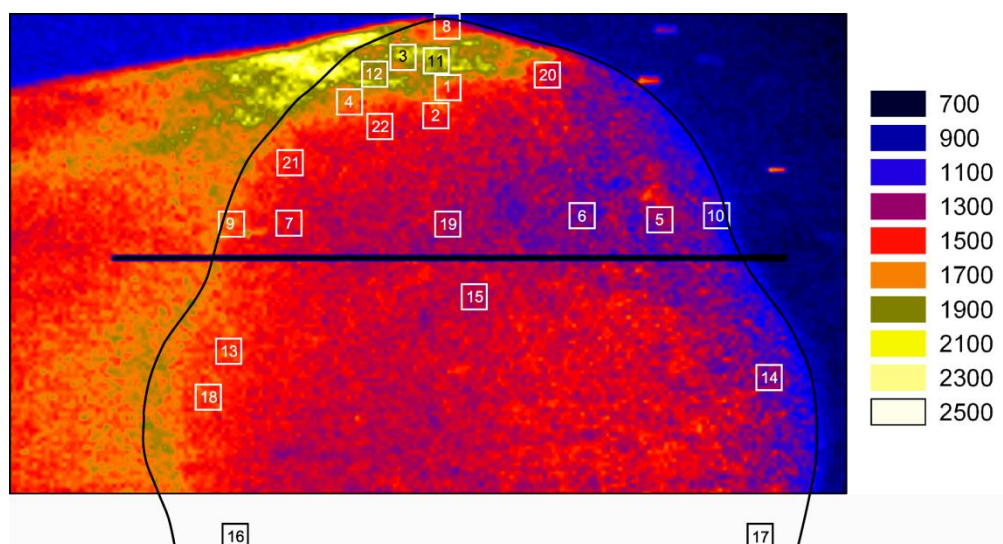


Figure 5.30: Arsenic S-XRF map of half of a transversely sectioned rice grain showing a high As concentration at the top of the grain. The size of the mapped area is 3 mm x 1.7 mm. The squares show the location and size of the NanoSIMS analysis areas and the black line indicates the edge of the grain. The smearing of the data to the left hand side of the map is an artefact due to the thickness of the sample and the horizontal black line is an artefact caused by the X-ray beam cutting out during the scan. Reprinted with permission from John Wiley and Sons.

The sample analysed with the synchrotron was also imaged with the SEM to investigate the damage caused by the synchrotron beam, which was immediately visible. Across the analysed area a regular array of holes could be observed as shown in Figures 5.31 and 5.32. These holes were at approximately 15 μm intervals and about 2 μm across which is consistent with the beam conditions used for the experiment. The holes are not normal to the surface of the rice grain, due to the orientation of the incoming X-Ray beam, therefore it is difficult to estimate the depth of the holes. It is clear to see that even a short dwell time of 1 sec/pixel has caused a significant amount of damage. Ideally SIMS should be performed on a flat surface, but in order to analyse exactly the same sample in the NanoSIMS this topography could not be avoided and any enhancements of ion signals from these regions were disregarded. In Figure 5.31 it is clear to see that the sample has cracked significantly. This is a result of the sample rapidly drying out as it was placed into the SEM vacuum after being exposed to ambient air for several weeks. This did not cause a problem for NanoSIMS analysis because there were large enough areas, as shown in Figure 5.32, which could be imaged while avoiding these large cracks.

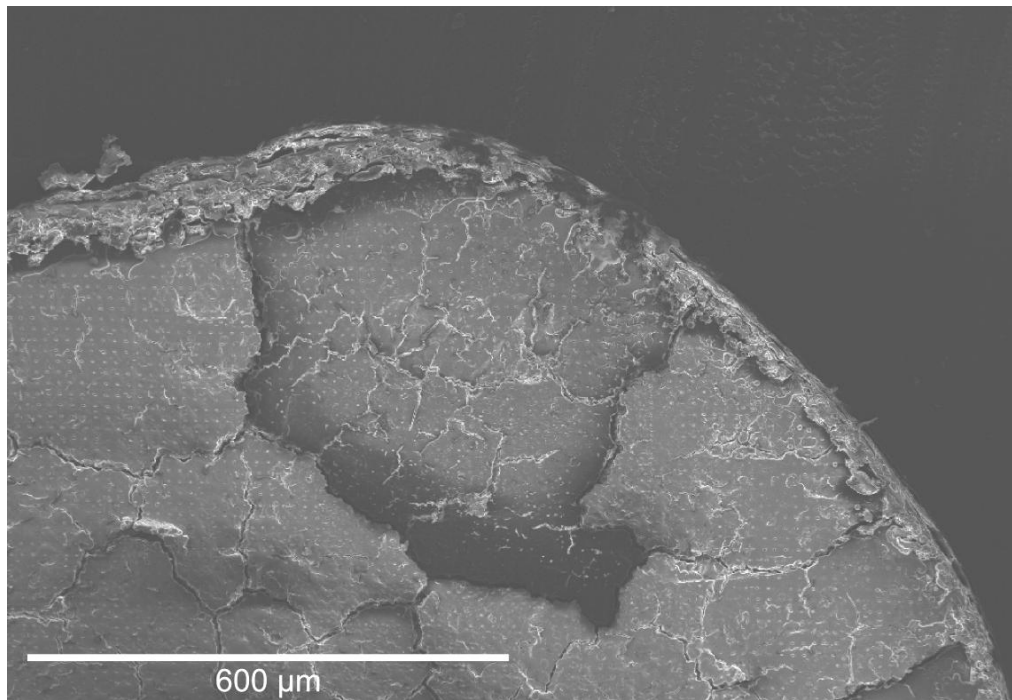


Figure 5.31: SEM image of the area analysed in the synchrotron. The sample has cracked in the SEM vacuum due to rapid desiccation however this did not affect NanoSIMS analysis

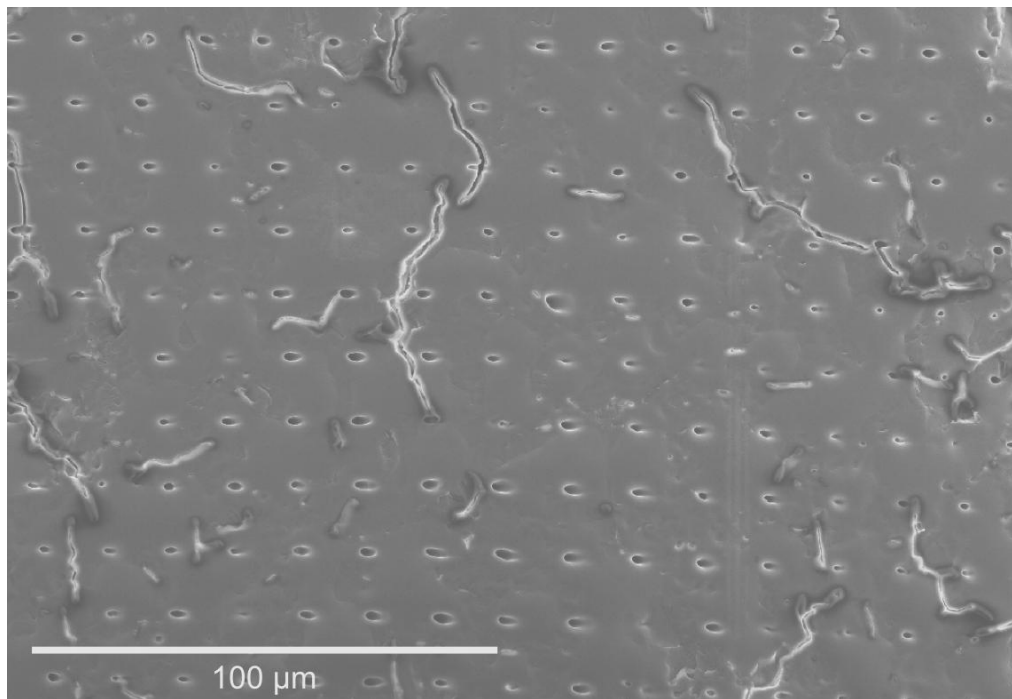


Figure 5.32: SEM image showing the separation of the analysis points and damage caused by the X-ray beam. Note the holes are not normal to the surface of the rice grain due to the orientation of the incoming X-Ray beam as shown in Figure 5.29. This image has been published in the following reference (Lombi et al., 2011). Reprinted from *Environmental and Experimental Botany*, 72(1), Enzo Lombi, Kirk G. Scheckel, Ivan M. Kempson, 'In situ analysis of metal(loid)s in plants: State of the art and artefacts', 3-17, 2011, with permission from Elsevier.

The areas analysed in the NanoSIMS are indicated by the white numbered squares in Figure 5.30. Areas were chosen based on where either high or low As signal intensity was observed in the As S-XRF map and to avoid the worst of the damage caused by the rapid desiccation. At each location a dose of $7 \times 10^{16} \text{ Cs}^+ \text{ ions cm}^{-2}$ was implanted then a 90 by 90 μm map (with a dwell time of 10 ms, a current of 29 pA and D1=1) was obtained to compare the As distribution directly with the S-XRF data.

In regions near the OVT, S-XRF showed a higher average As concentration, and higher resolution NanoSIMS analysis (D1=3) was able to identify micron-sized hotspots (localised regions of high As intensity) as shown in Figures 5.33, 5.34 and 5.35 from areas 1, 2 and 7 respectively. Distinct As hotspots were found in areas 1–4, 7 and 11–13 of Figure 5.30, and, in general, one or two hotspots were found in each 90- μm scan. With the exception of areas 7 and 13, no hotspots were found at any location other than the high-As region shown by S-XRF near the OVT. Because of the large step size of the S-XRF analysis it is possible that the highly localised hotspots in areas 7 and 13 lay between the analysis points, and hence these areas are shown in Figure 5.30 as being low in As.

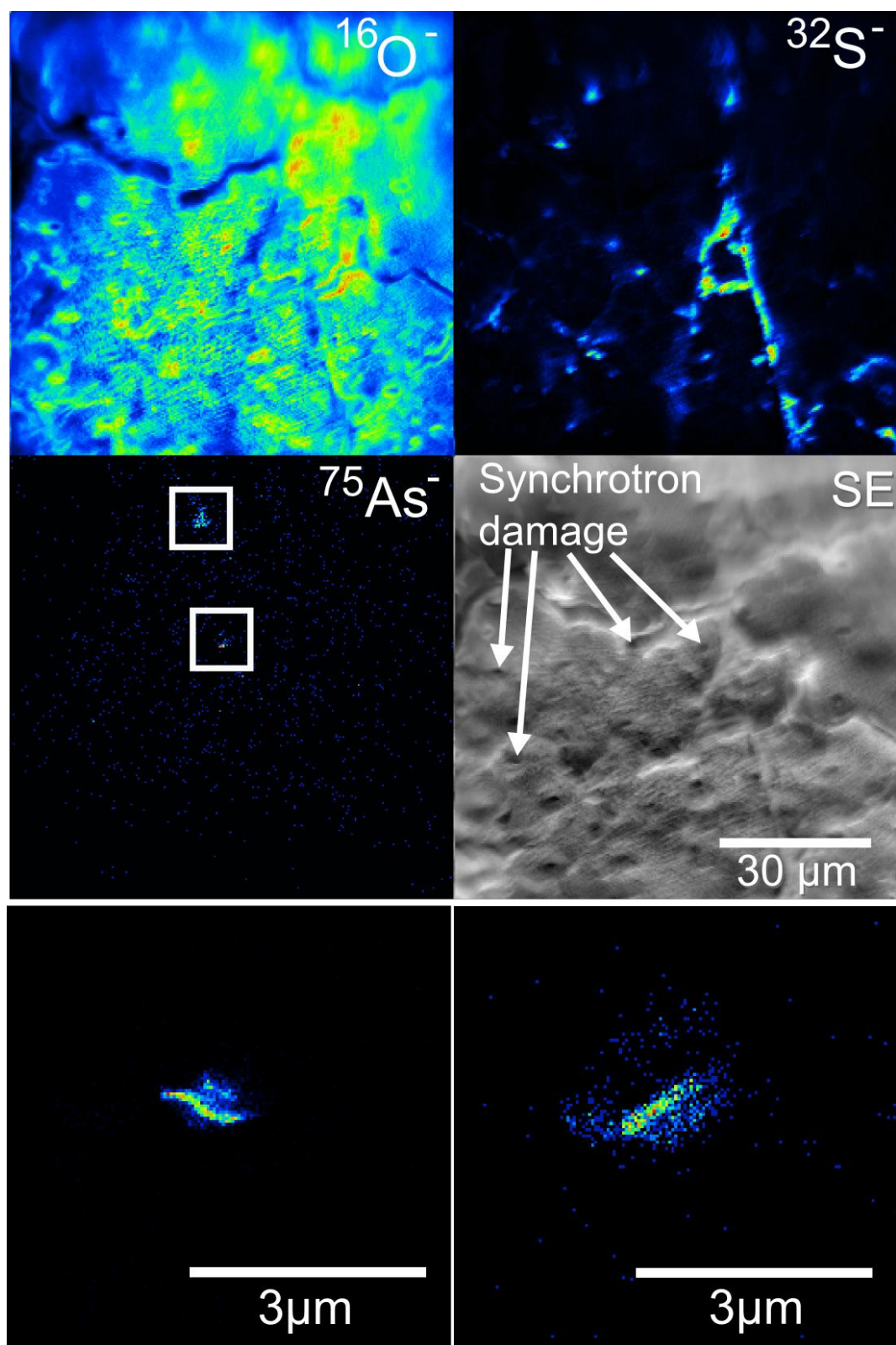


Figure 5.33: NanoSIMS ion maps of $^{16}\text{O}^-$, $^{32}\text{S}^-$, $^{75}\text{As}^-$ and the secondary electron image from area 1 of Figure 5.30. Two arsenic (As) hotspots were found in this area. Higher magnification scans ($10\ \mu\text{m}$) were taken of each of these; the upper hotspot is shown on the left and the lower on the right. Synchrotron damage (regular holes) can be seen in the SE image. Reprinted with permission from John Wiley and Sons.

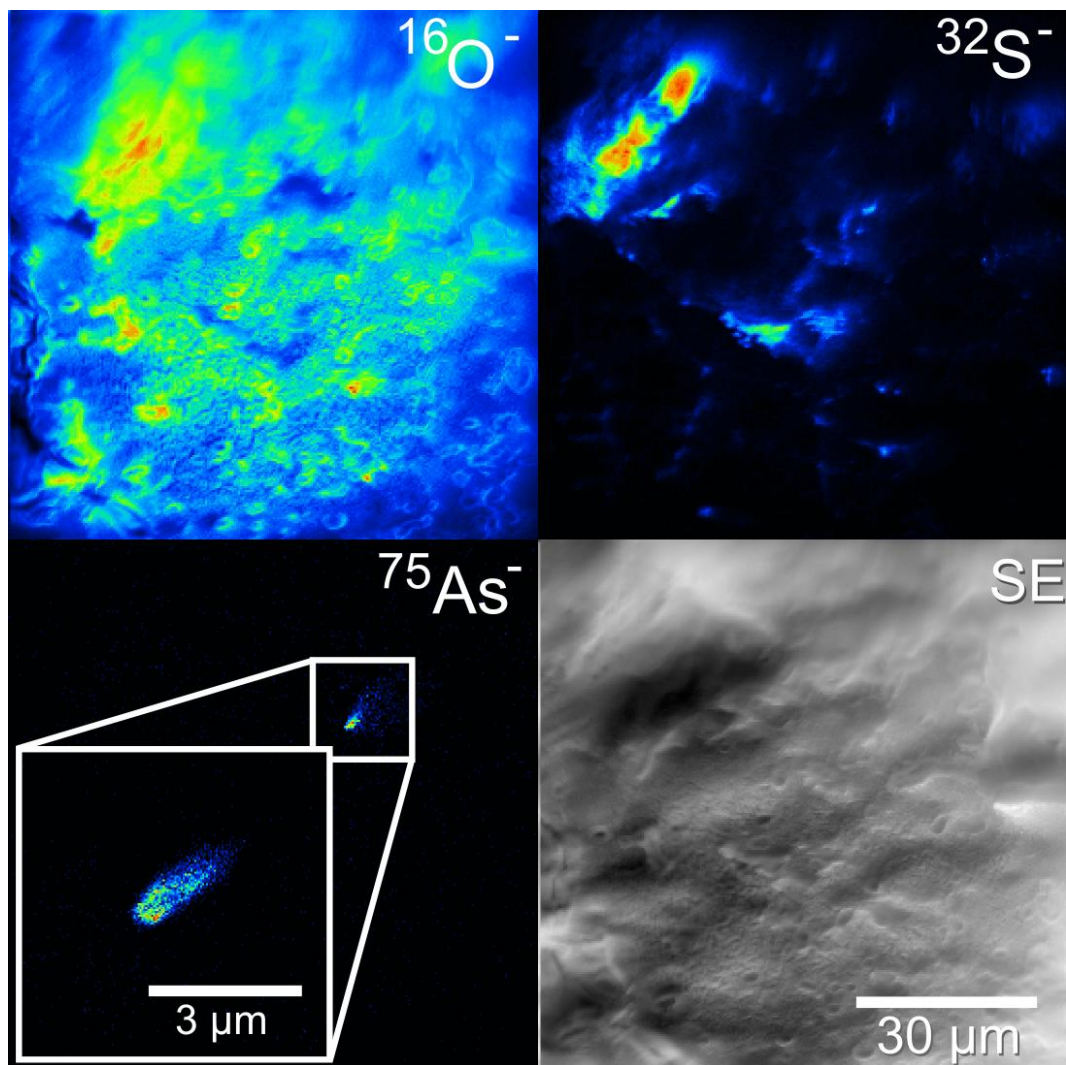


Figure 5.34: NanoSIMS ion maps of $^{16}\text{O}^-$, $^{32}\text{S}^-$, $^{75}\text{As}^-$ and the secondary electron image from area 2 of Figure 5.30. A higher magnification scan of the As-hotspot is shown as an insert in the As image.

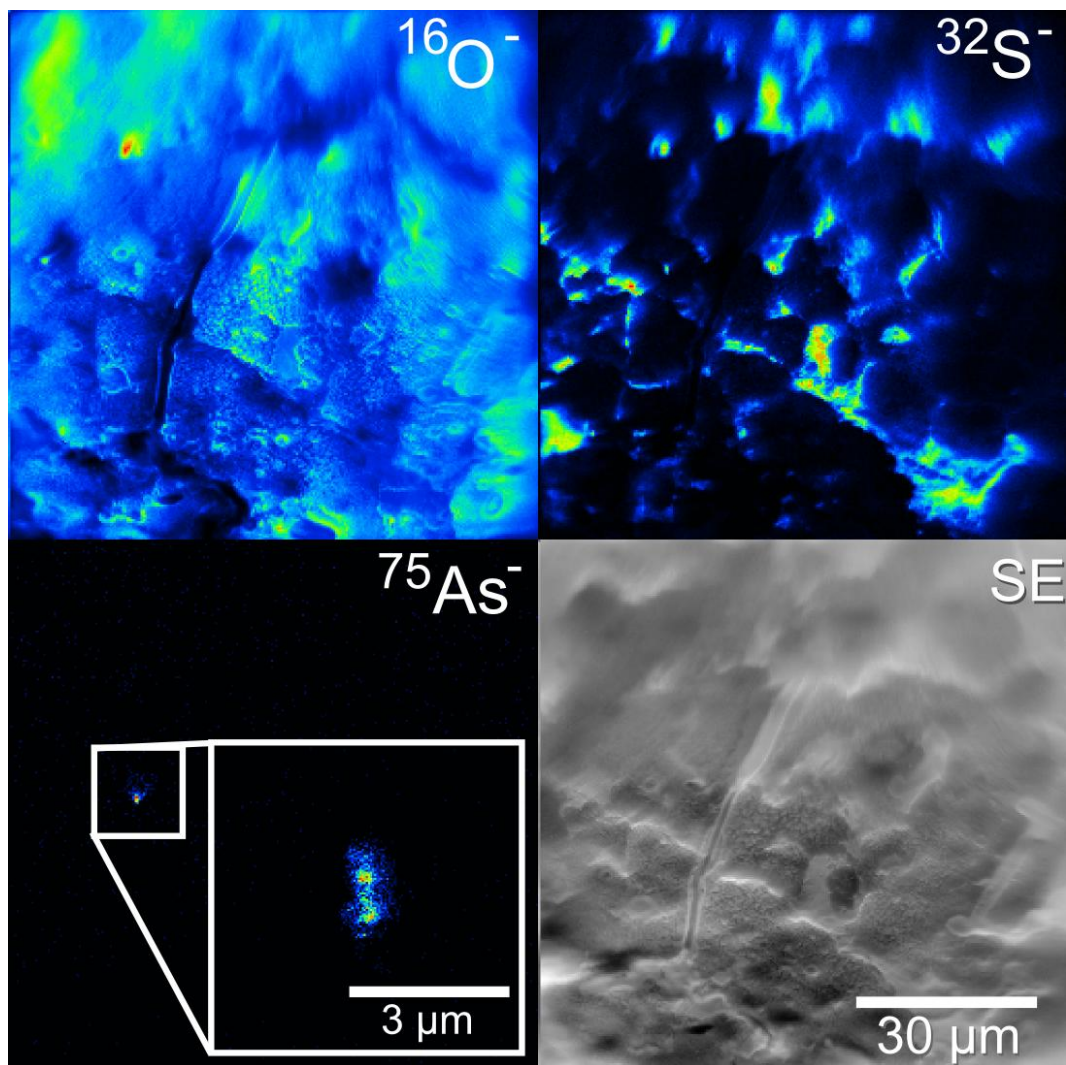


Figure 5.35: NanoSIMS ion maps of $^{16}\text{O}^-$, $^{32}\text{S}^-$, $^{75}\text{As}^-$ and the secondary electron image from area 7 of Figure 5.30. A higher magnification scan of the As-hotspot is shown as an insert in the As image.

In each area analysed, the total number of As counts from NanoSIMS images was normalised pixel by pixel with the total number of S counts in order to reduce errors caused by topography or small changes in primary ion current which could modify the yield of secondary ions. The $^{12}\text{C}^{14}\text{N}^-$ signal (commonly used in SIMS analysis for normalisation (Follet-Gueye *et al.*, 1998; Derue *et al.*, 2006a)) could not be used for this normalisation because in some images the acquisition was stopped because the signal intensity was so high that the detector was tripped off to avoid damage. Due to the similar distribution to $^{12}\text{C}^{14}\text{N}^-$ it was decided that $^{32}\text{S}^-$ would be the best alternative. The normalised data were grouped into three regions, those near the OVT (the high-As region identified in the S-XRF data), the aleurone and the endosperm (all areas away

from the high-As region), and averaged. Area 13 was excluded from the averages of the endosperm region because this area was anomalously high in both overall As counts and number of hotspots, as shown in Table 5.1.

Region (Analysed areas in Figure 5.30)	Average of total As counts/total S counts	Average number of hotspots per square micron
OVT (1-4, 11, 12, 20, 22)	$2.61 \times 10^{-4} \pm 1.5 \times 10^{-4}$	$3 \times 10^{-4} \pm 4 \times 10^{-4}$
Endosperm (5-7, 14-19, 21)	$1.37 \times 10^{-4} \pm 4.8 \times 10^{-5}$	$1 \times 10^{-5} \pm 4 \times 10^{-5}$
Aleurone (8-10)	$1.60 \times 10^{-4} \pm 2.5 \times 10^{-5}$	0
Area 13	2.40×10^{-3}	1.4×10^{-3}

Table 5.1: Average of As/S normalised NanoSIMS data and average number of hotspots per square micron for the OVT (region of high As concentration), the endosperm, the aleurone and area 13. Errors are 1 standard deviation. The numbers refer to the analysed NanoSIMS positions as indicated in Figure 5.30.

Both the normalised counts and the average number of hotspots per square micron show that there is approximately twice as much As in the region near the OVT as in the endosperm, with no enrichment of As in the aleurone layer. The As:S ratio for the OVT region was $2.61 \times 10^{-4} \pm 1.5 \times 10^{-4}$, twice that from the endosperm $1.37 \times 10^{-4} \pm 4.8 \times 10^{-5}$ (quoted errors are 1 SD). The As X-ray signal from the synchrotron data in the As-enriched region near the OVT is also approximately double that from the endosperm part of the grain, and hence there is good quantitative agreement in the ratio of As between the two regions from the two techniques. The lateral resolution of the synchrotron analysis is not sufficient to show details of the localisation of As in the As-rich areas. The combination of a large step size and large interaction volume means that several small hotspots may be included under one analysis point and therefore a high, but diffuse, As signal is presented in the synchrotron map. Furthermore, because of the penetration of the X-rays, hotspots of As located within the volume probed by the beam would also contribute to the measured signal. Therefore, if hotspots were located in the same area but at different depths the overall map would show a more homogeneous distribution of As. By

contrast, SIMS is inherently a surface analysis technique and therefore the signal is generated only from the top few nanometres of the sample surface. With the higher resolution capabilities of the NanoSIMS, it is quite clear that the higher average As concentrations were actually located in small As hotspots near the OVT. At present it is not clear why the As is localised in these micron-sized hotspots. There is no obvious subcellular structure which might be acting to localise this high As concentration to these micron-sized regions. The aleurone regions show a slightly higher As:S ratio than the endosperm. The selected area was 90 μm and hence included a fair amount of the subaleurone region of the endosperm which was shown previously to be higher in As concentration and therefore is likely to have increased the measured ratio.

Much of the previous research reviewed at the beginning of the chapter had indicated that As was predominantly localised to the aleurone and OVT regions of the rice grain. The NanoSIMS work in this chapter presents higher resolution and subcellular information regarding the As distribution allowing the mechanisms of uptake to be more clearly understood. For the soil-grown high As concentration grains which contain about 70% DMA the As is localised to the subaleurone protein regions with very little within the aleurone layer. The concentration of As decreases towards the centre of the grain as the protein concentration decreases. This fits with the data of Carey *et al.* (2010), who showed that when rice plants are only supplied with DMA the As is localised much more widely throughout the grain. Khan *et al.* (2010) also showed that as the As concentration in the grain increases a greater proportion will be in DMA form. The quantitative data of Figure 5.28 indicate that for these soil-grown rice grains there is little difference in the $^{75}\text{As}^-/^{12}\text{C}^{14}\text{N}^-$ ratios between the aleurone cells near the OVT and the subaleurone/aleurone regions and this is not unexpected considering the mixed speciation present in these samples with about 30% of the As present as inorganic As which is expected to be localised within the OVT region. This would also explain why the rice grain analysed with both the NanoSIMS and S-XRF shows some localisation of As in a region near the OVT. For the hydroponically grown rice grains which were treated only with As(III), As was found to be only

localised to the aleurone cells near the OVT with very little in the subaleurone/aleurone away from this region, this is again in agreement with Carey *et al.* (2010) and Lombi *et al.* (2009).

In contrast to the previously published data suggesting As localisation at the OVT, the NanoSIMS data reveal that much of the As in this region is in fact localised to the multilayered aleurone cells rather than in the OVT itself. Some As was also found to have spread into the endosperm region adjacent to this part of the grain in the form of micron-sized hotspots. The synchrotron does not have sufficient resolution to determine the subcellular localisation of As at the OVT or in the subaleurone region.

DMA is much more mobile in both xylem and phloem transport and Khan *et al.* (2010) state that DMA is more efficiently translocated from rice roots to shoots and from the shoot tissues to the grain therefore it is possible that DMA is more mobile within the grain itself leading to a much more dispersed As localisation in comparison to samples where inorganic As dominates. Both Lombi *et al.* (2009) and Carey *et al.* (2010) indicate that there could be a physiological barrier in the unloading of As from the OVT into the aleurone and that if DMA is more easily transported across this barrier it could explain the higher levels of DMA in the endosperm. This would also explain why in the literature consistently higher levels of inorganic As are observed in the bran. However, the NanoSIMS data presented here indicate that inorganic As is in fact unloaded into the multilayered aleurone cells adjacent to the OVT with some evidence of As in the aleurone cells away from the OVT. This suggests that there is not a physiological barrier to the aleurone but there must be a barrier preventing inorganic As (especially near the OVT) becoming more widespread throughout the grain. The distinctly different distributions for inorganic As and DMA suggests that they must be transported into and throughout the grain via different mechanisms.

6. ARSENIC AND SILICON IN RICE ROOTS⁴

6.1. INTRODUCTION AND PREVIOUS WORK ON RICE ROOTS

With As mainly entering the rice plant via its roots this is therefore an important part of the plant which must be studied in order to be able to understand the mechanisms of uptake which affect the final As concentration in the grain. The structure of rice roots is shown in Figure 6.1 indicating the main cell types.

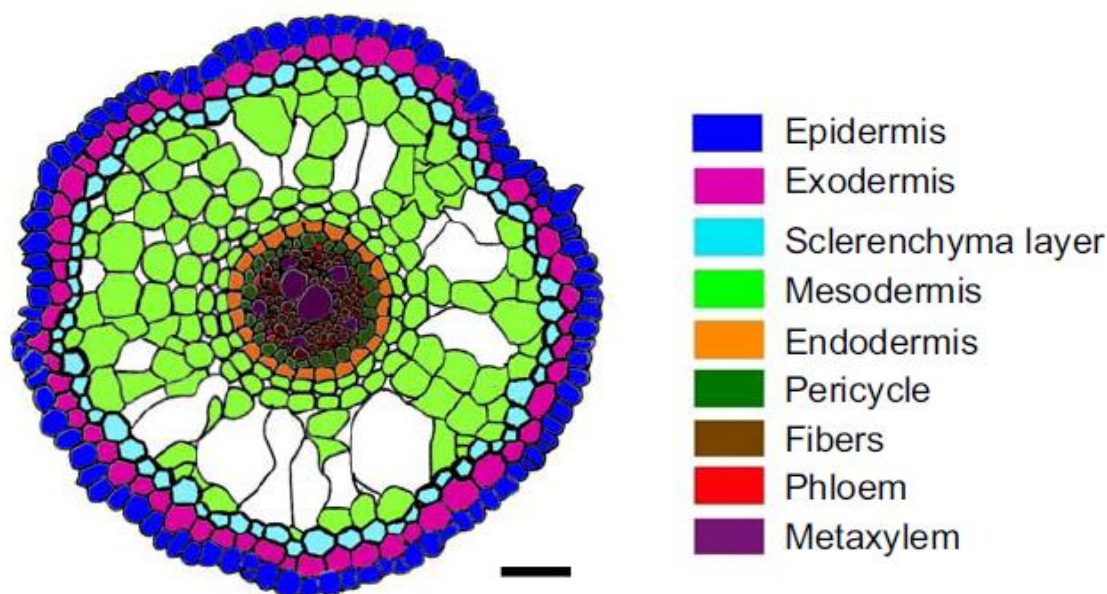


Figure 6.1: A schematic diagram showing the different cell types in rice roots (Rebouillat *et al.*, 2009) The white parts indicate large air spaces. Scale bar = 50 μ m. With kind permission from Springer Science + Business Media: Rice, Molecular Genetics of Rice Root Development, 2(1), 2008, J. Rebouillat *et al.* Page 20, Figure 3.

It is well known that arsenate is taken up by plant roots via phosphate transporters (Zhao *et al.*, 2009); however in submerged paddy field conditions arsenite is the dominant form of As in the soil solution (Bogdan & Schenk, 2008; Xu *et al.*, 2008). Arsenite, present in the environment predominantly as $\text{As}(\text{OH})_3$ due to its high pK_a (≈ 9.2), enters rice root cells through Lsi1, a Nodulin 26-like intrinsic protein (NIP) aquaporin (NIP2;1) (Ma *et al.*, 2008). Lsi1 is a major

⁴ I have published a substantial part of this chapter in the following reference and some of the text and images have been used verbatim in this chapter: **Katie L. Moore**, Markus Schröder, Zhongchang Wu, Barry G. H. Martin, Chris R. Hawes, Steve P. McGrath, Malcolm J. Hawkesford, Jian Feng Ma, Fang-Jie Zhao, Chris R. M. Grovenor. High Resolution Secondary Ion Mass Spectrometry Reveals the Contrasting Subcellular Distribution of Arsenic and Silicon in Rice Roots, *Plant Physiology*, 156(2), 2011, 913-924

influx transporter for silicic acid (Ma *et al.*, 2006) and is also permeable to a range of small neutral (undissociated) molecules including arsenite and methylated As species (Ma *et al.*, 2008; Li *et al.*, 2009a; Zhao *et al.*, 2010c). Lsi1 is localised in the exodermis and endodermis where the Casparian strips are formed, with the transporter found on the distal side of the plasma membranes. A second Si transporter, Lsi2, localised on the proximal side of same cells as Lsi1 and also in the plasma membrane, mediates the efflux of silicic acid from exo- and endodermal cells towards the stele as shown in Figure 6.2 (Ma *et al.*, 2007).

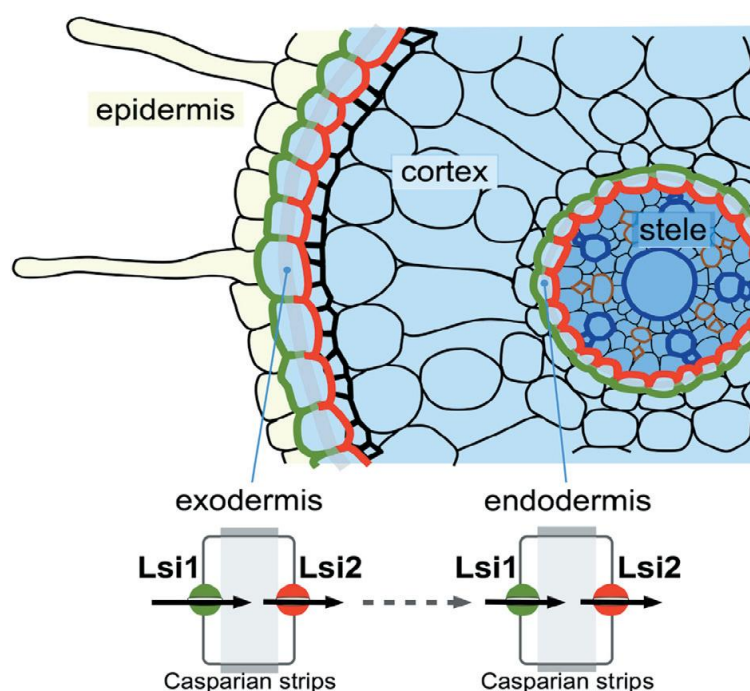


Figure 6.2: A schematic drawing showing the position of the Si influx (Lsi1) and efflux (Lsi2) transporters in the exodermis and endodermis (Zhao *et al.*, 2009). Reprinted with permission from John Wiley and Sons.

Lsi2 is also able to transport arsenite; mutation in the *Lsi2* gene was found to result in a marked decrease in the concentration of arsenite in the xylem sap, As accumulation in rice shoots and As concentration in rice grain compared with the wild-type cultivars (Ma *et al.*, 2008). Lsi2 appears to play a more important role in controlling As accumulation in the above-ground tissues than Lsi1. Increasing the Si concentration in the soil solution was found to decrease As accumulation in rice shoots and grain (Bogdan & Schenk, 2008; Li *et al.*, 2009b), probably through a competitive inhibition effect on arsenite transport via Lsi2 (Zhao *et al.*, 2010b). Rice has a high

requirement for Si as it plays important roles in the defence against biotic and abiotic stresses (Ma & Yamaji, 2006). The highly efficient pathway of Si uptake in rice inadvertently allows the accumulation of arsenite, mobilised under the anaerobic conditions of submerged paddy soils (Zhao *et al.*, 2010b).

Despite Si and arsenite sharing the same uptake pathway in rice roots, the translocation of Si from roots to shoots is much more efficient than that of As; a typical concentration ratio of Si in the xylem sap to that in the external medium is around 20 (Mitani & Ma, 2005) whereas for As this is only 0.3 – 0.5 (Zhao *et al.*, 2009). The reasons for this difference are not entirely clear, but may be attributed to the propensity for arsenite to be extruded to the external medium (Xu *et al.*, 2008; Su *et al.*, 2010), or being complexed by thiol-rich peptides such as phytochelatins (Raab *et al.*, 2005; Liu *et al.*, 2010). Arsenite-thiol complexes are believed to be sequestered in the vacuoles of root cells, thus further decreasing the mobility of As towards the xylem, although direct evidence for the subcellular localisation of As has not been presented for any non-hyperaccumulating plant species. There are a few studies concerning the Si distribution in the root cells of rice (Parry & Soni, 1972; Lux *et al.*, 1999; Gong *et al.*, 2006), showing that Si is preferentially accumulated in the cell walls. Two of these results are shown in Figure 6.3. SIMS has also recently been used to map silicon and germanium in root and leaf tissues of annual blue grass (*Poa annua*) and orchard grass (*Dactylis glomerata*) at subcellular resolution (Sparks *et al.*, 2010) showing Si also to be localised to the proximal side of the endodermis.

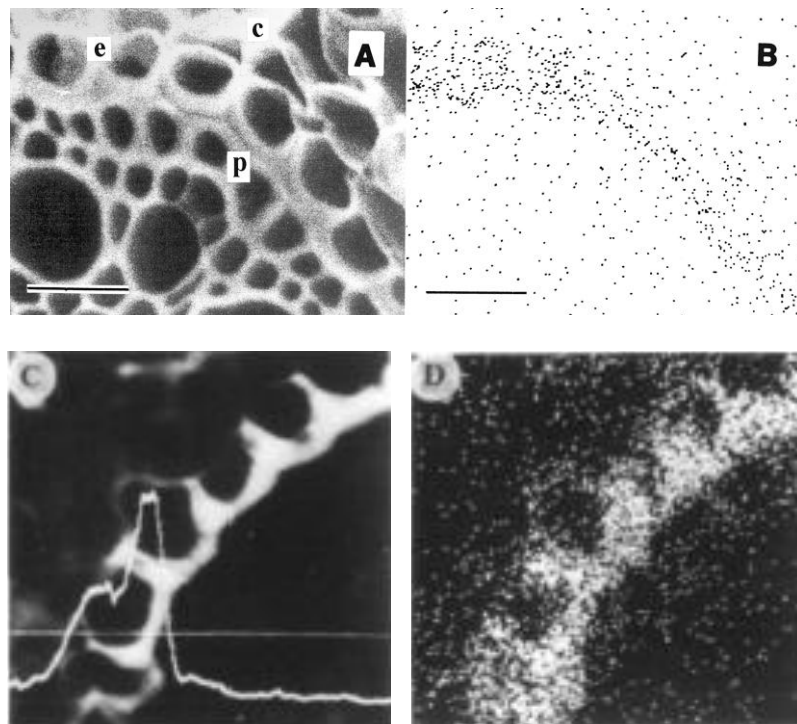


Figure 6.3: Scanning electron micrographs and the corresponding EDX maps of Si for rice roots focusing on the endodermis cells. A and B are from Lux *et al.* (1999) and C and D from Parry and Soni (1972). Both show Si localised to the proximal side of the endodermis. On images A and B the scale bar = 10 μm . C and D are presented in the original manuscript at a magnification of X 1940. Figures A and B: With permission from NRC Research Press. Figures C and D: Parry and Soni, *Electron-probe Microanalysis of Silicon in the Roots of *Oryza sativa* L.*, *Annals of Botany*, 1972, 36, 781-3, by permission of Oxford University Press.

Rice is an aquatic plant which releases O_2 into the rhizosphere, oxidising ferrous iron to ferric iron which is then precipitated as Fe oxide/hydroxides onto the root surface, forming an Fe plaque (Armstrong, 1967). The presence of this Fe plaque may act as a barrier to uptake of some potentially harmful metals or metalloids (Hansel *et al.*, 2002; Liu *et al.*, 2004). Both arsenate and arsenite are adsorbed onto Fe oxide/hydroxides, but the affinity is significantly less for arsenite (Liu *et al.*, 2004; Fendorf *et al.*, 2008). A study using excised rice roots showed that Fe plaque decreased arsenate uptake, but increased arsenite uptake (Chen *et al.*, 2005). Although it is apparent that Fe plaque serves as a strong sink for As, its impact on As accumulation elsewhere in the plant is not clear. X-ray tomography is a popular method in the literature for mapping the Fe plaque and adsorbed As. Two different studies both show very similar results of co-localisation of Fe and As on the outer epidermis of *Phalaris arundinacea* and rice roots, as shown in Figure 6.4 a) and b) respectively. In both cases the resolution is fairly limited which

means that subcellular localisation of neither element can be determined. (Hansel *et al.*, 2002; Seyfferth *et al.*, 2010).

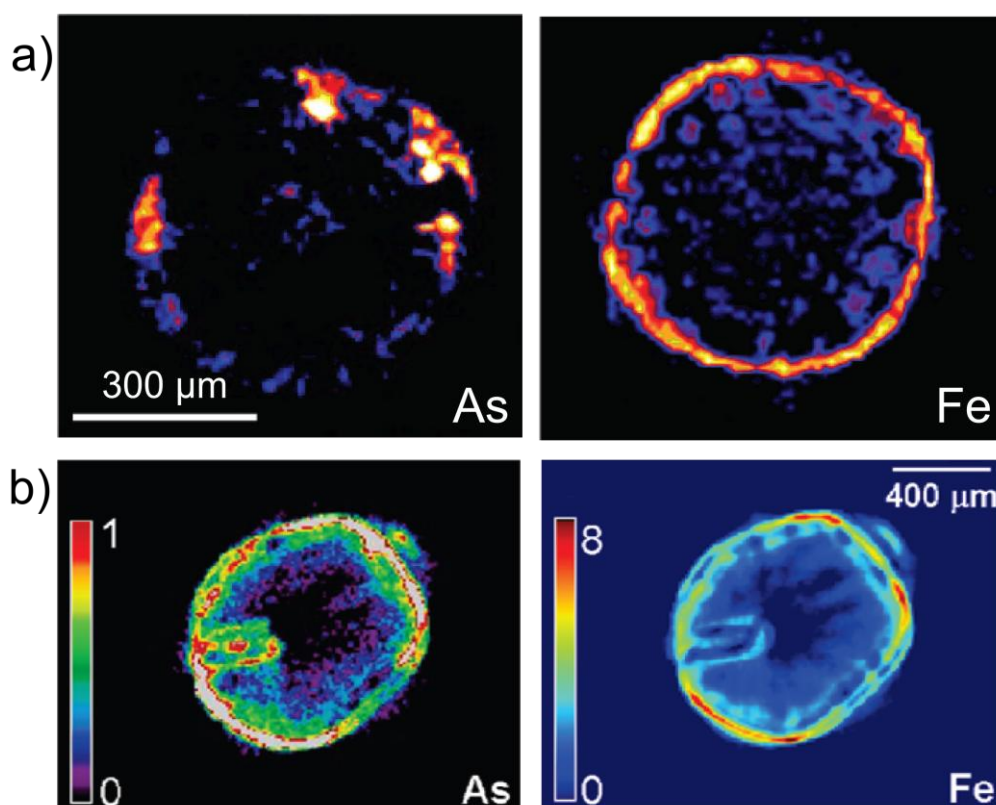


Figure 6.4: Cross-sectional X-ray microtomography of a) *P. arundinacea* and b) rice roots showing the co-localisation of As with the Fe plaque from soil-grown plants (Hansel *et al.*, 2002; Seyfferth *et al.*, 2010). Figure a) reprinted with permission from Hansel CM, La Force MJ, Fendorf S, Sutton S. 2002. Spatial and temporal association of As and Fe species on aquatic plant roots. *Environmental Science & Technology* 36: 1988-1994. Copyright 2002 American Chemical Society. Figure b) reprinted with permission from Seyfferth AL, Webb SM, Andrews JC, Fendorf S. 2010. Arsenic localization, speciation, and co-occurrence with iron on rice (*Oryza sativa* L.) roots having variable Fe coatings. *Environmental Science & Technology* 44: 8108–8113. Copyright 2010 American Chemical Society.

6.2. PROJECT AIMS

The aim of this project was to investigate the subcellular distribution of As and Si in rice roots. With the recent discovery that both of these elements share the same uptake pathway, via the Lsi1 and Lsi2 transporters, it is important in developing an understanding of the uptake mechanisms to know where As and Si are localised within the root and what effect knocking out the Lsi2 transporter has on the distribution of these elements. Lsi2 was chosen as this has a

greater effect on the grain As concentration than *Lsi1* (Ma *et al.*, 2008). The Fe plaque is a common feature on the surface of aquatic plants and is known to be a strong sink for As. Little is known about how As is localised onto this Fe plaque at the subcellular scale and which other elements are adsorbed onto this plaque.

Abbreviations used in this chapter:

WT = Wild type plant

***lsi*₂** = Mutant with *Lsi*₂ transporter not functional

+ Fe = With iron plaque

- Fe = Without iron plaque

As(V) = Arsenate (AsO_4^{3-})

As(III) = Arsenite (AsO_2^-)

6.3. SAMPLE PREPARATION

6.3.1. PLANT MATERIALS AND CULTURE

Two lines of rice (*Oryza sativa*) the *lsi2* mutant (Ma *et al.*, 2007) and its wild-type cv T-65, were used. All plant growth and culture was carried out at Rothamsted Research. Seeds were surface sterilised with 0.5% NaOCl for 15 min, rinsed thoroughly with deionised water, and placed on a nylon net floating on a 0.5 mM CaCl_2 solution. After germination, seedlings were transferred to 0.35 L pots (4 plants per pot) filled with ½ strength Kimura solution and pre-cultured for 3 weeks. The nutrient composition was as follows: 0.091 mM KNO_3 , 0.183 mM $\text{Ca}(\text{NO}_3)_2$, 0.274 mM MgSO_4 , 0.1 mM KH_2PO_4 , 0.183 mM $(\text{NH}_4)_2\text{SO}_4$, 0.5 μM MnCl_2 , 3 μM H_3BO_3 , 0.1 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 0.4 μM ZnSO_4 , 0.2 μM CuSO_4 , 40 μM NaFe(III)-EDTA, 2 mM MES (2-morpholinoethanesulfonic acid; pH adjusted to 5.5 with KOH). Nutrient solution was renewed every three days. The growth conditions were 16 h photoperiod with a light intensity of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 30°C/25°C day/night temperatures and 70% relative humidity. To induce Fe plaque formation, rice seedlings were placed in deionised water overnight, and then in 2 mM FeSO_4 (pH adjusted to 5.5 with KOH) for 24 h (Liu *et al.*, 2004). Plants were transferred into deionised water for 6 h to remove extra ferrous ions from the root surface. Another group of plants not

treated with FeSO_4 were used as the control. All seedlings were transferred to a nutrient solution with 1 mM silicic acid for two days before the As treatments were started. Both arsenate and arsenite were supplied at a concentration of 10 μM and the As exposure lasted for three days. Three of the treated seedlings (replicates) were used for NanoSIMS analysis. One replicate was analysed for total As concentration in the roots and shoots by inductively coupled plasma mass spectrometry (ICP-MS; Agilent ICP-MS 7500ce, Agilent Technologies, Santa Clara, CA, USA) following acid ($\text{HNO}_3\text{:HClO}_4$ 85:15) digestion.

In an additional experiment, WT rice plants were grown in the $\frac{1}{2}$ Kimura nutrient solution up to the flag leaf stage (one plant per 1 L pot). Plants were then exposed to 1 mM silicic acid and 10 μM arsenite for two weeks before the root samples were prepared for NanoSIMS analysis.

6.3.2. HIGH PRESSURE FREEZING AND EMBEDDING

Rice roots were sectioned under MES buffer. Segments of the root, 2-2.5 mm in length were taken at 2 cm from the tip of the root using a scalpel blade, taking care not to squash the root. Mature roots were cut at approximately 20 cm from the root tip from plants that were grown in the additional experiment. The subsequent HPF, freeze substitution and embedding processes were identical to those outlined in the experimental techniques (Chapter 3) except that the rice roots were oriented in the embedding moulds so that they could be transversely sectioned.

6.3.3. SUBSTRATES AND MICROTOMY

A diamond knife on a Reichert Ultracut E Ultramicrotome was used to create 1 μm thick sections for NanoSIMS analysis. Sections were picked up with a hair and were deposited initially onto a droplet of water on a Si wafer and stretched flat on a hotplate. Using Si as a substrate is a common method for mounting microtome sections for NanoSIMS analysis as the silicon substrate is very flat, conducting and allows the charge from the primary ion beam to be dissipated away (Guerquin-Kern *et al.*, 2005; Lechene *et al.*, 2006; Smart *et al.*, 2010). As the Si

distribution was of particular interest in these samples, the Si wafers were coated with 20nm of Pt to try to prevent any uncertainty in the origin of the Si signal. These samples were also sectioned relatively thickly with the aim that this would allow sufficient analysis time before the section was damaged, exposing the Si wafer. In the first NanoSIMS images from these samples it was quickly noticed that the detected Si was not just originating from the sample. The cell walls appeared to be weak points in the section and sputtered at a much quicker rate than the resin. In one experiment the Pt signal was monitored along with the Si signal in a depth profile to see how the two signals developed over time. This should establish whether some Si was detected before the Pt, and this Si would originate from the sample and not the wafer. Although there was a distinct change in the Si gradient in the depth profile when the cell walls started to disappear this did not exactly coincide with the Pt peak so there was still uncertainty in the origin of this Si. It was therefore decided that it was not possible to confidently determine the Si distribution when the sections were placed onto Si wafers, therefore a different substrate had to be used.

It was decided that platinum coated plastic substrates would be an alternative as these had been shown previously to be suitable for SIMS analysis (Guerquin-Kern *et al.*, 1997; Lau *et al.*, 2010). Ten millimetre diameter, 0.2 mm thick substrates were punched out of 13 mm Thermanox® cover slips so that they would fit into the NanoSIMS holders, and coated with 20 nm of Pt. The Pt coated substrates were checked in the NanoSIMS to confirm that there was no significant Si signal from the plastic. There were some scratches on the plastic, however these did not cause a problem as the sections just stretched over them and there was no obvious effect on the topography of the top surface of the thick sections. The microtomed sections were then coated with a further 10 nm of Pt to prevent charging. Care was needed to ensure that the substrate was placed flat inside the hole of the sample holder as the middle part can easily become bowed outwards which would affect focusing and astigmatism as well as ion transmission efficiency. A stub was placed behind the substrate to fix it in place and help keep the top surface as flat as possible.

Microtomed sections were examined with a light microscope, an Axioskop 2 MAT light microscope with an AxioCam digital camera (Carl Zeiss MicroImaging GmbH), to assess the structural preservation of the roots and identify areas of interest for NanoSIMS analysis.

6.4. RESULTS AND DISCUSSION

6.4.1. DETECTOR POSITIONING (HMR)

Although the concentrations of As were significantly higher in the roots than in the rice grains it was still necessary to tune the detectors on standards. The same standards were used as previously, GaAs for As, for Fe a polished stainless steel sample and a Si wafer for Si.

6.4.2. CHOOSING A PROTOCOL FOR IMPLANTATION OF Cs^+

The first NanoSIMS images of the rice roots were acquired after implanting Cs^+ and cleaning away the top Pt layer for 15 minutes with a raster size of 100 μm implanting a dose of approximately $6 \times 10^{15} \text{ Cs}^+$ ions cm^{-2} . This was a much shorter implantation time than most of the other experiments, however it was noticed that the $^{12}\text{C}^{14}\text{N}^-$ and $^{56}\text{Fe}^{16}\text{O}^-$ signals appeared rapidly and cellular features could be resolved even after short implantation times. It was also noticed on the initial samples mounted on Si wafers that Si signals quickly appeared at the cell walls hence these parts of the cells were quickly sputtered away and a low implantation time minimised damage to the sample.

In an overnight experiment collecting several successive images the distribution of $^{31}\text{P}^-$ and $^{75}\text{As}^-$ changed dramatically from the first to last image yet the $^{12}\text{C}^{14}\text{N}^-$ signal barely altered. The 1st and 16th images are shown in Figure 6.5 and Figure 6.6 respectively. It is clear in the $^{31}\text{P}^-$ image from the first of the consecutive images that there are only a few regions of high intensity and the $^{28}\text{Si}^-$ and $^{75}\text{As}^-$ images are almost entirely devoid of counts. With further imaging the phosphorous

image shows a high signal intensity throughout most of the stele and there is a distinct line of cells showing As accumulation yet the $^{12}\text{C}^{14}\text{N}^-$ signal has barely altered. The damage to the cell walls is clear in the $^{28}\text{Si}^-$ image of Figure 6.6, with the majority of the cells being outlined in Si, most of which is presumably originating from the underlying Si substrate. In order to allow a quantitative comparison of the As concentration in different treatments it was necessary to normalise the $^{75}\text{As}^-$ signal with another element to minimise the effect of topography and differences in primary ion current. It is therefore important that the ion yields are at steady state otherwise the ratio between different ions could change dramatically depending on the point along the yield/dose curve where the data is taken, making it impossible to reliably compare different data sets. For instance, the $^{75}\text{As}^-/^{12}\text{C}^{14}\text{N}^-$ ratio in the whole of Figure 6.5 is 1.0×10^{-5} yet this increased by a factor of six to 6.6×10^{-5} in Figure 6.6, the $^{31}\text{P}^-/^{12}\text{C}^{14}\text{N}^-$ ratio increased by just less than an order of magnitude between image 1 and image 16.

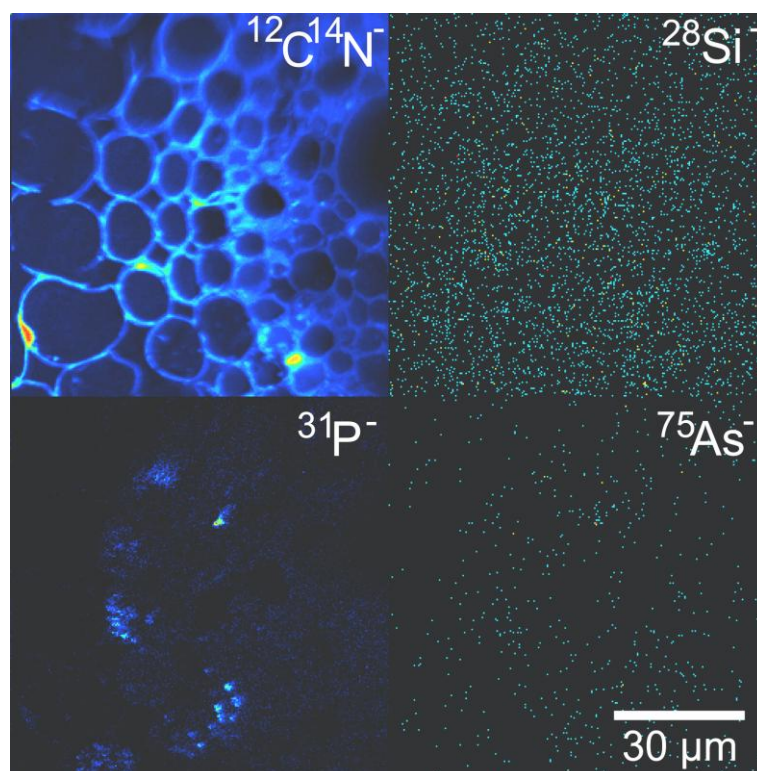


Figure 6.5: NanoSIMS maps of $^{12}\text{C}^{14}\text{N}^-$, $^{28}\text{Si}^-$, $^{31}\text{P}^-$ and $^{75}\text{As}^-$ from the 1st image of the stele region of an arsenate treated root deposited onto a Si wafer. Only a few areas in the $^{31}\text{P}^-$ image show high intensity and there are very few Si or As counts.

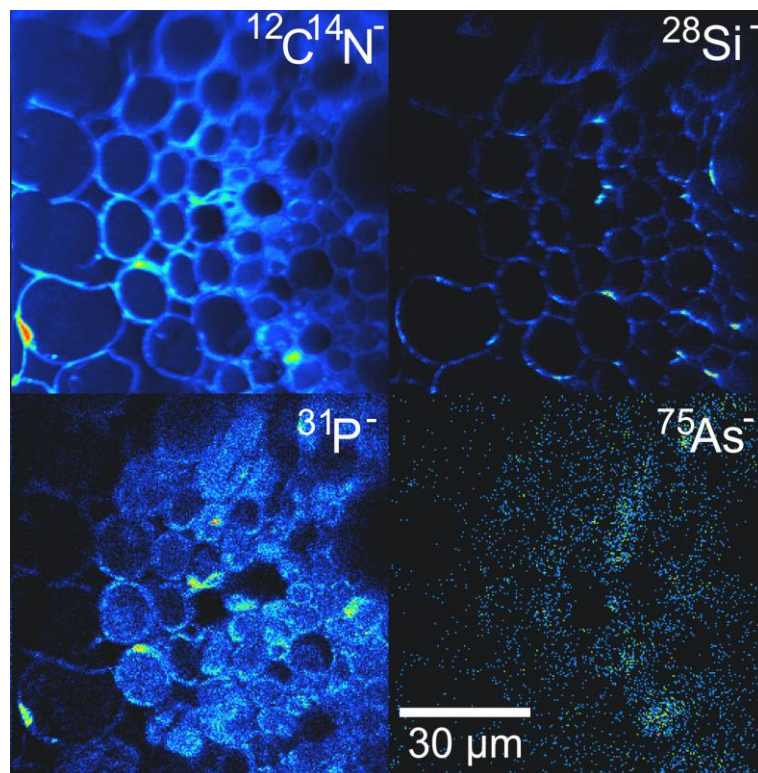


Figure 6.6: NanoSIMS maps of $^{12}\text{C}^{14}\text{N}^-$, $^{28}\text{Si}^-$, $^{31}\text{P}^-$ and $^{75}\text{As}^-$ from the 16th image of the stele region of an arsenate treated root deposited onto a Si wafer. Both the $^{31}\text{P}^-$ and $^{75}\text{As}^-$ signals have increased dramatically and there is significantly more definition in these images. The $^{28}\text{Si}^-$ signal has also increased dramatically indicating that the cell walls have been sputtered away preferentially.

6.4.3. DEPTH PROFILING

In order to work out when the microtomed sections reached steady state and determine which ions showed the most potential as normalisation signals, a standard depth profile was performed on one root sample. This allowed the intensity of the ions to be monitored with time and the dose needed to reach steady state could be calculated. In order to perform the experiment in a reasonable time and to minimise the reduction in primary ion current, a $20\ \mu\text{m} \times 20\ \mu\text{m}$ area was selected at the point where 3 cell walls meet from the same sample analysed in Figure 6.6. This small raster size resulted in a fast sputtering rate and hence the depth profile through the entire $1\ \mu\text{m}$ sample was measured relatively quickly. To ensure that there were enough arsenic counts in each image the count time per pixel was 30 ms and 33 images were acquired. Mass scans, secondary ion beam tuning and coarse focusing were performed on the area analysed in Figure 6.6 to minimise implantation in the area of interest before the depth profile. The beam was

defocused throughout the depth profile. Just prior to performing the tuning, the current at the sample was measured in FCo as 16.3 pA with D1=1 and this current was used to calculate the dose. The resulting depth profile is shown in Figure 6.7.

The $^{12}\text{C}^{14}\text{N}^-$ signal is immediately very intense and is essentially at steady state right at the start of the depth profile, but this is the only ion which behaves in this way. This is one of the reasons why this ion is used predominantly in SIMS experiments of biological samples as an imaging ion (Berry *et al.*, 1992; Lechene *et al.*, 2006), and why we generally use this ion to focus an image. As this area is not from a sample with an iron plaque the $^{56}\text{Fe}^{16}\text{O}^-$ signal probably originates from a surface contamination and this is consistent with the signal dropping away quite quickly at the start of the depth profile. The Si signal increases dramatically throughout the depth profile, and although it is not clear if this signal is originating from the root or the Si substrate, it is likely to be the latter. There is a steep increase in silicon signal when the sample has been completely sputtered away, after which the silicon signal is fairly constant when just the silicon wafer remains. $^{30}\text{Si}^-$ was chosen for this depth profile rather than the more abundant $^{28}\text{Si}^-$ to prevent the detector tripping off at 2×10^6 cps to avoid damage when the wafer surface was reached. This sudden increase allows an approximate depth scale to be put onto the graph as the thickness of the root section is known to be 1 μm . Phosphorous and As behave in a very similar manner but neither appear to reach steady state conditions. The signals rise at a much slower rate than $^{12}\text{C}^{14}\text{N}^-$, reach a peak in intensity and then start to drop away again before the section has been completely sputtered through, possibly indicating that the cellular material where these elements are located sputters at a faster rate than the resin. As these elements of interest do not reach steady state it is difficult to normalise them against another species which behaves differently as the position along the curve will make a large difference to the ratio, for example the $^{75}\text{As}^-$ signal varies by nearly 2 orders of magnitude. The variation in the ratio that would be seen in this situation is shown schematically in Figure 6.8.

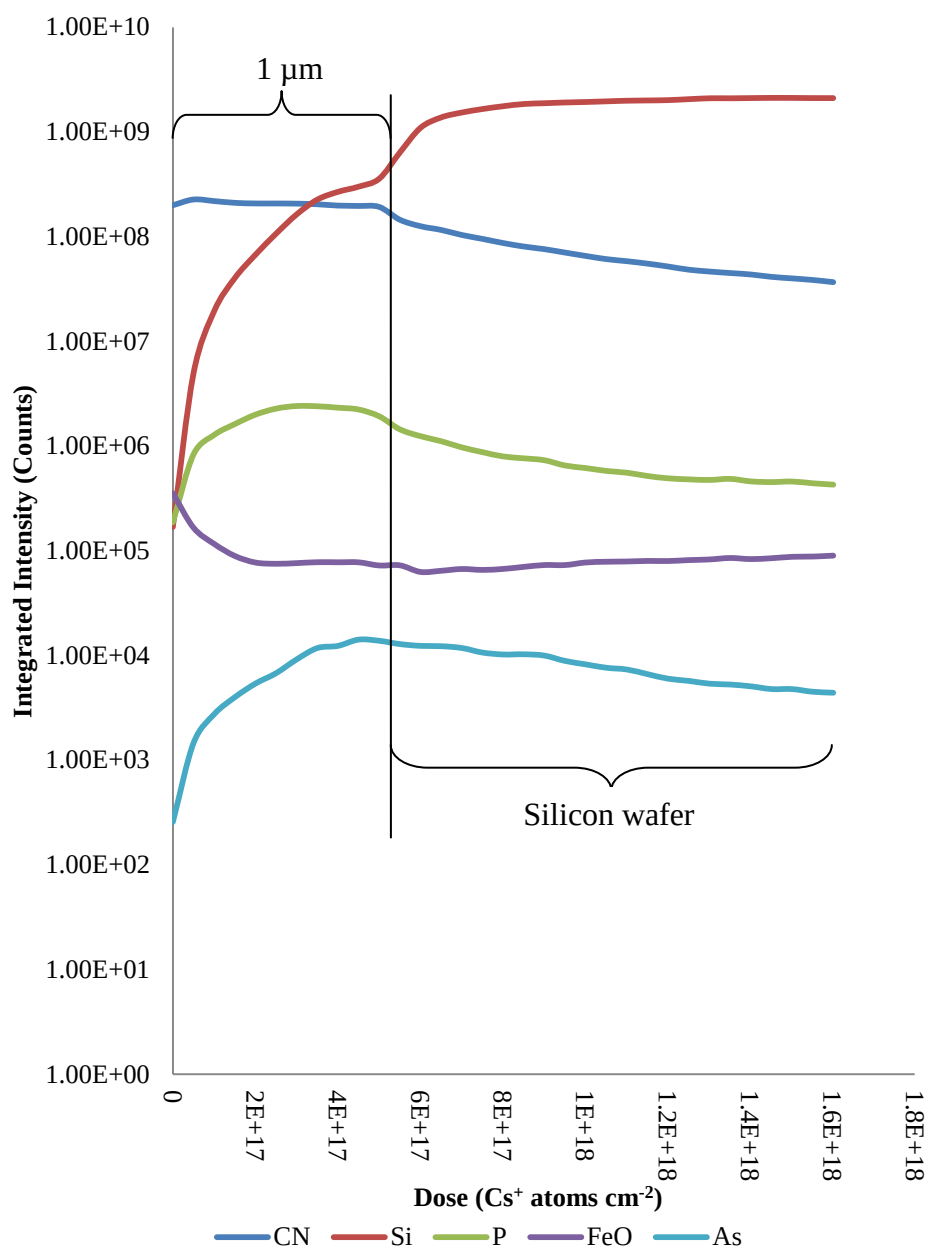


Figure 6.7: Depth profile from a $20\text{ }\mu\text{m}$ raster showing the change in signal intensity for $^{12}\text{C}^{14}\text{N}$, ^{30}Si , ^{31}P , $^{56}\text{Fe}^{16}\text{O}$, ^{75}As with dose. The area chosen was at the point where three cell walls intersected. Note the dramatic change in Si intensity which indicates when the section has been completely sputtered away.

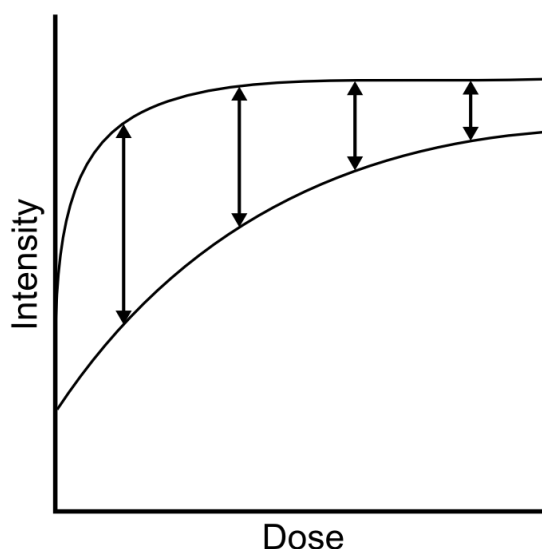


Figure 6.8: Schematic diagram showing how the ratio between two signal changes depending on dose. The upper curve is similar to the behaviour of $^{12}\text{C}^{14}\text{N}^-$ and the lower similar to $^{75}\text{As}^-$.

As $^{12}\text{C}^{14}\text{N}^-$ has a dramatically different behaviour to the ions of interest, several more depth profiles were performed to investigate the potential of the following ions as normalisation ions; $^{12}\text{C}^-$, $^{12}\text{C}_2^-$, $^{16}\text{O}^-$, $^{31}\text{P}^-$, $^{35}\text{Cl}^-$ and $^{16}\text{O}^1\text{H}^-$. As there were a few problems with interpreting this initial depth profile, for example knowing when and how the cell walls disappeared, when the sample was completely sputtered away and whether different matrices had different steady state conditions, subsequent depth profiles were performed by collecting a series of images, rather than as a standard depth profile where no positional information is recorded, so that different regions could be considered separately.

For the first experiment, $^{12}\text{C}^-$, $^{16}\text{O}^-$, and $^{12}\text{C}^{14}\text{N}^-$ were chosen as normalisation ions. The area, as shown in Figure 6.9, was again chosen to include 3 cell walls but for this experiment two cell walls coated with iron plaque were included. The sample treatment was WT + Fe As(V). The tuning, this time including focusing, was performed on an adjacent area so that the counts on a fresh surface could be measured from zero dose.

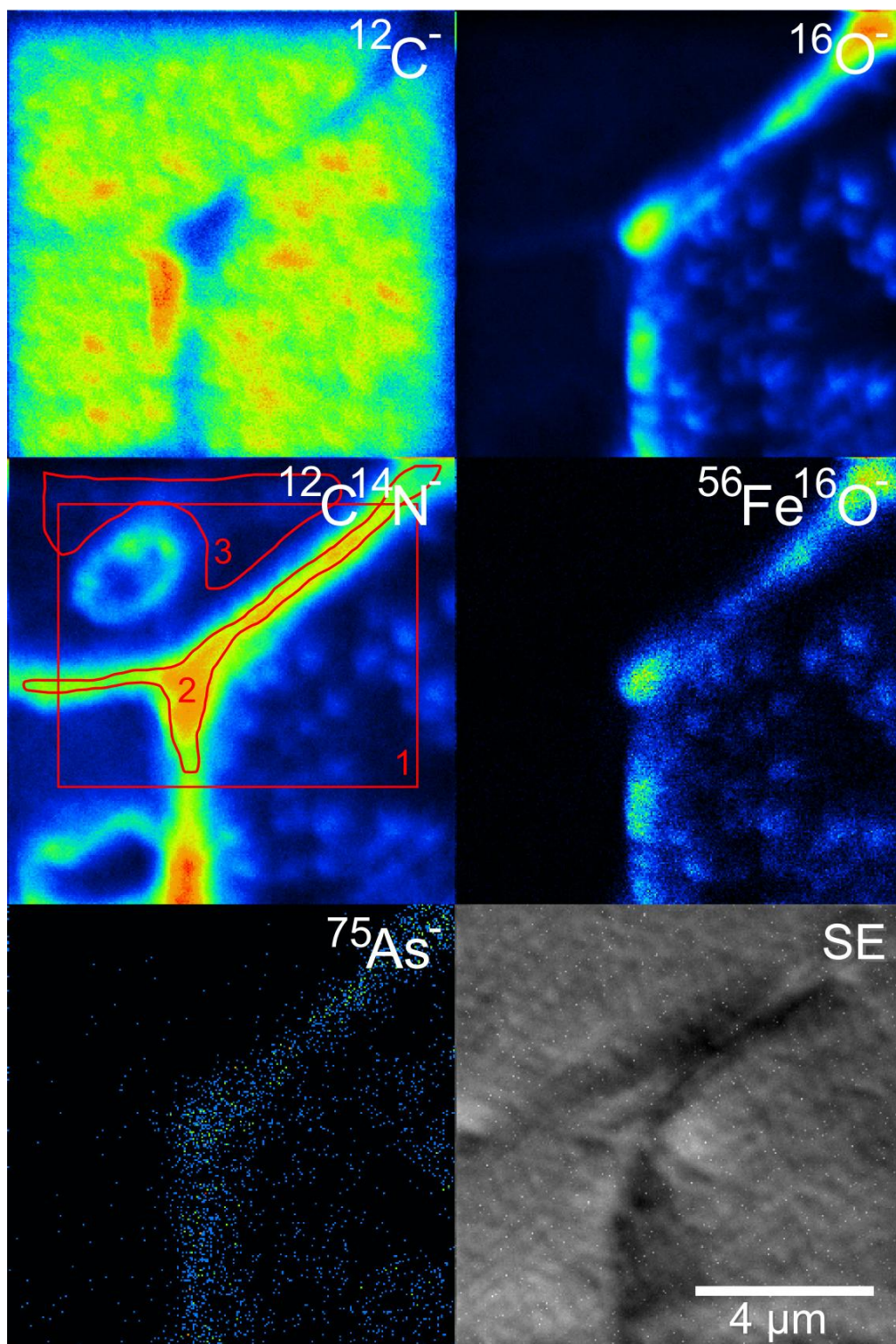


Figure 6.9: NanoSIMS maps of $^{12}\text{C}^-$, $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{56}\text{Fe}^{16}\text{O}^-$, $^{75}\text{As}^-$ and the secondary electron image. This is the second image from the depth profile (the first image has very few counts). The $^{12}\text{C}^{14}\text{N}^-$ image shows the three cell walls but only the two on the right have an iron plaque coating. The analysis areas for the depth profile are highlighted on the $^{12}\text{C}^{14}\text{N}^-$ image in red.

The size of the analysis area was $10\ \mu\text{m}$ and a count time of 10 ms per pixel was chosen so that each image was acquired fairly quickly and only a small amount of material was removed during

each image giving many points in the curve. The analysis was stopped when the sample had been completely sputtered through.

It was obvious from the images that the cell walls sputter away at a much faster rate than the surrounding resin, resulting in artefacts at the edges of the holes. Once holes had been created, it was easier to sputter away material from the edges and hence at these regions the resin was removed faster allowing the hole to spread rapidly across the sputtered area. By the 29th plane, Figure 6.10, corresponding to a dose of $3.2 \times 10^{17} \text{ Cs}^+ \text{ ions cm}^{-2}$, it was quite clear that the cell walls were being preferentially removed, although the first signs of holes were apparent 5 or 6 images before this. The Fe plaque on the two right hand cell walls has also completely disappeared which also suggests that the cell walls are preferentially removed before the surrounding material.

Before analysis was undertaken on these results the images needed to be aligned to correct for drift (caused by temperature changes in the NanoSIMS lab); this was performed automatically by ImageJ. Although this resulted in a loss of approximately 1/3 of the image over the whole experiment there was still sufficient area to perform analysis on. Three different regions of interest were selected, shown on the $^{12}\text{C}^{14}\text{N}^-$ image of Figure 6.9, to investigate how the sputtering behaviour depends on the area. First a box (area 1) was positioned over most of the analysis area (excluding the areas close to the image edge which are affected by the crater edge) to include both cell walls and cell contents embedded in resin. The second, area 2, was carefully selected by drawing an area which only included the cell walls and the final area (area 3) only included cell contents embedded in resin. The total number of counts from each area on each image was summed and plotted against the calculated dose density. The current on the sample was 2.8 pA with D1=2 and each image delivered a dose of $1.17 \times 10^{16} \text{ Cs}^+ \text{ ions cm}^{-2}$. The depth profiles from the three analysis areas are shown in Figures 6.11 - 6.13.

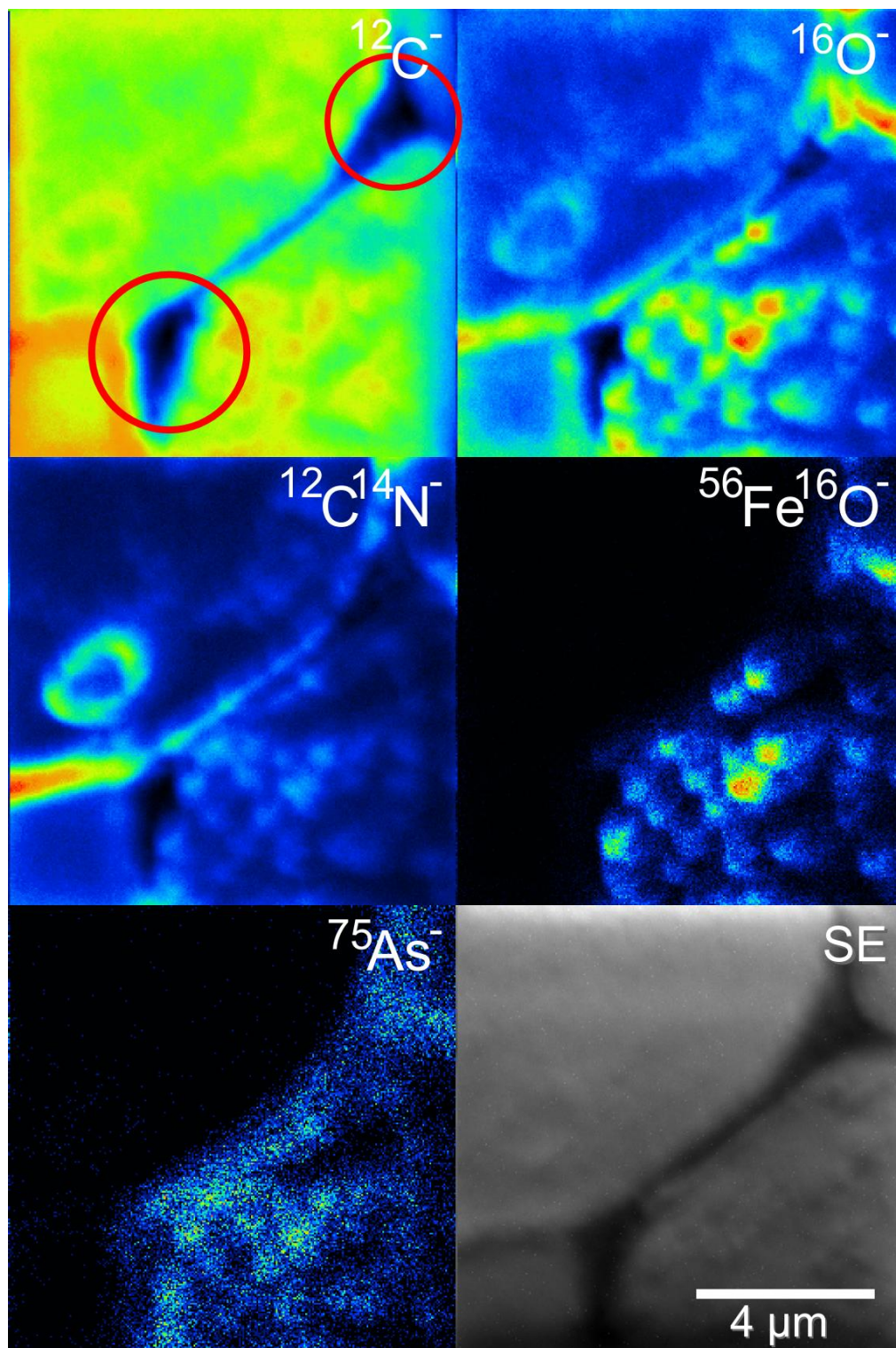


Figure 6.10: NanoSIMS maps of $^{12}\text{C}^-$, $^{16}\text{O}^-$, $^{12}\text{C}^{14}\text{N}^-$, $^{56}\text{Fe}^{16}\text{O}^-$, $^{75}\text{As}^-$ and the secondary electron image. This is the 29th image from the depth profile. A large hole has been generated on the lower cell wall where the three walls meet and another hole has started to form in the top right hand corner (indicated by the red circles). There was $\sim 2\ \mu\text{m}$ drift between the first image, Figure 6.9, and this image.

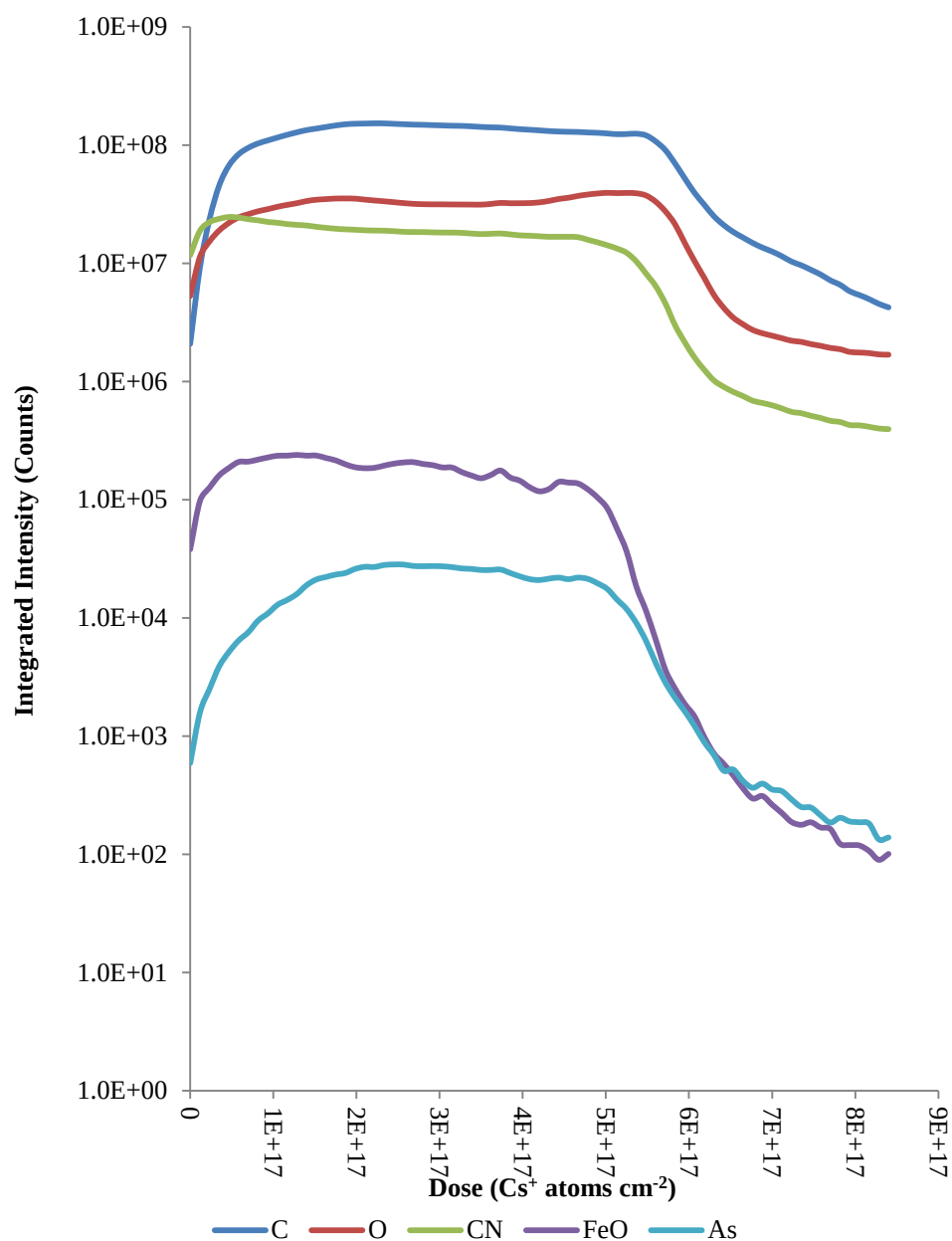


Figure 6.11: Summed counts from the whole area (area 1), as indicated in Figure 6.9, plotted against the calculated dose.

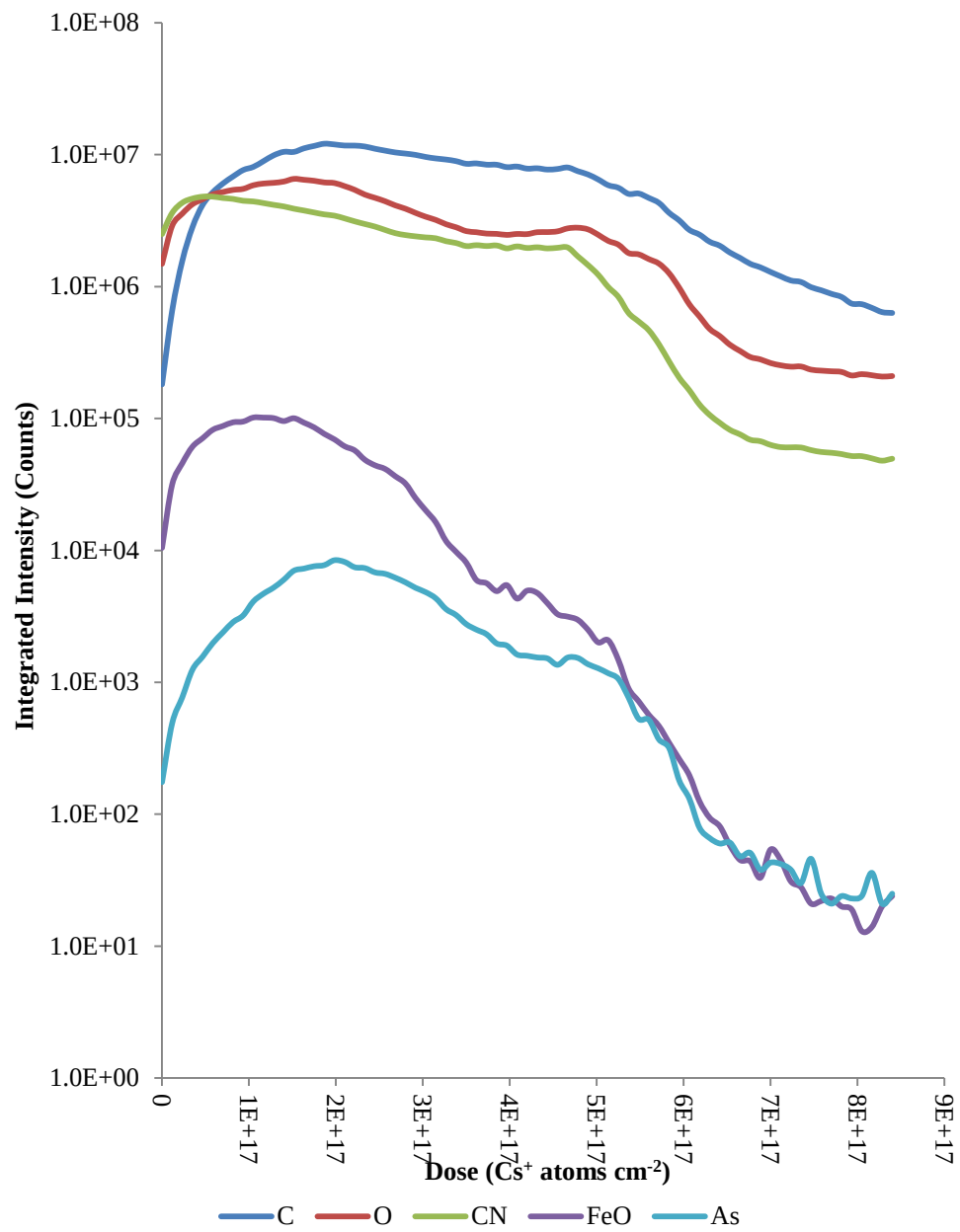


Figure 6.12: Summed counts from the cell walls (area 2), as indicated in Figure 6.9, plotted against the calculated dose.

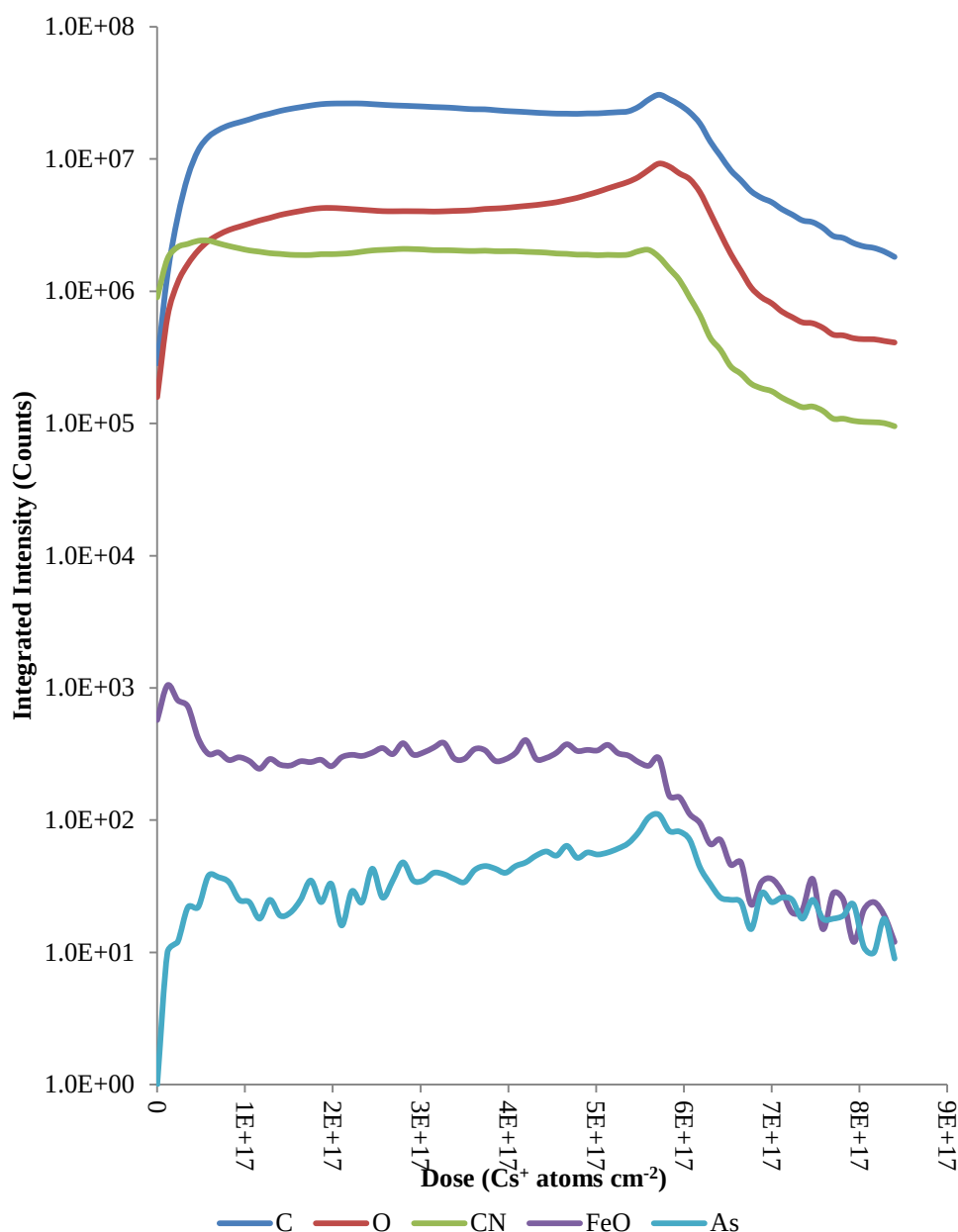


Figure 6.13: Summed counts from the resin (area 3), as indicated in Figure 6.9, plotted against the calculated dose.

If we just consider the depth profile in Figure 6.11 it would indicate that most of the ions reach steady state very quickly, well before a dose of 10^{17} Cs^+ ions cm^{-2} . Arsenic still behaves quite differently to the rest of the ions and does not reach steady state until 2×10^{17} Cs^+ ions cm^{-2} . The curves for $^{12}\text{C}^{14}\text{N}^-$, $^{56}\text{Fe}^{16}\text{O}^-$ and $^{75}\text{As}^-$ show a gradual decrease in intensity in the ‘steady state’ region, and this is likely to be caused by preferential removal of the cell walls from where the majority of these ions originate. $^{12}\text{C}^-$ and $^{16}\text{O}^-$ do not show this drop because these ions originate more homogeneously from the fixed cell interiors. This method is exactly the same as taking a

normal depth profile, which is a common approach, and the steady state dose found using this method is similar to that of Guerquin-Kern (2005). If, however, we just look at the data from the cell walls, Figure 6.12, we see a noticeably different profile, especially for $^{75}\text{As}^-$ and $^{56}\text{Fe}^{16}\text{O}^-$. At a dose of about $2 \times 10^{17} \text{ Cs}^+ \text{ ions cm}^{-2}$, a drop in intensity is observed for all ions. The consequence of the cell walls having a faster sputtering rate means that the signals never reach steady state, and this is most obvious in the $^{56}\text{Fe}^{16}\text{O}^-$ and $^{75}\text{As}^-$ profiles. Figure 6.13 indicates that the data from the cell contents reaches steady state at about $2 \times 10^{17} \text{ Cs}^+ \text{ ions cm}^{-2}$, and because it is essentially homogeneous material there is little variation with depth.

It is clear from this experiment that by just looking at an averaged area it does not give a good indication of what is actually happening in various parts of a sample of this kind where there are many different matrices, each with their own sputtering rates and ion yields. In addition by taking images we can see how the image drifts with time and correct for this therefore eliminating the possibility of analysing areas with different dose densities and avoiding crater edge effects.

If the ratioed $^{75}\text{As}^-$ data for the cell walls is plotted against dose it is possible to see what effect the different sputtering rates have on the normalised data, Figure 6.14. It is quite clear that the ratios change considerably with dose and that there is no point on any of the curves where the ratio is completely steady. There is little difference between $^{12}\text{C}^-$, $^{16}\text{O}^-$ and $^{12}\text{C}^{14}\text{N}^-$ as normalisation ions, but normalising with $^{56}\text{Fe}^{16}\text{O}^-$ is not possible because this ratio is continually changing and because $^{56}\text{Fe}^{16}\text{O}^-$ is localised to the same place as As this would not eliminate matrix effects. The $^{12}\text{C}^-/^{12}\text{C}^{14}\text{N}^-$ ratio can also be used to give an indication of the steady state conditions, suggesting that a dose of around $2 \times 10^{17} \text{ Cs}^+ \text{ ions cm}^{-2}$ might be a good choice, but this is at about the same point where the cell walls start to disappear.

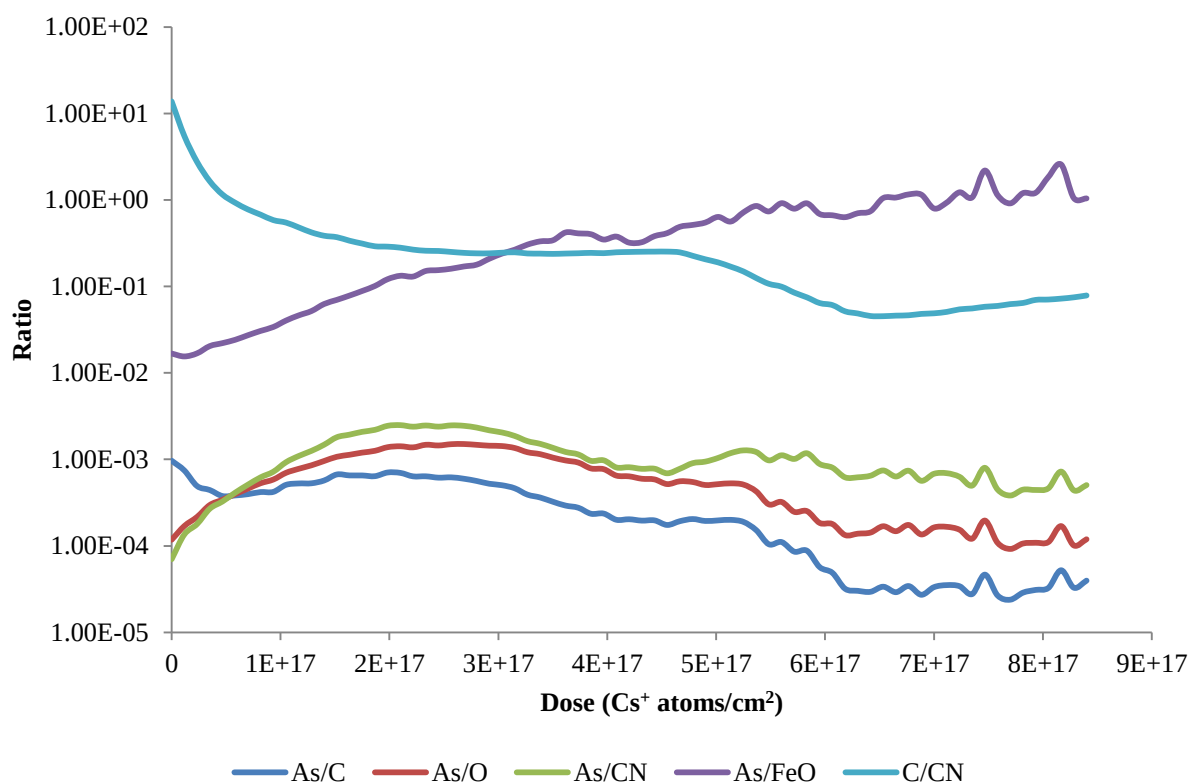


Figure 6.14: Normalised arsenic data showing how the ratio changes with dose density when normalising with C, O CN and FeO

Performing the same experiment on a new area, with $^{16}\text{O}^1\text{H}^-$, $^{31}\text{P}^-$ and $^{35}\text{Cl}^-$ as potential normalisation ions gives similar results hence none of these are suitable ions either.

As none of the ions had a similar behaviour to $^{75}\text{As}^-$, apart from $^{31}\text{P}^-$ which due to its similar distribution would not necessarily normalise out differences in primary ion current, it was decided that $^{12}\text{C}^{14}\text{N}^-$ was still the best normalisation ion for these samples. It was also apparent from the graphs that there was no point when the cell walls reached a steady state condition so a compromise had to be made. As it was not practical to perform this imaging depth profile type experiment for each region of interest, a significant amount of Cs^+ implantation must be performed with a large primary ion beam current before imaging. A trade off must be made between implanting enough Cs^+ to give enough counts and to get past the initial transient region and not implanting too much that the cell walls start to disappear.

It was decided that implanting up to the point just before the cell walls disappear is the most sensible option so that the initial images are taken on the up slope of the peak in intensity rather than being on the down slope when the cell walls are disappearing therefore allowing several images to be acquired from each area. A dose of $1.3 \times 10^{17} \text{ Cs}^+ \text{ ions cm}^{-2}$ was selected to implant enough Cs^+ to ensure that the sample was no longer in the initial transient region, and would put the sample into a region where the changes in $^{75}\text{As}^-$ intensity with an additional image worth of dose were approximately a factor of 2-4 as shown in Figure 6.12. Assuming a current of 100 pA with $D1=0$, implantation time would be 2 hours 25 minutes for a $60 \mu\text{m}$ area which is a reasonable time to allow subsequent analysis. Obviously not every sample behaves in exactly the same way, and different regions on the sample, e.g. the iron plaque compared to the stele, will sputter differently, but this protocol ensures that each sample is treated in the same way and that data from different samples are as comparable as possible.

6.4.4. CONFIRMATION OF SI SIGNAL FROM SAMPLE

The Pt coated plastic substrates were first tested using a *lsi2* mutant root section (*Lsi2* +Fe As(V) treatment). It was immediately observed that there were some cells outlined with Si, but to ensure that this signal was only originating from the root and not a contamination from the underlying substrate the Pt signal was observed. The sample was cleaned with a dose of $1.3 \times 10^{17} \text{ Cs}^+ \text{ ions cm}^{-2}$ ensuring that the top Pt coating had been removed. Figure 6.15 shows that although some $^{195}\text{Pt}^-$ signal originates from some of the same cells where the Si is observed, the distribution is not identical and the colour merge clearly indicates that the Pt and Si signals are not originating from the same points in the sample. Many cell walls in the image show a high Pt signal which do not show corresponding Si signal. This therefore confirms that the Si signal from the endodermis cells is originating from the sample and not the underlying substrate. There is also a low level of Pt detected across the whole image which is likely to be caused by implantation of the Pt coating during cleaning.

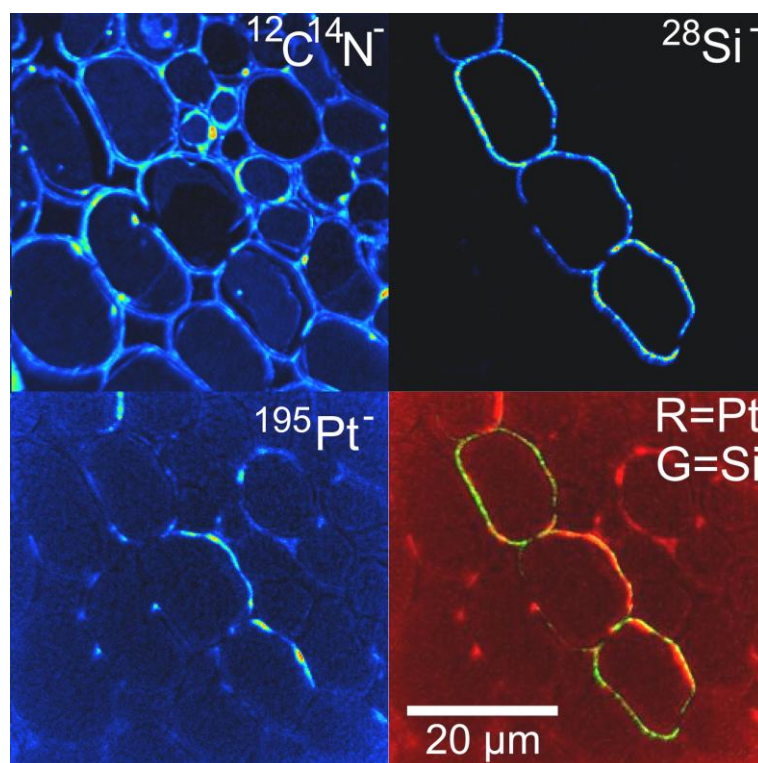


Figure 6.15: NanoSIMS ion maps of the stele of a Lsi2 +Fe As (V) treated root showing $^{12}\text{C}^{14}\text{N}^-$, $^{28}\text{Si}^-$, $^{195}\text{Pt}^-$ and the colour merge between $^{195}\text{Pt}^-$ (red) and $^{28}\text{Si}^-$ (green). The $^{195}\text{Pt}^-$ image shows where there are tears in the resin or where cell walls have been preferentially sputtered and hence the underlying Pt coated substrate is exposed.

6.4.5. ASSESSMENT OF FREEZING PRESERVATION

Transmission electron microscopy was used to assess the quality of preservation after high pressure freezing. The osmium tetroxide fixative used during the freeze substitution stage stains membranous structures, ribosomes and Golgi complexes. Although some contrast could be observed in the TEM from unstained sections it was decided to post stain with uranyl acetate (to stain nucleic acids) followed by lead citrate (to stain membranes, nuclear and cytoplasmic proteins, nucleic acids and glycogen) to improve contrast (Hayat, 2000).

The structural preservation of the roots was generally found to be quite high, giving some confidence that the *in vivo* elemental distributions would have also been well preserved which is encouraging for high resolution NanoSIMS chemical imaging. The most apparent damage was observed at the cytoplasm/cell wall interface. In all samples examined in the TEM it was obvious that in places the cytoplasm and rest of the cell contents had separated from the cell wall leaving

an empty space in the cell subsequently filled with resin during the resin impregnation stage. These tears are shown in Figure 6.16 a) and b), in particular in the cytoplasm at the bottom of the image, and occur across the whole stele region of the root. These regions were noticeably weaker under the stress induced by heating by the electron beam, tearing easily, and are also believed to be the weakest points in the NanoSIMS sections as these correspond to the shape and location of the regions where the underlying Pt signal is first exposed in Figure 6.15.

The cell walls in Figure 6.16 a) appear reasonably well preserved, showing a fibrous layered structure surrounding the cells, although some cell walls show signs of HPF damage. The high pressure has resulted in fracture of several cell walls and this has also caused damage to the neighbouring cell cytoplasm. This is a common artefact of HPF due to the very high pressure to which the delicate plant material is subjected (McDonald, 2007). Three breaks in cell walls are indicated by the white arrows in Figure 6.16 b), the lowest of which is very severe and could be a source of artefact in the NanoSIMS imaging with cellular material apparently mixing between adjacent cells.

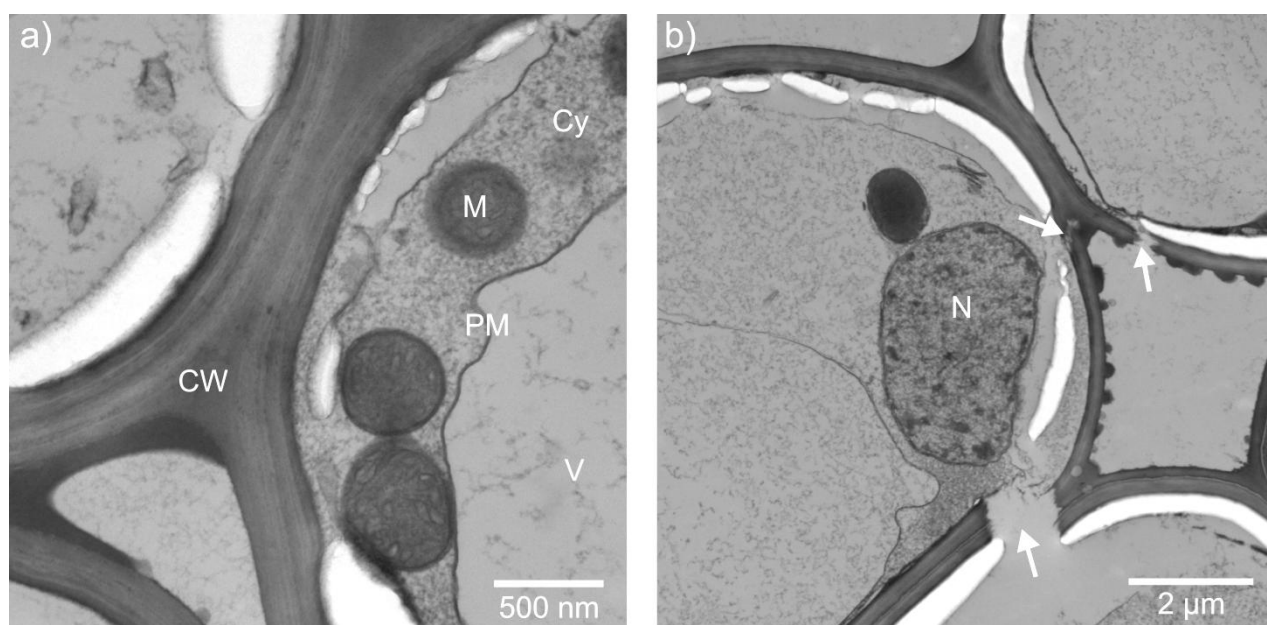


Figure 6.16: Transmission electron micrographs showing examples of high pressure freezing damage, a) cellular contents have been separated from the cell walls, shown by the bright parts in the image, b) cell wall fracture caused by the high pressure as indicated by the white arrows. CW, cell wall; Cy, cytoplasm; M, mitochondrion; N, nucleus; PM, plasma membrane; V, vacuole.

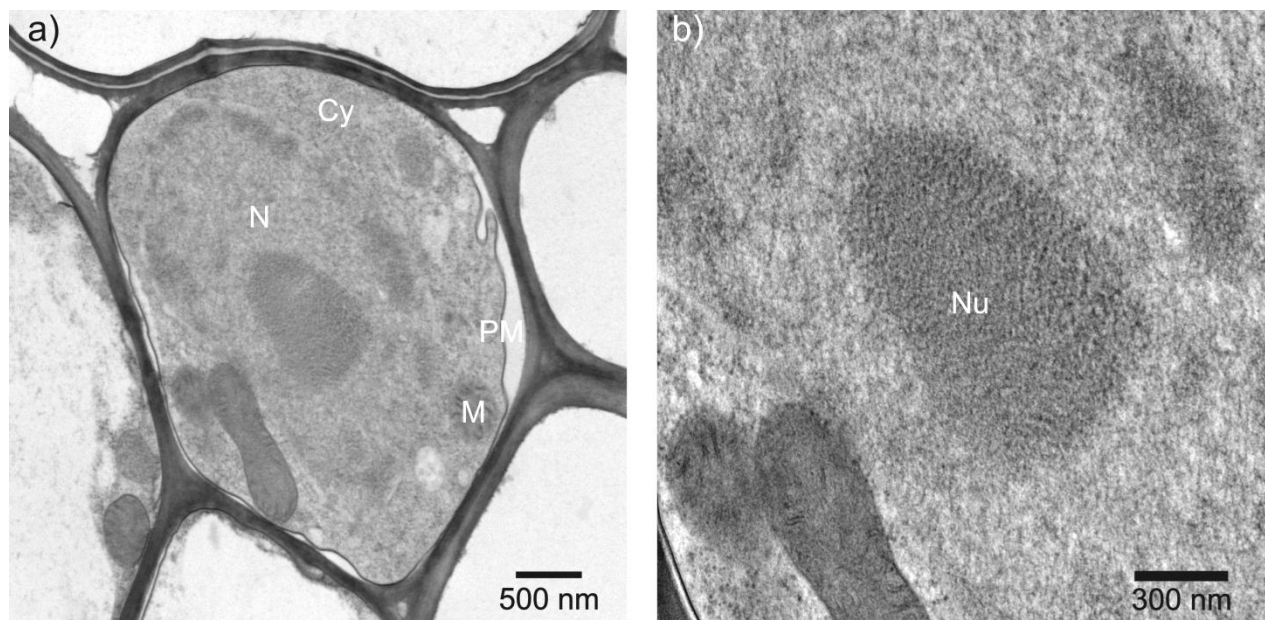


Figure 6.17: Transmission electron micrographs showing a) a cell showing high pressure freezing damage to a nucleus and b) the nucleolus clearly showing the characteristic fibrous damaged structures. Cy, cytoplasm; M, mitochondrion; N, nucleus; Nu, nucleolus; PM, plasma membrane.

Figure 6.16 b) also shows a well preserved nucleus, but an example of a damaged nucleus is shown in Figure 6.17. This nucleus shows the characteristic signs of freezing damage, a grainy structure, especially within the nucleolus as shown in Figure 6.17 b). It is not surprising that some cells did not freeze well considering that the stele region of the root is almost at the 200 μm depth limit where freezing is still considered to be high quality (Moor & Riehle, 1987; Hayat, 2000). Nevertheless very few observed cells appeared to be poorly preserved, hence it can be assumed that there is a satisfactory level of preservation across the root and that these samples are likely to be suitable for detailed NanoSIMS analysis.

A more stringent method of assessing the level of preservation of the frozen material is to check the distribution and ratios of the highly diffusible cations such as Na^+ , K^+ and Ca^+ as these are very difficult to preserve in their *in vivo* distribution. The distribution of $^{23}\text{Na}^+$ and $^{40}\text{Ca}^+$ was mapped on one root sample using the O^- beam. Calcium was found to be localised only in the cell walls whereas Na was only found in the vacuoles as shown in Figure 6.18. This distribution

is as expected for plant material (Derue *et al.*, 2006a) allowing us to be confident that the sample preparation, for this sample at least, has not significantly altered the *in vivo* distribution of elements. The K^+ distribution was not measured for these samples so it was not possible to determine the Na^+/K^+ ratio which can be used to give a useful indication of the level of sample preparation (Derue *et al.*, 2006a).

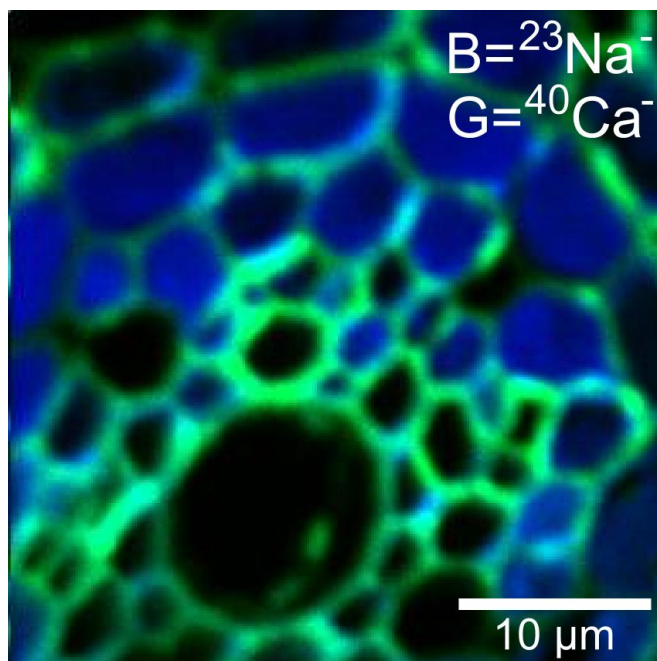


Figure 6.18: NanoSIMS duo image of the stele region of an *lsi2* mutant treated with arsenate showing Na^+ localised within the vacuoles and Ca^+ in the cell walls (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

6.4.6. EFFECT OF FE PLAQUE

Rice roots were coated with an orange-coloured Fe plaque after being treated with a ferrous sulphate solution for 24 has shown on the left of Figure 6.19.



Figure 6.19: Photograph showing the orange-coloured Fe plaque after treating with ferrous sulphate solution (left) and the white roots without this treatment (right).

NanoSIMS imaging of the outer cell layers of the roots revealed the distribution of this Fe plaque. The black and white ion-induced secondary electron image shown in Figure 6.20 shows the four outer cell layers of the root, the epidermis in the bottom right, the exodermis, the sclerenchyma layer and one layer of cells from the cortex in the top left. This image is typical of all the roots which were exposed to the ferrous solution irrespective of whether the sample was treated with As(V) or As(III). There was also no difference, as expected, between the wild-type and the *lsi2* mutant.

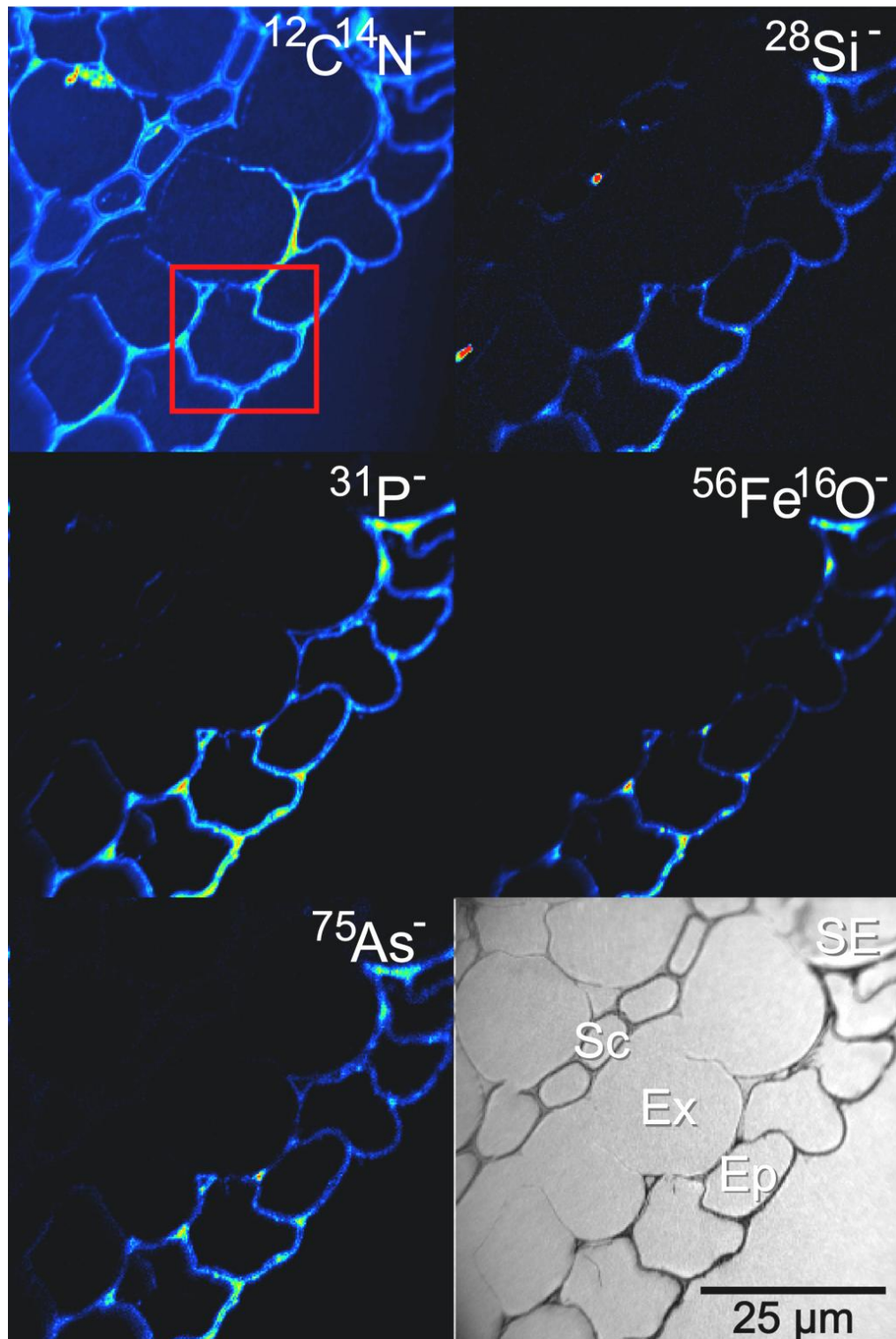


Figure 6.20: NanoSIMS image of the outer edge of an *lsi2* mutant root near the root tip treated with Fe plaque, Si and arsenate showing the outer four cell layers of the root with strong epidermal As co-localisation with the Fe plaque (represented by the $^{56}\text{Fe}^{16}\text{O}^-$ image). The two Si hotspots associated with the sclerenchyma cells probably result from surface contamination rather than the real distribution of Si within the root. Ep = Epidermis, Ex = exodermis, Sc = sclerenchyma, SE = secondary electron image. Maximum As signal is 125 counts (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

The $^{12}\text{C}^{14}\text{N}^-$ image reveals damage caused by the HPF. There are some breaks in the cell walls and membranes along either side of the sclerenchyma layer but this does not prevent imaging of the Fe plaque as the root is well preserved along the epidermis. The co-axial optics of the NanoSIMS means that the detected ions must have opposite polarity to the primary ion beam. The highest lateral resolution can be obtained using the Cs^+ beam meaning that the Fe plaque was detected as the $^{56}\text{Fe}^{16}\text{O}^-$ ion. Complementary analysis was undertaken with an O^- beam to map $^{56}\text{Fe}^+$ on another sample (Figure 6.21) and the same distribution was observed with both polarities so mapping the Fe plaque as $^{56}\text{Fe}^{16}\text{O}^-$ has not affected the observed distribution.

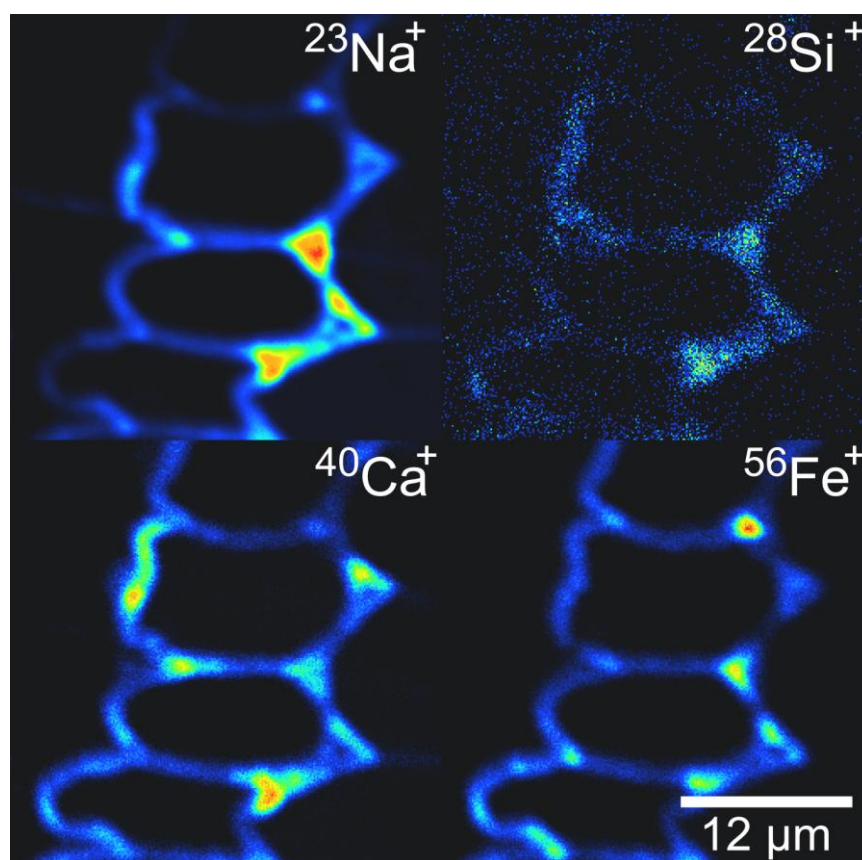


Figure 6.21: NanoSIMS O^- beam image of the outer edge of a WT root with Fe plaque taken from near the root tip and showing the outer epidermis with strong epidermal accumulation of Fe Plaque. Note the distribution of the Fe is almost identical to that shown in Figure 6.20 and that $^{23}\text{Na}^+$, $^{28}\text{Si}^+$ and $^{40}\text{Ca}^+$ also show strong accumulation on the Fe plaque.

The $^{56}\text{Fe}^{16}\text{O}^-$ was only located on the epidermis of the root, indicating that the formation of Fe plaque occurs exclusively outside the exodermal cells probably due to blockage by the Casparian strip. The $^{56}\text{Fe}^{16}\text{O}^-$ distribution was not homogeneous along the epidermis; there were distinct regions of accumulation along the outer edge of the epidermis and along the boundary of the

exodermis where entry to the root is blocked. With the exception of $^{12}\text{C}^{14}\text{N}^-$ all the other imaged elements, $^{23}\text{Na}^+$, $^{28}\text{Si}^-$, $^{31}\text{P}^-$, $^{32}\text{S}^-$ (not shown), $^{40}\text{Ca}^+$ and $^{75}\text{As}^-$ (in both the As(V) and As(III) treatments), showed similar accumulation on the Fe plaque. Although $^{23}\text{Na}^+$ would be expected within the vacuoles of the epidermal cells it is likely that the epidermal cells are dead hence the distribution would have been altered. Where stronger $^{56}\text{Fe}^{16}\text{O}^-$ accumulation was observed there was a similar stronger accumulation of As.

In contrast, roots which were not exposed to the ferrous solution did not show characteristic accumulation of Fe and As on the epidermis of the root. Very little As was detected on the outer surface of the roots, as shown in Figure 6.22, and there was a difference of 120 counts between the maximum As signal detected from the sample with Fe plaque to that without. There was also no enhanced accumulation of Si, P or S on the outer epidermis in comparison to the exodermal and sclerenchyma cells.

The formation of Fe plaque is a common feature of rice roots growing in submerged soils. The effect of the Fe plaque is very distinct and obvious in Figure 6.20 and noticeably absent in Figure 6.22. The Fe plaque resulted in a high accumulation of the As on the epidermis as would be expected with adsorption of As(V) or As(III) by the Fe oxyhydroxides (mainly ferrihydrite, (Hansel *et al.*, 2001)). Stronger $^{56}\text{Fe}^{16}\text{O}^-$ signals were associated with stronger As signal as would be expected considering the strong affinity of Fe plaque for As (Liu *et al.*, 2004; Fendorf *et al.*, 2008). This co-localisation has also been shown in rice roots with X-ray tomography (Seyfferth *et al.*, 2010). Higher resolution imaging of the epidermis cell indicated in Figure 6.20 and shown in Figure 6.23 a) allowed line scans to be taken across the epidermal cells. These line scans, an example of which is shown in Figure 6.23 b), indicated a higher $^{56}\text{Fe}^{16}\text{O}^-$ peak on the outside of the cell with a smaller peak on the inside (position of cell wall determined from the secondary electron image). The Fe plaque was therefore found to be predominantly localised outside the plasma membranes of the epidermal cells, but with some Fe detected inside the cell plasma

membrane. The Fe plaque is composed of fine needles about 10 nm thick and 200-300 nm long, as shown by TEM imaging (Figure 6.24), and this explains why the Fe plaque does not appear as a sharp line in the NanoSIMS images. This distribution is similar to that observed previously for rice roots (Green & Etherington, 1977), but is shown here at higher resolution. This TEM image also shows that some Fe plaque is present inside the cell, albeit with lower density, consistent with the NanoSIMS line scans. The reason for the Fe plaque penetrating the membrane of the epidermis cells is probably because these cells were dead.

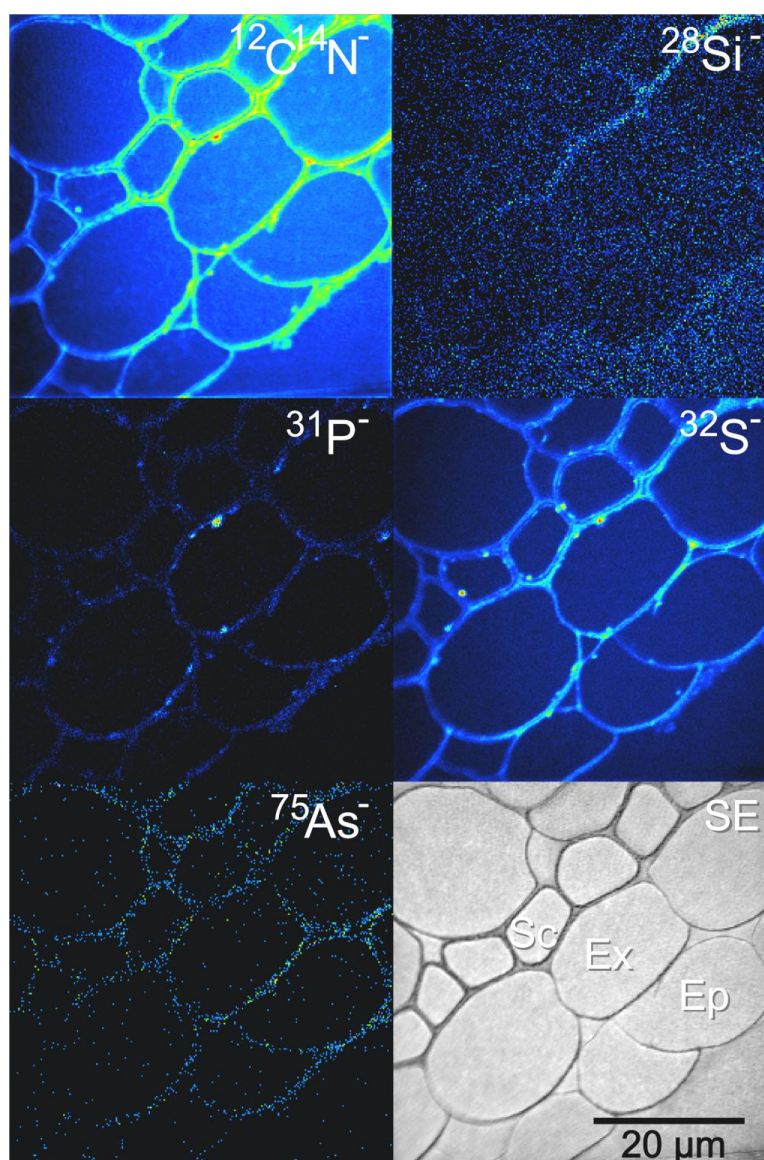


Figure 6.22: NanoSIMS image of the outer edge of an *lsi2* mutant root taken from near the root tip without Fe plaque and treated with Si and arsenate showing the outer four cell layers of the root. Without the Fe plaque on the epidermis there is very little As accumulation in this region. Ep = Epidermis, Ex = exodermis, Sc = sclerenchyma, SE = secondary electron image. Maximum As signal is 4 counts (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

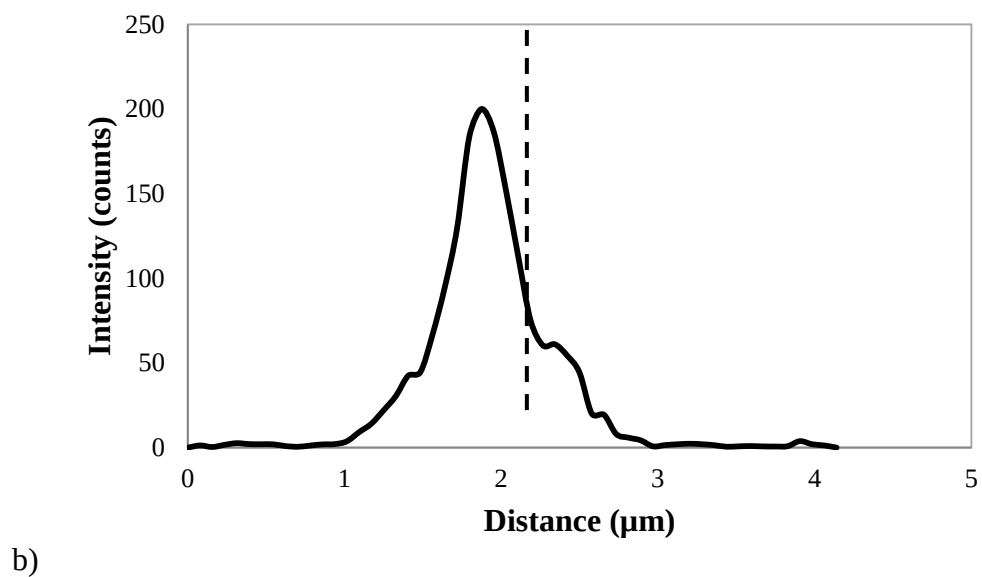
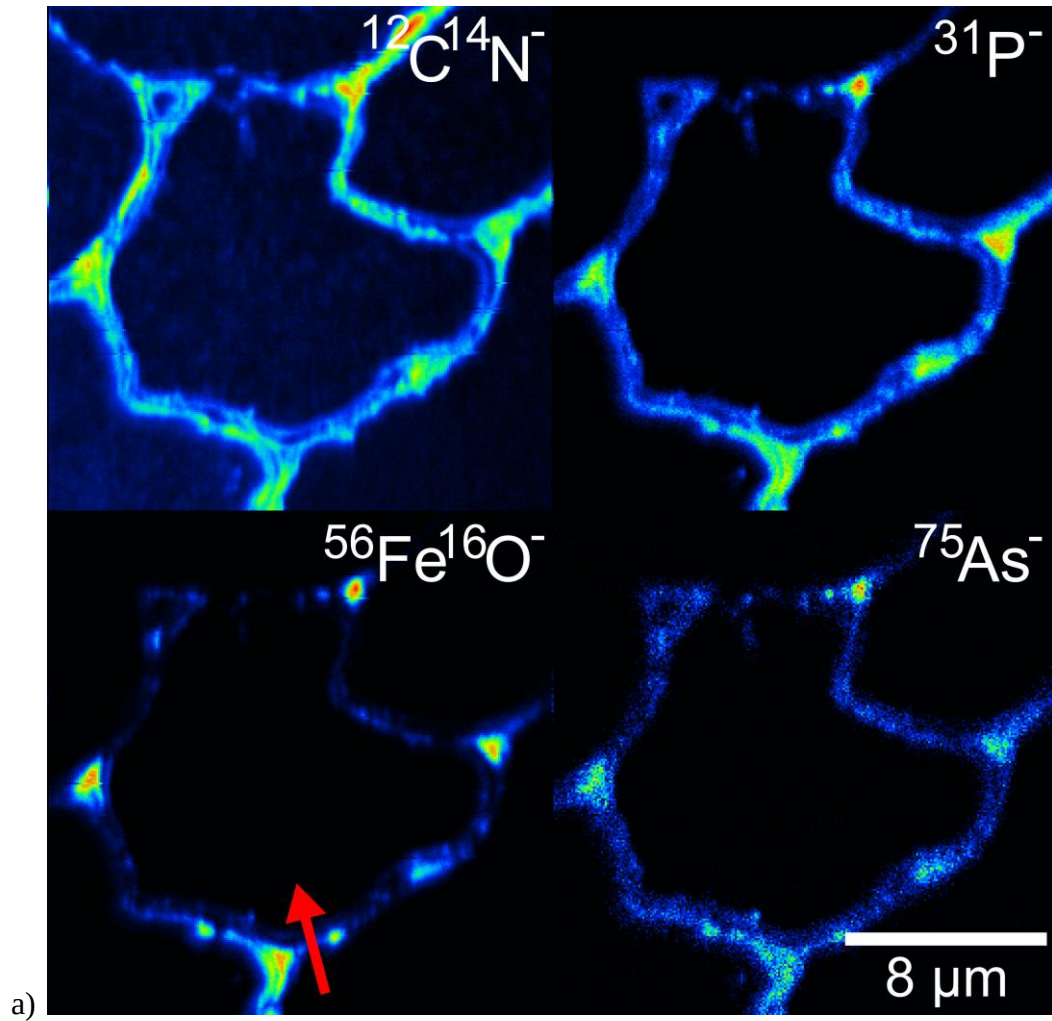


Figure 6.23: a) NanoSIMS image of the epidermis of an *lsi2* mutant root near the root tip with Fe plaque and treated with Si and arsenate. b) Line scan across the outer edge of the epidermis at the point indicated by the red arrow in a). The dashed line indicates the position of the cell wall. This indicates a high concentration of Fe plaque on the outer edge with a lower amount inside the cell.

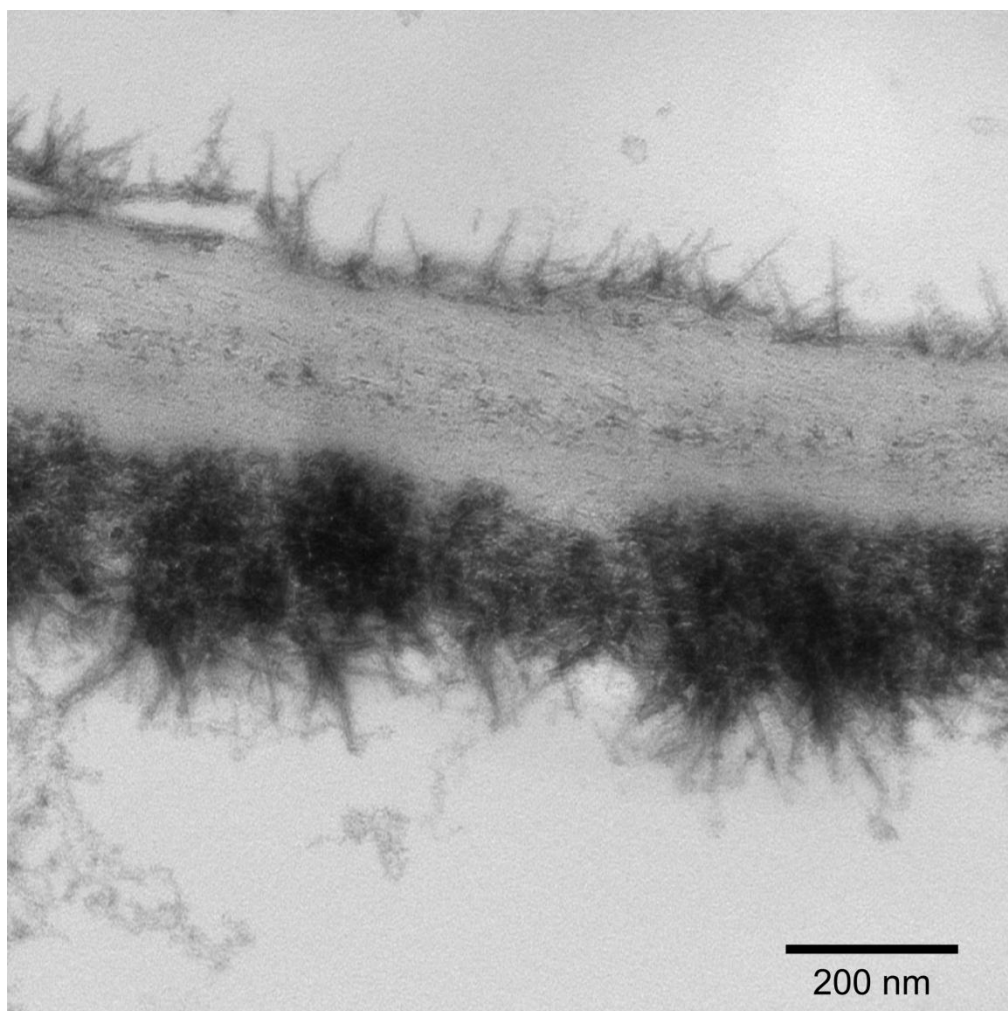


Figure 6.24: A TEM image showing the morphology of the Fe plaque on the outer epidermis of the root. The outer edge (bottom) shows a much higher density of Fe needles than the inside of the cell (top). This is likely to be seen if these cells are dead allowing Fe to pass into the cell (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

There was no observable difference in the distribution of As adsorbed onto the Fe plaque between the samples treated with arsenite or arsenate. It is likely that oxygen released by the roots of the arsenite treated samples into the rhizosphere resulted in some oxidation of arsenite to arsenate which was then adsorbed onto the root epidermis. Alternatively, adsorbed arsenite may be oxidised to arsenate on the Fe plaque. Other elements including P and Si show strong association with the Fe plaque. This work has presented detailed high resolution information on the morphology and localisation of Fe plaque and co-localisation of As and other elements with the plaque which can aid understanding of the mechanism of sequestration of As and other important elements at the root surface.

6.4.7. EFFECT OF LSI2 MUTATION ON EXODERMIS ELEMENTAL DISTRIBUTION

The exodermis contains one of the two Casparian strips present in rice roots, with the Lsi2 transporter located on the proximal side of the exodermis (Figure 6.2). The Lsi2 transporter mediates the efflux of Si and arsenite into the apoplast of the cortex cells which degenerate to form aerenchyma in the mature zone of roots (Ma *et al.*, 2007; Ma *et al.*, 2008). As this transporter is non-functional in the mutant, accumulation of As and Si in the exodermis might have been expected, however this was not observed. In no mutant samples was there either detectable As accumulation in the exodermis (Figure 6.22) or obvious enhancement of Si in these cells compared with the wild-type samples (Figure 6.25). Both the wild-type samples (Figure 6.25) and the *lsi2* mutant (Figure 6.20 and Figure 6.22) showed some Si accumulation along the sclerenchyma. It is not clear why the mutant did not accumulate more As in the exodermis than in the endodermis, but the lack of strong exodermal accumulation of Si and As in *lsi2* may be caused by efflux of these elements to the epidermal apoplast and the external medium via Lsi1, a passive transporter allowing bidirectional flux of Si and As(III) (Mitani *et al.*, 2008; Zhao *et al.*, 2010a).

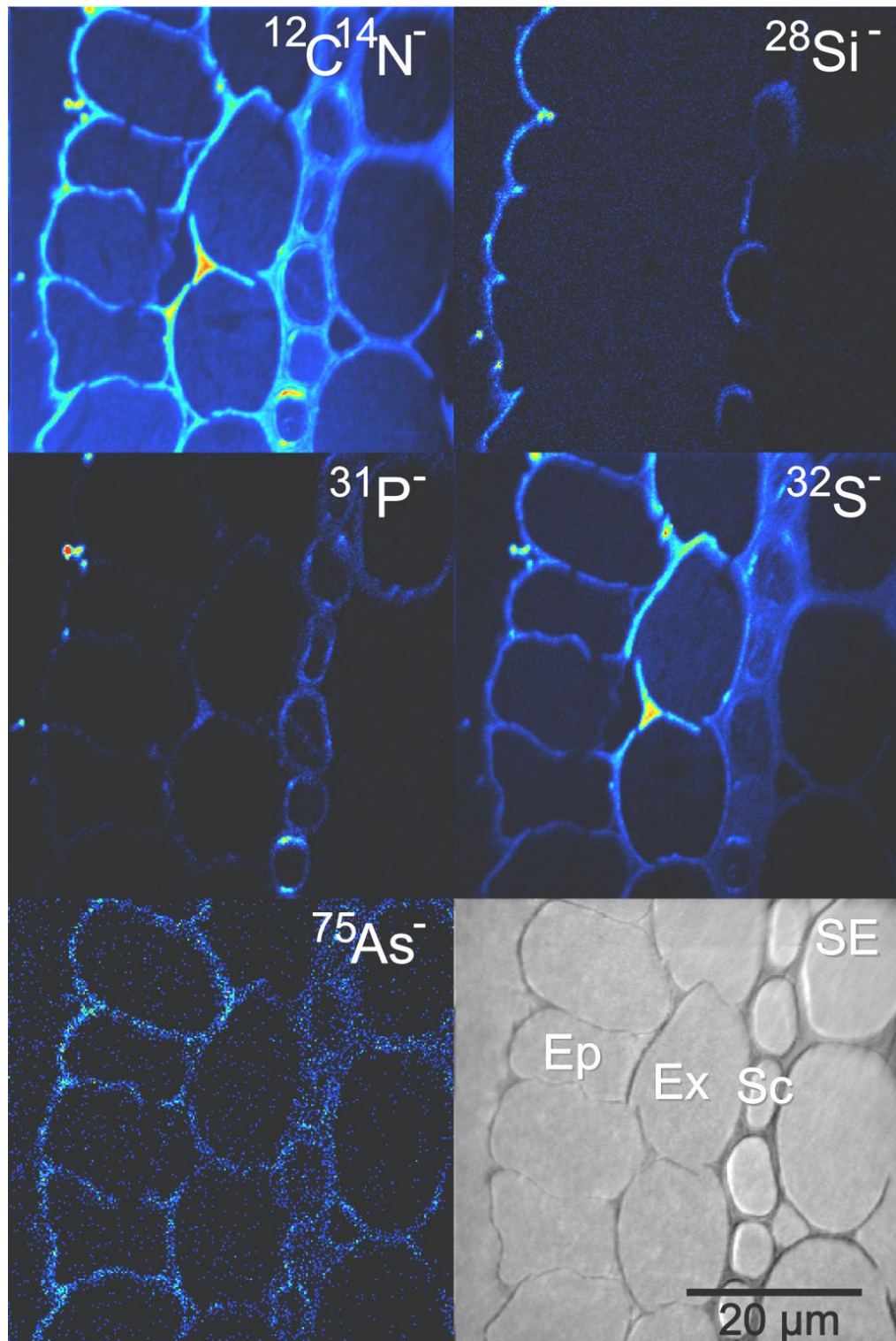


Figure 6.25: NanoSIMS image of the outer edge of a wild-type root taken from near the root tip without Fe plaque and treated with Si and arsenite showing the outer four cell layers of the root. There is no significant accumulation of As in the vacuoles of the exodermis. Ep = Epidermis, Ex = exodermis, Sc = sclerenchyma, SE = secondary electron image

6.4.8. EFFECT OF THE LSI2 MUTATION ON ENDODERMIS ELEMENTAL DISTRIBUTION

Imaging of the stele region of the *lsi2* mutant treated with ferrous sulphate solution, Si and As(V) revealed the distributions of As and Si at high resolution (Figure 6.26). One of the major xylem vessels is situated in the top right hand corner of the image as shown in the secondary electron image, and the endodermis where the second Casparian strip is situated can be seen across the centre of the image. The $^{12}\text{C}^{14}\text{N}^-$ image reveals many cellular details indicating the presence of the membranes, nuclei and vacuoles. Vacuoles can be observed in the endodermis, several of which have separated from the cell walls along with some of the cytoplasm, a sign of damage during the preparation/high pressure freezing process. The white arrows in the $^{31}\text{P}^-$ image, along with the high intensities in the $^{12}\text{C}^{14}\text{N}^-$ image, reveal the presence of two nuclei located in cells on either side of the endodermis. Conventional sample preparation procedures such as chemical fixation do not preserve the chemical distribution of diffusible species (Chandra *et al.*, 2000), for example ions in the vacuoles, however it can be seen that a uniform As accumulation in the endodermal vacuoles has been preserved by HPF (Figure 6.26) giving us confidence that the sample preparation has not significantly altered the As distribution.

Si was found to be localised in characteristic rings in the endodermal cell walls (see below for the evidence for cell wall localisation), with no obvious differences in intensity observed on the distal or proximal sides (Figure 6.26). The $^{75}\text{As}^-$ image reveals the accumulation of As in the vacuoles of the endodermis cells. This accumulation was possibly a result of the *Lsi2* mutation, since such a strong localisation was not observed in the endodermis of wild-type roots (see below and Figure 6.28). Imaging of the whole endodermis around the stele in a series of NanoSIMS images revealed a similar pattern of As accumulation in many vacuoles (Figure 6.27).

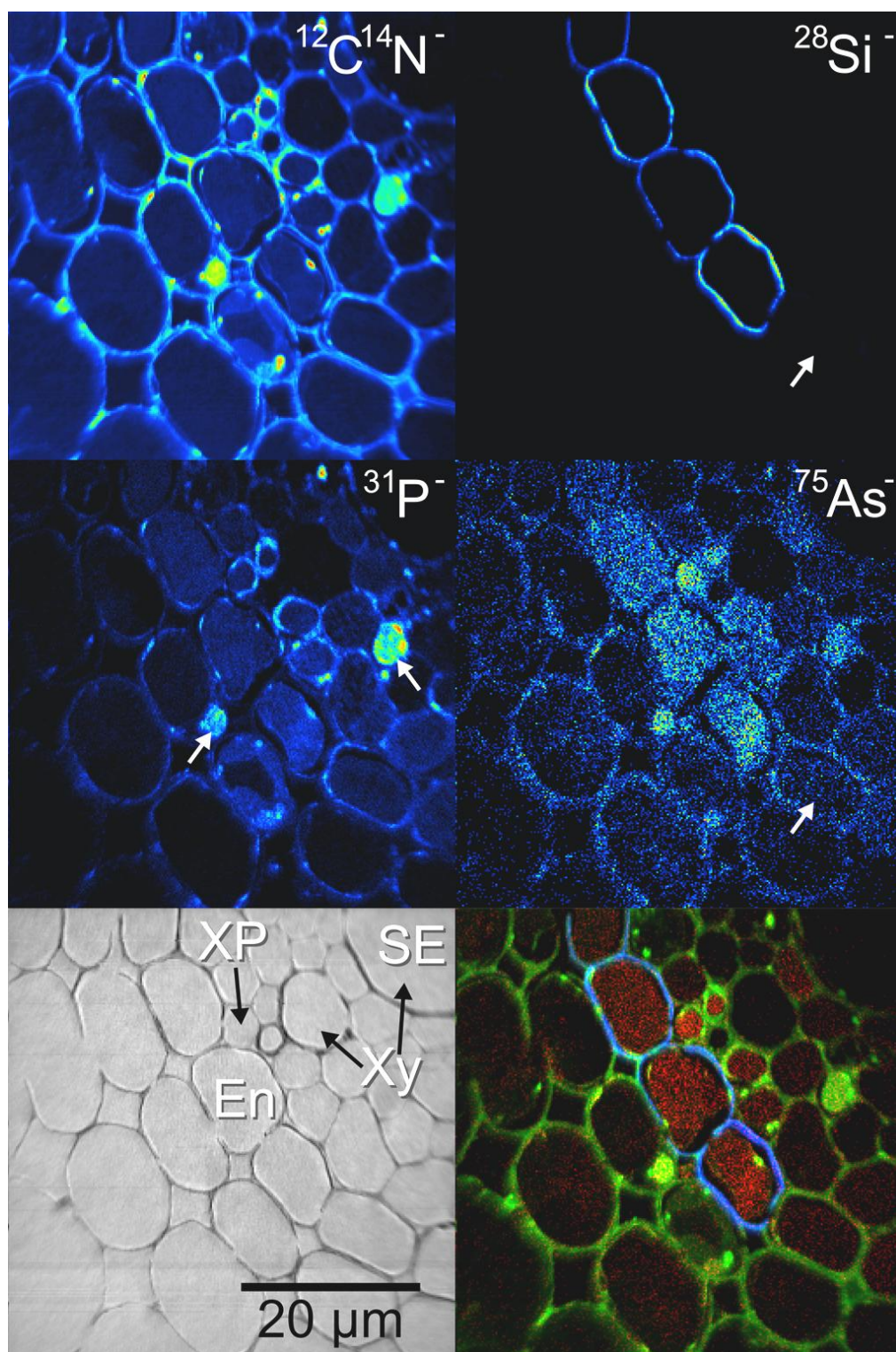


Figure 6.26: NanoSIMS image of the stele region of an *lsi2* mutant from near the root tip treated with arsenate showing As accumulation in the vacuoles of the endodermis with Si localised around the same cells. The color merge image shows the relative locations of $^{75}\text{As}^-$ (red), $^{12}\text{C}^{14}\text{N}^-$ (green) and $^{28}\text{Si}^-$ (blue). The white arrows on the $^{31}\text{P}^-$ image indicate the position of sectioned nuclei and the white arrows on the $^{28}\text{Si}^-$ and $^{75}\text{As}^-$ images indicate a cell with no Si or As accumulation. XP = xylem parenchyma, En = endodermis, Xy = xylem, SE = secondary electron image (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

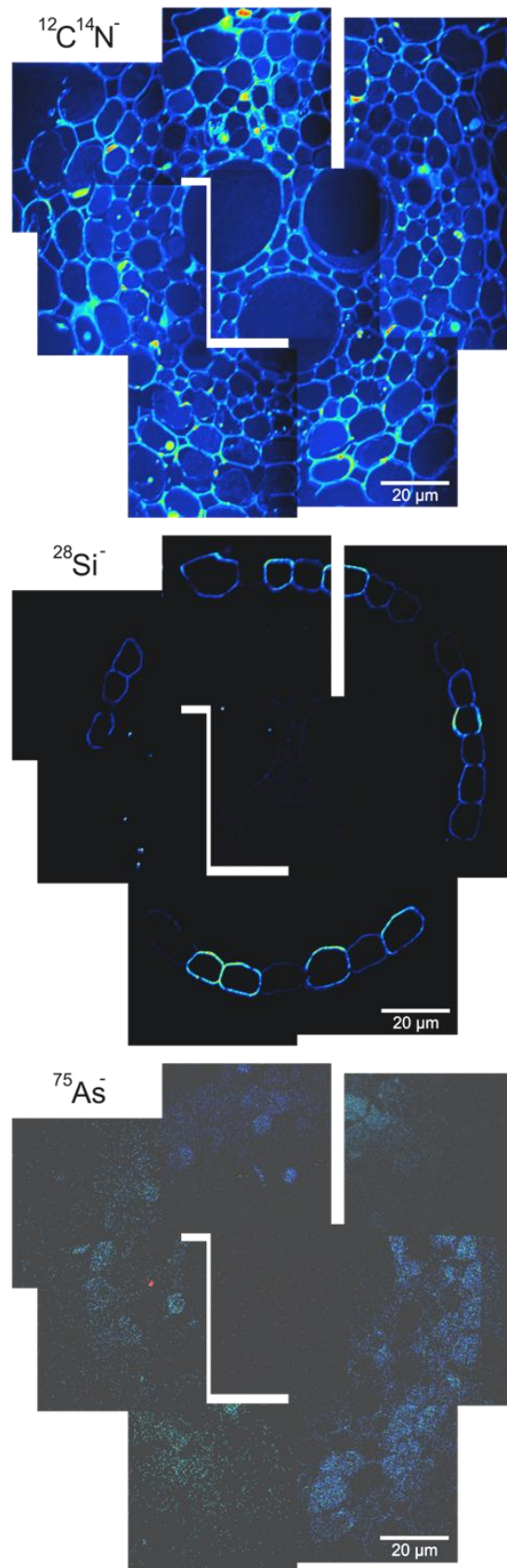


Figure 6.27: NanoSIMS images of the stele region of an *lsi2* mutant from near the root tip treated with arsenate showing As accumulation in the vacuoles of the endodermis with Si localised around the same cells. Not all endodermal cells are outlined with Si and this sometimes corresponds to a lack of As accumulation in the vacuoles of the same cells.

Not all of the endodermal cells, however, were outlined with Si, which in some cases coincided with no accumulation of As in the vacuole of that cell. This is shown by the white arrows in the $^{28}\text{Si}^-$ and $^{75}\text{As}^-$ images for the lower endodermal cell in Figure 6.26 and can be seen quite clearly in Figure 6.27 in particular on the left of the image and in the top right of the whole root image.

In contrast to the *lsi2* mutant, analysis of the wild-type samples did not show any strong As accumulation in the endodermis of the roots (Figure 6.28), which is as expected because the *Lsi2* transporter is able to efflux As into the stele for loading into the xylem. Arsenic was only localised to the cell walls and membranes with some accumulation in the metabolically active regions of the cells, for example in the nucleus of the middle endodermal cell as indicated by the white arrow. Surprisingly, there was no difference in the Si distribution between the wild-type and the *lsi2* mutant. In both cases the Si was localised in the endodermal cell walls, again with no obvious differences between the concentration at the proximal and distal sides.

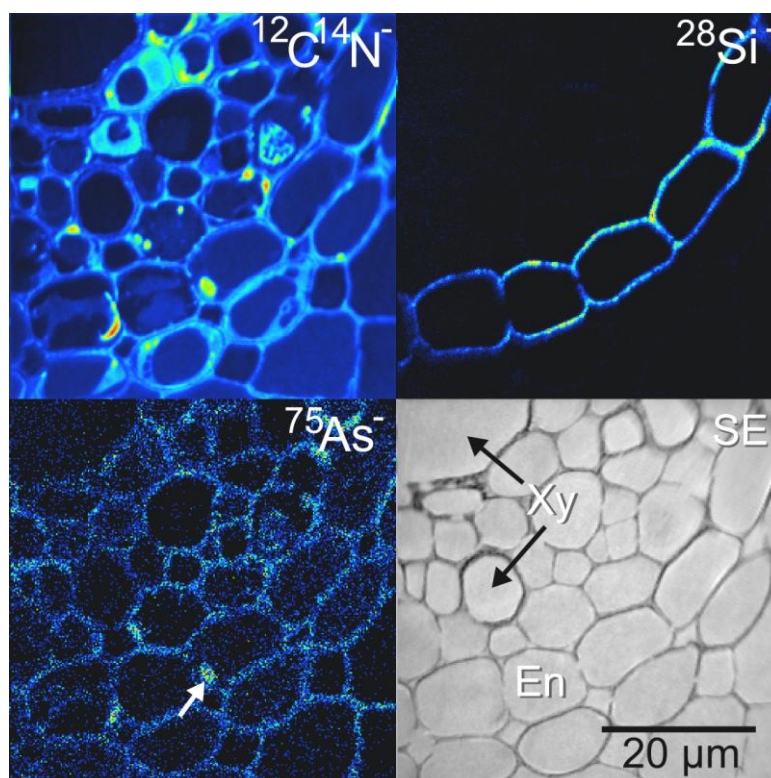


Figure 6.28: NanoSIMS image of the stele region from a wild-type root, near the root tip treated with arsenate showing no significant As accumulation in the vacuoles of the endodermis. Si is localised around the endodermal cells with the same distribution pattern as in the *lsi2* mutant (Figure 2). En = endodermis, Xy = xylem, SE = secondary electron image (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

To my knowledge, this study is the first time that accumulation of As in the vacuoles of root cells has been observed in a non-hyperaccumulator species. Previously, vacuolar accumulation of As has only been reported in the fronds of the As hyperaccumulator fern *Pteris vittata*, which contained As concentrations 1 to 2 orders of magnitude higher than those in the rice roots in this study (Lombi *et al.*, 2002; Pickering *et al.*, 2006). Vacuolar sequestration of As has been proposed as an important mechanism of As detoxification (Bleeker *et al.*, 2006; Zhao *et al.*, 2009). In As hyperaccumulating ferns As is stored in the fronds mainly as uncomplexed arsenite (Lombi *et al.*, 2002; Wang *et al.*, 2002; Zhang *et al.*, 2002; Pickering *et al.*, 2006). Recently, an arsenite efflux transporter (PvACR3) localised to the tonoplast in As hyperaccumulators has been shown to mediate arsenite transport into the vacuoles and to play an important role in As detoxification (Indriolo *et al.*, 2010). In contrast, As is stored mainly as arsenite-phytochelatin complexes in As non-hyperaccumulators (Raab *et al.*, 2005; Liu *et al.*, 2010), and these are likely to be the main forms of As observed in the vacuoles of rice roots. Recently two ABCC-type transporters have been identified in *Arabidopsis thaliana* as the major vacuolar transporters for arsenite-phytochelatin complexes required for As detoxification (Song *et al.*, 2010). Indeed, in the cells showing strong As accumulation in the endodermal vacuoles, there were also elevated S signals (Figure 6.29) suggesting that the majority of As in rice roots is stored as arsenite-phytochelatin complexes. It would be interesting to investigate As localisation in the roots of a hyperaccumulator (e.g. *Pteris vittata*) to test the hypothesis that a lack of or a weak vacuole sequestration in roots is a component of the As hyperaccumulation phenotype typified by an extremely efficient translocation of As from roots to fronds.

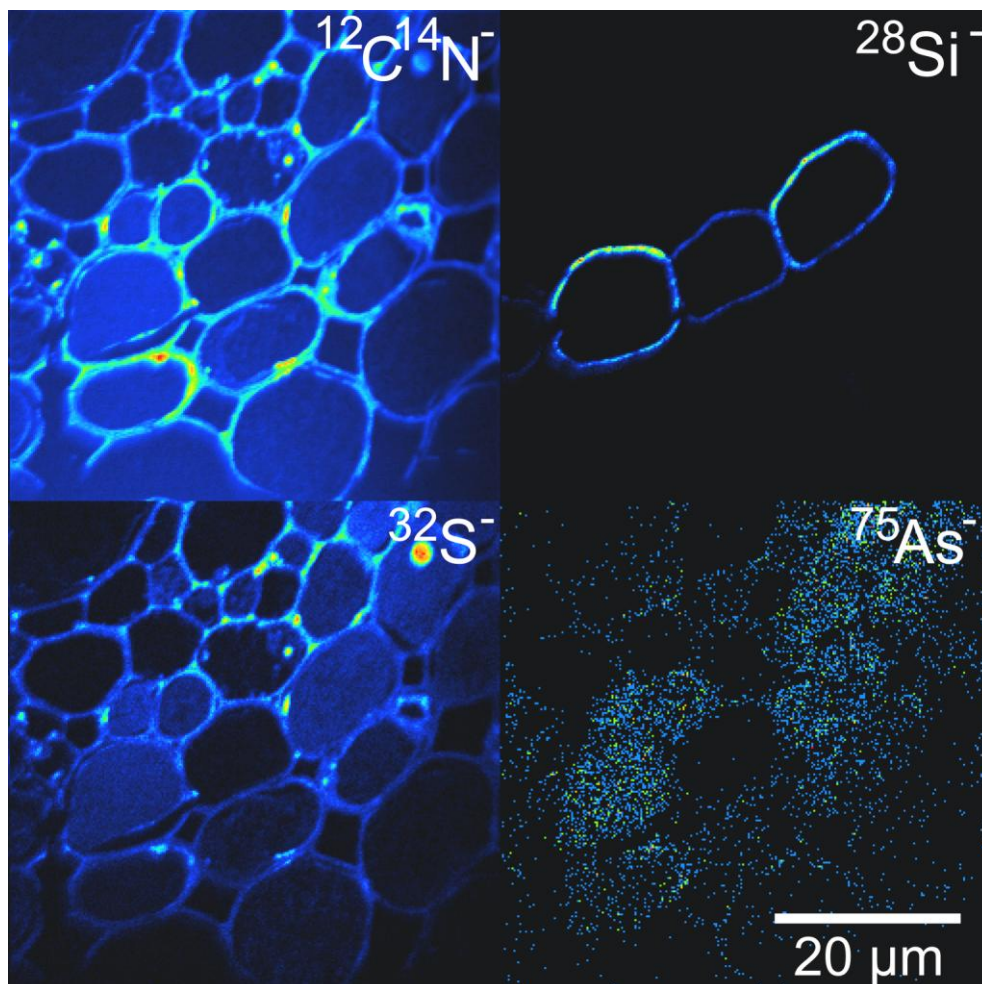


Figure 6.29: NanoSIMS image of the stele region of a *lsi2* mutant root treated with Si and arsenate. The cells that show As accumulation in the vacuoles of the endodermis also show elevated S signals suggesting that As is stored as arsenite-thiol complexes (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

In the present study, a great difference in the As localisation was not observed between the As(V) and As(III) treatments, both treatments showed accumulation of As in the endodermis vacuoles. This similarity can be explained by a rapid reduction of arsenate to arsenite in root cells; Xu *et al.* (2007) showed that more than 90% of the As taken up by rice roots from the arsenate treatment was reduced to arsenite within 1 day of As exposure.

6.4.9. ARSENIC ACCUMULATION IN THE PERICYCLE AND XYLEM PARENCHYMA

A markedly different distribution of As to that shown above was observed in the *lsi2* mutant treated with Si and As(V). Instead of As accumulation in the endodermis, As accumulated in the pericycle, the layer of cells immediately inside the endodermis (Figure 6.30). This also

corresponded with a complete lack of Si detected in the endodermis. It is likely that this was from a younger portion of the root where the Casparian strip had not formed so that the Si transporters are less expressed in this region (Yamaji & Ma, 2007). The maximum Si signal from this region is 4 counts, significantly lower than the 500-1000 counts detected from a sample showing Si localisation in the endodermis.

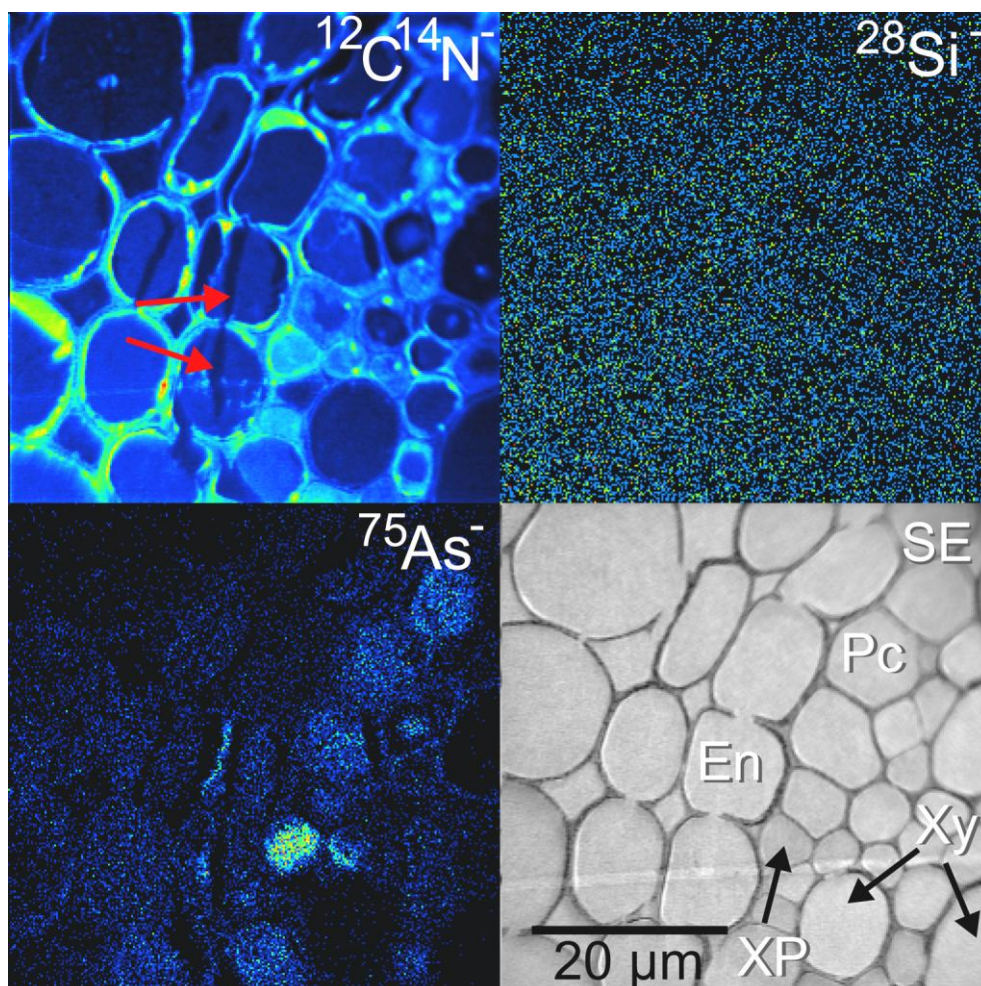


Figure 6.30: NanoSIMS image of the stele region of an *lsi2* mutant from near the root tip treated with Si and arsenate showing As accumulation in the vacuoles of the pericycle. No localisation of silicon was detected in this region. En = endodermis, Xy = xylem, XP = xylem parenchyma, Pc = pericycle SE = secondary electron image. The red arrows on the $^{12}\text{C}^{14}\text{N}^-$ image indicate where there has been high pressure freezing damage resulting in a split across the cells subsequently filled with resin (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

In order to test whether the observed As and Si distributions were affected by the proximity of the analysed sections to the root tip, the Si and As distribution were imaged in a mature root zone (~20 cm from the tip) of a WT root. This revealed that although there was some accumulation of

As in the vacuoles of the endodermis, a very high As intensity was detected in the pericycle cell vacuoles similar to that observed in Figure 6.30. The higher As signal in this sample was partly due to a longer period of As exposure (2 weeks). This strong As signal was also colocalised with a very strong S signal (Figure 6.31) suggesting that this As is again stored as arsenite-phytochelatin complexes.

The *lsi2* mutant generally showed a more pronounced accumulation of As in the vacuoles of the endodermal cells whereas the wild-type showed accumulation in the pericycle cells. This difference can be attributed to the disruption in the mutant of the Lsi2 transporter, which is localised to the proximal sides of the exo- and endodermis (Ma *et al.*, 2007), and hence arsenite efflux into the stele is restricted. For the root shown in Figure 6.30, where As accumulation was observed in the pericycle of *lsi2* young root zone, knocking out the Lsi2 transporter may have little effect because in this section of root the Casparian strip may not be fully developed, allowing solutes to pass freely through the endodermis into the stele. This would allow accumulation of As inside the stele and would explain the lack of Si accumulation, as the transporters are less expressed in this region. A strong As accumulation in the pericycle cells is observed in the mature root zone from a wild-type plant along with some endodermal accumulation in the vacuoles, as well as a preferential accumulation of Si in the proximal side of the endodermal cell walls (Figure 6.31 and Figure 6.33). These patterns could be attributed, at least in part, to the function of Lsi2. Accumulation of As in the pericycle in the wild-type plants may explain why little difference was observed in the root As concentration between the wild-type and *lsi2* mutant samples (see below in Figure 6.35 and (Ma *et al.*, 2008)); when Lsi2 is functional As accumulates in the pericycle and when this transporter is knocked out As accumulates in the endodermal vacuoles.

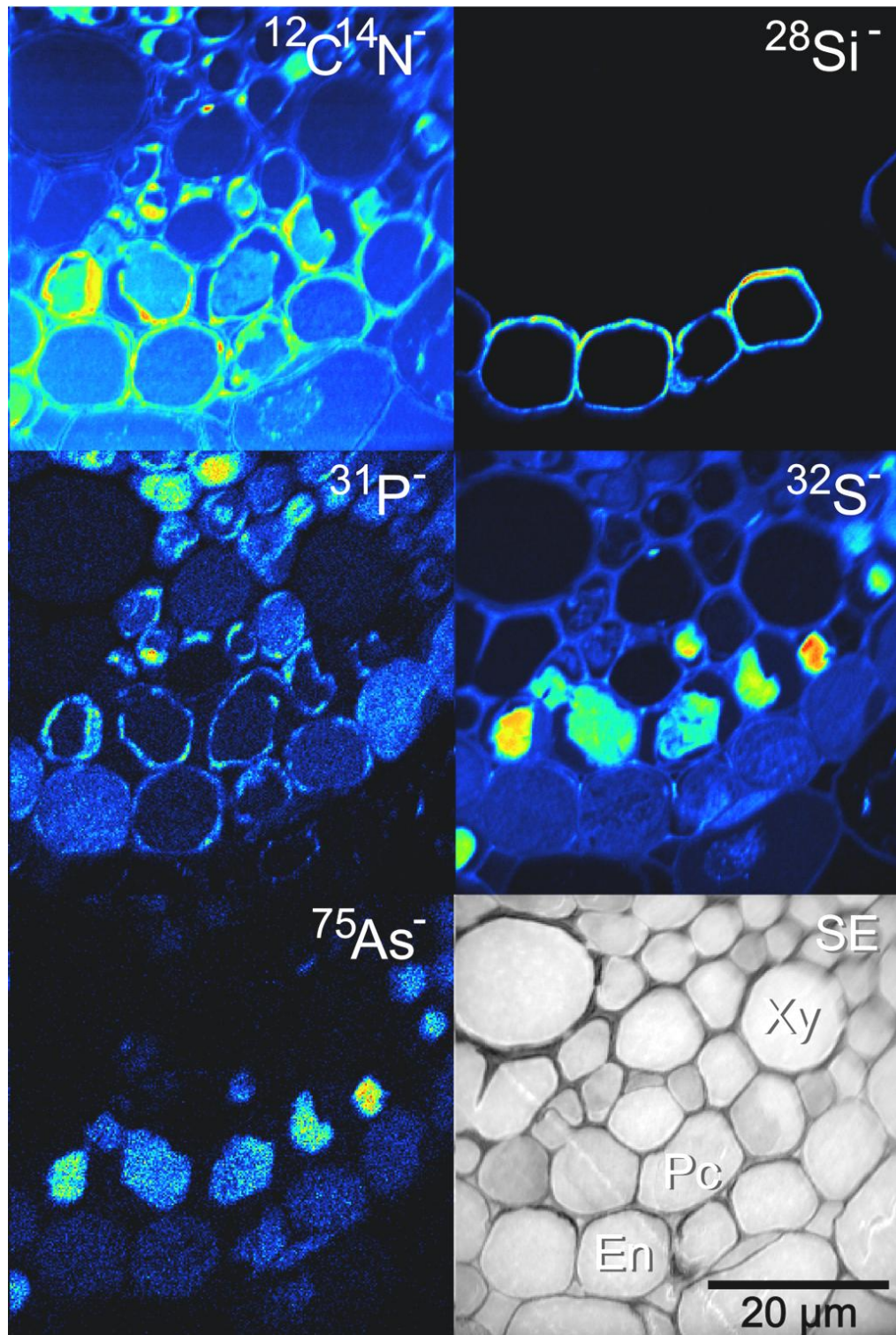


Figure 6.31: NanoSIMS image of the stele region of the mature zone of a wild-type root treated with Si and arsenite showing some As accumulation in the endodermis and very strong As accumulation in the vacuoles of the pericycle colocalised with a strong S signal. The proximal side of the endodermal cells is noticeably thicker in the SE image corresponding with a higher Si concentration. En = endodermis, Xy = xylem, Pc = pericycle SE = secondary electron image (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

In addition to As accumulation in the endodermis, another recurring feature, irrespective of whether the sample was treated with As(III) or As(V) and also in the mature root zone, was accumulation of As in the xylem parenchyma. Some examples of this are presented in Figure 6.32. As may be sequestered into the xylem parenchyma vacuoles so that As is not freely transported to the shoots. This observation was more apparent in the *lsi2* mutant samples and the treatments without Fe plaque (Figure 6.32), which is also consistent with increased As concentration in the shoots with no Fe plaque treatments.

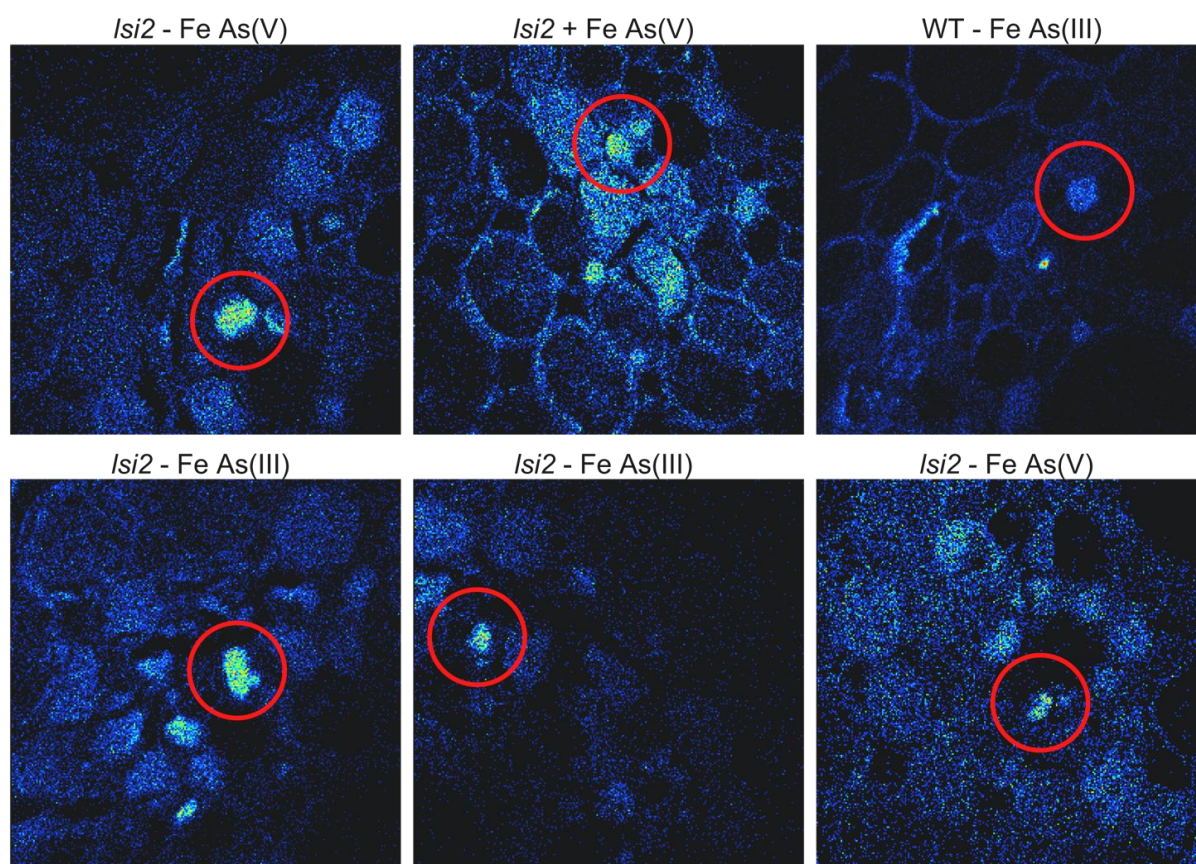


Figure 6.32: NanoSIMS images of $^{75}\text{As}^-$ showing As accumulation in xylem parenchyma cells near the root tip, as indicated by the red circles. This accumulation is more common in the *lsi2* mutants however it has also been observed in one wild-type (top right). Only the image in the top middle is from a sample treated with a ferrous solution. All images are 50 μm x 50 μm (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

These xylem parenchyma cells also contained a high concentration of S suggesting that in common with the endodermal and pericycle vacuoles the majority of As in rice roots is stored as arsenite-phytochelatin complexes. The NanoSIMS data presented here shows that the

endodermis, pericycle (especially in the mature root zone) and xylem parenchyma cells are the main locations of As storage in rice roots whilst the outer cells store little As. A concentration effect may be expected as a result of the radial transport of solutes from the outer cells to the stele. Alternatively, this pattern may reflect the sites where arsenite-thiol complexation and subsequent vacuolar transport are most active.

6.4.10. SILICON IS LOCALISED TO THE CELL WALLS OF THE ENDODERMIS

As it was not clear from the NanoSIMS images in which part of the cell the endodermal Si was localised, complementary TEM analysis was undertaken on adjacent thin sections of the root as TEM can provide cellular information at a higher resolution than the NanoSIMS. An ultrathin section was cut for TEM analysis followed by a 1 μm section for the NanoSIMS analysis, (ultrathin TEM sections were not sufficiently mechanically stable for NanoSIMS analysis). The TEM indicated that the majority of the endodermal cell walls contained a thin line of dark features (indicated by white arrows in Figure 6.33B, C). The distribution of these features was similar to that observed for Si in the NanoSIMS, being entirely absent in some cell walls. To determine if these dark features were in fact rich in Si, neighbouring cell walls were identified, one containing these features and the other without. The same cells were then located in the NanoSIMS section and a high resolution image was acquired. Figure 6.33A shows two endodermal cells, the left has dark features in the cell walls, the right does not. This is shown more clearly in the higher resolution images (Figure 6.33B, C). These higher resolution images also show further evidence of the damage which has been caused by the HPF process. Breaks in the cell walls can be seen (Figure 6.33A, C) and the higher resolution images clearly show that the contents of the cell, including the cytoplasm in some cases, have pulled away from the cell walls. Apart from the obvious fractures the cell walls appear to have remained intact allowing us to be confident that the Si distribution has not been altered. The NanoSIMS image (Figure 6.33D) clearly shows that the dark features in the TEM images are in fact Si rich. Only the left

hand cell contains Si (shown in blue) corresponding exactly with the dark features in the TEM images of Figures 6.33B and C. The $^{12}\text{C}^{14}\text{N}^-$ signal, represented in green, is elevated in the cell walls, membranes and cytoplasm with a lower signal from the vacuoles. At the top of the NanoSIMS image the red arrow indicates the position and direction of a line scan taken across the cell wall (Figure 6.33E). Both the image and the line scan show that the $^{12}\text{C}^{14}\text{N}^-$ signal originates from the cell wall and the lower peak comes from the vacuolar membrane with the $^{28}\text{Si}^-$ peak falling inside these two peaks. A similar distribution can be found in all the samples analysed, both WT and the *lsi2* mutant. From the TEM images it is clear that the higher $^{12}\text{C}^{14}\text{N}^-$ signal relates to the darker cell wall material and that the lighter grey cell wall where the Si is localised yields very little $^{12}\text{C}^{14}\text{N}^-$ signal.

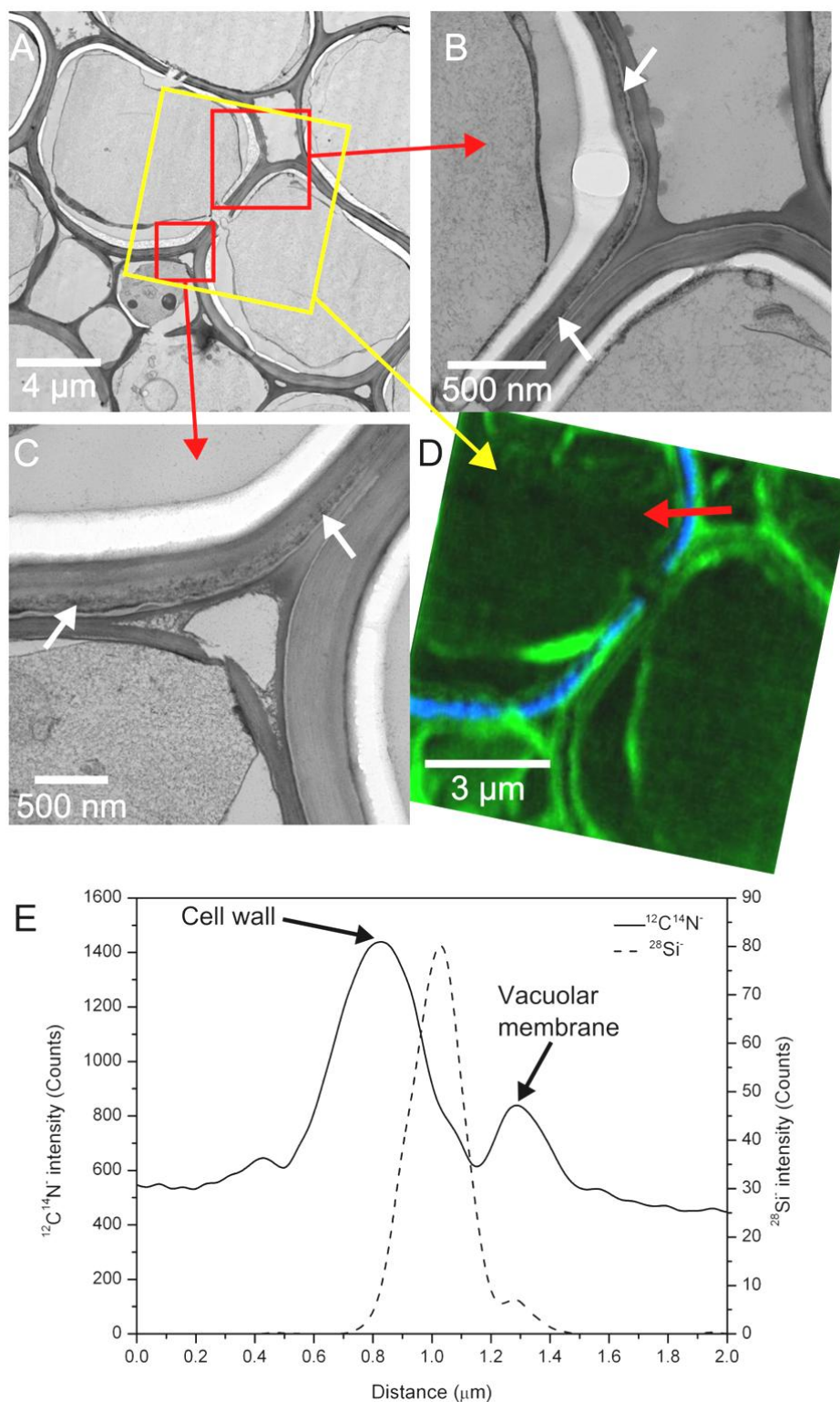


Figure 6.33: Combined TEM and NanoSIMS analysis indicating that Si is localised to some but not all endodermal cell walls. An overview of two endodermal cells is shown in A with the red squares indicating the location of the higher magnification TEM images shown in B and C. White arrows indicate the position of the dark Si-rich features. The NanoSIMS image, D, is taken from an adjacent section and the relative positions of the $^{12}\text{C}^{14}\text{N}^-$ and $^{28}\text{Si}^-$ signals are shown in green and blue respectively. The red arrow in D indicates the position of the line scan in E showing that the $^{12}\text{C}^{14}\text{N}^-$ and $^{28}\text{Si}^-$ signals originate from different locations in the cell wall (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

Imaging of the mature zone of wild-type roots, approximately 20 cm from the tip, showed an almost identical distribution of Si, i.e. Si is localised to the cell walls and completely surrounds the endodermis. The only differences were that the dark features observed in the TEM extended throughout the entire cell wall, rather than a thin line towards the outside edge of the cell, and that the proximal side of the endodermal cell walls was distinctly thicker than the distal side. The TEM and NanoSIMS images from the same cell are shown in Figure 6.34. NanoSIMS imaging revealed that the Si signal originated from a much thicker region of the cell wall and that the intensity was much higher on the proximal side as also shown in Figure 6.31.

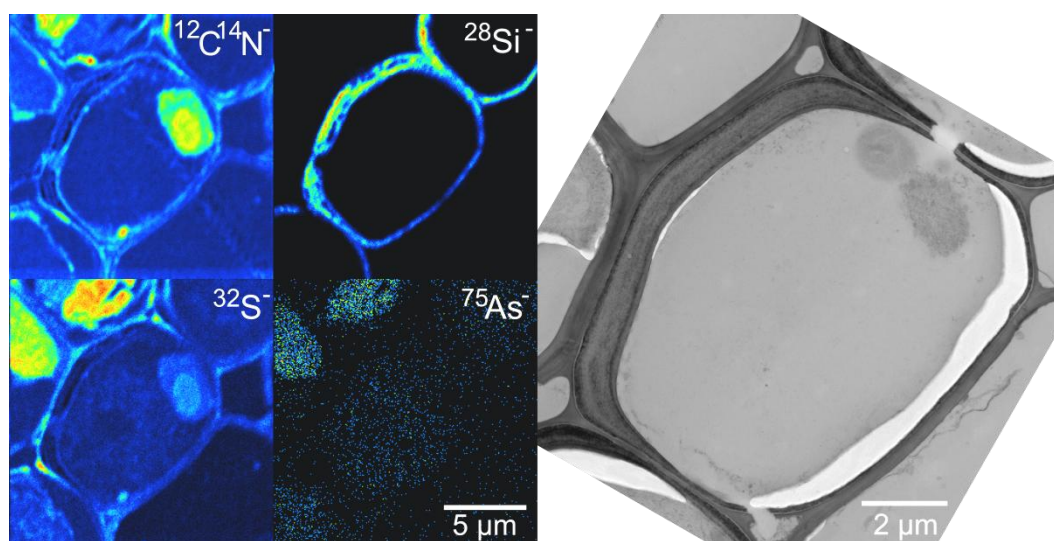


Figure 6.34: A NanoSIMS and TEM image of the same endodermal cell from the mature region of a wild-type root. The proximal side of the cell wall is noticeably thicker and contains a higher concentration of Si than the distal side. The endodermal cells show some accumulation of As however the pericycle shows a much stronger localisation of As (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

It is interesting that although Si and As are transported across the plasma membranes by the same transporters they have very different distributions in the endodermal cells. The difference between As and Si may be because of the absence of transporters to transport Si into the vacuole for storage, and this would also explain, at least partly, the much higher root-to-shoot translocation of Si than As in rice. The lack of a difference between *lsi2* and wild-type was unexpected, but perhaps not surprising considering that the two lines of rice differ significantly only in the shoot Si concentration and not in the root Si concentration (Ma *et al.*, 2007).

A few previous studies, using techniques with a lower lateral resolution than the results presented here, have shown Si localisation in the endodermis of rice roots (Parry & Soni, 1972; Lux *et al.*, 1999; Gong *et al.*, 2006). However, in these studies, with the exception of Parry and Soni (1972), Si appears to localize only to the proximal side of the endodermis, rather than completely surrounding the cell as observed in our studies. Similarly Sparks *et al.* (2010) mapped the Si distribution in the roots of two grass species (*Poa annua* and *Dactylis glomerata*) and found the same distribution with the Si localised only to the proximal side of the endodermis. Gong *et al.* (2006) stated that in their study the Si was found to be a component of the root cell walls and Parry and Soni (1972) concluded that more Si was localised on the inner tangential walls, which is consistent with the observations of the present study. A possible explanation for the discrepancy between this study and others could be related to the concentration and duration of the Si supply to the plants. In the previously published studies the duration of the Si supply was longer or the concentration was higher than the relatively low concentration of 1 mM used in this study. For the wild-type samples Si can be transported across the endodermis by Lsi1 and Lsi2 and if the Si concentration is very high, strong proximal accumulation of Si in the endodermal cell walls could be caused if Si efflux from the endodermis is greater than Si loading into the xylem as observed in the mature root zone (Figure 6.31 and Figure 6.34) and the previous studies of Parry and Soni (1972), Lux *et al.* (1999), Gong *et al.* (2006) and Sparks *et al.* (2010). For the *lsi2* mutant roots in this study an accumulation on the distal side of the endodermis may not be observed due to the low concentration and short duration of Si supplied. It is also possible in the X-ray microanalysis studies of Gong *et al.* (2006) and Lux *et al.* (1999) that there was insufficient resolution and/or sensitivity to detect the lower Si concentration in the distal side, clearly revealed by the NanoSIMS, and hence the actual Si distribution could be similar in all these samples.

6.4.11. QUANTITATIVE ANALYSIS OF AS CONCENTRATIONS

Substantial care has to be taken to ensure reliable composition data is achieved by SIMS analysis. It is well understood in the SIMS community, for instance, that it is very difficult to quantify SIMS data, mainly due to the well known matrix effect (Wilson *et al.*, 1989). Complex protocols are needed to perform robust quantitative SIMS analysis, ideally requiring establishment of relative sensitivity factors specifically for the material in question (Derue *et al.*, 2006b). This becomes very difficult with biological materials because there tend to be large variations in density and composition between different components of the cell, e.g. the nucleus is structurally and chemically very different to the cell walls. However, this matrix effect is most severe when analysing elements at concentrations greater than $1000 \mu\text{g g}^{-1}$, while at lower concentrations an approximately linear relationship between the measured and actual concentration holds (Vickerman & Gilmore, 2009). For this study therefore, with As concentrations in the few hundred $\mu\text{g g}^{-1}$ range, the effect of the different biological matrices on yield of As has been assumed to be negligible, and that variations in the local As signal detected can be assumed to be representative of the actual concentration differences in the sample. Even so, the effect of surface topography and differences in the primary ion current prevent a direct comparison of the As counts from each of the analysed areas. To quantify the NanoSIMS data from different samples the total number of $^{75}\text{As}^-$ counts was normalised with the total number of counts in the corresponding $^{12}\text{C}^{14}\text{N}^-$ image. Normalization with $^{12}\text{C}^{14}\text{N}^-$ has been found to be a useful approach to avoid these potential sources of error and allow a direct comparison between different samples and treatments (Follet-Gueye *et al.*, 1998; Derue *et al.*, 2006a). Normalization was repeated for all of the analysed regions of interest, and where possible the results were averaged over several roots from the same treatments. For some treatments the sample preservation was relatively poor leading to fewer analyzable roots and hence there are larger errors in these data sets. The total As concentration of whole roots was determined by ICP-MS from one replicate, and because the NanoSIMS only analyzes a few cells the NanoSIMS data

have been collated by adding values from the edge of the roots to the data from the centre of the roots to get representative ratios from the whole section. The ratios from the NanoSIMS were then compared with the ICP-MS data as shown in Figure 6.35. For a comparison between the two data sets, the NanoSIMS data for the wild-type sample treated with ferrous Fe and As(III) has been scaled to match that of the ICP-MS data for this treatment. The errors on the NanoSIMS data have been calculated by adding together the standard deviations for the edge and stele data.

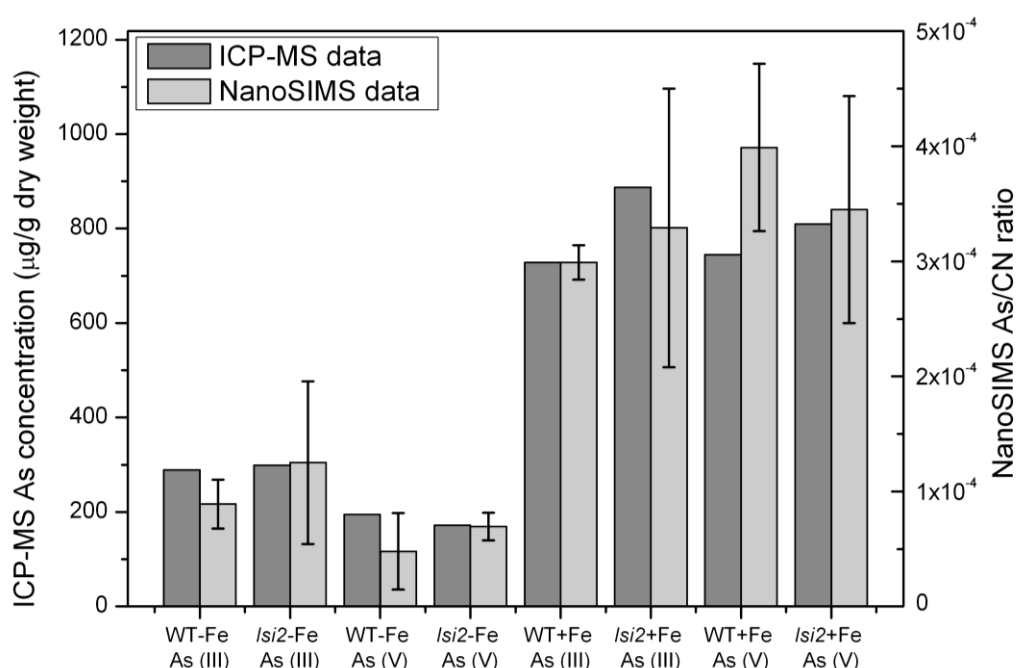


Figure 6.35: ICP-MS and NanoSIMS data for each treatment. ICP-MS data shows the total concentration of As in the roots. The NanoSIMS data shows the summed (edge and stele) $^{75}\text{As}/^{12}\text{C}^{14}\text{N}^-$ ratio and has been scaled to the wild-type (WT) + Fe As (III) ICP-MS data for comparison. Errors on the NanoSIMS data are the summed standard deviations from the edge and stele regions. ICP-MS analysis was performed on one replicate of the root samples (Moore *et al.*, 2011). Copyright American Society of Plant Biologists, www.plantphysiol.org

As shown in Figure 6.35, the NanoSIMS data show good agreement with the ICP-MS data for bulk As concentration variations. This suggests that the sample preparation has not significantly altered the As concentration, and that our methodology can be used to quantify the NanoSIMS data. Different roots with the same treatment show similar results both in terms of the elemental distribution and the normalised ratio, also suggesting that the sample preparation has not altered the elemental distributions.

7. CONCLUSIONS

This thesis has demonstrated that NanoSIMS is a powerful technique for identifying the location of important trace elements in cereal grains and roots. To my knowledge this is the first time that the precise location of part-per-million concentrations of Se and As have been analysed with subcellular resolution in these types of samples and several new features have been observed.

Chapter 4 presented a detailed study of the distribution of Se in mature and immature wheat grains. Sample preparation of mature wheat grains was found to be relatively simple, and was also found to be an appropriate method for preparing rice grains. Selenium is located relatively uniformly across the aleurone, apart from the presence of Se-rich hotspots, which could have been caused by the very high Se content in the grains analysed in this study. In the mature grains Se is localised to the protein matrix surrounding the starch granules in the starchy endosperm with a concentration that decreases from the subaleurone to the centre of endosperm. In the immature wheat grains Se is localised to protein bodies in the endosperm. The similar distribution patterns of Se and S suggest that the two elements are transported to and deposited in the grain via the same pathways and its colocalisation with $^{12}\text{C}^{14}\text{N}^-$ suggests that Se is predominantly associated with proteins. Mapping of the elemental distribution within the embryo region of mature grains was not possible due to charging. Although previous research had indicated that the embryo was a region of high Se concentration, this was not observed in the immature wheat grains. As Se is strongly bound to proteins, simple chemical fixation is all that is required to preserve its distribution without the need to use complex high pressure freezing. Iron was shown to be localised to the phytate granules which explains the low bioavailability of Fe in cereal grains.

NanoSIMS imaging of rice grain containing elevated levels of As in Chapter 5 provides the first subcellular study of As in rice grain. For the majority of the high concentration, soil-grown

grains analysed in this work As was found to be predominantly localised to the high-protein subaleurone region with the concentration decreasing towards the centre of the grain and with very little in the aleurone layer itself. The S-XRF experiment on the soil-grown high As concentration grains revealed a high As region near the ovular vascular trace which the NanoSIMS showed at higher resolution to consist of micron-sized As-regions. It is believed that this is the first time S-XRF and NanoSIMS analysis has been used on the same sample, and there is good quantitative agreement for relative As concentrations between the two techniques. In the samples treated with As(III) in hydroponic culture the As was found to be highly localised to the multilayered aleurone cells near the OVT with a high local concentration. Away from the OVT, in contrast to the soil-grown high As grains with a high DMA content, there was little As detected in the subaleurone region, it was confined to the aleurone and appears to be localised in the phytate granules. In grains with high DMA content As is predominantly localised to the subaleurone region with a decreasing concentration towards the centre of the grain. The distribution of As in rice grain appears to depend strongly on the chemical speciation, i.e. DMA or inorganic As.

Chapter 6 has shown the potential of using high pressure freezing, high resolution secondary ion mass spectrometry and TEM to localize As in the roots of rice plants and revealed a contrasting subcellular distribution of As and Si in the roots even though these two elements are transported across the plasma membranes by the same transporters. To my knowledge, this study is the first to show vacuolar accumulation in a non-hyperaccumulating plant and showed that HPF is an effective method to preserve this vacuolar distribution of As in rice roots. Where the Fe plaque forms on the root epidermis, e.g. under anaerobic conditions, it serves as a strong sink for both As(V) and As(III). Inside rice roots, As is stored in the vacuoles of the endodermis, pericycle and xylem parenchyma cells. Colocalisation of S with As in these vacuoles supports the notion that As is stored in the form of arsenite-phytochelatin complexes. Mutation of the Lsi2 transporter, making it non functional, results in stronger vacuolar accumulation of As in the endodermal cells

compared with the wild-type where accumulation in the pericycle is observed. Silicon is localised to the cell walls of the endodermis both near the root tip and in the mature root zone, and this distribution is apparently not strongly affected by the *Lsi2* mutation. TEM analysis allowed the quality of preservation to be determined, and assisted with interpreting the NanoSIMS data allowing the precise localisation of Si to be determined.

This thesis has shown the potential benefits of using physical science techniques, in particular high resolution SIMS analysis, for the study of biological problems. It is hoped that this work will contribute towards improving the understanding of the mechanisms of uptake of Se and As into cereal grains and how As is taken up and localised in rice roots.

7.1. SUGGESTIONS FOR FURTHER WORK

Although analytically very challenging, it would be interesting to investigate the distribution of Se and As in the grain samples at lower concentrations than were used for these experiments to see if the same features are observed in samples with As concentrations similar to those more commonly found in paddy fields of Bangladesh ($< 0.5 \text{ mg kg}^{-1}$) and in fields where normal concentrations of Se fertiliser have been applied ($\sim 0.35 \text{ mg kg}^{-1}$). The Se-rich hotspots may not be present in grains with lower concentrations and subaleurone localisation of As may not be observed in grain with lower concentrations of As. A more thorough investigation of the effect of different As concentrations and the speciation of As supplied to the plant has on the subcellular localisation would enable a more systematic approach to understanding the mechanism of As uptake and unloading into rice grain. Combined synchrotron and NanoSIMS studies are very powerful and further work could enable a detailed understanding of these mechanisms giving information regarding the whole grain distribution and speciation of these trace elements (synchrotron) as well as subcellular information and the distribution of important lighter elements (NanoSIMS).

It became apparent while undertaking the rice root experiment that the distance from the root tip from which the section was taken is critical to the observed Si and As distribution. Unfortunately during the course of this work there was only enough time to perform one experiment on mature rice roots. This indicated that the distance from the root tip (i.e. different root developmental stages) may in fact be important, and it would be interesting to investigate this further to see the effect of the formation of the Casparian strip, look at the root tip itself as well as lateral roots and various points along the root in the more mature regions. The effect of other mutants would also be very interesting such as the *lsi1*, *lsi6* Si transporters (in particular in the root tip) and phosphate transport mutants (transporting arsenate). It would also be interesting to investigate As localisation in the roots of a hyperaccumulator (e.g. *Pteris vittata*) to test the hypothesis that a lack of or a weak vacuole sequestration is a component of the As hyperaccumulation phenotype typified by an extremely efficient translocation of As from roots to fronds.

Very little is known about how As is distributed in the above-ground biomass and how it is transported to the rice grain. A key controlling point appears to be the nodes of the rice stem, where solutes (such as As and Si) coming from the roots are split into two streams, one through the enlarged vascular bundle to the leaves and the other through the diffuse vascular bundle to the grain. *Lsi6* has recently been identified as a key controlling transporter for distributing Si in rice nodes and it would be interesting to investigate the effect knocking out this transporter has on the As distribution in the node. It would also be interesting to look at phosphate mutants so that the mechanism of uptake can be better understood in this complex region of the plant. High-resolution imaging techniques in combination with plant transporter mutants could be very useful in revealing the functions of various transporters.

In order to more accurately quantify the As and Se concentrations in the grain and root samples analysed here it would be necessary to develop relative sensitivity factors for each of the materials in question. This would be a significant undertaking, and although it was beyond what

was required for the majority of the work in this project, would be incredibly valuable in establishing high resolution SIMS analysis as an analytical tool in trace element analysis. It would also be useful to undertake more work with the duoplasmatron to analyse the distributions of Ca^+ , K^+ , Na^+ and Mg^+ , as these are important elements which can be used to give an indication of the quality of the sample freezing and preservation.

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