Specimen Preparation and Calibration for NanoSIMS analysis of Biological Materials

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Abstract

In order to achieve reliable and reproducible analysis of biological materials by SIMS it is critical both that the chosen specimen preparation method does not modify substantially the in-vivo chemistry that is the focus of the study and that any chemical information obtained can be calibrated accurately by selection of appropriate standards. In Oxford we have been working with our new Cameca NanoSIMS50 on two very distinct classes of biological materials; the first where the sample preparation problems are relatively undemanding – human hair – but calibration for trace metal analysis is a critical issue, and the second – marine coccoliths and hyperaccumulator plants – where reliable specimen preparation by rapid freezing and controlled drying to preserve the distribution of diffusible species is the first and most demanding requirement, but worthwhile experiments on tracking key elements can still be undertaken even when it is clear that some redistribution of the most diffusible ions has occurred.

Keywords: NanoSIMS, Biological Materials

1. Dynamic SIMS Analysis of Biological Materials

D-SIMS microscopy has not yet been applied extensively to biological materials even though for some 20 years it has been known that the technique offers significant analytical advantages, including particularly exceptionally high sensitivities and high specificity. When combined with the sub-micron lateral resolution of the latest generation of instruments, this technique offers the potential to provide insight into many areas of biological research where inter-cellular chemical variations in 3-dimensions have critical and as yet poorly understood effects. D-SIMS analysis in the Life Sciences was first discussed in detail in 1982 by Burns et al. [1] and the whole of Volume 74 in the journal Biology of the Cell was dedicated to D-SIMS analysis of biological materials.

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2. Preparation of Biological Samples for SIMS

Investigation of the distribution of inorganic macronutrient elements such as potassium, calcium and magnesium at the sub-cellular level in biological systems is one of the most challenging problems in cell biology. These physiologically important ions are highly diffusible in nature and play important roles within the cell. Due to the high vacuum requirements of analytical techniques like SIMS, live cells cannot be analyzed. Therefore, cells must be preserved as close as possible to their native state so that analysis reveals the real chemical makeup of the living cell, not artefacts caused by the preparation process. This poses a classical problem for chemical analysis by physical science techniques, and makes sample preparation the most critical step in sub-cellular ion studies with SIMS because loss or redistribution of these easily diffusible ions is frequent, occurs rapidly and is difficult to prevent after sectioning and during preparation. Thus careful fixation of the tissue is a prerequisite, and an ideal sample preparation should fix the cells in such a way that it preserves the *in vivo* ion distribution of the cell and does not alter the cell matrix. The preparation of biological samples for SIMS has been an area of intense research and debate in recent years [see for instance 1-4]. There are two approaches for preparation of biological samples; chemical and cryogenic fixation.

3. Chemical Fixation

Conventional methods of biological sample preparation for morphological analysis by transmission electron microscopy (TEM) depend on aqueous chemical fixation and resin embedding, but these cannot be used effectively for chemical analysis by SIMS because chemical fixation destroys the native chemical composition of the living cell. In a study of the easily diffusible mobile ions potassium, calcium and magnesium in a soybean leaf, Grignon et al [5] demonstrated that after chemical fixation redistribution and absorption occurred on certain cell organites of the leaf. A glutaraldehyde-based fixative procedure was used and potassium, calcium and magnesium were shown to co-localise at the periphery of the cell, whereas the physiological cytoplasmic distribution of potassium and magnesium could only be analysed correctly after sample freezing in liquid propane. Chemical fixation methods are thus only really suitable for the analysis of elements bound to macromolecules, DNA-interacting drugs and other covalently bound tracers not highly diffusible ions [6]. Chandra asserts that although some publications support less rigorous sample preparation methodologies, only the strictest cryogenic sample processes should be employed for the localization of diffusible ions with SIMS [3].

4. Cryo-fixation

During rapid freezing, the integrity of the specimen depends on the rapidity of the cooling. Very high speed freezing is essential to limit the size of the ice crystals which damage cell membranes, and the time lapse between excision of tissue and freezing must be minimal. The most commonly used techniques freeze-fix the samples in liquid nitrogen, propane or isopentane, but these frozen materials can then be processed in several ways. The specimen may be analysed as it is with the ice still in place or the ice may be removed by freeze substitution or freeze drying and then resin embedded for analysis.

If a microscope with a suitable cryogenic stage (and appropriate fast entry airlocks to avoid warming) is available, then the frozen sample can be analysed with the vitreous ice still in place. Rather little is known about the ionisation process in direct SIMS examination of frozen hydrated specimens, but it has been found that analysis of frozen hydrated cells under a dynamic primary

beam preferentially removes water along the z-direction. This effect also enhances other analyte signals and may cause false imaging contrast in ion images [7].

The analysis process is simplified if the ice can be removed from the sample without modifying the local concentration of diffusible ions, although inevitably there must be at least a redistribution of ions as a result of the volume change as the ice is removed. Freeze substitution procedures remove ice from the frozen cell interior at low temperatures (-80 °C) using solvents such as ether, acrolein, or acetone. This kind of process may preserve the native distribution of some ionic and molecular species, but successful freeze substitution depends critically on the maintenance of a dry environment at all stages of the procedure [5]. Alternatively, freeze-drying (or lyophilization) is a technique where a frozen sample is dried in a slow and controlled manor by the sublimination of water vapour in a vacuum.

After one of these two processes to remove water ice, it is common in the preparation of TEM specimens to resin embed the sample. This inevitably causes a major alteration of the cell matrix which is not desirable for the specimens designed for chemical analysis by SIMS [8]. However, resin embedded samples have flatter surfaces (which is important for avoiding topological artifacts in the SIMS images) and local charging effects can be minimized because a tissue equivalent resin ensures a uniform matrix between the cells and in the interstitial spaces [5]. More critically, this procedure does cause loss and relocation of rapidly diffusing ions, especially when using hydrophobic resins.

Figures 1 and 2 show two examples from our recent work in Oxford of the kinds of artifacts that can be created by these well-established freezing and resin impregnations techniques. For the first example, specimens of *Alyssum lesbiacum* (Candargy) Rech. f.* [family Brassicaceae] were grown in the Department of Plant Sciences in Oxford from seed collected from the island of Lesbos. After germination, plants were cultivated on a 1:1 mixture of perlite and serpentine topsoil (to a depth of 250 mm) from the Lizard Peninsular in Cornwall. Terminal shoots of *Alyssum lesbiacum* leaf tissue were frozen ultra-rapidly in liquid propane and held at -196°C immediately after removal from the plant. The frozen tissue was then dehydrated slowly over 5 days at high vacuum (10^{-4} Pa) by molecular distillation drying at the triple point of water in a Life Cell Molecular Distillation Drying apparatus. The heating rate was 10 °C h^{-1} from –196 °C to –150 °C. The rate was then slowed to 1 °C h^{-1} from –150 °C to –20 °C through the range at which most sublimation occurs. Once at room temperature, the samples were infiltrated with Spurr Resin over a period of two months, during which period they were manually agitated daily. They were then polymerised under ultraviolet light over a period of two days at a temperature of –20 °C. Sections from the embedded blocks were then dry sectioned using a RMC MT7 ultramicrotome with a Drukker diamond knife to a thickness of 100 nm. The sections were transferred to 3 mm TEM copper grids, which had a formvar plus carbon support film [9].

The optical micrograph in Figure 1(a) shows the extremely good preservation of the structure of this plant section. Details like the characteristic hairlike trichomes on the leaf surface plugged into a layer of rounded epidermal cells are clearly seen, with the oblong mesophyll cells under the surface. Some intracellular structures, such as chloroplasts, are also visible. These samples are also sufficiently stable to undergo, with care to avoid excessive ion beam exposure, extended analysis in the Cameca NanoSIMS50 in Oxford. Figure 1(b) shows a CN^- ion image which reveals many intracellular features. A region containing spiral thickening in the xylem tissue is visible. Cross-sections through the lignin thickening around the inside of the xylem element can be seen, as can starch grains within the chloroplasts of adjacent mesophyll cells. While the internal morphology of the plant cells is clearly well preserved, Figure 1(c) shows that the distribution of diffusible species is not. In this pair of images, taken with the oxygen incident beam, the Na and K signals are primarily from the exterior membranes of the cells, suggesting serious migration of these ions from the positions characteristic of living cells during the drying and resin impregnation process even though rapid freezing was used at an earlier stage in the specimen preparation.

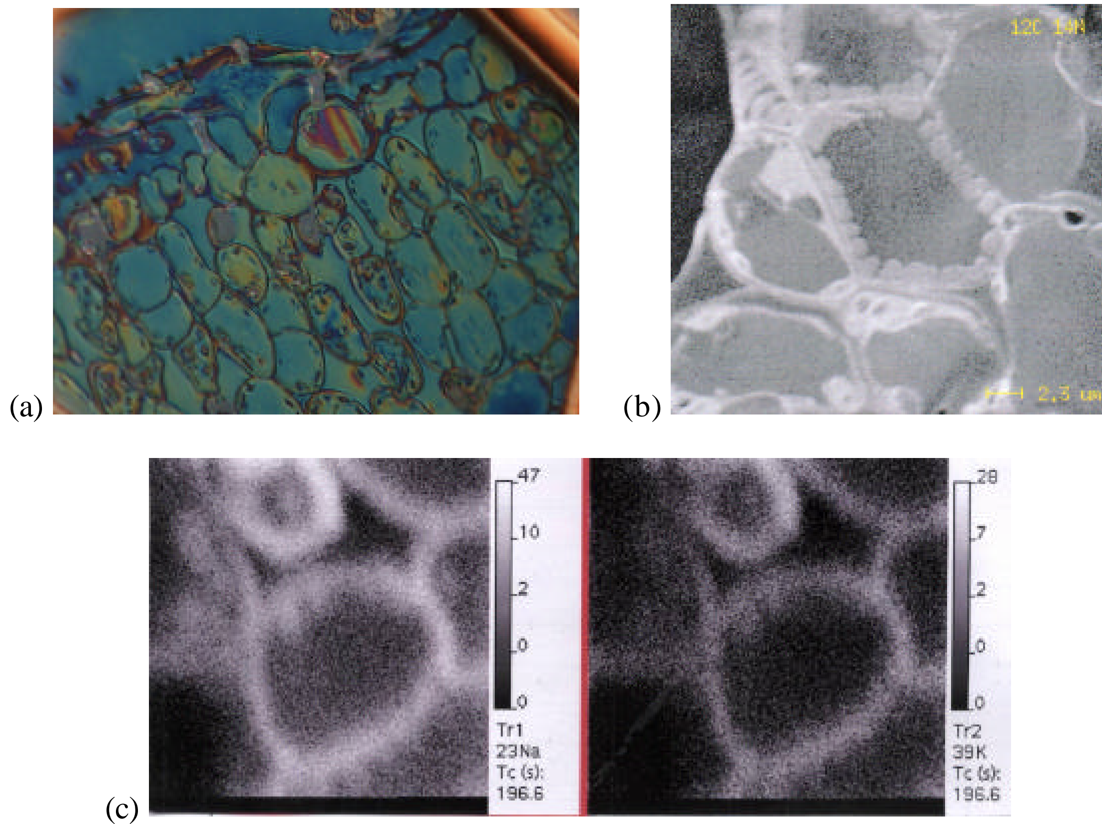

Fig. 1. (a) and (b) Optical micrograph and NanoSIMS CN^- images (taken using the Cs^+ incident beam) of freeze dried and resin substituted samples of *Alyssum lesbiacum* showing the good preservation of cell morphology. (c) Na and K ion NanoSIMS images of the same samples taken with the incident oxygen beam demonstrating the migration of diffusible species to the cell walls during specimen preparation.

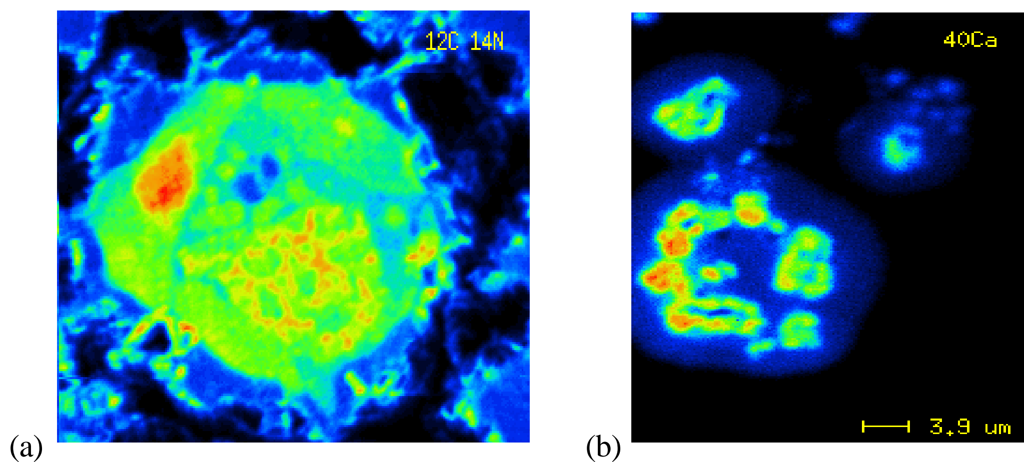

Fig. 2. (a) NanoSIMS CN^- image (using the Cs^+ incident beam) of *Coccolithus pelagicus* prepared by cryofixation and resin embedding showing the good preservation of internal cell morphology. (b) $^{40}Ca^+$ ion NanoSIMS image of the same kind of sample taken with the incident oxygen beam demonstrating the apparent spreading of the Ca outside the external calcite platelets as a result of the specimen preparation process.

A second example of the problems that can arise with this kind of specimen preparation is illustrated in Figure 2. We have been studying the sequestration of Ca, Mg and Sr in Coccolithophores, unicellular, calcifying oceanic phytoplankton, in order to build a mechanistic understanding of ion transport during biomineralisation and how this impacts the shell chemistry of marine organisms. We have studied thin (1µm) sections of *Coccolithus pelagicus* prepared by cryofixation and resin embedding. Figure 2(a) shows a CN- image of one of these cells, and the

excellent preservation of the internal structures and external calcite platelets is clear. However, the $^{40}Ca^+$ image in Figure 2(b) indicates that, at least in some specimens, the sample preparation has resulted in the spreading out of the Ca ions into the surrounding resin matrix. Clearly this kind of artifact will make a study of the trafficking mechanisms of diffusible ions very difficult. However, as will be shown below, it is still possible to perform reliable and revealing chemical analysis on the mineral components of these organisms.

5. Freeze Fracturing

An alternative freeze-fracture-drying procedure has been developed by Chandra and Morrison for SIMS studies of cell cultures. The cells to be studied are cultivated directly on a silicon wafer. During sample preparation, a second piece of silicon is placed on top of the cells which are prevented from squashing by the addition of polystyrene balls. This sandwich is then fast frozen as described above, and the two pieces of silicon are prized apart with the intent of fracturing some of the cells in half. The whole sample is then freeze dried without the use of any resin infiltration step [2]. Chandra and coworkers have used this specimen preparation strategy to work on some key topics in cancer research; including the mapping of Ca stores in cells undergoing mitosis and cytokinesis by the use of ^{44}Ca tracer ions introduced to distinguish natural physiological Ca from that introduced during treatment, and the location of B in the body during boron neutron capture therapy (BNCT). One key issue that has to be addressed with this kind of sample, however, is the intrinsic roughness of the surface of samples which will introduce severe topographic contrast in the images. Figure 3 shows an example of a Coccolithophore specimen prepared in this way.

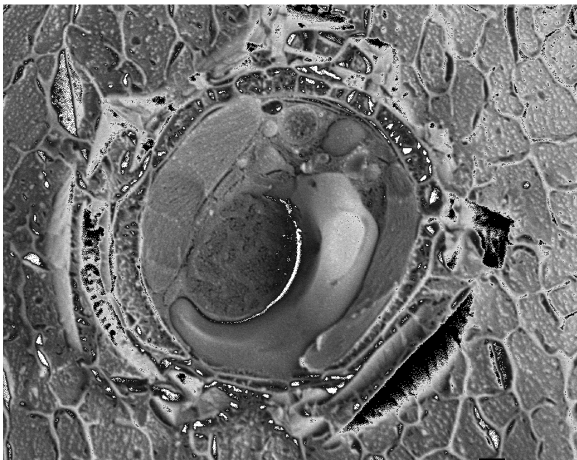

Fig. 3. An example of an individual coccolithophore prepared by freeze-fracture by Dr. Alison Taylor and Prof. Colin Brownlee of the Marine Biological Association in Plymouth. The significant surface relief usually resulting from this method of sample preparation is clearly seen.

6. Validation of Mobile Ion Fixation

Although the preservation of structural integrity in biological specimens can be easily checked by optical or TEM imaging and in the kind of SIMS ion images shown in Figures 2 and 3, the preservation of chemical integrity at the sub-cellular scale is much more difficult to evaluate. One very effective way to be sure that the specimen preparation method has not modified the *in vivo* local concentration of diffusible ions is to measure potassium/calcium abundance ratios [2, 3, 5]. For instance, in a typical living plant cell the potassium concentration is highest in the cytoplasm and calcium is most abundant in the cell walls. This distribution should be reflected in the preserved sample, and if it is not then ion redistribution has occurred. Several groups have reported that this kind of analysis has been successfully used to validate their sample preparation methods [10, 11].

7. Recent NanoSIMS analysis in Oxford on biological samples

7.1. Analysis of Human hair

Recently reported results on the analysis of hair samples using a NanoSIMS [12] illustrate very well how the distribution of trace elements can be easily measured in this kind of sample. Similar experiments by Kempson and Skinner [13] using TOF-SIMS have mapped a larger number of ionic species, but with a much lower spatial resolution that makes it possible only to distinguish variations in local chemistry between the surface and interior of the hair. Because the hair is dead tissue, specimen preparation is extremely simple – a conventional microtome is all that is required to prepare a reasonably flat cross-sectional surface for analysis, as shown in the ion image in Figure 4(a). We have been using normal human hair samples to test and calibrate the ability of the NanoSIMS in the study of dilute concentrations of metallic elements in biological samples. Figures 4(b and c) shows an example of this kind of experiment, where the iron in melanin granules in Asiatic hair has been mapped with the Duoplasmatron oxygen ion beam. A key technical issue in this experiment is to avoid the interference between the $^{40}Ca^{16}O^{+}$ and the $^{56}Fe^{+}$ ion signals, separated by only 0.02765 mass units which requires a mass resolution better than 2000. The approximate natural concentration of iron in hair is a few parts per million, and we are currently attempting to quantify the iron content in the melanin granules by the use of appropriate dilute standards. Copper is also present in the melanin granules at about the same concentrations, and can also be mapped in similar experiments.

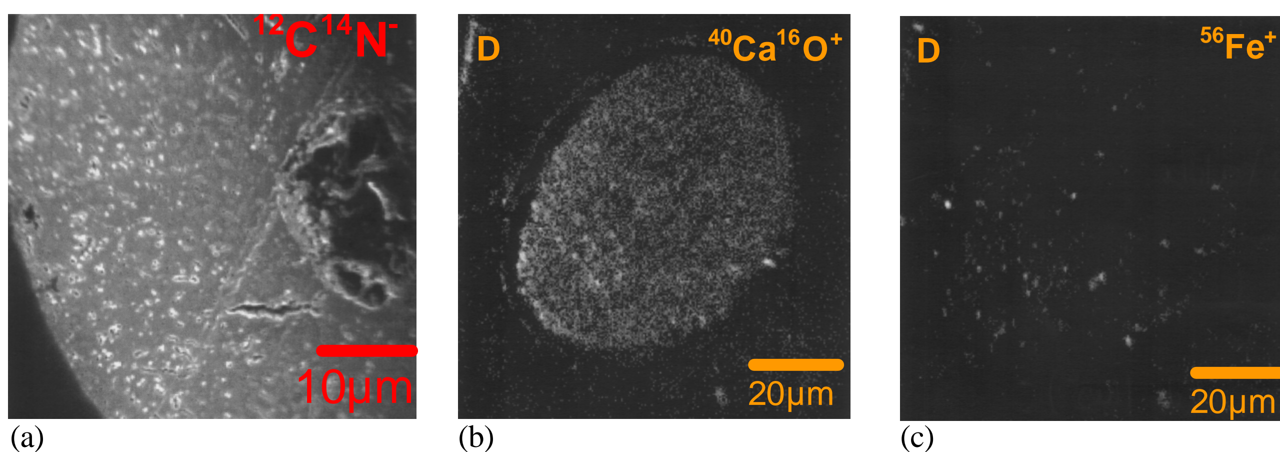

Fig. 4. (a) CN^- ion image of an asiatic hair taken with the Cs+ incident beam. The melanin granules are taken as in Hallegot et al [12] to be the brightly imaging regions, but noting that topographic contrast from the edge of the pores created by nuclear remnants also contributes to these bright spots. (b) and (c) CaO^+ and Fe^+ ion images of a different hair section showing that the two ion species at very similar masses give quite different characteristic distributions, confirming that peak overlap is not contributing significantly to the iron image.

7.2. Biomineralisation in marine coccoliths

We have also been studying the distribution of Mg, Sr and Ca in the calcite platelets, which are precipitated intracellularly in a specialized vesicle before extrusion onto the cell surface, in similar *Coccolithus pelagicus* specimens to those shown in Figure 2. Figure 5 illustrates that mapping MgO, SrO and CaO molecular ions reveals the unexpected fact that, whilst Sr and Ca map on top of one another and therefore respond very similarly during biomineralisation, the Mg and Ca do not co-locate in the external platelets. It appears that the Mg is concentrated into a rind around the final edge of the precipitating platelet. A working hypothesis is that the coccolithophore may employ the calcite precipitation inhibiting properties of Mg, by enhancing the concentration of Mg

in this vesicle in order to quench precipitation when the platelet has reached the required size, before extrusion. The high spatial resolution achievable in the NanoSIMS is very important in this experiment, and we believe that chemical modifications during specimen preparation are unlikely to affect the platelet chemistry to any significant extent.

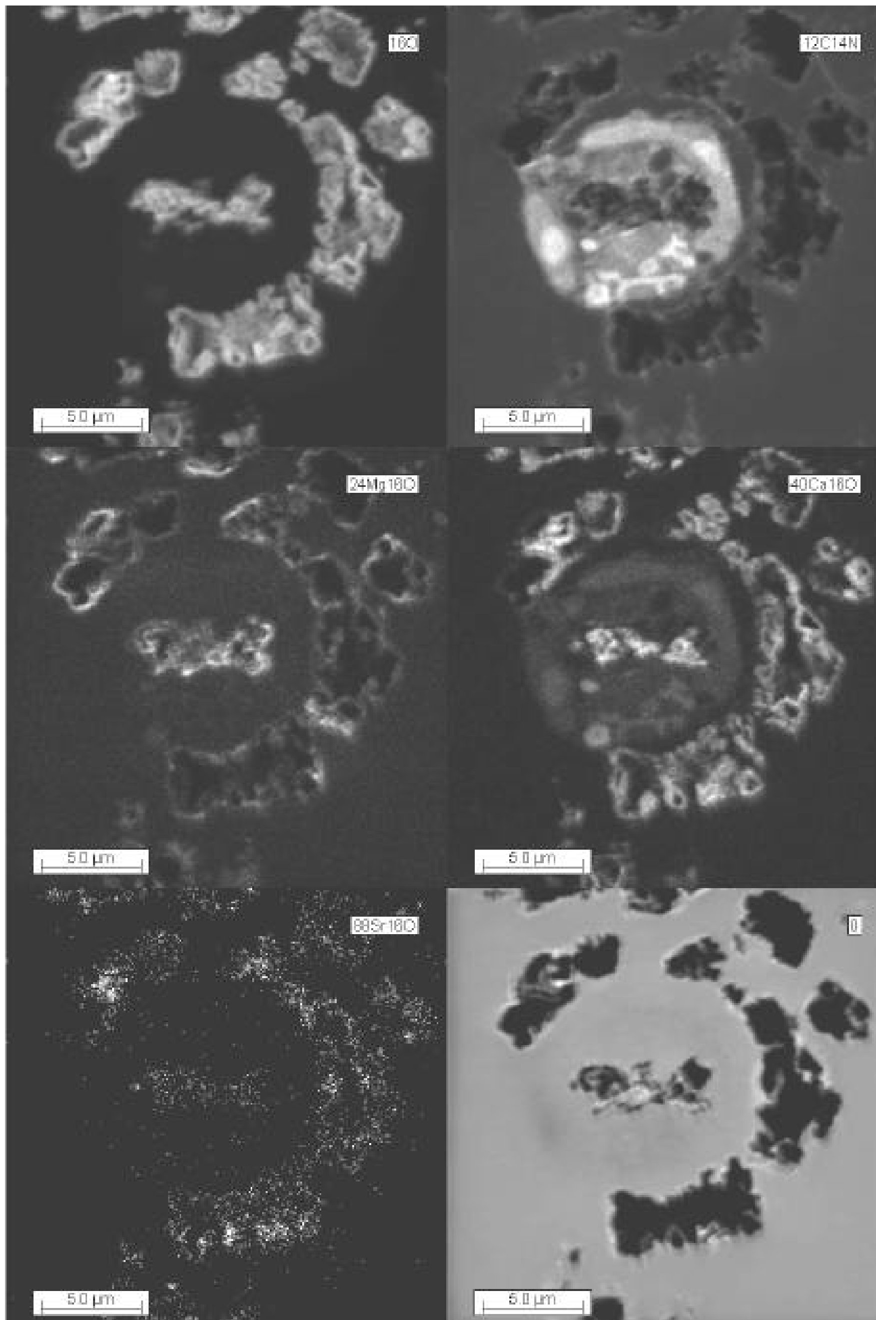

Fig. 5. NanoSIMS images taken using the Cs^+ incident beam of a *Coccolithus pelagicus* prepared by cryofixation and resin embedding. The oxy gen and CN^- images at the top clearly reveal the external platelets and the central cell. Once again, the internal processes are clearly to be seen in the CN^- image. The separation of $^{24}Mg^{16}O^-$ and $^{40}Ca^{16}O^-$ in the two central images is striking. The internal structures of the central cell are also well revealed in the $^{40}Ca^{16}O^-$ image on the right. The two lower images are from $^{88}Sr^{16}O^-$ and the secondary electron image respectively. The $^{88}Sr^{16}O^-$ appears to be co-located with the $^{40}Ca^{16}O^-$.

7.3. Ni sequestration in hyperaccumulator plants

A final example of recent work in Oxford is on the location of metals in the hyperaccumulator plants described above and in Figure 1. We are trying to explore the mechanisms of metal trafficking in these plants and the localisation within the plant structure of the micronutrients. Previous work on hyperaccumulator plants conducted in Oxford University has shown that uptake of nickel is associated with a specific and proportional synthesis of the aminocarboxylic acid histidine, which strongly coordinates the incoming nickel and facilitates its translocation through

the plant [14]. It is thought that when nickel is accumulated in the shoot, it is sequestered partly in the central cell vacuole in the form of soluble complexes with carboxylic acid. However, a considerable fraction of the nickel accumulates in the peripheral cells of the shoot, and nickel may reach very high concentrations in the epidermal hairs, know as trichomes, which are unicellular stellate structures that cover the surface of species such as *Alyssum lesbiacum.* In the previous work in Oxford, leaf cross-sections of *Alyssum lesbiacum* were investigated by Krämer et al. [9] using proton induced X-ray emission (PIXE) with a spatial resolution approximately 1 µm. The results indicated that nickel is sequestered to a considerable degree in the epidermal trichomes, but the technique does not allow the finer sub-cellular scale analysis needed to explore the mechanisms of sequestration. In contrast, Küpper et al [15] reported the nickel to be predominantly localised in the vacuoles of epidermal cells rather than the cell wall in the leaves of *Alyssum bertolonii*, *Alyssum lesbiacum* and *Thlaspi goesingense* using energy dispersive x-ray analysis of rapidly frozen hydrated material performed on a scanning electron microscope with a cryostage attachment. Their EDX results also apparently indicated exclusion of nickel from trichomes of *Alyssum bertolonii* and *Alyssum lesbiacum*, but, confusingly, staining with dimethylglyoxime appeared to indicate the presence of Ni in the basal part of the trichomes.

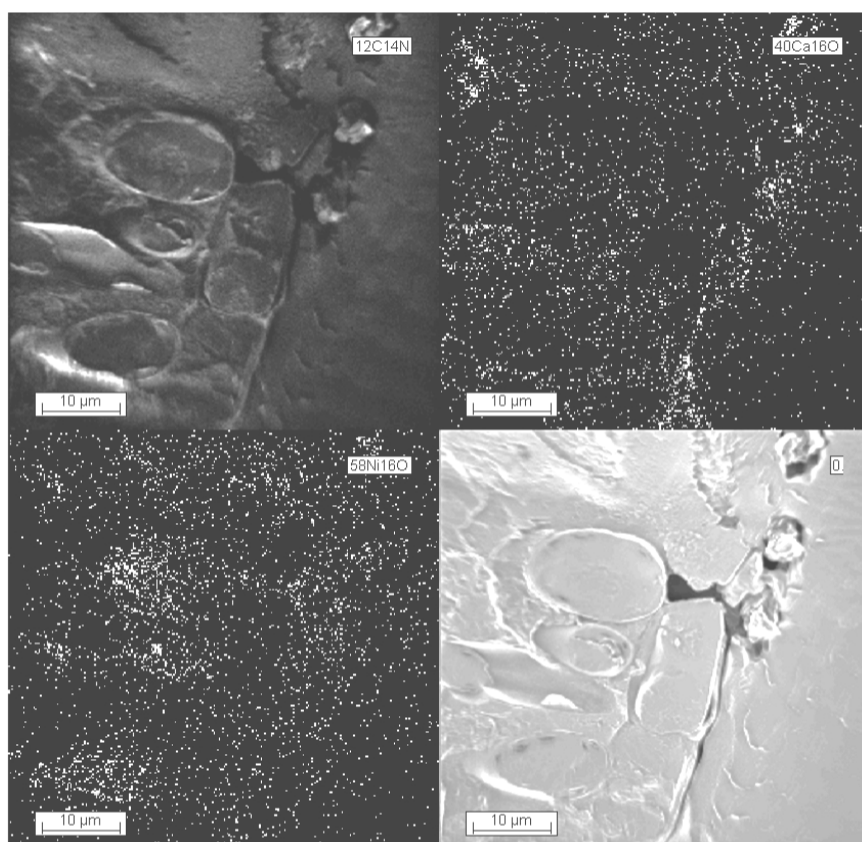

Fig. 6. NanoSIMS CN^- images (taken using the Cs^+ incident beam) of freeze dried and resin substituted samples of *Alyssum lesbiacum* showing a region of the leaf surface containing a large trichome with root structure below the surface of the leaf. The top two images are from CN^- and $^{40}Ca^{16}O^-$ ions respectively, while the lower two images are from $^{58}Ni^{16}O^-$ and the secondary electron signal. The Ca seems to be preferentially located in the trichome branches while the Ni is more located in the root structure.

We have been able to show with the higher spatial resolution available in the NanoSIMS that the counts in the $^{58}Ni^{16}O^-$ ion image are concentrated in the basal part, or root, of the trichome while the $^{40}Ca^{16}O^-$ appears most concentrated in the trichome 'branches' that lie on the leaf surface, Figure 6. These observations may help explain the discrepancies between the observations of the two previous groups, but the work is as yet at a rather preliminary stage. Because the trichome structures are dead before excision of the leaf and sample preparation, we expect the redistribution of the sequestered metallic species to be less of a problem than with the diffusible species in Figure 1.

8. Conclusions

By selecting appropriate samples, it is possible to study some interesting problems in biological materials where redistribution of diffusible species during specimen preparation is not a critical problem. We have here shown several examples of analyses carried out using the Oxford NanoSIMS where the distribution of metallic species in relatively robust parts of a biological system can be studied reliably and reproducibly, and can assist in generating improved models for sequestration and biomineralisation processes. The final aim of all these projects is to explore the mechanisms of trafficking of the metallic species at much higher resolution, but this will require improvements in the specimen preparation protocols.

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