Elemental Analysis of Biological and Cryogenic Samples

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Introduction

Commercial production of large-area silicon drift detectors (SDDs) heralds an exciting new time in biological elemental analysis. Improved low-energy performance and higher collection efficiencies mean EDS is no longer limited by low count rates and poor solid angles. High count rates can be achieved on all types of materials, including soft biological tissues, without altering normal SEM operating conditions, meaning biological analysts can now acquire localized *in-situ* elemental analysis in seconds rather than hours. In this article we present comparative results from a variety of biological and cryogenic samples, analyzed with both a traditional 10 mm^2 EDS detector and an Oxford Instruments X-Max^N 150 mm^2 SDD detector.

Materials and Methods

Silicon drift detectors. Energy Dispersive Spectrometry (EDS), based on the conventional Si(Li) detector, has been used for many years to analyze the chemical composition of materials in scanning electron microscopy (SEM) and transmission electron microscopy (TEM) microscopes. Around 10 years ago, researchers were introduced to a new type of EDS detector. Known as a silicon drift detector (SDD), this detector provided fast mapping and high count rates to everyone who could generate sufficient counts from their sample—and, as a bonus, there was no need to remember to fill up the liquid nitrogen dewar because the new detectors were LN_2-free.

Large-area SDDs. Now, a new generation of large-area SDD detectors for SEM and TEM have been released with active areas of up to 150 mm^2. Further improvements in light element performance and collection efficiencies mean that analysts can now gather up to 15 times the counts achieved on an old 10 mm^2 detector without changing any of their other collection conditions (Figure 1) and crucially, without affecting resolution and performance. This greatly improves the EDS analysis prospects for biological and cryogenically frozen samples and allows rapid *in-situ* X-ray mapping of large areas. An X-ray "map" is defined as an image showing the intensity of a specific element at each pixel in the spectrum—element maps can be shown individually, on top of the electron image, or layered together to show the elemental distribution of several elements simultaneously.

Fast elemental mapping. Analysis is no longer limited to single-point spectra. Now, the combination of a large-area sensor with the superior count rates offered by the SDD and improved software capabilities means that fast mapping

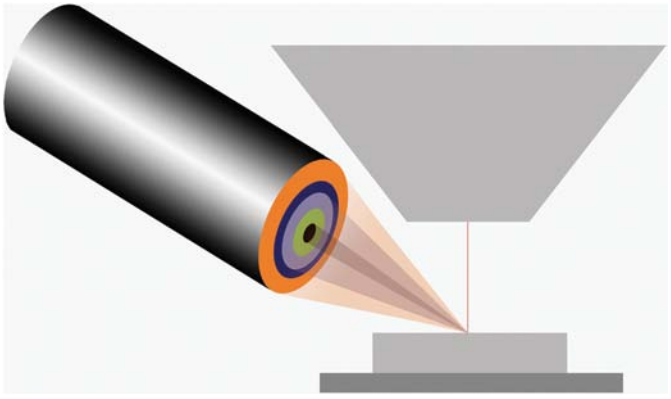

Figure 1: A traditional 10 mm^2 detector (black) has a small collection angle and captures few counts. Modern large-area SDDs range up to 150 mm^2 in area (orange) and capture up to 15 times as many counts in the same acquisition time.

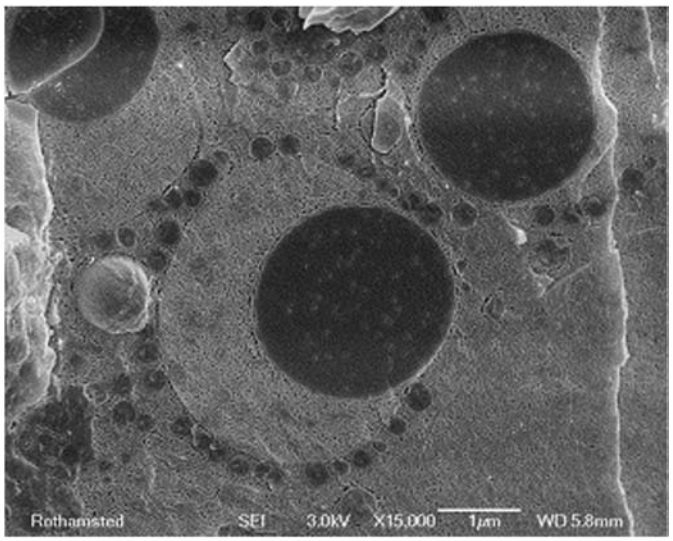

Figure 2: SEM secondary electron image showing the internal structure of a wheat aleurone cell. Image courtesy of Jean Devonshire, Rothamsted Research.

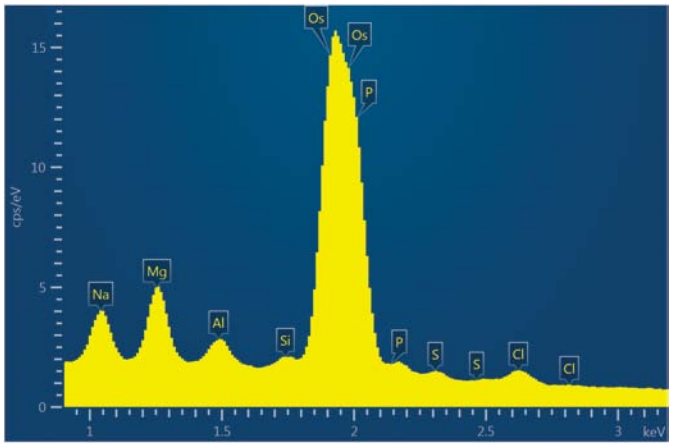

Figure 3: EDS sum spectrum of a wheat seed, acquired while scanning the image of Figure 4b, showing the overlap between the Os Mα and P Kα X-ray peaks. SDD sensor active area = 150 mm^2; acquisition time = 3 minutes.

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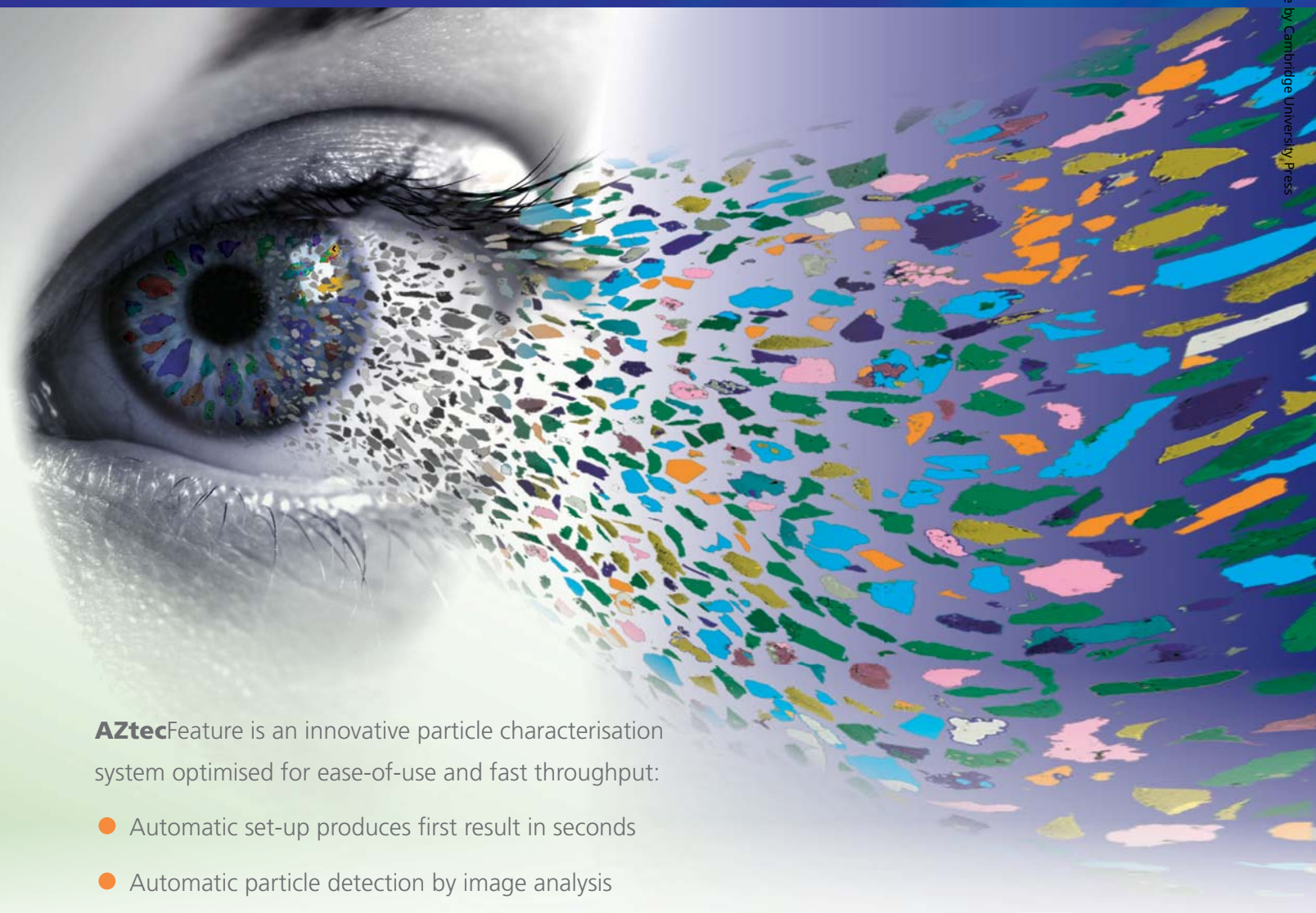

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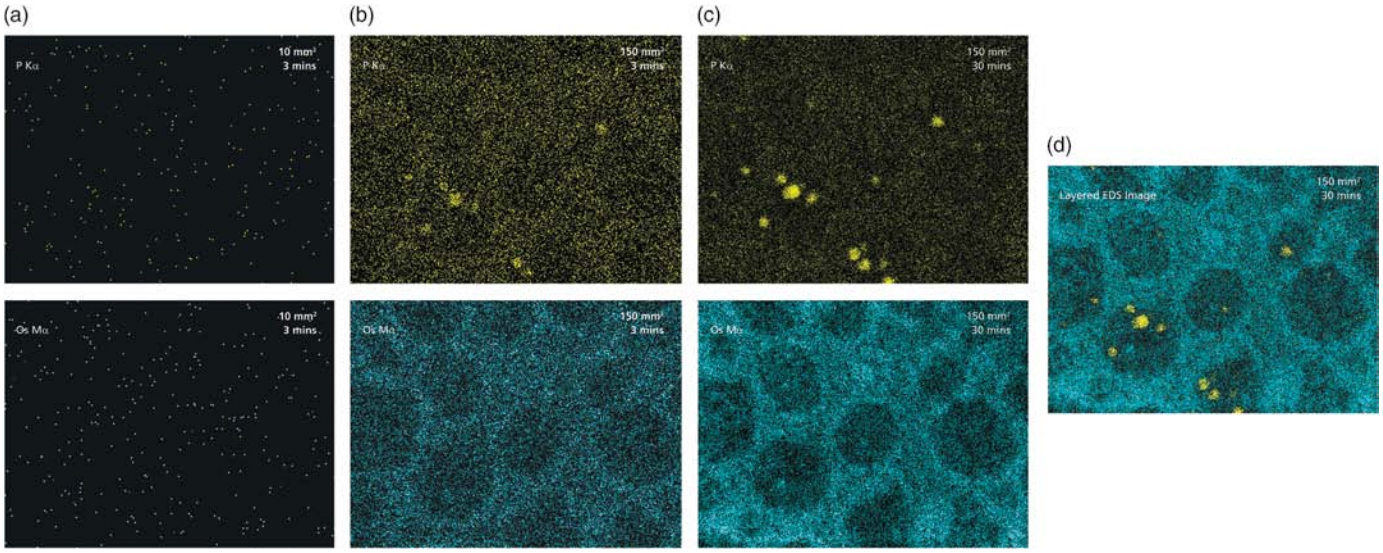

Figure 4: X-ray maps of wheat aleurone cell acquired at a high count rate. The top row shows locations of high intensity for the P K-series X rays, and the bottom row shows the distribution of the Os M-series. (a) Traditional 10 mm² detector, 3-minute maps, (b) 150 mm² detector, 3-minute maps, (c) 150 mm² detector, 30-minute maps, (d) EDS layered image showing combined 30-minute Os and P maps. Image width = 12.8 µm. All the data were collected with an accelerating voltage of 4 kV.

becomes an integral part of the everyday analysis tool. In conjunction with the ability to map large areas of the sample (millimeters across), all data are saved as a Spectrum Image (SI) datacube (*x* by *y* by *Energy*), meaning it can be collected, analyzed, and re-analyzed offline [1].

Hard biological specimens. Until recently, the excitement of fast EDS analysis eluded the biological analyst. Because of low fluorescent yields and significant absorption effects, a light organic matrix produces fewer X-ray counts than traditional inorganic or solid-state materials would. Historically, small detector solid angles resulted in poor X-ray count rates, and X-ray mapping was typically very slow with a poor signal-to-background ratio. Point analysis was typically the only way to get results—a hit-or-miss technique on biological samples, which were often inhomogeneous and poorly understood.

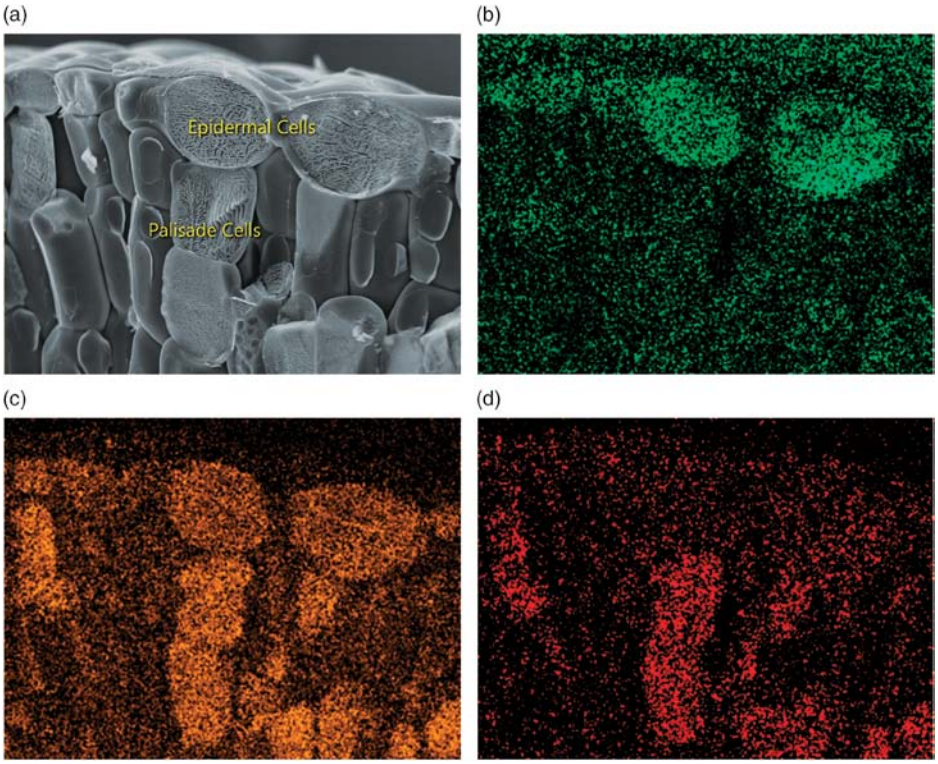

Figure 5: X-ray maps from *Thlaspi caerulescens* (Alpine Pennycress), a plunge-frozen fully hydrated sample, acquired with a 150 mm² detector over 20 minutes at 15 kV. (a) SEM secondary electron image. (b) Zn Kα map (green), (c) P Kα map (orange), and (d) Ca Kα map (red). Zinc is concentrated in the epidermal cells and calcium is located mostly in the palisade cells. The sample was gold-coated to reduce charging. Image width = 179 µm.

Throughput on inorganic samples is traditionally improved by increasing beam current to increase count rate. This method works well for hard biological specimens, such as bones and teeth, which are not damaged by the beam, and takes full advantage of the high-count-rate capabilities of a SDD detector to gather spectral data and produce maps quickly and efficiently.

Soft biological samples and cryogenic EDS. Soft biological samples such as botanicals, thin tissue sections containing nanoparticles, and foodstuffs can be extremely sensitive to electron beam damage and would be destroyed by increasing the beam current. Such samples are traditionally prepared by cryogenically freezing the sample in liquid nitrogen and maintaining it at those temperatures in the microscope. To preserve the microstructure and prevent frost damage, beam currents and accelerating voltages must be kept low (<5 kV). With Si(Li) detectors, the only way to increase total counts from these samples was to count for a long time, increasing the risk of specimen drift, contamination, and beam damage.

The introduction of modern large-area SDD detectors changed soft-matter

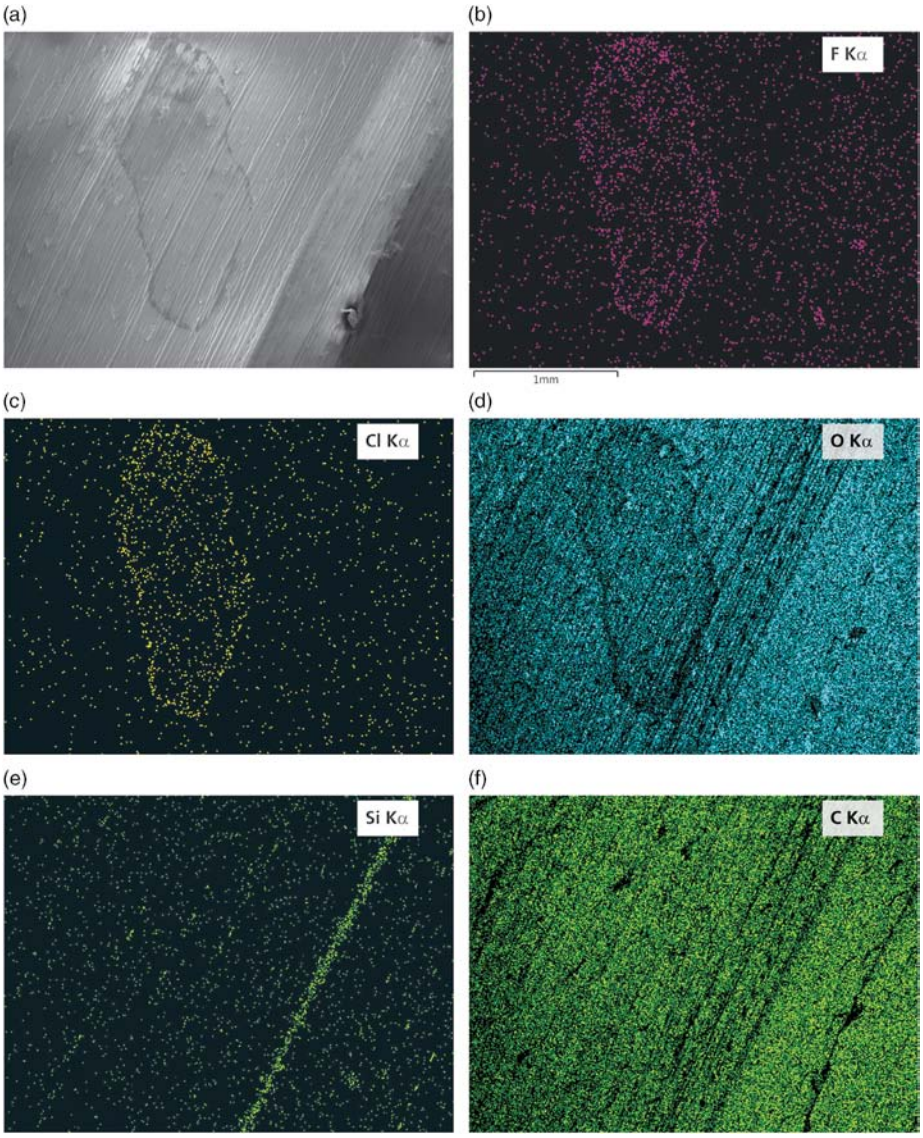

Figure 6: Fast EDS maps taken on a 150 mm^2 detector at 6 kV. Data were collected in just one frame with a total collection time of under 3 minutes. The F and Cl signals indicate the presence of fungicides on the surface of the leaf. The Si was unexpected, showing natural silica accumulation structures in the plant. Image width = 3.2 mm.

SEM biological analysis. Without changing any of the operating conditions, more counts can be collected from the sample, even on cryogenically frozen samples. Now count rate is no longer a problem, even at the low kV and low beam currents favored by biological analysts. Fast EDS mapping and spectrum imaging makes the advantages of SDD technology available to all SEM and TEM analysts.

Results

X-ray mapping. Figure 2 shows a wheat seed, which has been embedded into a Spurr type resin and stained with OsO_4 to highlight the fatty acids in the oily bodies surrounding the phospho-rich structures in the center of the aleurone cells. This is a fairly standard biological sample preparation technique. The aleurone cell layer was then analyzed with a large-area EDS detector (150 mm^2). The Os Mα and P Kα peaks overlap in the low-energy EDS spectrum (Figure 3), but the improved light element performance of SDD detectors means the elements are easily deconvolved and separated at each pixel to ensure well-defined, accurate elemental X-ray maps are obtained (TruMaps). All map data are stored as a SI data cube ($x \times y \times Energy$) meaning that data can be reinterrogated as many times as necessary without the need to recollect. This, combined with the speed of collection, is particularly of interest when the sample has a limited lifespan and is not permanently fixed (that is, cryogenically fractured samples). The sample can be analyzed quickly to minimize beam damage, and all the collected data are saved for analysis, meaning that any areas of interest not identified immediately can still be looked at in detail at a later date.

X-ray maps at high count rate. Large-area EDS detectors make it possible to collect these data in just a fraction of the time it would have previously taken. Figure 4 compares the maps collected at 4 kV from a traditional 10 mm^2 Si(Li) detector (Figure 4a) to those acquired with a large-area 150 mm^2 SDD detector (Figure 4b). The same setup and acquisition times (3 min.) were used so that the only variables were the size of the EDS sensor (10 mm^2 vs. 150 mm^2) and the count rate capability of the detector. Data collected with the 150 mm^2 detector are clearly superior in both counts and image definition. The lipids are clearly delineated in the Os map (blue) and easily identified as separate structures from the P-rich storage areas (in yellow), unlike the images seen from the 10 mm^2 detector.

Longer counting time (30 minutes) yields further improvements in signal-to-noise and even offers sufficient detail in the Os map to enable individual oily bodies to be identified without the need of an electron image (Figure 4c). These maps were collected while employing drift correction software and exhibit no sample drift despite the relatively long acquisition time. Combining both the Os and P maps into a single layered image (Figure 4d) illustrates the elemental distributions found across the sample.

Cryogenic X-ray mapping. Alpine Pennycress (*Thlaspi caerulescens*) is a well-known phytoremediator [2]. It absorbs zinc, cadmium, and other potentially toxic minerals dissolved in soil water. The toxic elements are absorbed into a central vacuole, which keeps them away from the rest of the organelles. By plunge-freezing a leaf in liquid nitrogen, from a plant that had been grown in a Zn-rich environment, it becomes possible to map the chemical changes in the plant in as natural a state as possible.

It has been shown in the past that EDS is a suitable analysis tool to look at the localization effects of phytoremediation [3], but previous research was limited by low count rates and long analysis times. This, of necessity, restricted researchers to a few spectral point analyses only. The result was limited information about the true distribution of these elements throughout the

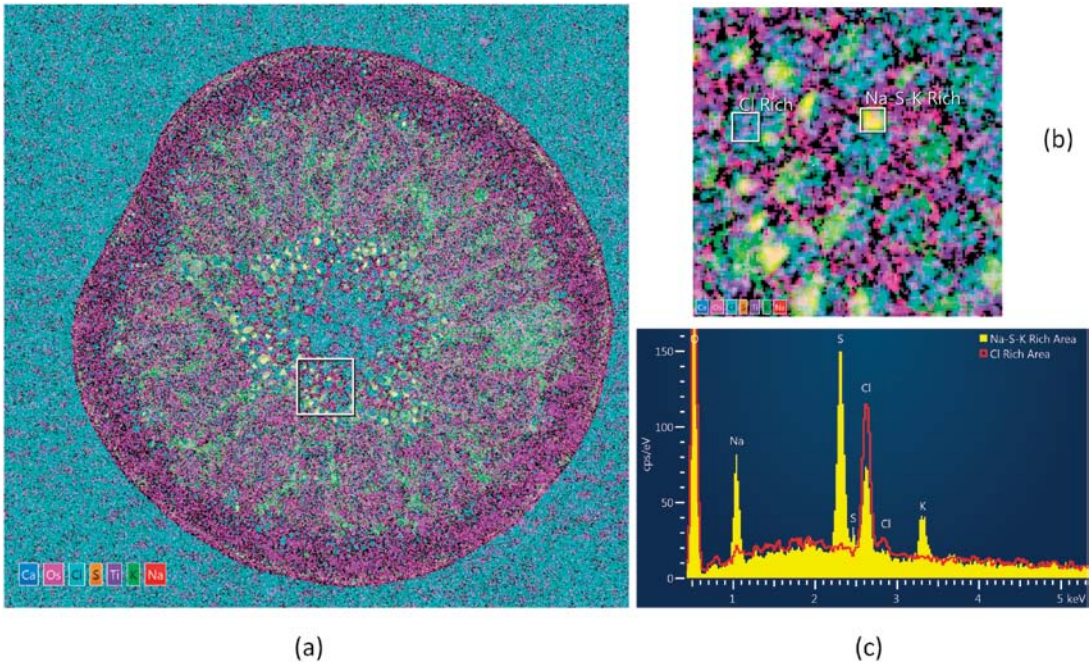

Figure 7: (a) Large-area map of the stem of a wheat plant. This image is composed entirely of X-ray elemental information acquired at 5 kV. There is no electron image behind the layered X-ray maps from K, S, Cl, Si, Na, and Ca. Image width = 2.8 mm. (b) A magnified section (box in (a)) illustrating the interrogation of data at higher magnification. Image width = 275 µm. (c) X-ray spectra from the boxes in image (b): S-rich region (yellow) and Cl-rich region (red).

whole sample. Analysis with a large-area SDD detector, however, enabled rapid mapping of a large area of sample in a short time.

Figure 5 shows that Zn is absorbed into the epidermal cells, whereas Ca is localized in the palisade cells. In just 20 minutes, these elemental distribution maps show where further investigations would be of use. Without a large-area detector and under the same conditions, this would have taken much longer—up to 5 hours.

Fast biological X-ray mapping. Figure 6 shows a wheat leaf, which was live-surface treated with a mixture of isopyrazam (F-rich) and cyproconazole (Cl-rich) fungicides then plunge-frozen in liquid nitrogen. It was not coated or treated in any other way prior to analysis and was simply analyzed on a cryostage with a JEOL 5600LV at 6 kV in just 1 frame, with a total collection time of under 3 minutes.

The Cl and F maps clearly show the presence of the fungicides on the surface whilst the detection of Si was a surprise. Further research showed that Poacaea monocots such as wheat are natural silicon accumulators [4]. The long epidermal Si-rich cells give the plant structural strength while the tiny Si-rich spines constitute a natural defence against herbivore grazing.

Large-area X-ray maps. Mapping of elements over large specimen areas is particularly important in heterogeneous materials such as biological tissues because elemental compositions may vary widely across the sample. While reducing the magnification to look at a larger area of sample would enable analysts to get a rough idea of elemental distribution, further analysis at higher magnification would mean having to map the same area twice, potentially increasing the risk of contamination and beam damage.

Large-area EDS mapping software allows analysts to identify an area on their sample for mapping, set the resolution and magnification of individual EDS maps, define a number of fields, and then map each field individually. When all the fields have been mapped, they may be montaged into a single dataset that can be manipulated and magnified to see small details not easily visible in the low-magnification map. As with all EDS spectrum images, data can be reconstructed at any time should a specific point in the map need further investigation.

Figure 7 shows a large-area map of a wheat plant stem. This image is composed solely of X-ray information from the elements present with no electron image behind. The advantage of this is that chemical information can be viewed cleanly and simply without topographical contrast effects confusing the analysis. This is an excellent example of how important it is to look at the elemental distributions and their context in relation to the whole sample. The smallest irregularities in the sample can be easily identified and examined in detail (Figures 7b and c)—without any need to re-collect the data or damage the sample any further.

Conclusion

Biological EDS is no longer limited by low count rates and poor solid angles. Changes in detector design and improved collection efficiencies have resulted in higher count rates without the need to change SEM operating conditions. Large-area SDD detectors offer biological analysts a new way of collecting important elemental information about their samples. There is no longer any need to destroy the sample to get an elemental analysis because X-ray map data can collected in minutes rather than hours. High count rates mean fast elemental analysis even on soft or cryogenic samples, low beam damage, and large-area mapping for visualization of chemical distributions on a scale of millimeters.

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