10.1071/FP10095_AC

Accessory Publication: Functional Plant Biology, 2010, 37(11), 1011–1040.

Accessory Publication

Our standard protocol for cryo-analytical SEM (CEDX)

(1) *Preliminary study* – Before beginning any CEDX analysis of unfamiliar material we do preliminary optical microscopy of the region of interest. Hand-cut sections and even thin sections of embedded tissues help in selecting and observing closely the region to be analysed. A thorough understanding of the tissue architecture saves both time and money when doing the analyses.

(2a) *Selection of material* – Ensure that the samples are representative of the conditions for which you want the analyses (e.g. from well-watered, or desiccating plants in the field, or seedlings in a Petri dish).

(2b) *Cryo-fixation* – Freeze the desired regions with liquid nitrogen (LN_2), or cryo-pliers (Fig. S1) cooled with LN_2, disturbing the functioning plant as little as possible. Remove the frozen pieces and store at LN_2 temperature till used. (Frozen samples can be transported long distances in a "dry shipper" at LN_2 temperature.)

(3) *Cryo-planing* – A smoothly planed face is essential for accurate quantitation. Trim frozen tissue to ~2 mm lengths under LN_2. Place cryo-glue on an appropriate Al stub (Fig. S2a) and quickly remove the sample from the LN_2 using cooled forceps, orient it in the glue and re-freeze immediately. Transfer the stub and specimen in LN_2 to a cryo-microtome. Plane to a smooth face in the desired plane at ~ –90°C (the optimum temperature can vary with the nature of your material, e.g. presence of oils and resins will require a lower temperature). For the initial 'rough' planing the sections cut (and discarded) can be relatively thick (ca. 0.5–0.2 μm) with cutting speeds 1–1.5 mm s^{-1}. For final polishing of the face much slower speeds are used (e.g. 0.3 mm s^{-1}) and only thin (60–80 nm) slices removed. Particularly during this slow final polishing, it is important to watch the specimen face carefully and gently remove any debris that accumulates on the face or the knife edge. A fine sable-hair brush must be used. If debris is accumulating in spaces in the tissue at the block face, an anti-static gun used during cutting may help. Glass knives are used to trim to the desired plane, and for many specimens glass-planed surfaces are satisfactory, but for

hard specimens a diamond knife may be necessary for the final polishing. Planed specimens can be stored in LN_2 or used immediately.

(4) *Transfer to the microscope* – Remove the stub with its planed sample from the microtome and quickly affix it in place on a stage adapter (Fig. S2b) under LN_2. Slide the adapter into the sloped mount (Fig. S2c, d) under LN_2. Attach the rod of the cryo-transfer apparatus to the adapter and quickly move it to the SEM preparation chamber and thence to the cold stage. During transfers avoid exposure of specimens to air as much as possible.

(5) *Etching* – While watching the specimen at 1 kV, etch at –90°C till cell outlines are barely detectable. Some parts of a complex plant tissue etch faster than others. The judgement of etching progress is personal and subjective and should be done by a practised operator constantly watching the loss of ice, varying the area observed, and the magnification. Etching at –90 ºC should not last longer than about 3 min. Over-etched specimens cannot be used for accurate quantitation. The observer is aiming for the minimum change compatible with certain knowledge of the anatomy of the specimen. When the cell outlines have just appeared the heater is turned off and the stage is cooled rapidly to –160°C by increasing the flow of LN_2, and the specimen returned to the preparation chamber.

(6) *Coating* – Appropriate coating of the specimen is also necessary for accurate quantitation. We use Al (or C for Al quantitation). Coating with Al is also an art that needs practice, maintaining the right current through the heating coil for the right time, while the specimen is moved about in the Al shower. This usually needs two operators. It may be necessary to modify the commercial evaporator in the cryo-preparation chamber, with a spiral of resistance wire in which is placed a rolled piece of high purity Al foil of predetermined mass. To get a shiny mirror coating with Al requires a higher vacuum than normally available in the conventional preparation chamber. A turbo pump should be used to attain at least 10^{-6} Torr.

(7) *Microscope settings recommended for CEDX analysis* –

(a) Choose an appropriate accelerating voltage. It needs to be high enough to excite X-ray lines for the elements you are interested in observing. We use 15 kV.

(b) Use the working distance as dictated by your EDS geometry. This information should be provided by your EDS manufacturer. For our detector for example this is 15.3 mm.

(c) It is important to use an instrument with good beam stability. Maintain a fixed beam current; if possible measure this using a Faraday cup and a micro-ammeter. Check regularly during your session to ensure the chosen value is maintained. Any change in this value will translate into changes in measured X-ray intensities which are independent of any element concentration you are trying to measure. We use a probe current of 300 pA.

(d) Maintain a cobalt, copper or nickel standard in the chamber. These all emit low ('L' lines) and high energy ('K' lines) X-rays which are useful for checking for drift in the offset and gain settings of the spectrometer. Your software will probably provide a calibration routine for this purpose. It's a good idea to check this at the start of each day.

(7) *Collecting spectra* –

(a) Count for a fixed live time (e.g., 100 s).

(b) Make sure the sample is not charging when collecting X-ray data, otherwise the measured intensities can be severely in error. You may need to recoat the sample.

(c) Collect spectra using raster dimensions that cover the tissue compartment that you want to analyse (e.g. vacuole, cytoplasm, wall, nucleus, plastid, vessel lumen) without overlap of adjoining compartments. This will eliminate error due to the heterogeneity caused by ice crystals and sequestered solutes, by giving a mean reading for the whole compartment.

(8) *Quantitation* – The use of frozen standards is critical for accurate quantification. We include 5% colloidal graphite in the standard solutions. Because the signal received from one element is influenced by the presence of other elements, the calibration solutions contain the same molarities of Na, Mg, P, S, Cl, K. Thus a series of solutions is made up, each of all these elements together at 0 mM, 12.5 mM, 25 mM, 50 mM, 100 mM, 300 mM, 500 mM, and the one series of measurements provides calibration curves for all these elements. Convex drops of each thoroughly-mixed solution (use a vortex mixer) are quickly set over holes in stubs individually and immediately frozen in liquid ethane. The convex drops are planed, etched, and coated identically to the specimens.

We collect 10 spectra per drop, using the same instrument parameters for the standards and the unknown tissue specimens. Peak intensities are determined by either using the net integrated counts for each of the peaks of interest or alternatively it may

also be possible to derive a parameter known as the k-ratio for each of the elements of interest depending on your software. The k-ratio, defined as $k = I_{unknown}/I_{standard}$, is simply a scaled version of the above-mentioned net integrated counts. The advantage of using k-ratios over using net integrated counts is that peak fitting as well as background removal routines are sometimes more sophisticated when using the k-ratio approach (depending on your software). This leads to more accurate estimates of (relative) peak intensities. A sample calibration curve relating k-ratios to [Ca] in the standards is given in Fig. S3. In our experience the calibration curves for each element are linear only up to 300 mM. The 500 mM points usually fall below the projected line, and we do not claim the same accuracy for concentrations above 300 mM. Once prepared, unplaned frozen standard drops can be stored indefinitely in a cryo-store, and planed and processed later as required. Repeat calibrations are done about twice yearly.

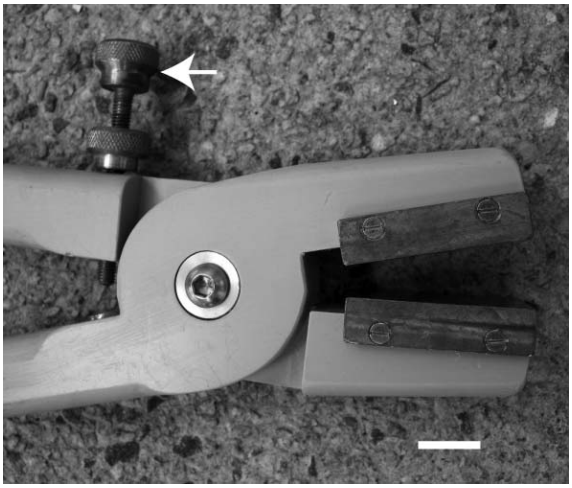
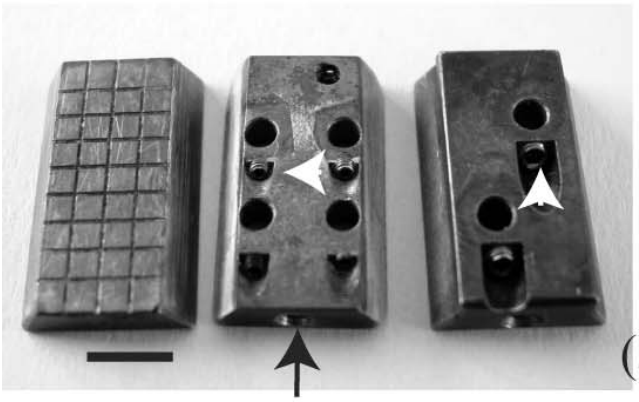
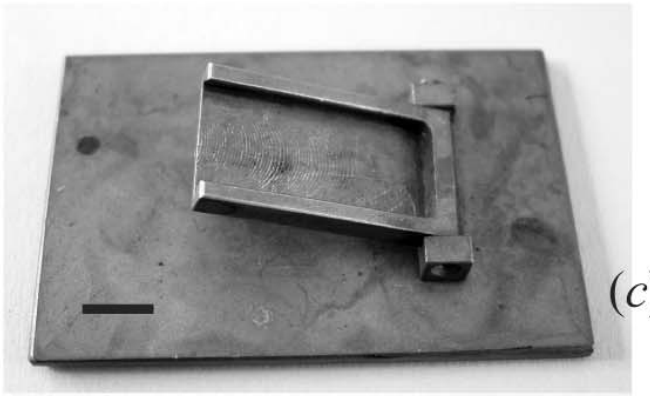


Fig. S1. Purpose-made cryo-pliers with thick, polished copper jaws. The spacing between the jaws is adjustable with the screw system (arrow) to accommodate specimens of different thickness. Bar = 10 mm.

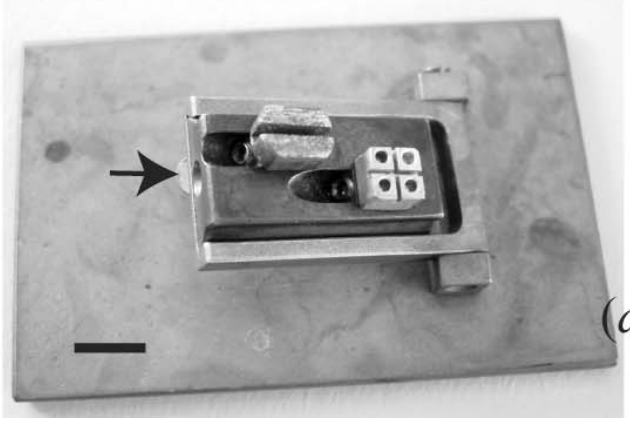
(*a*)



(*b*)



(*c*)



(*d*)

Fig. S2. Purpose-made hardware for handling frozen specimens.

(*a*) Aluminium stubs that can accommodate a variety of specimen sizes for either transverse or longitudinal fracturing or planing and viewing. Stubs fit into the cryo-microtome chuck, and into the holes in the adapters in (*b*). Bar = 6 mm.

(*b*) Adapters that accommodate cryo-specimens and slot into the microscope cryo-stage. The one on the left is for whole mounts, the others have holes for two or four stubs, each secured by a grub screw (arrowheads). The small hole at the top end of the middle adapter can hold a standard. Each adapter accommodates the rod of the cryo-transfer device in a threaded hole in one end (arrows). Stubs with the frozen, planed specimens are inserted into the adapters under LN_2. Bar = 6 mm.

(*c*, *d*) The mount (*c*) for the adapters (*b*), is slanted upwards so that the cryo-transfer rod clears the edge of the LN_2 bath, for threading into the adapter (*b*, *d*). The specimens can then be transferred to the CSEM. Bars (*c*) and (*d*) are 6 and 10 mm respectively.

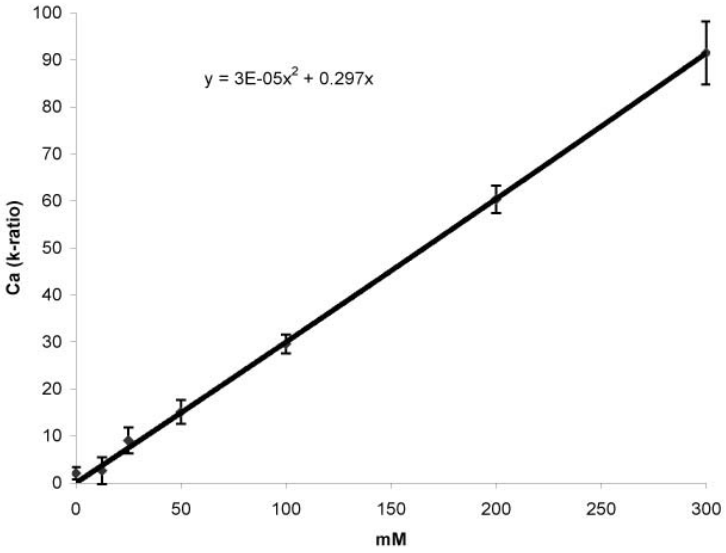


Fig. S3. A typical calibration curve for quantification relating k ratio to [Ca] in a cryo-planed and Al-coated carbon-slurry standard.