Imaging the Primary Cell Wall

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Introduction

As the son of an artist, I grew up surrounded by drawings, paintings, and sculpture. Perhaps for that reason, making images is central to my work as a scientist. The problem I study, organ morphogenesis, requires analysis of the cell wall. Fertile approaches stretch out in many directions (see the rest of this book) but I have been drawn to architectural problems, revealing how components are integrated into a whole. For structural appreciation, imaging is paramount.

For imaging the cell wall, my laboratory has developed or enhanced several methods. I will describe some of these here, and discuss advantages and limitations, without attempting to review cell wall imaging comprehensively. The first section is on immunocytochemistry at the light-microscope level. The second and third sections are on high-resolution imaging based on field-emission scanning electron microscopy. The first is convenient and allows many samples to be examined and a relatively large area of tissue to be viewed; the second and third are technically more demanding but resolve structure at virtually the level of macromolecules.

Butyl-methyl-methacrylate embedding for immunocytochemistry

Cell wall researchers will be familiar with the use of antibody probes to localize polysaccharide as well as protein epitopes in the cell wall (Knox 1997), but are probably less familiar with the use of butyl-methyl-methacrylate as an embedding matrix. I first encountered this resin while attempting to localize microtubules (Baskin et al. 1992). This methacrylate is easy to section, dry or wet; however, its primary advantage for immunocytochemistry is that following sectioning, the majority of the embedding matrix can be removed with a brief incubation in acetone, in contrast to nearly all other plastic embedments, which can be removed partially if at all only with harsh treatments. By removing the embedment, access to the antigen for the antibody is enlarged. Removability is shared by paraffin and wax but these preserve most samples poorly compared to plastic. At the electron-microscope level, adequate access for antibody to antigen is

provided by various plastics that are sufficiently porous to allow antibodies to penetrate 10 nm or so into the section, thus sampling an appreciable proportion of the volume of an ultra-thin section (60 nm thickness). However, for the semi-thin sections (1 to 2 µm thick) typically used in light microscopy, a 10 nm penetration depth amounts to a negligible proportion of the section volume, and the greater penetration gained by removing the embedment becomes a significant advantage.

Butyl-methyl-methacrylate, like most methacrylates, polymerizes via a free radical-based mechanism. This is useful because it means that polymerization can be catalyzed by ultraviolet light, thus avoiding denaturation caused by high-temperature polymerization. However, early efforts to use this methacrylate mix were frustrated by oxidative damage to the sample that lowered antigenicity, damage presumably mediated by free radicals. I found that adding the free-radical scavenger dithiothreitol to the resin allowed polymerization but blocked the attack on the sample (Baskin et al. 1992). Subsequently, this resin has been used to localize tubulin and other antigens in a variety of samples (e.g., Herman et al. 1994; Stadler et al. 1995; Hoffman et al. 1998; Palmer et al. 2001).

In general, embedding in butyl-methyl-methacrylate is straightforward (Baskin and Wilson, 1997). However, the small size of arabidopsis roots (ca 0.15 mm diameter) makes them easy to lose while changing solutions. To retain the roots, I use a method that is not only convenient but also turns out to be beneficial for sample preservation. Originally, I encased each root in a small droplet of low-gelling-temperature agarose (Baskin et al. 1992), but this is messy and exposes the sample to heat, albeit briefly. Then, I modified a method from cryofixation where samples are mounted on a Formvar film (Baskin et al. 1996). A chemically fixed root tip is placed on a Formvar-coated wire loop, a second Formvar film secures the root tip on the loop. The Formvar films are readily permeated by solvents and small molecules. Between Formvar films, the thin arabidopsis root tip is prevented from bending or twisting. I call this “mechanical fixation” and beyond being convenient, it seems to enhance sample preservation.

Loops are made in advance and coated by casting Formvar rectangles (measuring a little more than the loop diameter on one side and a

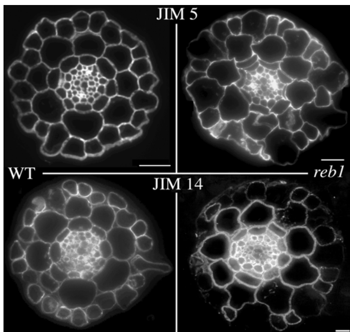
little more than twice the loop diameter on the other) and plunging the loop into the water over the rectangle so that the plane of the loop bisects the long axis of the rectangle. The Formvar rectangle wraps around the wire loop and the coated loop is removed at once from the water. Such loops remain stable for months. To secure a sample, the procedure is repeated: After the sample has been fixed and rinsed, a loop (already Formvar coated) is placed horizontally on a drop of water (or buffer) and the sample placed on the Formvar. Excess sample is trimmed if needed, and the loop (with sample) is plunged onto a new Formvar rectangle, thus encasing the sample between Formvar layers, held by the loop. Several loops can be placed in a vial and solutions exchanged without losing the sample. The loop is embedded with the sample, and removed during trimming. I use fine copper wire (36 gauge), which can be trimmed along with the block.

My colleagues and I have recently taken advantage of this methacrylate to characterize cell wall epitopes present in the *rhd1*/*reb1*/*cst2* mutant of arabidopsis (Andème-Onzighi et al. 2002). We found that selected arabinogalactan-protein epitopes were differentially expressed within the epidermis between root-hair forming cells (trichoblasts) and non-root-hair forming cells (atrichoblasts) and suggested that these proteins may be involved in repressing bulge formation in atrichoblasts (Figure 1).

Field-emission scanning electron microscopy for cell wall ultrastructure

The most common approach to cell wall ultrastructure is transmission

Fig. 1 Transverse sections of arabidopsis roots embedded in butyl-methyl-methacrylate and stained with anti-cell wall antibodies.



Top panels show JIM5, which recognizes pectin, bottom panels show JIM 14, which recognizes arabino-galactan epitopes. Left hand panels show wild type (Columbia), right hand panels show *reb1*. Note that JIM 5 stains more or less ubiquitously, as JIM 14 stains the wild type; however, JIM 14 staining in the trichoblasts of *reb1* appears specifically decreased. Bars = 25 µm (top), 15 µm (lower right). Figure modified from Andème-Onzighi et al., 2002).

electron microscopy, with samples embedded, sectioned, and stained with heavy metals. Such microscopy illuminates the ultrastructure of the wall, particularly to show its thickness and lamellation, and the sections can be used for immunocytochemistry; however, this approach suffers from the fact that the heavy metals do not stain cellulose or other polysaccharides reliably, and consequently images can be difficult to interpret (Emons 1988).

An alternative instrument for ultrastructure is field-emission scanning electron microscopy. The field-emission gun allows the microscope to scan the sample with a narrow diameter beam, with consequent improvement in resolution. As with all scanning electron microscopy, the image largely reflects the topography of the sample so it is useful for showing the structural arrangements of components. Additionally, sample preparation for field-emission scanning electron microscopy is faster and easier than for transmission electron microscopy, requiring dehydration and critical-point drying, but no embedding or ultra-thin sectioning.

A further difference is that for transmission electron microscopy the cell wall is usually viewed in cross-section whereas for field-emission scanning electron microscopy the wall is usually viewed from the surface. Surface views are particularly important for delineating architectural relations. Surface views can also been obtained for transmission electron microscopy by casting a metal-carbon replica from exposed cell walls (e.g., Iwata and Hogetsu 1989; McCann et al. 1990); however, replica making is technically difficult, and in field-emission scanning electron microscopy the sample itself is imaged rather than a replica.

Since the early development of the field-emission gun, wood anatomists have taken advantage of the instrument to examine the orientation of microfibrils at the innermost surface of tracheids and other cell types in wood (Hirakawa and Ishida 1981; Abe et al. 1994). Such cells are ideal for field-emission scanning electron microscopy because the cell walls are thick and strong and cell contents are absent or easily removed to expose the surface for imaging. In contrast, the use of field-emission scanning electron microscopy to image the primary cell wall has only been rather recently reported (Vesk et al. 1996; Crow and Murphy 2000).

An early and influential application of field-emission scanning electron microscopy to the primary cell wall was developed at Australian National University in Geoffrey Wasteneys' lab (Sugimoto et al. 2000). I happened to be there on sabbatical and was able to learn the procedure at first hand and to explore related methods. Again, the object of interest is the arabidopsis root and its small size requires heroic efforts. In Sugiomoto's method, roots are fixed, cryroprotected, frozen in liquid nitrogen, mounted on a cryo-ultramicrotome, and sectioned while frozen. About a dozen sections are discarded and the remaining portion of the root, now cut open, is warmed up, incubated

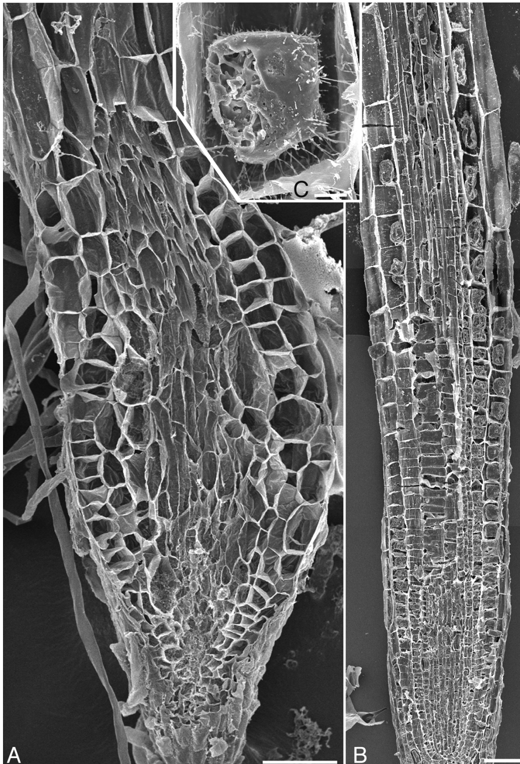


Fig. 2 Survey views of arabidopsis roots prepared for field-emission scanning electron microscopy.
A: Image of the *rsw4* mutant prepared by the cryo-ultramicrotme method. B: Image of wild type prepared by the polyethylene-glycol method. C: Higher magnification of an epidermal cell from B showing that many Hechtian strands are preserved. Bars = 60 µm (A); 30 µm (B); 3 µm (C).

in bleach to remove the cytoplasm, dehydrated, critically point dried, sputter-coated with platinum, and viewed. I tried out this method on radially swollen mutants (*rsw4* and *rsw7*; Wiedemeier et al. 2002) and an example is shown here (Figure 2A). Inside the emptied cells, the cell wall can be imaged at high resolution (not shown, but see Sugimoto et al. 2000).

While the method produces striking images, and has been used successfully by others (Zhong et al. 2002), it has some difficulties. The bleach, required to remove the cytoplasm, may also attack the cell wall. The pieces of root left after sectioning are minuscule and difficult to process for dehydration and sectioning. Finally, the cryo-ultramicrotome is a specialized and expensive piece of equipment. Therefore, I sought alternatives.

One alternative is butyl-methyl-methacrylate. The acetone treatment mentioned above while sufficient to give antibodies access nevertheless leaves an insoluble residue (ca. 10% of the original section mass) that obscures all but the grossest topography in the field-emission scanning electron microscope. Because an objective in my research is to assess the orientation of cellulose microfibrils, I examined methacrylate sections that were treated in acid mixtures commonly used to remove all organic matter other than cellulose, including a mixture of acetic and hydrochloric acids (Updegraff 1969), and a mixture of acetic acid and hydrogen peroxide (Wolters-Arts and Sassen 1991). Semi-thin sections (1.75 µm) were collected on coverslips, incubated in acetone to remove the majority of the methacrylate and then incubated in acid at 100 ^{o}C, usually for 1 h. Subsequently, sections were rinsed, dehydrated, critically point dried, sputter-coated and imaged.

The results were disappointing. Low magnification images (Figure 3A and B) resemble sections viewed in polarized light microscopy or treated with Calcofluor White and viewed in fluorescence microscopy, indicating that the overall cell pattern survived extraction. However, when cell walls present in face view were examined at high magnification, the microfibrils were invariably disorganized (Figure 3C). Each type of extraction gave similar results. Even though the sections are fixed to glass for the extraction, the hot acid presumably causes the cell wall to swell and allows some microfibrils to rearrange

in the boiling reagent. Indeed, acid-extracted samples viewed in cross section in the transmission electron microscopy have swollen and distorted cell walls (Emons 1998).

As an alternative, I embedded roots in low molecular weight polyethylene glycol. Polyethylene glycol is water soluble allowing it to be fully extracted after sectioning. Additionally, I plasmolysed roots in sucrose prior to fixation and embedding so as to be able to expose

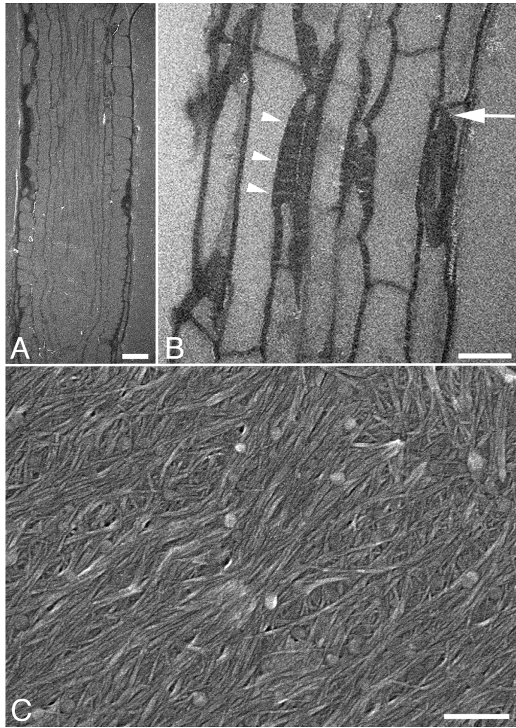


Fig. 3 Arabidopsis roots embedded in butyl-methyl-methacrylate, sectioned at 1.75 µm, acetone-treated to remove methacrylate, and treated with peroxide/actetic acid at 100 °C for 1h to remove everything except cellulose.

A: Survey view showing the section outlined by its remaining cell walls. The quiescent center is roughly at the bottom of the panel. B: View of elongation zone cells where some regions of cell wall are present in the plane of the section (dark areas). Faint transverse striations (white arrowheads) are probably pit fields. C: High magnification (region as indicated by arrow in B) showing microfibrils, which appear thick and significantly disorganized. Bars = 30 µm (A); 20 µm (B); 250 nm (C).

the cell wall surface for imaging without bleach. After infiltration and embedding, sections were collected onto coverslips, incubated in water to remove the embedment, and then prepared for field-emission scanning electron microscopy (dehydrated, critical-point dried, sputter coated). An example of a wild-type root is shown (Figure 2B). The method is sufficiently gentle to retain many Hechtian strands linking the cell wall to the protoplasts (Figure 2C). While the cell wall can be imaged in large cells in regions not covered by the protoplast, in small cells, the whole wall is obscured. Therefore, sections were incubated in salt and dilute protease, which removed protoplasts completely.

As with cryo-ultramicrotomy, polyethylene-glycol embedding allowed the cell wall surface to be imaged at high resolution (Figure 4A and B). Some tissues had distinct cell wall textures, for example the root cap cell walls microfibrils appear thick and stiff (Figure 4C). By incubating sections in various reagents it was possible to examine their effects. Bleach treatment changed the appearance of the cell wall, making the microfibrils narrower and less encrusted (Figure 4D). Removing pectin with calcium chelation changed the appearance modestly (Figure 4E), perhaps straightening microfibrils, whereas removing pectin with a pectolyase (which presumably removes more pectin than chelation does) caused microfibrils to appear to merge (Figure 4F). Others have also seen this merging in pectin-depleted samples (Sugimoto et al. 2000; Pagant et al. 2002), but the phenomenon has yet to be explained.

Although polyethylene-glycol embedding succeeded, it was difficult. The blocks are so soft that sectioning them was only possible at about 16 ^{o}C, provided that the relative humidity was less than around 70%; even then, many sections were lost. Furthermore, sectioning radially swollen mutants disrupted the integrity of the tissue, perhaps because their cell walls are weak. I tried polyethylene glycols only up to a molecular weight of 1450. Larger glycols have been used in transmission electron microscopy to provide “resinless embedding” (Penman and Penman 1997), and these should prove easier to section. On the other hand, the larger the molecular weight the more difficult to remove completely with water. Nevertheless, high molecular weight glycols might give better results for field-emission scanning electron

microscopy views of arabidopsis cell walls.

Imaging cell walls in organs with large cells

Arabidopsis is an object of intense scrutiny, nevertheless other plants continue to be useful subjects for research and techniques for imaging cell wells in such species should be developed. In working on maize roots, I found a reliable way to prepare cell walls for field-emission scanning electron microscopy imaging that, when compared to the difficulties described above for arabidopsis, is amazingly simple. All

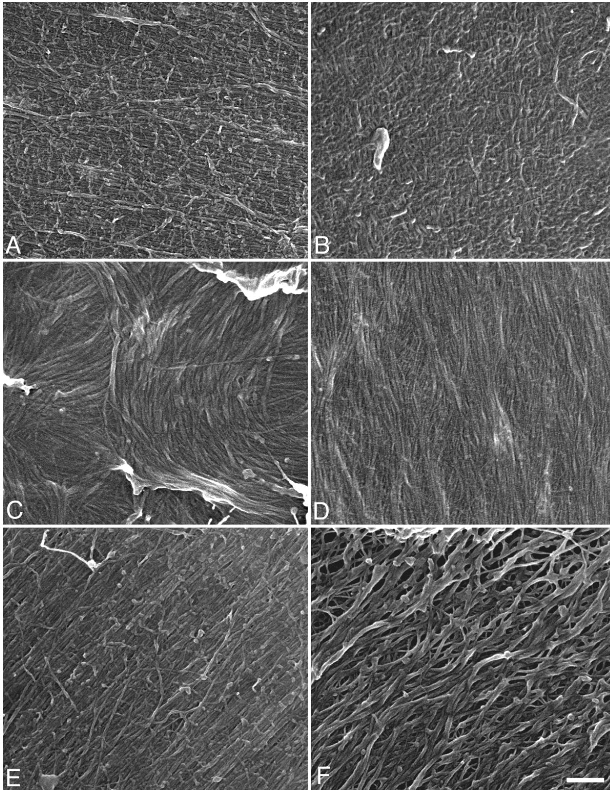


Fig. 4 High magnification field-emission scanning electron micrographs of polyethylene-glycol embedded arabidopsis roots.

A, B: Epidermal cells in the growth zone with different textures. C: Central columella cell. D – F: Sections treated as described after removing the polyethylene glycol and before dehydration. Images are from the growth zone. D: Bleach (0.5 % for 8 min). E: CDTA (50 mM for 16 h). F: Pectolyase (0.1 % for 1 h; Seishin Y-23). Bar = 200 nm.

that is required is to section the root, without fixation, in dilute buffer. Sectioning without fixation is accomplished readily for an organ the size of a maize root on a Vibratome, an instrument that vibrates a razor blade through a sample, producing sections on the order of 100 µm thick. However, if a Vibratome is not available, bisecting the organ by hand suffices. The sectioned material is incubated in buffer for a short period (10 to 60 min) and then fixed, dehydrated, critically point dried, sputtered with platinum, and viewed in the field-emission scanning electron microscopy (Figure 5).

Sectioning before fixation ejects cytoplasm and the buffer incubation washes out any residual material (Figure 5A). The method works similarly for cucumber and tobacco hypocotyls and inflorescence

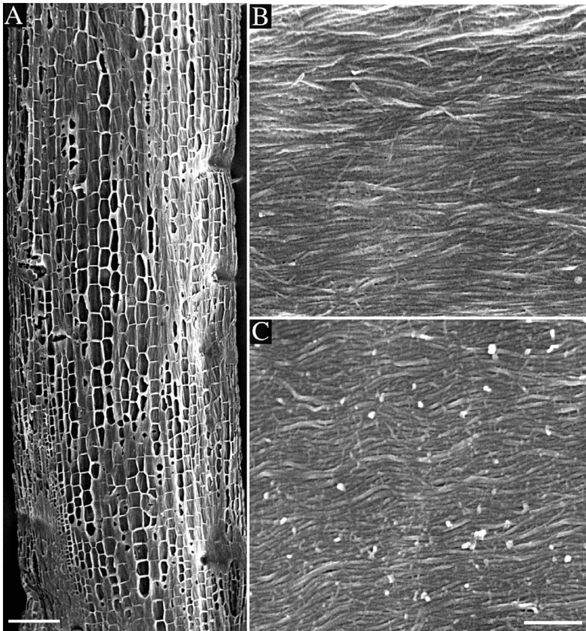


Fig. 5 Field-emission scanning electron micrographs from cutting sections prior to fixation.

A: survey view of a cucumber hypocotyl indicating the widespread absence of cytoplasm (no bleach required). B, C: High magnification images of cortical parenchyma from a maize root (B) and a cucumber hypocotyl (C). Samples cut on Vibratome (maize) or free-hand (cucumber). Bars = 300 µm (A); 250 nm (B, C).

stems of arabidopsis; however, when arabidopsis roots are cut with a razor blade under water, the plasma membrane is retained tenaciously inside the cells, precluding this approach. In images from larger organs, the microfibrillar texture as well as cross-links of various kinds are clear (Figure 5B and C). A consistent feature of cell walls prepared in this way, as well as of arabidopsis roots prepared as above, is that the microfibrillar texture appears as a single lamina with little or no visibility of underlying layers. Additionally, the diameters of the microfibrils vary continuously between roughly 8 and 40 nm. Given that the diameter of the crystalline cellulose microfibril, presumably as synthesized by a single rosette, is 3 to 5 nm (Davies and Harris 2003), it appears as though the cellulose microfibrils are sheathed in matrix polysaccharides and proteins to make structures that are thicker but still highly fibrillar.

These images depict cell walls that presumably remain close to their native composition, with the only material lost being that extracted by a low ionic-strength wash before fixation or by ethanol or liquid CO_2 after fixation. However it is an open question to what extent the architecture remains native, because dehydration and critical point drying themselves could rearrange cell wall components, particularly those architectural features that depend on non-covalent bonds. To resolve this question, it will be necessary to use the capability of the field-emission scanning electron microscopy to image frozen samples or to turn to other techniques such as atomic force microscopy so that the ultrastructure of the cell wall can be imaged in a hydrated state.

In conclusion, images may inspire artists to create for our enjoyment and scientists to experiment for our enlightenment.

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