Mechanical fixation techniques for processing and orienting delicate samples, such as the root of *Arabidopsis thaliana*, for light or electron microscopy

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Despite improvements in live imaging, fixation followed by embedding and sectioning for light or electron microscopy remains an indispensible approach in biology. During processing, small or delicate samples can be lost, damaged or poorly oriented. Here we present a protocol for overcoming these issues when, along with chemical fixation, the sample is fixed mechanically. The protocol features two alternatives for mechanical fixation: the sample is encased either in a rectangular block of agarose or between Formvar films suspended on a wire loop. We also provide methods for key steps all the way through to sectioning. We illustrate the method on the root of *Arabidopsis thaliana*, an object that is ~0.15 mm in diameter and difficult to process conventionally. With this protocol, well-oriented sections can be obtained with excellent ultrastructural preservation. The protocol takes about 1 week.

INTRODUCTION

Imaging cells within a tissue has become easier thanks to advances in confocal microscopy, which allows optical sectioning. However, even with the best objectives, the penetration of the laser decreases markedly with the distance of the cell of interest from the coverslip and the resolution of individual structures within the cell becomes increasingly limited with depth. Furthermore, the penetration of large reagents like antibodies is also progressively limited as the sample thickness increases. To take advantage of the high resolution of transmission electron microscopy (TEM), the investigator must cut sections, even to examine a single cell. For all of these reasons, studies with sectioned material are essential, both in their own right and as complementary methods to whole-mount examination of intact organs.

In our laboratories, the primary focus is the root of *A. thaliana*, an accessible and streamlined organ being used by a large number of laboratories as a model for studying plant growth and development[1]. For example, this model has enabled fine-scale analysis of gene expression, with individual cell types being sampled as a function of position[2–5]. However, we are still limited in our understanding of how the cellular anatomy of the root changes with development and how cellular connectivity correlates with developmental transitions.

Assaying cells at high resolution in their developmental and anatomical context, requires sectioning. When processed for sectioning, a thin sample such as the *A. thaliana* root (~150 μm) usually becomes curled or twisted. When the root is bent, it is difficult to obtain sections that include the meristem, elongation zone and maturation zone, and therefore it is difficult or even impossible to ascertain the anatomical position of the sectioned cells within the organ. To overcome this difficulty, a method is required that minimizes the bending of the sample during processing so that oriented sections may be cut reliably.

Published methods for improving the ability to obtain well-oriented sections are available for animal tissue culture cells growing in monolayers on a flat substrate[6]; however, these depend on tight adhesion between the cell and the substrate and do not apply to a free structure, such as a thin root. To the best of our knowledge, most protocols for a sample like a root rely on manipulating the sample after it is fully infiltrated with not-yet-polymerized embedding resin. Specifically with respect to the *A. thaliana* root, an ingenious method is available wherein the infiltrated root is placed within custom-made molds just before embedding[7–9]. Although that method undoubtedly allows well-oriented sections to be obtained, it has limitations. First, the method often requires a second embedment, which adds to the time of the protocol. Second, the method involves manipulating infiltrated samples, which could damage them or expose the investigator to toxic fumes from the embedding resin. Third, the method makes no provision for dehydration and infiltration, during which small samples like the *A. thaliana* root are easily lost or damaged. Di Laurenzio *et al.*[10] mention pre-embedding in agarose after fixation for embedment in paraffin and subsequent *in situ* hybridization, but they offer no specific details on the method.

Here we describe a simple and effective step-by-step protocol for handling small samples, exemplified by the *A. thaliana* root. The protocol includes two alternative methods to mechanically protect the sample (either in an agarose block or between Formvar films), modified dehydration and infiltration procedures to enable thorough penetration despite the agarose or Formvar, and techniques for optimal sectioning and staining. We discuss challenges we have encountered and provide solutions that have been tested in our laboratories. A complete account of procedures for embedding and sectioning is beyond the scope of this article, but interested readers should refer to a relevant text[11,12]. This protocol has been used in several of our previous publications[13–17] and the improved version given here is in standard use in our research groups.

We developed this protocol for a plant root but nothing in it is plant-specific, and the protocol should apply to any small or delicate sample, be it plant, animal or fungal, which is subject to loss or damage during processing or for which the final orientation of sections needs to be controlled. In general, our protocol should be helpful for any sample that is thin or flat, such as an insect wing,

Box 1 | Choice of chemical fixative

Although widely used, chemical fixatives are often misunderstood[24]. Formaldehyde is maximally soluble in water at a concentration of 37–40% (wt/vol; so-called formalin). Diluted by roughly tenfold, it is perhaps the most commonly used fixative, and is suitable for anatomy and histology at the light microscope level, but is not recommended for TEM or immunological work because it contains impurities and gives less satisfactory preservation compared with dilute stock solutions. Commercial formalin may contain 10–15% (vol/vol) methanol, which impairs fixation for TEM and can also interfere with immunological methods. In water, formaldehyde forms polymers, typically containing 8–100 formaldehyde units, collectively referred to as paraformaldehyde. The polymeric form has reduced fixation activity, and the larger species tend to precipitate. Typically, formaldehyde solutions used for TEM and for light-level immunocytochemistry are specifically prepared to avoid impurities and to minimize paraformaldehyde formation. Note that formaldehyde solutions can be made directly in the laboratory from paraformaldehyde powder; however, doing so runs the considerable risk of inadvertent exposure and is not recommended.

Formaldehyde penetrates membranes and tissues rapidly. A brief fixation in formaldehyde can considerably reduce autolysis and improve the structural integrity of the sample. However, it reacts with proteins rather slowly, and formaldehyde-based cross-links are rather weak. Therefore, treatment with formaldehyde alone seldom provides sufficient fixation for TEM. To improve preservation, fixatives typically contain glutaraldehyde. Unlike formaldehyde, the cross-linking of proteins by glutaraldehyde is all but irreversible. However, glutaraldehyde penetrates membranes and tissues rather slowly, which could allow sample deterioration prior to the onset of strong fixation. Fortunately, mixing these fixatives allows the formaldehyde to diffuse rapidly into the cells and stabilize structure and glutaraldehyde subsequently to establish a more robust fixation.

However, the strong fixation from glutaraldehyde comes at a cost. It can cause autofluorescence, can mask antigens, and can lead to nonspecific binding of antibodies because of free aldehyde groups introduced by glutaraldehyde fixation; all of these properties hinder immunological methods. Therefore, for immunocytochemistry at the light microscope level exclusively, it is often advantageous to omit glutaraldehyde. Moreover, for TEM to detect a given antigen, it often requires some trial and error to determine a suitable mixture of these two aldehydes. The concentrations given here can be used as starting points for untried samples or antigens.

Neither of these aldehyde fixatives preserves lipids well; therefore, in addition to them, osmium tetroxide is widely used for TEM. Osmium is a strong oxidant and reacts rapidly with unsaturated fats, providing good fixation of membranes and lipids. It causes relatively little extraction of tissues but it penetrates them slowly. It is nearly always used in TEM when heavy metals will be the only kind of stain applied; however, osmium reduces or even eliminates antigenicity and therefore its use is often omitted for immunological stains. Although it is not as effective as osmium, uranyl acetate maintains phospholipids and membrane structure to some extent, particularly when given *en bloc* before embedding.

a biofilm or a brain slice in which the behavior of identified cells has been recorded. In addition, the protocol could be used for cell suspensions (e.g., a protozoa culture) and thereby remove the need for centrifugation.

When compared to conventional protocols for processing material for light microscopy or TEM[18], our protocol differs mainly in that the mechanical fixation necessitates a longer schedule to ensure adequate dehydration and infiltration; this schedule includes longer steps and more finely graded steps. When adapting our protocol for other samples, the precise schedule for dehydration and infiltration will probably require modification. In contrast to the reduced damage and increased orientation offered by our protocol, the increased length of the protocol will decrease the throughput of samples and might decrease the antigenicity of sensitive antigens.

Experimental design

Chemical fixation. The goal of fixation is to arrest all biological activity and cause as few responses as possible. A good way to accomplish this is with cryofixation; however, this method requires specialized apparatus and has limitations, particularly for multicellular samples. For most tissues and antigens, chemical fixation with aldehydes provides acceptable preservation, with formaldehyde and glutaraldehyde being the most widely used (**Box 1**). We find fixative A suitable for histology in either light microscopy or TEM and fixative B useful for immunological detection in either case (see REAGENT SETUP).

We prefer to use seedlings whose roots are less than ~2.5 cm long at the time of fixation, because longer roots get tangled during processing. Customarily, seedlings are fixed by transferring them into a few milliliters of fixative. However, lifting them off of their growth substrate might elicit an unwanted mechanical response. In addition, the transferred seedlings usually float on the surface of the fixative, which hinders uniform fixation. We get excellent results by pouring the fixative solution directly onto the growth plate: this avoids mechanical disturbance and submerges the seedlings, which remain adhered to the agar growth medium.

Another issue for fixation is the choice of buffer. Classically, microscopists used phosphate buffers, whereas more recently they have preferred organic buffers, such as cacodylate, PIPES or HEPES. Organic buffers tend to have a high buffering capacity, but the need for strong buffering is seldom demonstrated, and usually the reasons for buffer selection in a given protocol are undefined. Cacodylate contains arsenic and its use should be restricted to protocols in which it performs demonstrably better than alternatives. We find that 0.1 M phosphate buffer at pH 6.8 gives good preservation and retention of antigenicity.

Other variables related to fixation are time and temperature. Samples can be underfixed, wherein preservation is poor and antigenicity is reduced, or overfixed, wherein the sample becomes difficult to infiltrate and again antigenicity is reduced. Compared with room temperature (20–25 °C) fixation, fixation of samples at 4 °C tends to reduce extraction of cell contents, to slow autolytic processes and to reduce tissue shrinkage. In contrast, 4 °C will slow the process of fixation itself. We fix for 1–2 h at 4 °C, pre-embed, and then fix for an additional 12–18 h (i.e., overnight) at 4 °C in fresh fixative.

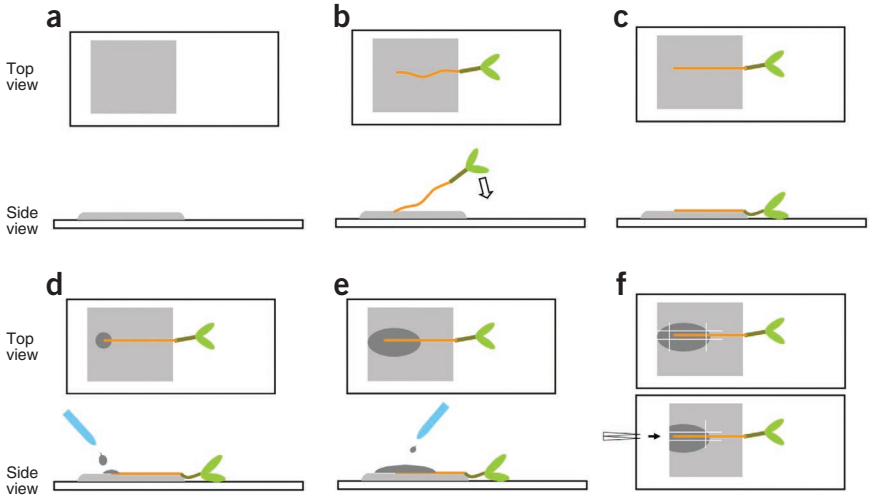

Figure 1 | Schematic illustrating the procedure for agarose embedding. (**a**) Put one drop of 0.6% (wt/vol) agarose onto a clean glass plate and spread it out into a thin layer (approximately 2 cm × 3 cm). Allow a few minutes for the agarose to harden. (**b**,**c**) Pick up a seedling with forceps and let the root tip touch the agarose layer first, and then slowly and gently drag the seedling away from the tip to straighten the root, eventually laying down the whole seedling. (**d**,**e**) Place one drop of agarose next to the root tip and let the agarose spread around the tip. Repeat for two more drops. (**f**) Use a single-edged razor blade to slice the agarose and use forceps to transfer the agarose block containing the root to a vial containing fixative. See also **Supplementary Video 1**.

Mechanical fixation. The crux of our methods for obtaining straight roots is to reinforce them at an early step in the processing. We use two methods for this reinforcement, both of which give good results. The first is agarose embedding (**Fig. 1** and **Supplementary Video 1**). Here the root tip is encased in 0.6% (wt/vol) agarose. We use a low-melting point agarose (melting point ~36 °C) to avoid damage from heat. We found that 0.6% (wt/vol) agarose provides sufficient mechanical support for the subsequent processing, and after osmium fixation, 0.6% (wt/vol) agarose becomes hard enough to be handled by forceps. However, higher concentrations of agarose, particularly those >1% (wt/vol) hinder dehydration and infiltration.

The other method is the use of wire loops coated with Formvar (**Fig. 2** and **Supplementary Video 2**). This method was apparently developed for plunge freezing to allow a sample to be suspended on a thin surface of low heat capacity (i.e., the Formvar film) when plunged into a cryogenic fluid for optimal heat removal[19]. The loop holds the sample for all steps, including embedding, and is removed at last when the block is trimmed before sectioning. This is possible because when samples, including the root of *A. thaliana*, are placed on a loop and cryofixed, they adhere tightly to the Formvar; subsequent freeze substitution and infiltration can be accomplished with few lost samples. However, to handle a chemically fixed root, it is necessary to hold the root on the loop by encasing it in a second Formvar film (**Fig. 2**).

In addition to facilitating the cutting of well-oriented sections of the root, Formvar loops also appear to enhance preservation. Baskin and collaborators[20] compared chemical and cryofixation of the *A. thaliana* root and reported that cryofixation was superior in several respects, provided that samples were assayed by light microscopy. However, although the cryofixed roots were processed on wire loops, the chemically fixed ones were processed in agarose pellets (different to those described here). Subsequently, the Baskin laboratory started using wire loops for chemically fixed samples and discovered that many (though not all) of the good properties attributed to cryofixation apparently depended on the wire loop[13]. We hypothesize that immobilizing the sample on, or between, Formvar films restricts the twisting and bending that can occur during processing and thereby maintains the integrity of the sample. We refer to this effect as mechanical fixation.

Choice between the methods of mechanical fixation. The relative merit of agarose versus Formvar loops will depend on the investigator and the project. One notable difference is that many or all of the agarose blocks for a treatment can be processed together in one vial, whereas each loop should be placed in its own vial or loops will stick together. As agarose is a common material in most laboratories, it might be a good place to start. However, if the samples will be used for TEM, the Formvar loop method is preferred because it affords better exchange of solutions during processing,

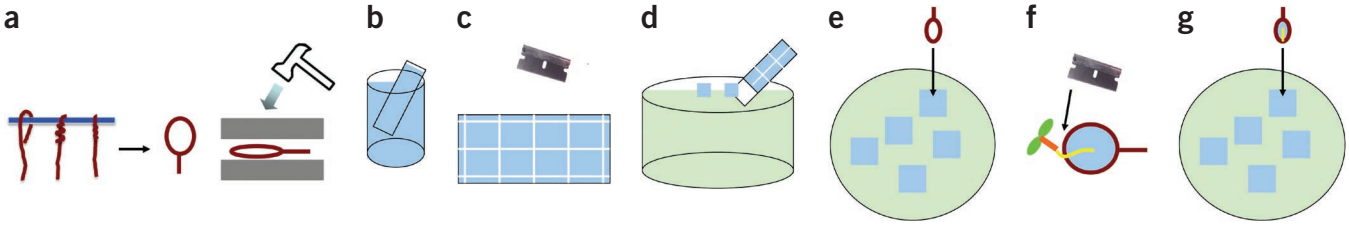

Figure 2 | Schematic illustrating the procedure for making Formvar-coated wire loops (**a**) Form a loop of copper wire (36 gauge) and flatten it between two pieces of smooth steel. (**b**) Immerse a glass slide in 0.25% (wt/vol) Formvar (in ethylene chloride or chloroform), and stand the slide up on end for ~2 min to dry. (**c**) Score the Formvar in a pattern of small rectangles with a sharp blade. (**d**) Immerse the glass side slowly and gently into a beaker filled with water. The Formvar rectangles float and separate. (**e**) Plunge a loop into the middle of a rectangle so that the Formvar surrounds the wire loop. (**f**) Once the loop has dried (usually at least 24 h), place a root tip onto the Formvar surface and cut away the excess of the root with a razor. (**g**) Coat the assembly with another Formvar layer by plunging, as in **e**. See also **Supplementary Video 2**.

Box 2 | Choice of epoxy: Epon or Spurr's?

When choosing an embedding resin, many factors need to be taken into consideration, such as solubility in water, viscosity for penetration, sample volume change during polymerization, structural preservation, sectioning quality and stability under the electron beam[11,12]. Epoxy resins are most frequently used for examining cellular structure, whereas acrylic resins are commonly used for immunological methods.

Epoxy resins require the addition of an accelerator for polymerization and the resin hardness can be adjusted by changing the proportions of various components. They maintain the fine cellular structure well, with little change in volume during polymerization, and they are stable under the electron beam. However, they have relatively high viscosity and require prolonged infiltration. In addition, the density of polymerized epoxy and its hydrophobicity limit antibody access. When antibody recognition is required, acrylic resins are usually chosen. They penetrate samples well and can be polymerized by ultraviolet light at or below room temperature, thereby minimizing heat denaturation of antigens. However, they strongly extract cytoplasmic content and often change the volume of the sample during polymerization.

Epon 812 and Spurr's are two commonly used types of epoxy resins. Epon 812 (related products called Embed 812 or Poly-Bed 812) tends to give higher image contrast than Spurr's but has higher viscosity, which hinders the penetration. This has proved particularly relevant for plants for which improved infiltration through dense cell wall material is possible with Spurr's, thus giving it a decisive advantage.

which is important for thorough infiltration. Formvar is tough and permeable, standing up to organic solvents and posing only a modest barrier to dehydration and infiltration. However, care must be taken when using the wire-loop method, particularly with strong or unusual treatments. For example, the Formvar film is dissolved by propylene oxide. In addition, copper itself is attacked by LR White embedding medium (London Resin Company).

Osmication. A standard procedure for TEM, treatment with osmium tetroxide preserves membrane structure and increases contrast. When immunological methods are planned at the TEM level, preliminary experiments are valuable to determine to what extent the epitopes of interest are masked by osmication. It is sometimes possible to find a low concentration (or short treatment time) that gives beneficial effects of osmium (contrast, membrane preservation) and allows immunostaining.

***En bloc* staining.** This term refers to staining samples with heavy metal for TEM prior to sectioning. Although many EM protocols omit this step, we found it is helpful, along with the customary on-grid staining, to obtain clear membrane structures and well-contrasted ultrastructure. *En bloc* staining should be performed just after osmium postfixation and before dehydration. In addition, phosphate ions can precipitate heavy metal stains, such as uranyl acetate, and thus when phosphate buffers are used, samples must be thoroughly rinsed in water (Step 8) before the *en bloc* stain.

Dehydration. Common embedding agents are immiscible in water and therefore samples are dehydrated before infiltration. The two most commonly used dehydrating agents are ethanol and acetone. Ethanol is cheaper than acetone but has relatively weaker dehydration capability. Acetone is a strong dehydrant but can extract cytoplasmic contents. Ethanol is sufficient for light microscopy, but for TEM we recommend dehydrating in acetone. Dehydration should be conducted with stepwise increases in the concentrations of the dehydrant to minimize tissue shrinkage (Step 11). In addition, performing all steps at 4 °C can reduce extraction of cellular contents.

Infiltration and embedding. There are different types of embedding resins that can be considered. For TEM it is typical to use epoxy resins such as Epon or Spurr's[11,12] (**Box 2**). Here we use Spurr's because its low viscosity compared with Epon facilitates penetration into mechanically fixed roots. The hardness of the polymerized resin can be adjusted as needed for various samples by changing the ratio of the components[21,22], and we have selected values that work well for the *A. thaliana* root. Epoxy resins such as Spurr's give excellent preservation of ultrastructure but usually mask antigens. In rare cases, etching the epoxy sections can be used to obtain good antigen recognition for immunological work[23,24]. More commonly, for immunocytochemistry in TEM, acrylic resins are used, especially LR White. With heat-sensitive antigens, low temperature embedding in one of the Lowicryl resins can be useful. Finally, if the immunostaining study will be done with light microscopy exclusively, then butyl-methyl methacrylate should be considered, because this resin is easy to work with and extractability after sectioning offers superior access to antigens for antibodies[13].

Customarily, samples are aligned in the mold before embedment. However, the alignment of specimens in this way, especially for a long thin object like the *A. thaliana* root, is often imperfect because the investigator has a limited ability to control the position of the sample and because movement can occur between placement and polymerization. Therefore, after polymerization we trim the block down as appropriate and glue the reduced piece to a metal stub, thereby achieving a greater control over the orientation of the root.

MATERIALS

REAGENTS

▲ **CRITICAL** For all reagents, the user should read the MSDS and follow the indicated precautions regarding use and handling.

- Na_2HPO_4 (BioExpress, cat. no. 0404-500G)
- NaH_2PO_4 (BioExpress, cat. no. 0571-500G)
- NaOH Pellets (BioExpress, cat. no. 0583-500G)
- Formaldehyde (16% (wt/vol)), methanol free, ultrapure (Polysciences, cat. no. 18814-20) ▲ **CRITICAL** As stock, we use 16% (wt/vol) formaldehyde, obtained as an aqueous solution in sealed ampoules from the manufacturer. After opening, we remove any unused stock solution from

the ampoule and store it in a tightly capped tube at 4 °C in the dark for several months.
- Glutaraldehyde (GA): 8% (wt/vol) EM grade (Polysciences, cat. no. 07710) ▲ CRITICAL As stock, we use 8% (wt/vol) GA, obtained as an aqueous solution in sealed ampoules from the manufacturer. After opening, we remove any unused stock solution from the ampoule and store it in a tightly capped tube at −20 °C in the dark for several months.
- Agarose (Genemate, cat. no. E-3120-500) ▲ CRITICAL This agarose has a low melting point (36 ± 1.5 °C).
- Chloroform (Fisher, cat. no. C298-1)
- Formvar (EMS, cat. no. 15800)
- Osmium tetroxide (4% (wt/vol)), EM grade aqueous stock (Polysciences, cat. no. 0972A-20) ! CAUTION Osmium tetroxide is toxic and reactive; handle it with gloves and in a fume hood. There is potential for eye damage from vapors.
- Uranyl acetate (EMS, cat. no. 22400) ! CAUTION It is slightly radioactive and a heavy metal hazard. Check with your institution for guidelines regarding storage and disposal.
- Acetone (HPLC grade; Fisher, cat. no. A949-1)
- Spurr's low viscosity embedding kit (Polysciences, cat. no. 01916-1; kit contains ERL4221 epoxy resin, diglycidyl ether (DER), nonenyl succinic anhydride (NSA) and 2-dimethylaminoethanol (DMAE)) ! CAUTION It is toxic; handle it with gloves and in a fume hood.
- Lead citrate (Sigma-Aldrich, cat. no. 15334)
- Toluidine blue (Fluka, cat. no. 89640)
- Sodium borate (J.T. Baker, cat. no. 1303-96-4)

EQUIPMENT
- 12-well culture plate (BioExpress, cat. no. T-3026-6)
- Fontax tweezers (EMS, cat. no. 72701-F)
- Glass scintillation vials (EMS, cat. no. 72634)
- Glass slides (75 × 25 mm; Corning, cat. no. 2947-75X25)
- Coverslips (Fisher Science, cat. no. 12-540B)
- Fume hood (Labconco)
- Water filtration system (Millipore; to prepare Milli-Q water)
- Filter (0.22 µm; Whatman, cat. no. 6780-2502)
- LABNET mini incubator (BioExpress, cat. no. I-5110)
- Light microscope (Olympus BX51)
- Glass knife maker (LKB, Type 7801B)
- Glass knife boat (EMS, cat. no. 71007)
- Flat mold (EMS, cat. no. 62352-15)
- Sorvall MT-2 ultra microtome
- 300-mesh uncoated copper grids (EMS, cat. no. G300-cu)
- Tabletop orbital shaker, IKA MTS-4 (IKA, cat. no. 3208000)
- Vial rotator (TAAB, cat no. Type N, RO51)
- Uninsulated copper wire (36 gauge; ARCOR, cat. no. WL585135 M B36)
- Vials for wire loops, polypropylene 'omni-vials' (Wheaton, cat. no. 225402)
- Nail polish (most brands purchased from the local drug store work fine) ▲ CRITICAL Avoid polishes with added hardeners or drying agents.
- Dental wax (Ted Pella, cat. no. 109)
- Aluminum mounting stubs (EMS, cat. no. 70145-10)
- Steel blocks, custom-made (~20 cm × 5 cm × 3 cm)
- Formvar loops (see EQUIPMENT SETUP)
- Hammer (standard, as used by carpenters)
- Wire cutter (standard, as used by electricians, for fine wire)
- Conical tubes (e.g., Falcon tubes)
- Parafilm
- Razor blade

REAGENT SETUP

Phosphate buffer To make this buffer, we mix mono- and dibasic solutions of sodium phosphate as follows: 51% of phosphate stock A: 0.2 M $NaH_2PO_4 \cdot H_2O$ (27.6 g per liter); 49% of phosphate stock B: 0.2 M $Na_2HPO_4 \cdot H_2O$ (28.4 g per liter). This mixture produces a 0.2 M solution of pH 6.8 without requiring titration. The buffer can be made anytime and stored at room temperature for months, but fixatives should be added immediately before use.

Fixative A Aldehyde fixative A is prepared by using 0.5% (wt/vol) formaldehyde, 3% (wt/vol) GA and 0.1 M phosphate buffer (pH 6.8).

Fixative B Aldehyde fixative B is prepared by using 4% (wt/vol) formaldehyde, 0.5%(wt/vol) GA and 0.1 M phosphate buffer (pH 6.8).

! CAUTION Fixative is toxic. Use caution when working with fixatives. Work in a fume hood and use correct disposal methods. Fixatives should be freshly prepared and used immediately with minimal storage time because of fast deterioration.

Low-melting agarose Weigh the agarose and add it to 10 ml of double-distilled (dd) H_2O in a 50-ml Falcon tube. Microwave the tube on full power for ~15 s, mix well and place the tube in a 60 °C incubator (if no incubator is available, microwave the tube again for 15 s to liquefy the agarose). A solution of agarose can be stored at room temperature for weeks and used repeatedly many times. Cut ~0.5 cm off the pipette tips to facilitate applying agarose to samples.

Osmium tetroxide fixative Mix 1 part osmium tetroxide stock solution (4%, wt/vol) with 3 parts phosphate buffer solution. Unused OsO_4 should be stored in small glass bottles (not plastic), sealed well, at −20 °C. Because of the reactivity of osmium, we recommend storage in nested containers and the generous use of Parafilm to seal them. Storage beyond 6 months is not recommended. ! CAUTION It is toxic and reactive. Use caution when working with osmium. OsO_4 is volatile; the gas can quickly damage both the eyes and lungs. Always work in a fume hood and use correct disposal methods.

Spurr's resin Weigh the following components into a 50-ml Falcon tube: 8.2 g of ERL4221, 2.83 g of DER, 11.8 g of NSA and 0.2 g of the accelerator DMAE. Add DMAE last and only after completely mixing the previous components. The mixed resin can be stored at 4 °C for up to 1 week. The complete mixture is driven to polymerize rapidly at 60 °C, but it will polymerize slowly even at room temperature. Therefore, we make one solution of Spurr's without DMAE for infiltration ('I-Spurr's') and a second, complete solution for embedding ('E-Spurr's'). ! CAUTION The components of Spurr's resin are toxic and suspected carcinogens. Work with them in a fume hood and use proper disposal methods. Excess amounts of the mixture can be polymerized prior to disposal. ▲ CRITICAL Stir resin gently to avoid forming bubbles because they hinder infiltration. If bubbles form, remove them by applying a gentle vacuum.

Toluidine blue Prepare the toluidine blue solution by adding 4 g of toluidine blue crystals to 100 ml of Milli-Q H_2O. Prepare sodium borate solution by adding 4 g of sodium borate in 100 ml of Milli-Q H_2O. Mix the two solutions at 1:1 and filter the solution through a syringe with a 0.22-µm pore filter. The solution can be stored at room temperature for months.

CO_2-free water To prepare CO_2-free water, microwave ~1 liter of Milli-Q water in a beaker for 10 min and let it cool to room temperature; it should be covered with Parafilm to prevent absorbing CO_2 from the air. This must be freshly prepared before use.

Uranyl acetate Add 0.4 g of uranyl acetate powder to 20 ml of CO_2-free water in a 50-ml Falcon tube and shake the tube gently until the powder is dissolved. Uranyl acetate is photoreactive and can precipitate in the light. Wrap the tube with aluminum foil to block the light. Withdraw ~20 ml into a syringe, attach a 0.22-µm filter, and cap tightly. Store the sealed syringes at 4 °C in the dark. Solutions remain usable for many months. ! CAUTION Heavy-metal stains are toxic; use with care.

Lead citrate Add ~0.03 g of lead citrate to 5 ml of 1 N NaOH (made in CO_2-free Milli-Q water) in a 50-ml Falcon tube. Add CO_2-free water to 20 ml. Close the tube tightly and shake it vigorously until all the lead citrate is dissolved. Withdraw ~20 ml into a syringe, attach a 0.22-µm filter, and cap tightly. Store the sealed syringes at 4 °C in the dark. Solutions remain usable for many months. ! CAUTION Heavy-metal stains are toxic; use with care.

Formvar Dissolve Formvar in ethylene chloride or chloroform to make a 0.25% (wt/vol) solution (Formvar solutions can also be purchased). The solution can be stored at room temperature indefinitely. Although great care must be taken to keep Formvar clean in order to uniformly coat grids for use during TEM, when making loops we have found that no special precautions are needed.

EQUIPMENT SETUP

Glass knives Prepare glass knives by following the knife-making machine's instructions. Carefully inspect the edge of the knife under a stereomicroscope. A sharp knife will have a fine, straight edge. Adjust the height of the boat and edge of the knife so they are the same level. Attach the boat to the knife using nail polish or molten dental wax. If you use dental wax to affix the boat to the knife, a heated metal spatula works well to melt the wax onto the boat. Ensure that the boat is securely sealed to the knife.

Formvar loops The length of the loop plus stem (usually 3–4 cm) should be a little less than the height of the vials to be used for dehydration and infiltration (**Supplementary Video 2**). Cut copper wire into lengths that are

about 2 cm longer than the desired final length. Loop one end of the copper around a cylindrical metal piece, such as the shaft of a screwdriver, and twist the excess around the stem, forming a stable loop structure (**Fig. 2a**). Generally, a loop diameter of 3 mm is convenient. Flatten the loop between two smooth steel blocks. Dip a glass slide into the Formvar solution and let it dry for a few minutes (**Fig. 2b**). Once it is dry, place the slide on a clean, flat surface and hold it firmly at one end. Score the Formvar film along the two long edges of the slide (as close to the edge as possible) and also up the middle longitudinally. Then score along the short edge opposite to that being held and make additional transverse scores, forming rectangles (approximately 4 mm × 10 mm for 3-mm wire loops; **Fig. 2c–e**). While holding the slide from the same place and with the cut surface facing up, slowly dip the slide into a container of water, keeping the slide at ~45° to the water surface. The Formvar rectangles should detach from the slide and float on the surface of the water. Plunge a wire loop into the middle of the rectangle so that Formvar surrounds the wire loop. Store loops by plugging the stem into a piece of foam or clay at room temperature. Once they have dried, cover them with a glass container (e.g., a 1-liter beaker). They are fragile but if undisturbed can be used after months and even years of storage.

The water surface tension occasionally fails to detach the Formvar rectangles from the slide. There are several ways to facilitate the detachment of Formvar rectangles from the slide. First, be sure the outside edges of rectangles (along the slide edges) are well scored (**Fig. 2c**). Second, a slide can be made less sticky by soaking for a few minutes in acetone and air drying, by rapidly sliding lens paper back and forth over the slide's surface, or by gently touching the slide to one's nose or forehead.

PROCEDURE

Chemical fixation ● TIMING 2 h

1| Fix seedlings either by transferring whole seedlings with forceps from the growth plate to ~5 ml of fixative solution in one well of a multiwell plate or (preferred) by pouring the fixative directly onto the seedlings on the growth plate (10 ml for a standard 10-cm diameter Petri dish). Fix for 2 h at room temperature with gentle orbital shaking (~30 r.p.m.).

2| Wash seedlings three times, for 10 min each time, in phosphate buffer without fixative. When using a multiwell plate, transfer seedlings to a new well with fresh solution. When fixing directly on the plates, carefully remove the solution with a transfer pipette and add ~10 ml of fresh solution to the plate.

Mechanical fixation ● TIMING 1 h

3| Mechanical fixation can be achieved using either the agarose method (option A; see also **Fig. 1** and **Supplementary Video 1**) or Formvar loops (option B; see also **Fig. 2** and **Supplementary Video 2**).

(A) Agarose method

▲ CRITICAL Except for the agarose, the materials and surfaces for option A can be at room temperature. Avoid air bubbles during pre-embedding because they impair dehydration and infiltration.

(i) Put one drop (~200 µl) of molten agarose onto a clean surface (e.g., glass plate or plastic Petri dish lid) and immediately cover it with a coverslip (18 mm × 18 mm). Wait for 10–15 s and remove the coverslip. The agarose forms a flat thin layer (**Fig. 1a**).

(ii) By using forceps, pick up a single seedling from Step 2 and let the root tip touch the now-solid agarose layer and then lay the seedling down slowly, gently dragging the seedling to straighten the root (**Fig. 1b,c**). If desired, several roots can be aligned side by side so they can be processed simultaneously.

(iii) Immediately place a small drop of molten agarose (~15 µl) in the vicinity of the tip and let it spread to the root tip (**Fig. 1d**). Wait for several seconds and then add another drop of agarose in the same way. Wait for several more seconds and apply a third, somewhat larger drop (100–150 µl) of agarose to ensure that the root and about 5 mm or more on all sides is covered (**Fig. 1e**).

▲ CRITICAL STEP Add the first two drops slowly and do not drop the agarose directly onto the root or the root tip might float up or bend. Wait for several seconds so the two drops become solidified to anchor the roots.

(iv) After the agarose block becomes well solidified (approximately 5–10 min), use a thin double-edged razor blade to trim a block containing the root tip (**Fig. 1f**, top), scraping away the excess agarose from around all sides of the root (**Fig. 1f**, bottom). Use forceps to remove the block containing the root tip by coming in under the block and lifting up. Try to avoid squeezing the sample. Place the sample in a vial containing fixative solution (Step 4). For the *A. thaliana* roots, the dimensions of the agarose block was sufficient to increase the sample's rigidity but not large enough to unduly hinder infiltration (for example, see **Supplementary Video 1**).

▲ CRITICAL STEP The agarose blocks should be immersed in a volume of the fixative sufficient to generously cover the samples but not so much as to fill the vial; otherwise, exchange of the contents between sample and solution will be restricted. For agarose embedment, all samples of a given treatment can be collected in a single vial. Although it is not essential, we strongly recommend rotating the samples slowly (~60 r.p.m.) during all subsequent steps.

(B) Wire loop method

▲ CRITICAL All of the surfaces and material for option B can be at room temperature. Be sure to prepare the blank Formvar loops at least 1 d in advance so they are thoroughly dry before this step (see EQUIPMENT SETUP).

(i) Float a new set of Formvar rectangles, as described in EQUIPMENT SETUP. This can be done during the chemical fixation (Step 1).

(ii) Place a loop on a raised glass slide or other clean surface so that the loop rests on a droplet of water and the stem is free in the air. By using forceps, lift a seedling and place the root tip in the center of the loop and then place the root so that it lies along the surface of the Formvar (**Fig. 2f**). Cut the rest of the root away at the copper. If desired, a second root can be added. Then, slide the loop sideways off of the surface and plunge it onto a Formvar rectangle (**Fig. 2g**). Transfer the loop with sample to a vial with fresh fixative.
▲ **CRITICAL STEP** Place each wire loop in a separate vial, containing a volume of solution sufficient to cover the loop but not so much as to fill the vial; otherwise, exchange of the contents between sample and solution will be restricted. Although it is not essential, we strongly recommend rotating the samples slowly (~60 r.p.m.) during all subsequent steps.

Second chemical fixation ● TIMING overnight

4| Incubate the samples (agarose blocks from Step 3A(iv) or wire loops from Step 3B(ii)) in fresh fixative for 12–18 h (i.e., overnight) at 4 °C. Apply gentle vacuum to improve the penetration of fixatives if bubbles are visible near the sample.
▲ **CRITICAL STEP** Overnight fixation is preferred because a thorough fixation will improve dehydration, infiltration and embedding. Fixation for more than 24 h is not recommended, particularly for immunological methods because the long fixation can interfere with antigenicity.

Osmication ● TIMING 4 h

5| Wash the samples three times for 10 min each time in phosphate buffer without fixative.
▲ **CRITICAL STEP** Trace amounts of GA can reduce the effectiveness of the OsO_4.

6| Incubate the samples in ~2 ml of 1% (wt/vol) OsO_4 for 3–4 h at 4 °C.
▲ **CRITICAL STEP** This step should be omitted for light microscopy.

***En bloc* staining ● TIMING 4 h or overnight**

7| Transfer the samples to fresh vials.

8| Wash at least five times in distilled water (10 min each time).

9| Incubate in ~2 ml of 2% (wt/vol) uranyl acetate at room temperature for 2–3 h. Keep the samples out of the light.
■ **PAUSE POINT** This step can also be done at 4 °C overnight.

10| Wash the samples at least three times (10 min each time) in distilled water.

Dehydration ● TIMING 2.5 h or overnight

11| Use the same vial but replace solutions as follows: 30% (vol/vol) acetone, 50% (vol/vol) acetone, 70% (vol/vol) acetone, 90% (vol/vol) acetone, 100% acetone, 100% acetone and 100% acetone for 20 min each time. Carry out this step at 4 °C, with rotation. Alternatively, ethanol can be used at the same concentrations and for the same times. For dehydration, mix acetone (or ethanol) with ddH_2O.
■ **PAUSE POINT** Samples can be left in 100% acetone overnight.

Infiltration ● TIMING 4.5 d

12| Use the same vials and replace solutions, as follows: 10:1, 100% acetone/I-Spurr's, 4 h; 5:1, 100% acetone/I-Spurr's, 4 h; 3:1, 100% acetone/I-Spurr's, overnight; 2:1, 100% acetone/I-Spurr's, 8 h; 1:1, 100% acetone/I-Spurr's, overnight; 1:2, 100% acetone/I-Spurr's, 12–16 h; 1:3, 100% acetone/I-Spurr's, overnight; 100% I-Spurr's, 3–5 h; 100% I-Spurr's, 3–5 h; 100% I-Spurr's, overnight. Maintain samples at 4 °C, except during solution exchange, which should be done in a fume hood.

13| Replace the solution with 100% Spurr's with accelerator (E-Spurr's) and incubate for 8–12 h at room temperature.

Polymerization ● TIMING 1 d

14| Place a few drops of E-Spurr's into a flat mold.

15| For agarose samples, remove them all from the vial into a small dish and separate. By using forceps, transfer each one to a mold, taking care to straighten the blocks. For wire-loop samples, holding the stem with forceps, immerse the loop into the E-Spurr's in the mold, use a wire cutter to cut the stem, allowing the loop to be positioned within the mold as needed. It is not necessary to be overly concerned with root alignment at this step.

16| Once samples are placed, fill the remaining space in the mold with E-Spurr's and reposition the samples if necessary.

17| Carefully transfer the mold to a 60 °C oven for 24 h for polymerization.

Trimming ● TIMING 4 h to 1 d

18| Remove the block from the mold and trim the resin block so that only a rectangular region containing the root remains. Trim the resin block roughly using a single-edged razor blade. For wire-loop samples, the copper can be cut through using the razor with care. Alternatively, the resin above the copper can be cut away and the wire loop lifted out from the resin.

19| Glue the trimmed block onto an aluminum stub.
▲ CRITICAL STEP Try to keep the root parallel to the aluminum stub surface.

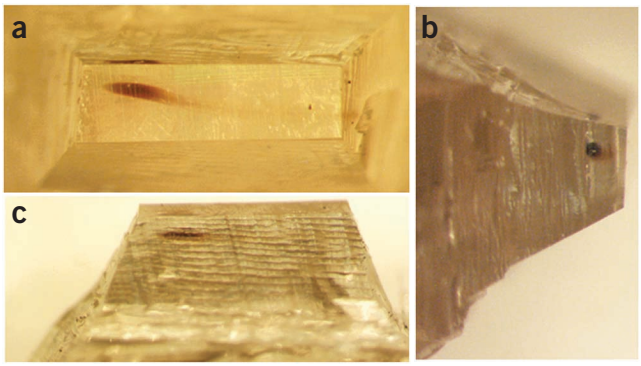

Figure 3 | An *A. thaliana* root embedded in Spurr's resin after initial trimming. This resin block is trimmed under a dissecting microscope and microtome, using a double-sided razor blade and glass knives. (**a**–**c**) View of the block from different sides: top (**a**), flanking side (**b**) and front side (**c**).

20| Use a sharp double-edged razor blade to cut away excess resin from the region of interest. The block face for sectioning should be trimmed into a trapezoid shape that facilitates sectioning.

21| Roughly trim the surface of the block using a glass knife so the root is visible from above the block (**Fig. 3**). If the trapezoidal shape is damaged, retrim the block using a double-edge razor blade.

Sectioning ● TIMING 4 h to several days

22| Use a glass knife to cut semithin sections (~2 µm) until the root or the desired region of the specimen is exposed.

23| Once the semithin sections begin to include the root, check the alignment as follows: collect a section on a glass slide, add a drop of the toluidine blue, heat (use a warming tray at 80 °C or ethanol burner) for a minute or two, remove excess stain with running water, and examine using a simple compound microscope at ×10 or ×20.
▲ CRITICAL STEP If the root is embedded in Spurr's resin, be sure to use toluidine blue solution containing 1% (wt/vol) sodium borate to enhance staining and elongate the staining time to 10 min.
? TROUBLESHOOTING

24| Cut ultrathin sections (~70 nm). Orient the long edge of the trapezoidal block face so that it reaches the knife first, as doing so will reduce compression and allow the section to be detached from the knife during sectioning. To release the surface tension of the water, put in several drops of 70% (vol/vol) ethanol. For sectioning, when a diamond knife is not available, use freshly made glass knives because they become dull over time even without use. Old or slightly dull knives can be used for trimming and semithin sections.
? TROUBLESHOOTING

25| To collect sections, pick up a copper grid using sharp forceps and collect the section(s) by inserting the grid in the water under the section ribbon and slowly removing them from the water at a 45° angle. Dry the grid by blotting (section side upward) on a filter paper. Store the grids in a grid box or small Petri dishes before staining. We routinely use 300-mesh grids. A coarser mesh allows larger contiguous areas of the sample to be imaged, but at the cost of being more fragile in the electron beam. To look at larger sample areas, or for those samples that infiltrate poorly or are otherwise susceptible to damage from the electron beam, the grid can be coated with Formvar for support[10], although doing so will lead to some loss of contrast.
▲ CRITICAL STEP To extend wrinkled or compressed sections before collecting them, wave a piece of filter paper soaked in chloroform or a heat pen close to the floating sections for a few moments without touching them. Note that extensive compression might be due to a dull knife, inadequate infiltration or to incomplete polymerization of the resin. Be sure to keep track of which side of the grid the sections are on. The dull side is preferred because it allows the sections to adhere more securely.
? TROUBLESHOOTING

Heavy-metal staining ● TIMING **1 h**

26| Cover the outsides of two Petri dishes (9 cm) with aluminum foil and place a strip of Parafilm in the bottom of each dish (**Fig. 4**).

27| Place several, independent drops of the uranyl acetate solution on the Parafilm of one dish and float each grid on a drop, section-side down. Cover with the lid of the Petri dish and incubate for 15–20 min.

? TROUBLESHOOTING

28| Rinse the grids by quickly immersing them 15–20 times in a beaker of CO_2-free ddH_2O.

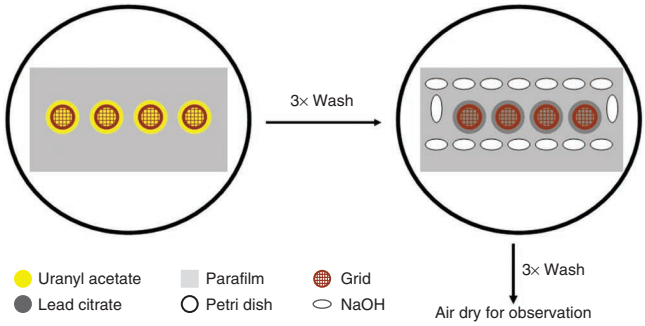

Figure 4 | Schematic illustrating heavy-metal staining. Note the large number of NaOH pellets (~4 per grid) used with lead staining.

29| Place drops of the lead citrate solution on the Parafilm of the other Petri dish from Step 26 and surround them with NaOH pellets. Float each grid from Step 28 on a drop, immediately cover with the lid of the Petri dish and incubate for 5–10 min.

▲ **CRITICAL STEP** Keep the Petri dish containing lead citrate covered as much as possible and avoid breathing on it.

? TROUBLESHOOTING

30| Rinse the grids by quickly dipping them four to six times into a beaker of CO_2-free Milli-Q H_2O.

31| After the last rinse, blot off the excess water on filter paper and place the grids on dry filter paper; air-dry them for 10–15 min.

▲ **CRITICAL STEP** Move the grids quickly between solutions and do not let the sections dry until after the final rinse.

32| The samples are now ready for TEM imaging.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
23	Root is not straight	The agarose layer is not flat	Use less agarose and spread it as much as possible
		Fomvar loops are not flat enough	Make loops of smaller diameter and be sure to flatten them well before use
24	Block face gets wet and sections slip back for each sectioning run	Too much water in the boat; water tension is too high	Reduce the water level in the boat. Add a few drops of 70% (vol/vol) ethanol
		Block face is too large (Step 19)	Retrim the block to make the surface as small as possible
		Sectioning speed is too low	Increase the cutting speed
		Knife is blunt and angle is too low	Use a new knife; adjust the knife angle
	Knife is cutting every other section	Knife or sample is loose	Ensure that the knife and sample are held firmly
		Knife is blunt	Move to a sharp area on the knife
		Sectioning method needs improvement	Reduce cutting speed Adjust knife clearance angle Reduce the size of the block face

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
25	Wrinkled section	Cutting compression	Use chloroform or a heat pen to spread the sections
27, 29	Sections float off from the grids during staining	Section is too thick and size is too large	Trim the block surface to include only samples or regions of interest Use Formvar-coated grids
32	Sections are unstable or break under electron beam	kV is too high	Reduce accelerating voltage to 70–80 kV
		Insufficient infiltration	Improve fixation, dehydration procedure Prolong each step of infiltration Use a rotator Use resin mixture without accelerator for infiltration Apply gentle vacuum for 15–20 min during the last step of infiltration
		Agarose or resin contains air bubbles	Work slowly with the agarose Minimize agitation
	Sections are contaminated with precipitate	Uranyl acetate precipitate in light	Keep uranyl acetate in the dark
		Lead citrate precipitate in air	Prepare fresh stain and filter before use Do not rinse after staining Avoid breathing on grids Use more NaOH in dish during staining
	Wavy or disrupted membrane structures, swollen organelles, holes in the sections and poor cellular morphology	Poor fixation	Check the pH of the buffer Make sure formaldehyde and glutaraldehyde are fresh Apply vacuum for 5–10 min during fixation
		Uneven penetration of resin	Improve infiltration Use a rotator (or faster rotation) Use longer steps for infiltration Use more gradual steps (especially at the beginning)
	Contrast of images is weak	Not enough staining	Prolong the incubation in osmium tetroxide and the *en bloc* uranyl acetate step Prolong staining of sections in uranyl acetate and in lead citrate

● TIMING

Steps 1 and 2, chemical fixation: 2 h
Step 3, mechanical fixation: 1 h
Step 4, second chemical fixation: overnight
Steps 5 and 6, osmication: 4 h
Steps 7–10, *en bloc* staining: 4 h or overnight
Step 11, dehydration: 2.5 h or overnight
Steps 12 and 13, infiltration: 4.5 d
Steps 14–17, polymerization: 1 d
Steps 18–21, trimming: 4 h to 1 d
Steps 22–25, sectioning: 4 h to several days
Steps 26–32, heavy-metal staining: 1 h

ANTICIPATED RESULTS

As seen in a semithin section, this method allows longitudinal sections to be obtained that span as much as a millimeter or more of the root (**Fig. 5**). In this way, cells in the meristem, zone of rapid elongation and mature zone can be sampled

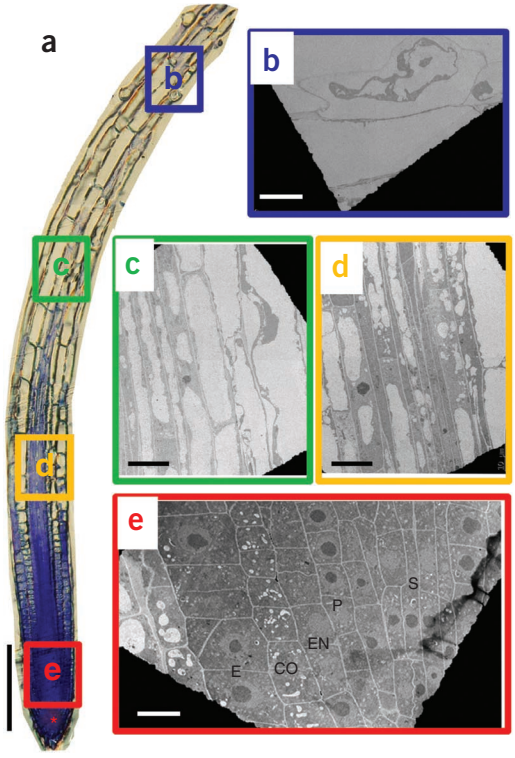

Figure 5 | Imaging defined regions of the *A. thaliana* root in longitudinal sections. (**a**) Reference semithin section stained with toluidine blue and used to guide and refine further trimming for TEM. Scale bar, 200 µm. Boxed areas show distinct regions of the root, with corresponding TEM images. (**b**) Mature zone. (**c**) Mid-elongation zone. (**d**) Apical region of the elongation zone. (**e**) Meristem. Red asterisk shows the approximate position of the quiescent center, a convenient reference point in this object for distance measurement. In **e**, the different cell types, including epidermis (E), cortex (CO), endodermis (EN), pericycle (P) and stele cells (S), are easily distinguished and extrapolated to basal regions with reference to **a**. Scale bars in **b**–**e**, 10 µm.

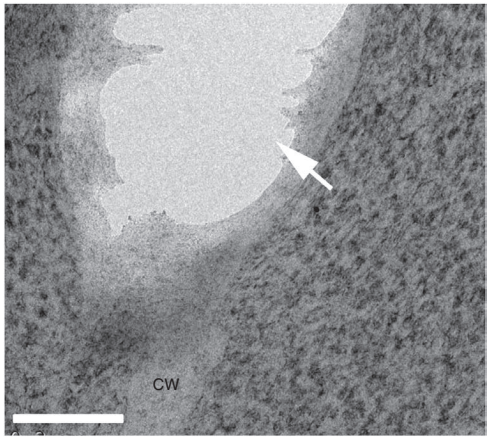

Figure 6 | An example of section damaged by the electron beam. This kind of instability is often caused by inadequate infiltration. CW, cell wall. Arrow marks the area that was broken under the electron beam. Scale bar, 200 nm.

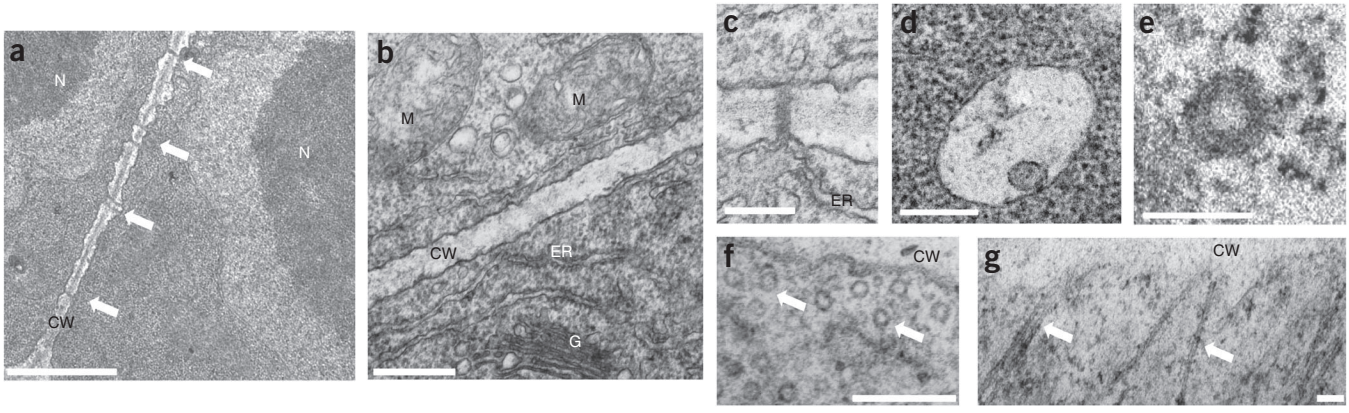

Figure 7 | Examples of ultrastructure seen in positionally defined cells. (**a**) The junction between two pericycle cells (~70 µm from quiescent center). Note the density of plasmodesmata (arrows). Scale bar, 2 µm. (**b**) The junction between two endodermal cells (~100 µm from quiescent center). Endoplasmic reticulum (ER), mitochondria (M) and Golgi (G) are evident. Scale bar, 500 nm. (**c**) A magnified image of a single plasmodesma between two pericycle cells (~70 µm from quiescent center). Note the association with the ER. Scale bar, 100 nm. (**d**) Stele cell (~50 µm from quiescent center) containing a late endosome (also called a multivesicular body). Scale bar, 100 nm. (**e**) Stele cell (~50 µm from quiescent center) containing a putative clathrin-coated vesicle. Scale bar, 100 nm. (**f**,**g**) Epidermal cells in the elongation zone (~1,200 µm from quiescent center). Arrows point to the cortical microtubules shown in the cross-section (**f**) and the longitudinal section (**g**). Scale bars, 200 nm. CW, cell wall; N, nucleus.

reliably from the same root; indeed from the same section if only light microscopy is to be used. For TEM, where optimal block length is typically <1 mm, the well-oriented root makes it straightforward to define the positions from which the sectioned material was obtained after further trimming (**Fig. 5**). In well-oriented, longitudinal sections, cell types are reliably identifiable (**Fig. 5e**).

At higher magnification, poor infiltration can make the sample unstable under the electron beam, causing tears in the section and loss of the region of interest (**Fig. 6**). However, when processing is handled correctly, our protocol is consistent

with good preservation (**Fig. 7**), as judged by the appearance of organelles (nucleus, mitochondrion, Golgi, endoplasmic reticulum) and observed ultrastructure (e.g., plasmodesmata, cortical microtubules).

Moreover, because the anatomical context is defined, ultrastructure can be observed as a function not only of cell type but also of position in the organ. With this protocol, we quantified plasmodesma diameter in stele cells and found that within the meristem this diameter decreased concomitant with an increase in the deposition of callose; an accumulation that correlates with blocked movement of the SHORT-ROOT (SHR) transcription factor between these cells[17]. In addition, the protocol allowed us to localize a binding partner of the SHR transcription factor to the endoplasmic reticulum and specific vesicles within the stele[16]. This protocol should facilitate any research in which cell structure or ultrastructure needs to be assessed in the context of an intact organ.

Note: Supplementary information is available in the online version of this article.

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AUTHOR CONTRIBUTIONS S.W., T.I.B. and K.L.G. developed the protocol and designed the experiments. S.W. performed the experiments. S.W., T.I.B. and K.L.G. wrote the manuscript.

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1. Dolan, L. *et al.* Cellular organisation of the *Arabidopsis thaliana* root. *Development* **119**, 71–84 (1993).
2. Petersson, S.V. *et al.* An auxin gradient and maximum in the *Arabidopsis* root apex shown by high-resolution cell-specific analysis of IAA distribution and synthesis. *Plant Cell* **21**, 1659–1668 (2009).
3. Brady, S.M. *et al.* A high-resolution root spatiotemporal map reveals dominant expression patterns. *Science* **318**, 801–806 (2007).
4. Nawy, T. *et al.* Transcriptional profile of the *Arabidopsis* root quiescent center. *Plant Cell* **17**, 1908–1925 (2005).
5. De Smet, I. *et al.* Bimodular auxin response controls organogenesis in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **107**, 2705–2710 (2010).
6. Oorschot, V., de Wit, H., Annaert, W.G. & Klumperman, J. A novel flat-embedding method to prepare ultrathin cryosections from cultured cells in their *in situ* orientation. *J. Histochem. Cytochem.* **50**, 1067–1080 (2012).
7. Beeckman, T. & Viane, R. Embedding thin plant specimens for oriented sectioning. *Biotech. Histochem.* **75**, 23–26 (2000).
8. De Smet, I. *et al.* An easy and versatile embedding method for transverse sections. *J. Microsc.* **213**, 76–80 (2004).
9. Leroux, O., Van der Kinderen, G. & Viane, R.L. A sandwich-embedding method for oriented sectioning. *J. Microsc.* **227**, 79–82 (2007).
10. Di Laurenzio, L. *et al.* The *SCARECROW* gene regulates an asymmetric cell division that is essential for generating the radial organization of the *Arabidopsis* root. *Cell* **86**, 423–433 (1996).
11. Glauert, A.M. & Lewis, P.R. *Biological Specimen Preparation for Transmission Electron Microscopy* (Portland Press, London, UK, 1998).
12. Ruzin, S.E. *Plant Microtechnique and Microscopy* (Oxford University Press, New York, NY, 1999).
13. Baskin, T.I. & Wilson, J.E. Inhibitors of protein kinases and phosphatases alter root morphology and disorganize cortical microtubules. *Plant Physiol.* **113**, 493–502 (1997).
14. Andème-Onzighi, C., Sivaguru, M., Judy-March, J., Baskin, T.I. & Driouich, A. The *reb1-1* mutation of *Arabidopsis* alters the morphology of trichoblasts, the expression of arabinogalactan proteins and the organization of cortical microtubules. *Planta* **215**, 949–958 (2002).
15. Wu, S. *et al.* A conditional mutation in *Arabidopsis thaliana* separase induces chromosome non-disjunction, aberrant morphogenesis and cyclin B1;1 stability. *Development* **137**, 953–961 (2010).
16. Koizumi, K., Wu, S., MacRae-Crerar, A. & Gallagher, K.L. An essential protein that interacts with endosomes and promotes movement of the SHORT-ROOT transcription factor. *Curr. Biol.* **21**, 1559–1564 (2011).
17. Vatén, A. *et al.* Callose biosynthesis regulates symplastic trafficking during root development. *Dev. Cell* **21**, 1144–1155 (2011).
18. Graham, L. & Orenstein, J.M. Processing tissues and cells for transmission electron microscopy in diagnostic pathology and research. *Nat. Protoc.* **2**, 2439–2450 (2007).
19. Lancelle, S.A., Callaham, D.A. & Hepler, P.K. A method for rapid freeze fixation of plant cells. *Protoplasma* **131**, 153–165 (1986).
20. Baskin, T.I., Miller, D.D., Vos, J.W., Wilson, J.E. & Hepler, P.K. Cryofixing single cells and multicellular specimens enhances structure and immunocytochemistry for light microscopy. *J. Microsc.* **182**, 149–161 (1996).
21. Newman, G.R. & Hobot, J.A. Resins for combined light and electron microscopy: a half century of development. *Histochem. J.* **31**, 495–505 (1999).
22. Ellis, E.A. Solutions to the problem of substitution of ERL 4221 for vinyl cyclohexene dioxide in Spurr low viscosity embedding formulations. *Microsc. Today* **14**, 32–33 (2006).
23. Groos, S., Reale, E. & Luciano, L. Re-evaluation of epoxy resin sections for light and electron microscopic immunostaining. *J. Histochem. Cytochem.* **49**, 397–406 (2001).
24. Kiernan, J.A. Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: What they are and what they do. *Microsc. Today* **8**, 8–12 (2000).