Chapter 7

Fluorescence Imaging of the Cytoskeleton in Plant Roots

Julia Dyachok, Ana Paez-Garcia, Cheol-Min Yoo, Karuppaiah Palanichelvam, and Elison B. Blancaflor

Abstract

During the past two decades the use of live cytoskeletal probes has increased dramatically due to the introduction of the green fluorescent protein. However, to make full use of these live cell reporters it is necessary to implement simple methods to maintain plant specimens in optimal growing conditions during imaging. To image the cytoskeleton in living Arabidopsis roots, we rely on a system involving coverslips coated with nutrient supplemented agar where the seeds are directly germinated. This coverslip system can be conveniently transferred to the stage of a confocal microscope with minimal disturbance to the growth of the seedling. For roots with a larger diameter such as *Medicago truncatula*, seeds are first germinated in moist paper, grown vertically in between plastic trays, and roots mounted on glass slides for confocal imaging. Parallel with our live cell imaging approaches, we routinely process fixed plant material via indirect immunofluorescence. For these methods we typically use non-embedded vibratome-sectioned and whole mount permeabilized root tissue. The clearly defined developmental regions of the root provide us with an elegant system to further understand the cytoskeletal basis of plant development.

Key words Actin, Arabidopsis, *Medicago truncatula*, Microtubules, Green fluorescent protein, Living cells, Roots, Immunofluorescence, Fixed plant material, Sectioning

1 Introduction

An important requirement for research on the plant cytoskeleton is the ability to routinely and reliably image its organization in the cell. This goal has been facilitated in large part by advances in fluorescence microscopy not only with regard to the development of advanced microscope systems but also through the introduction of reagents that allow for in vivo labeling of cytoskeletal elements [1–3]. Microtubules and actin filaments (F-actin), the two major components of the cytoskeleton, can now be visualized readily in living plant cells using an array of green fluorescent protein (GFP) constructs (e.g., [4–6]). Despite the rapid implementation of live cell imaging tools, methods for optimal fixation of plant tissues for cytoskeletal studies will remain an essential tool for plant biologists. This is because these fixation approaches not only verify results

Ray H. Gavin (ed.), *Cytoskeleton: Methods and Protocols*, Methods in Molecular Biology, vol. 1365,
DOI 10.1007/978-1-4939-3124-8_7,

from live cell probes, they also have led to the discovery of novel cytoskeletal structures not always revealed by in vivo cellular reporters (e.g., [7–9]). Furthermore, live imaging of the cytoskeleton has for the most part been limited to cells located on the plant surface such as epidermal cells, trichomes, and root hairs (e.g., [10–12]). In cells located within the plant interior especially those from plant species with thicker organ systems, it is necessary to utilize conventional methods of sectioning and permeabilization to allow access of the cytoskeletal label (e.g., [12–14]). In this chapter we describe procedures for fluorescent labeling of the cytoskeleton in plant roots for observation with confocal microscopy. We focus on plant roots because they provide an elegant system for studying the cytoskeletal basis of plant development [15]. We first describe procedures for fluorescent antibody labeling of non-embedded root sections and permeabilized whole roots. We then outline methods for preparing Arabidopsis and larger roots of the model legume *Medicago truncatula* for live cell imaging of fluorescently labeled cytoskeletal structures.

2 Materials

2.1 Vibratome Sectioning and Whole Mount Immunolabeling of Fixed Roots

1. 37 % formaldehyde.
2. Paraformaldehyde (16 % solution).
3. Dimethyl sulfoxide (DMSO).
4. PME buffer: 50 mM *P*IPES (piperazine-*N,N′* bis [2-ethanesulfonic acid]), 2 mM *M*gCl_2 and 10 mM *E*GTA. Adjust pH of PME to 7.0 using 10 N NaOH.
5. Phosphate buffered saline (PBS), pH 7.2 for diluting antibodies and pH 8.5 for preparing the mounting medium. PBS buffer: 135 mM NaCl, 25 mM KCl, 5 mM Na_2HPO_4, and 2 mM KH_2PO_4. To prepare PBS with a pH of 8.5, omit the KH_2PO_4 and adjust the pH with 10 N NaOH.
6. Cellulase YC and Pectolyase Y23.
7. 1 % (v/v) Triton X-100 in PME buffer 8. Mowoil 4-88 or any mounting reagent (e.g., Citifluor and VECTASHIELD).
8. Vibratome.
9. Superglue.
10. Double edged razor blades and fine forceps.
11. Bovine serum albumin and normal goat serum.
12. Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). To prepare 0.16 M MBS stock solution, dissolve 50 mg MBS in 1 DMSO. MBS stock solution can be stored at −20 °C for several months.
13. Methanol prechilled to −20 °C.

14. PEMT: 0.05 % (v/v) Triton X-100 in PME buffer.
15. PBST: 0.05 % (v/v) Triton X-100 in PBS buffer, pH 7.0.
16. Tissue culture inserts (TCIs, Corning Netwell Plate, 12-Well Cluster, 500 μm pore size).
17. Rotating shaker table.
18. Actin and tubulin antibodies.
19. Fluorescently labeled phalloidin.

2.2 Preparing Arabidopsis Seedlings for Live Cell Imaging of Roots

1. Agar or Agargel (*see* **Note 1**).
2. Murashige & Skoog (MS) basal salt mixture.
3. 48 × 64 × 0.13–0.17 mm coverslips.
4. Sterilized pointed end toothpicks.
5. Polystyrene sterile petri dishes.
6. Sterilized filter paper.

2.3 Preparing Medicago truncatula Seedlings for Live Cell Imaging of Roots

1. Sandpaper.
2. Whatman filter paper circles 70 mm diameter.
3. Polystyrene sterile petri dishes.
4. Germination paper 18 × 13 in.
5. Plastic trays.

3 Methods

3.1 Immunolabeling of Fixed Roots

In both sectioning and whole mount permeabilization of roots, the general methods for fixation to preserve cytoskeletal structures are roughly similar. The only major difference is that the latter requires specialized sectioning equipment while the former relies on extensive digestion of the root with cell wall degrading enzymes.

3.1.1 Immunolabeling the Cytoskeleton in Vibratome Sectioned Roots

1. For plant species with large roots (ca. 300 μm to 1 mm in diameter) such as *Zea mays* or *Medicago truncatula*, excise the terminal 5 mm of the primary root with a razor blade and immerse root segments in 3.7 % formaldehyde and 5 % DMSO in PME buffer (v/v) (*see* **Note 2**). We typically use clear 20 mL scintillation glass vials with rubber-lined caps to hold the fixative containing the root segments. Place uncapped vials containing fixative and the collected root tissues in a small vacuum dessicator jar equipped with a T connector. Apply a light vacuum for 10–20 min. After releasing the vacuum most of the root samples should sink to the bottom of the vial.
2. After incubating samples in fixative for 2 h, slowly aspirate the fixative with a pipette and dispose excess fixative in clearly marked waste bottles in a fume hood. Wash the samples by

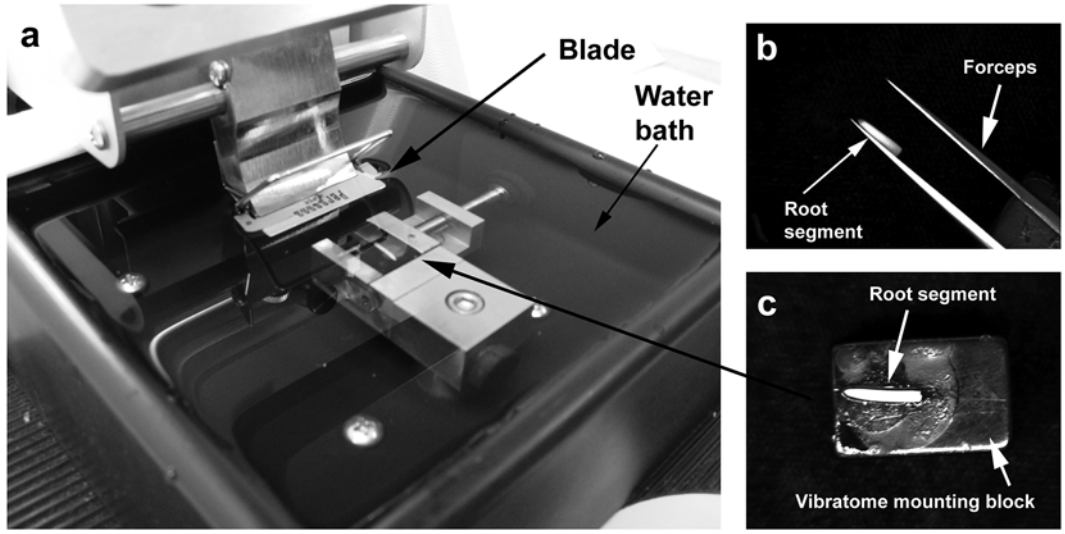

Fig. 1 Handling root tissues for vibratome sectioning. (**a**) The specimen holder of a vibratome 1000 showing the blade and block holder. (**b**) A segment of a fixed maize primary root on the tip of a pair of fine forceps. (**c**) Handling fixed root tissue as shown in (**b**) facilitates mounting the root on superglue coated vibratome block

immersing them in PME buffer for 3–5 min. Repeat this process three times to fully remove residual fixative.

3. For larger roots (i.e., >300 μm in diameter), longitudinal or cross sections can be easily obtained using a vibratome 1000 (Fig. 1a). To prepare root segments for vibratome sectioning, gently lift the excised root segment with a fine pair of forceps and blot excess liquid using filter paper or kimwipes (Fig. 1b). Spread a thin layer of superglue onto the surface of the vibratome mounting block and carefully position the root segment onto the block (Fig. 1c). To obtain good longitudinal sections, the long axis of the root must be perpendicular to the cutting surface of the blade (Fig. 1a). When the root contacts the thin layer of superglue, it takes about 60 s or less for the glue to polymerize allowing the root segment to firmly attach to the block (Fig. 1c; *see* **Note 3**).
4. Break a double edged razor blade in half and fasten half of the broken blade to the vibratome. Fill the vibratome specimen bath with deionized water and secure the block with the adhered root segment onto the block holder making sure that the root sample is fully immersed in water (Fig. 1a). Set the vibratome to section at 50–100 μm thickness following the manufacturers' instructions (*see* **Note 4**). Depending on the size of the root, one can obtain about 3–8 good quality longitudinal sections for cytoskeletal labeling.
5. Collect sections from the vibratome water bath using the wide end of a Pasteur pipette or a pair of fine forceps and transfer

sections to polystyrene petri dishes containing PME buffer. Alternatively, sections can be directly placed onto 22 × 22 coverslips or glass slides. Use a small piece of filter paper to remove excess PME buffer by touching the liquid adjacent to the root sections. Allow sections to partially dry onto the surface of the slide or coverslip (*see* **Note 5**).

6. When sections are secured onto the coverslip, apply a cocktail of 1 % cellulose YC in PME buffer for 10 min. The cellulose solution should cover the root sections during incubation. Slowly decant the cellulose solution and wash sections three times with PME buffer. During the washing steps, PME buffer can simply be added onto the surface of the coverslips containing the root sections and decanted (*see* **Note 6**). After cell wall treatment, incubate samples in 1.0 % Tritron X-100 in PME for 15–20 min and wash three times with PME buffer.
7. Apply primary antibody diluted in PBS, pH 7.2 onto the surface of the coverslips containing the root sections and place in a humid chamber for 2–3 h. Wash three times with PME buffer and apply secondary antibody conjugated to a fluorescent dye that specifically recognizes the primary antibody. Incubate sections in a humid chamber for another 2 h (*see* **Notes 7** and **8**).
8. Prepare the mounting medium while samples are incubating in the secondary antibody. This is accomplished by preparing 20 % Mowiol 4-88 in PBS, pH 8.5 (v/v). A 2 mL volume of mounting media is sufficient for mounting about 10–20 samples. The Mowiol-PBS solution can be prepared in 20 mL scintillation vials similar to those used for tissue fixation (*see* **Step 1** above). To dissolve the Mowiol crystals, stir the solution for 2 h using a small magnetic stir bar (*see* **Note 9**).
9. After secondary antibody treatment, wash samples with PME buffer three times and allow the sections to partially dry. Blot any excess liquid with filter paper and use the wide end of a Pasteur pipette to collect mounting medium (*see* **Note 10**). Carefully drop Mowiol onto the surface of the coverslip and mount sections on clean glass slides. After allowing the Mowiol to polymerize overnight, root sections can then be observed with a confocal microscope. Examples of the quality of cytoskeletal labeling from vibratome-sectioned roots is shown in Fig. 2 (*see* **Note 11**).

3.1.2 Immunolabeling of the Cytoskeleton in Whole Mount Arabidopsis Roots

1. Gently pick 3–4-day-old *Arabidopsis* seedlings growing on nutrient supplemented gel media (*see* Subheading 3.2 below for tips on growing Arabidopsis seedlings) with a fine pair of forceps and directly immerse seedlings into the fixative. For preserving F-actin in Arabidopsis roots we use 2 % (v/v) paraformaldehyde, 0.1 % (v/v) Triton X-100, 400 μM MBS in PME buffer, while for microtubules we use 3.7 % (v/v) paraformaldehyde.

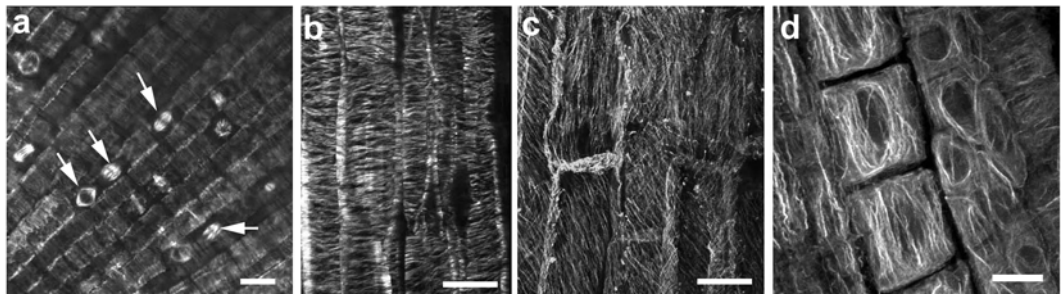

Fig. 2 Confocal microscopy of the cytoskeleton in root vibratome sections. (**a**) Microtubules in the meristematic region of a *Medicago truncatula* root. Arrows indicate mitotic figures. Transverse and oblique cortical microtubules in the elongation (**b**) and maturation zone (**c**) of a maize primary root. (**d**) F-actin organization in the vascular region of maize roots. Bars = 20 μm (**a**, **d**); 10 μm (**b**, **c**)

We perform fixation in 20 mL glass scintillation vials, adding 1–5 mL fixative per vial. Incubate samples in fixative for 1 h, applying a light vacuum for the initial 30 min. Place uncapped vials containing fixative and collected seedlings in a small vacuum dessicator jar equipped with a T connector. Open the vacuum vent slowly and keep it open until the hissing sound subsides. Close the vent and continue incubation. Release the vacuum slowly after 30 min and continue incubation so that the total time of fixation is about 1 h.

2. Subsequent steps, including washes, permeabilization and immunolabeling, are carried out in tissue culture inserts (TCIs), on a rotating shaker table. Upon fixation carefully transfer seedlings with fine forceps into a TCI inserted in a well of a 12 well plate containing 2 mL PEMT (*see* **Note 12**). Rinse seedlings in PEMT three to four times for 10 min each.
3. Permeabilize samples for 1 h in 1 % Triton X-100 in PME, followed by rinsing three times in PEMT, 5 min each.
4. For cell wall digestion, incubate samples for 20 min in 0.05 % (w/v) Pectolyase Y-23, 0.1 % (v/v) Triton, 1 % Bovine serum albumin, 0.4 M mannitol in PME. Wash by incubating three times in PEMT for 10 min each.
5. Permeabilize samples for 10 min in cold (−20 °C) methanol (*see* **Note 13**). When labeling microtubules, the cold methanol treatment should be omitted. Wash three times in PBS, 5 min each.
6. Incubate samples in blocking solution (5 % normal goat serum in PBS) for 1 h.
7. Primary and secondary antibodies are diluted in blocking solution (*see* **Note 14**). Primary antibody, mouse monoclonal anti-chicken gizzard actin ("C4," Chemicon, Temecula, CA) is used at a dilution of 1:1000. For microtubules, we use the

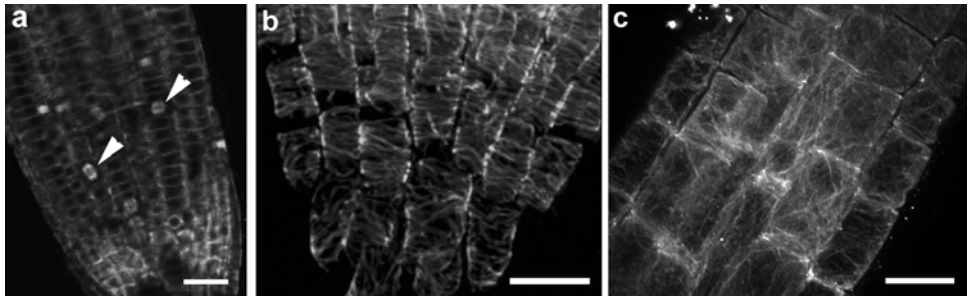

Fig. 3 Confocal microscopy of the cytoskeleton in whole mount Arabidopsis roots. (**a**) Low magnification image of microtubule labeling in the root meristem. *Arrowheads* indicate mitotic figures. (**b**) Transverse cortical microtubules in the root cap. (**c**) F-actin organization in the root distal elongation zone. Bars = 20 μm

monoclonal rat anti-yeast tubulin antibody (YOL1/34, Accurate Chemicals, Westbury, NY; *see* Subheading 3.1.1). Incubate samples with the primary antibodies for 16 h (usually overnight) at room temperature on a rotating shaker table.

8. Wash samples with PBST three to four times for a total duration of 1 h.
9. Add secondary antibodies (fluorescently labeled monoclonal anti-mouse for actin or anti-rat for microtubules) to the samples. We use Alexa Fluor 488 goat anti-mouse or goat anti-rat as the secondary antibody, diluted 1:200 in blocking solution (*see* **Note 15**). Incubate samples with the secondary antibodies for 5–6 h at room temperature on a rotating shaker table.
10. Wash samples with PBST three to four times for the total duration of 1 h and once with PBS for at least 10 min. Seedlings can be mounted for immediate observation or stored in PBS at 4 °C for up to 1 week (*see* **Note 16**).
11. For observation, mount seedlings in VECTASHIELD and image with a confocal microscope. Examples of the quality of cytoskeletal labeling from whole mount Arabidopsis roots is shown in Fig. 3.

3.2 Preparing Arabidopsis Roots for Live Cell Imaging

Most of the live cell imaging work on the plant cytoskeleton has been conducted using the model plant Arabidopsis. This is because Arabidopsis is readily transformed with various GFP cytoskeletal reporters and the smaller roots of Arabidopsis make sample preparation for live cell imaging very convenient. However, a number of precautions have to be taken to ensure that growth of the root is not compromised by excessive physical handling of the seedling. If at all possible, imaging of live Arabidopsis roots should be done directly on the media where the seeds are germinated.

1. Surface sterilize the seeds by immersing them for 3 min each in 95 % ethanol, 20 % bleach and sterilized deionized water.

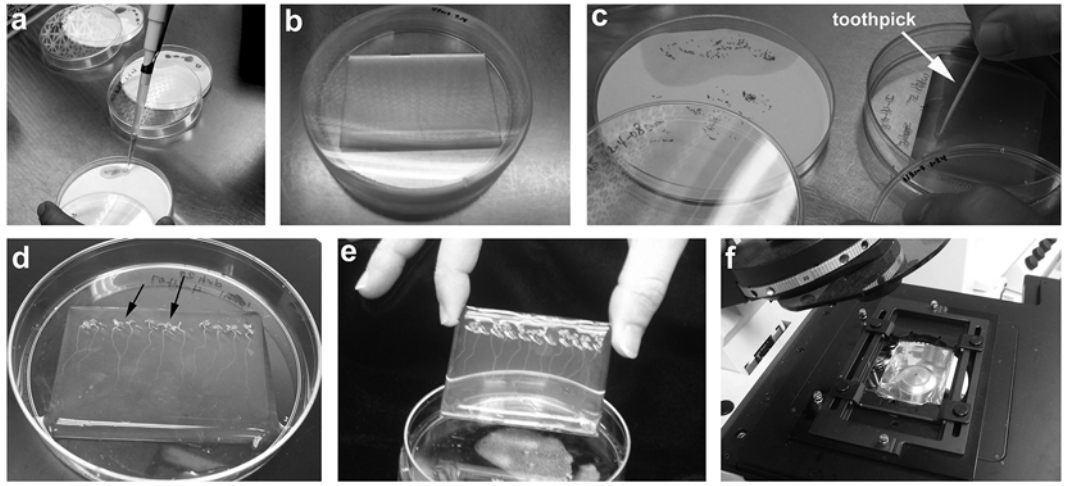

Fig. 4 Arabidopsis seed sterilization and planting for live cell confocal imaging of the cytoskeleton. (**a**) Spreading sterilized seeds on filter paper. (**b**) Agar–coverslip setup for planting Arabidopsis seeds. (**c**) Sterilized seeds can be picked individually using pointed toothpicks and planted directly on the agar–coverslip system. (**d**) Seven-day-old Arabidopsis seedlings (*arrows*) growing on the agar–coverslip system. The agar–coverslip system with the growing seedlings can be easily picked up by gently pressing the bottom of the polystyrene petri dish (**e**) and directly transferred to the stage of the confocal microscope for imaging (**f**)

Rinse the seeds in deionized water at least three times after bleach treatment. Transfer seeds to sterilized 90 mm filter paper in 100 × 15 mm polystyrene petri dishes, and spread the seeds evenly on the filter paper using a 1 mL pipette (Fig. 4a). Let the seeds dry in a laminar flow bench overnight. The seeds can be planted immediately after spreading onto the filter paper-lined polystyrene petri dishes but for long term storage they must be dried thoroughly prior to sealing with Parafilm.

2. Prior to seed sterilization a coverslip–agar medium for germinating seeds must be prepared in advance. This system consists of sterilized coverslips with about a 1 mm layer of nutrient supplemented polymerized 0.5–1 % agar. The coverslip agar system is placed inside 100 × 15 mm polystyrene petri dishes, wrapped with Parafilm, and kept at 4 °C for future use (Fig. 4b).
3. To prepare the coverslip-agar system, autoclave 0.5× MS salts in 0.5–1 % agar (v/v) supplemented with 0.5 mg/mL pyridoxine-HCl, 0.5 mg/mL nicotinic acid and 1 mg/mL thiamine. The vitamins can be dissolved in water as 1000× stock, filter-sterilized and stored at 4 °C prior to use. Add 0.10 g/L myo-inositol, 0.5 g/L MES, and 1 % sucrose to the MS vitamin solution. Adjust pH to 5.7 with 10 M KOH, add agar and autoclave. After autoclaving, carefully add the agar-MS solution on top of the coverslip using a pipette until the entire surface of the coverslip is covered with agar (Fig. 4b; *see* **Note 17**).

4. When the agar has fully polymerized and cooled, pick Arabidopsis seeds individually with a sterile toothpick and gently push the seed into the agar media so that the seed touches the bottom of the coverslip (Fig. 4c). This helps the roots grow straight inside the medium facilitating microscopic observation. Dried seeds can be easily picked up with a moist toothpick. This is done by immersing the tip of the toothpick into the agar and gently touching the seed.
5. After planting, seal the petri dish with Parafilm and transfer to a growth chamber. The petri dish should be positioned at a 60° angle from the horizontal to allow the roots to direct their growth toward the surface of the coverslip. It takes about 3–4 days for the roots to be ready for imaging (Fig. 4d).
6. When the roots have reached the desired age for imaging, remove the coverslip containing the seedlings by gently pressing the bottom of the polystyrene petri dish and lifting the coverslip making sure that the polymerized agar does not slide off (Fig. 4e).
7. Mount the coverslip directly on the stage of a confocal microscope for observation (Fig. 4f).

3.3 Preparing Medicago truncatula Roots for Live Cell Imaging

1. To induce germination, *Medicago truncatula* seed have to be scarified either mechanically using sandpaper or chemically with sulfuric acid [19]. For imaging *Medicago truncatula* roots we typically use mechanical scarification since not a lot of seedlings are needed. Place about 20 dry seed on the surface of a square piece of sandpaper (Fig. 5a). Rub the seed with a second piece of sand paper to abrade the seed coat (Fig. 5b).
2. Transfer abraded seeds to a petri dish lined with moist filter paper (Fig. 5c) and leave overnight at room temperature in darkness. The following day, the imbibed seeds are ready for transplanting (Fig. 5d).
3. Live imaging of roots is more convenient if they are straight. To obtain seedlings with straight roots, place a layer of germination paper in a plastic tray. Standard cafeteria trays can be used for this purpose. Moisten the germination paper with tap water and carefully arrange seeds side by side along a straight line (Fig. 5e).
4. Cover seeds with another layer of germination paper and add additional water if needed. Use a second plastic tray to the cover the seed and position the entire set-up vertically on a plastic reservoir with water about 2 in. deep. Capillary action will keep the germination paper moist for a few days.
5. After 2–3 days, roots that are about 3–4 cm in length should be available for imaging (Fig. 5e). Select seedlings with straight roots and mount in a glass slide (Fig. 5f).

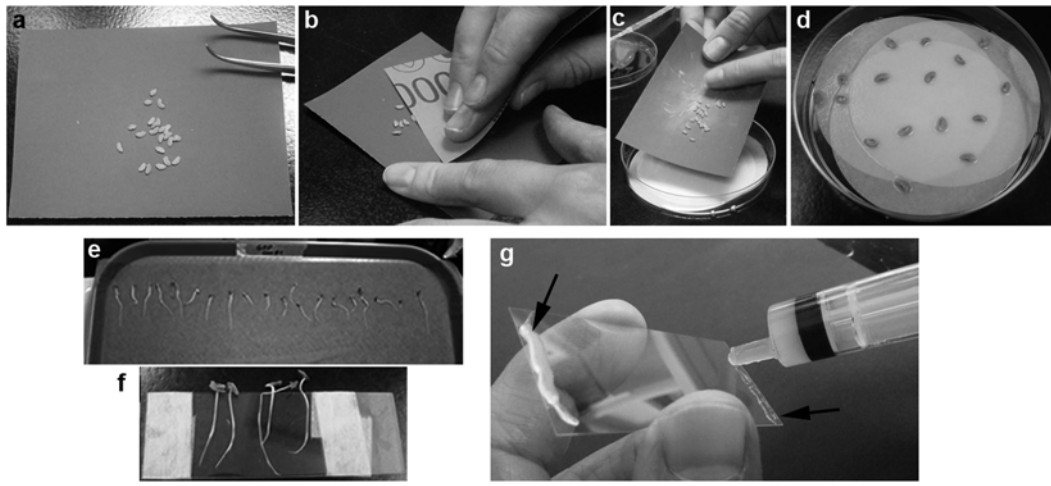

Fig. 5 Processing *Medicago truncatula* seeds for live cell imaging of roots. Mechanical scarification of dry seed with sandpaper treatment (**a**, **b**). Transfer of scarified seed to a petri dish lined with two or three layers of moist filter paper (**c**). Imbibed seeds ready for planting (**d**). Three-day-old seedlings growing on moist germination paper with primary roots fully emerged (**e**). Seedlings selected for imaging are transferred to a glass slide (**f**) and secured with a coverslip lined with vacuum grease (*arrows*, **g**)

6. Add a small amount of water to the roots and select a cover glass with the appropriate size. For *Medicago truncatula* roots, a 50 mm × 22 mm cover glass is typically used (*see* **Note 18**).
7. Apply vacuum grease evenly to opposite sides of the cover glass and gently cover the roots (Fig. 5g; *see* **Note 19**). Examples of cytoskeletal structures in living Arabidopsis and *Medicago truncatula* roots are shown in Fig. 6.

4 Notes

1. There are several companies that sell agar for plant culture, which can be used for preparing the agar–coverslip system. For imaging live Arabidopsis roots, however, we found that Agargel (A3301: Sigma-Aldrich) gives the most reliable images when overlayed on the coverslip.
2. In our experience, root microtubules have been preserved adequately with fixative solution containing 3–4 % (v/v) formaldehyde or paraformaldehyde [16]. On the other hand we have resorted to lower concentrations of formaldehyde (1.5–2 %) to optimally preserve the delicate F-actin structures in roots [14, 17, 18].
3. Make sure that superglue is spread evenly and thinly on the vibratome block surface so that only the outer cells of the root are in contact with the superglue. Typically a touch of superglue

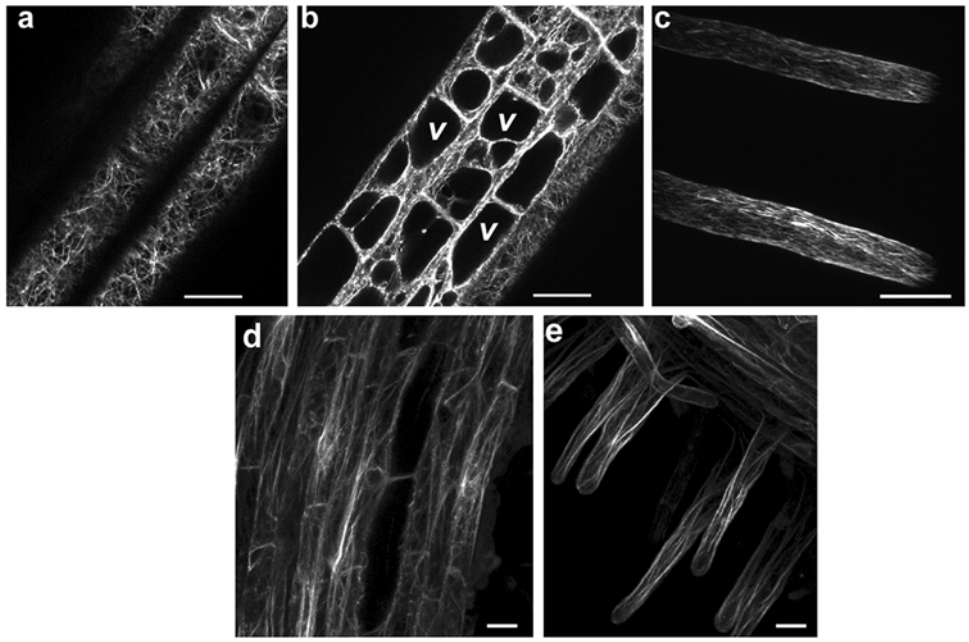

Fig. 6 Live cell imaging of Arabidopsis and *Medicago truncatula* roots expressing F-actin and microtubule GFP reporters. Cortical (**a**) and cytoplasmic (**b**) F-actin networks in epidermal cells from the root elongation zone of Arabidopsis. Images in (**a**) and (**b**) were taken at two different focal planes. Vacuoles (*v*). (**c**) Longitudinal cortical microtoubules in growing Arabidopsis root hairs imaged with a spinning disk confocal microscope. Confocal *Z*-stacks of epidermal cells in the root maturation zone (**d**) and root hairs (**e**) of *Medicago truncatula* stably expressing a UBQ10::GFP-ABD2-GFP construct [6]. Bars = 20 μm

is sufficient for the root tissue to adhere to the block surface. If too much glue is used, sectioning and subsequent labeling steps can be compromised.

4. Without any embedding support, vibratome sections of plant tissues typically break apart at a thickness of less than 40 μm.
5. The sections can be left on the lab bench to allow remaining PME buffer to evaporate. However, make sure that sections remain moist. Do not allow sections to dry completely as this could lead to distortion of cells during imaging. One way to secure the sections onto coverslips for subsequent antibody labeling is to use a thin film of bactoagar as originally proposed in Brown and Lemmon [13]. Dissolve 0.75 g bactoagar in 100 mL deionized water in a 250 mL beaker. Microwave the solution until it comes to a boil. When contents of the beaker start to rise, turn off the microwave and briefly swirl the contents of the beaker. Microwave the solution for a second time to completely dissolve the bactoagar granules. A loop can be constructed using copper or chromium-nickel wire. Material for making such loops can easily be found in the lab. For example, we have successfully used wire from the base of test tube

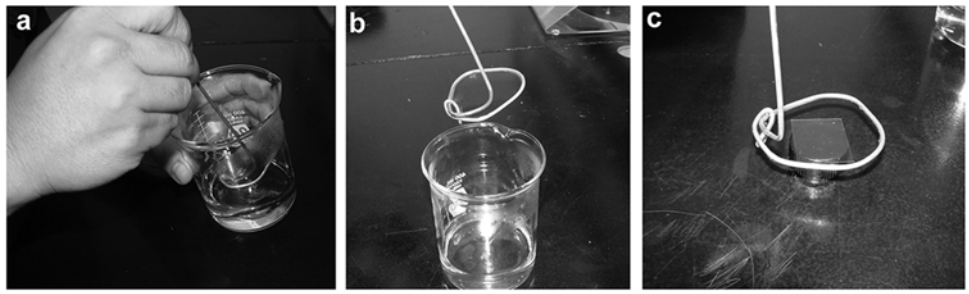

Fig. 7 Procedure for securing root vibratome sections on coverslips for indirect immunofluorescence labeling of the cytoskeleton. A thin film of agar is collected on a loop from molten 0.75 % agar solution (**a**, **b**). The coverslip containing the root section is placed on top of a cap of a scintillation vial and the loop with the thin agar film is passed over the coverslip to hold the root section in place (**c**)

brushes to make agar casting loops. To secure the sections, allow the agar film to solidify on the loop and slowly cover the sections with the agar film. Placing the coverslip on the caps of vials facilitates layering of the agar film onto the sections (Fig. 7). Layering of the agar film requires patience since the thin agar film can rupture easily. All solutions for labeling the sections can easily penetrate the agar film.

6. During subsequent treatments and washing steps one has to be careful that sections do not detach from the coverslip. Although the thin agar film described in **Note 5** typically prevents sections from floating away during sample treatment, there are cases when the bactoagar film will slide off because of constant exposure to the liquid solutions. When this happens, one has to take extra care that samples are not lost during the various washing steps.
7. Overnight incubation in primary antibody has sometimes led to improvements in the quality of cytoskeletal labeling and also allows flexibility with the labeling schedule if time becomes an issue. When overnight incubation in the primary antibody becomes necessary, the samples can be kept at 4 °C. A humid chamber can be constructed using 9 mm round polystyrene petri dishes lined with moist filter paper. Coverslips or glass slides containing the root sections can be laid on top of rubber lined caps of scintillation vials to prevent moisture from spreading onto the sample (Fig. 7c). Laying coverslips on top of vial caps also facilitates casting of the thin bactoagar film used for securing sections (*see* **Note 5**).
8. The choice of primary antibodies is critical for the success of labeling. There are a number of commercially available anti-tubulin and actin antibodies. For microtubules, we have been successful with the Rat anti-yeast tubulin (monoclonal rat and yeast tubulin antibody YOL1/34, Accurate Chemicals,

Westbury, NY) and anti-actin antibodies (C4, Chemicon, Temecula, CA). As an alternative to antibodies, fluorescently conjugated phalloidin can be used to label F-actin in roots [14, 17]. Commercial primary and secondary antibodies and fluorescently conjugated phalloidin typically come with recommendations for dilutions. However, it is advisable to test the dilution that is most effective for the plant tissue being studied. Antibodies and fluorescently conjugated phalloidin should be aliquoted into small volumes and stored at –20 °C prior to use to avoid repeated freezing and thawing.

9. Do not worry if residual Mowiol crystals remain after 2 h of stirring. Just be sure that you do not include any of the crystals when mounting your samples.
10. The Mowiol mounting medium will be viscous after 2 h of stirring and will occasionally contain air bubbles. Only a small drop (ca. 100–200 μL) of the Mowiol solution is needed to mount one coverslip. Be sure to avoid air bubbles when mounting your samples as this could affect the quality of imaging.
11. Because vibratome sections are thick the best images are obtained using a confocal microscope.
12. Solutions (2 mL per well) are added and removed directly to and from the well using a pipette. TCIs with sample seedlings are transferred from one solution to another by moving between wells. Tapping the TCI gently on a paper towel before inserting it into the well facilitates solution exchange. This arrangement allows for better preservation of fragile seedlings during the procedure.
13. Place a 12-well plate with methanol (2 mL per well) at –20 °C 1 h in advance.
14. To minimize the volume of antibody solution, TCI bottoms can be sealed with Parafilm before inserting into the well. Antibody solution (350–400 μL) is then added directly to the TCI with the sample. Seedlings should be fully submerged into the antibody solution. After the incubation with the antibodies, remove Parafilm from the bottoms of TCIs with samples and tap TCIs gently on a paper towel to remove the antibody solution. Proceed with washes.
15. Sealing TCI bottoms with Parafilm can also be used at this stage to minimize the volume of antibody solution required to cover samples fully.
16. During storage, keep plates with samples covered in aluminum foil to prevent loss of fluorescent signal.
17. Surface tension of the molten agar will form a dome on the surface of the coverslip. Avoid pouring excess agar on the coverslip as this will break the dome and cause the agar to flow out of the coverslip.

18. Observation of the cytoskeleton in living *Medicago* roots is more challenging because of the thicker roots. Imaging is typically limited to the epidermis and outer cortical cells. It is important to select a region of the root that is pressed closely to the surface of the coverslip. To overcome difficulties in imaging thick roots, some investigators have resorted to tissue-specific promoters and sectioning of live roots [20] or have used non-destructive clearing methods [21].
19. The vacuum grease serves two purposes. First, it keeps the cover glass securely attached to the glass slide and as a result, a confocal microscope with an inverted platform can be used. Second, it prevents roots from being crushed as the vacuum grease acts as a spacer between the glass slide and cover glass.

Acknowledgements

Work on root biology and the cytoskeleton in the Blancaflor laboratory is supported by the Samuel Roberts Noble Foundation Forage 365 initiative and National Aeronautics and Space Administration (NASA grant NNX12 AM94G).

References

1. Li J, Blanchoin L, Staiger CJ (2015) Signaling to actin stochastic dynamics. Annu Rev Plant Biol 66:415–440. doi: 10.1146/annurev-arplant-050213-040327
2. Eng RC, Wasteneys GO (2014) The microtubule plus-end tracking protein ARMADILLO-REPEAT KINESIN1 promotes microtubule catastrophe in Arabidopsis. Plant Cell 26: 3372–3386
3. Rocchetti A, Hawes C, Kriechbaumer V (2014) Fluorescent labelling of the actin *cytoskeleton* in *plants* using a cameloid antibody. Plant Methods 10:12
4. Marc J, Granger CL, Brincat J, Fisher DD, Kao T-H, McCubbin AG, Cyr RJ (1998) A GFP-MAP4 reporter gene for visualizing cortical microtubule rearrangements in living epidermal cells. Plant Cell 10:1927–1940
5. Vidali L, Rounds CM, Hepler PK, Bezanilla M (2009) Lifeact-mEGFP reveals a dynamic apical F-actin network in tip growing plant cells. PLoS One 4:e5744
6. Dyachok J, Sparks JA, Liao F, Wang Y-S, Blancaflor EB (2014) Fluorescent protein-based reporters of the actin cytoskeleton in living plant cells: fluorophore variant, actin binding domain and promoter considerations. Cytoskeleton 71:311–327
7. Lovy-Wheeler A, Wilsen KL, Baskin TI, Hepler PK (2005) Enhanced fixation reveals the apical cortical fringe of actin filaments as a consistent feature of the pollen tube. Planta 221:95–104
8. Collings DA, Wasteneys GO (2005) Actin microfilament and microtubule distribution patterns in the expanding root of Arabidopsis thaliana. Can J Bot 83:579–590
9. Wilsen KL, Lovy-Wheeler A, Voigt B, Menzel D, Kunkel JG, Hepler PK (2006) Imaging the actin cytoskeleton in growing pollen tubes. Sex Plant Reprod 19:51–62
10. Yoo CM, Quan L, Cannon AE, Wen J, Blancaflor EB (2012) AGD1, a class 1 ARF-GAP, acts in common signaling pathways with phosphoinositide metabolism and the actin cytoskeleton in controlling Arabidopsis root hair polarity. Plant J 69:1064–1076
11. Yoo CM, Blancaflor EB (2013) Overlapping and divergent signaling pathways for ARK1 and AGD1 in the control of root hair polarity in *Arabidopsis thaliana*. Front Plant Sci 4:528
12. Dyachok J, Shao MR, Vaughn K, Bowling A, Facette M, Djakovic S, Clark L, Smith L (2008) Plasma membrane-associated SCAR complex subunits promote cortical F-actin accumulation and normal growth characteristics in Arabidopsis roots. Mol Plant 1:990–1006

13. Brown RC, Lemmon BE (1995) Methods in plant immunolight microscopy. In: Galbraith DW, Bohnert HJ, Bourque DP (eds) Methods in cell biology. Academic Press, San Diego, CA, pp 85–107
14. Blancaflor EB, Hasenstein KH (2000) Methods for detection and identification of F-actin organization in plant tissues. In: Staiger C, Baluška F, Volkmann D, Barlow PW (eds) Actin: a dynamic framework for multiple plant cell functions. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 601–618
15. Blancaflor EB, Wang Y-S, Motes CM (2006) Organization and function of the actin cytoskeleton in developing root cells. Int Rev Cyto 252:153–198
16. Blancaflor EB, Zhao L, Harrison MJ (2001) Microtubule organization in root cells of *Medicago truncatula* during development of an arbuscular mycorrhizal symbiosis with *Glomus versiforme*. Protoplasma 217:154–165
17. Blancaflor EB, Hasenstein KH (1997) The organization of the actin cytoskeleton in vertical and graviresponding primary roots of maize. Plant Physiol 113:1447–1455
18. Hou G, Mohamalawari DR, Blancaflor EB (2003) Enhanced gravitropism of roots with a disrupted cap actin cytoskeleton. Plant Physiol 131:1360–1373
19. Garcia J, Barker DG, Journet E-P (2006) Seed storage and germination. In: Mathesius U, Journet EP, Sumner LW (eds) The *Medicago truncatula* handbook. ISBN 0-9754303-1-9. http://www.noble.org/MedicagoHandbook/
20. Ivanov S, Harrison MJ (2014) A set of fluorescent protein-based markers expressed from constitutive and arbuscular mycorrhiza-inducible promoters to label organelles, membranes and cytoskeletal elements in *Medicago truncatula*. Plant J 80:1151–1163
21. Warner CA, Biedrzycki ML, Jacobs SS, Wisser RJ, Caplan JL, Sherrier DJ (2014) An optical clearing technique for plant tissues allowing deep imaging and compatible with fluorescence microscopy. Plant Physiol 166: 1684–1687