Chapter 35

# STEEDMAN'S WAX FOR F-ACTIN VISUALIZATION

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**Abstract:** Actin filaments are visualised by means of indirect immunofluorescence in plant tissues that were fixed in formaldehyde, embedded in low-melting polyester wax and sectioned on a microtome. The technique described here avoids usage of detergents and organic solvents and is also compatible with immunolocalization of many other antigens.

## 1. INTRODUCTION

An actin cytoskeleton in plant cells can be visualised by several methods. Depending on the questions to be answered, the investigator can choose to study actin *in vivo*, using transgenic plants expressing GFP fusion proteins (see Kost et al., this volume) or via microinjection of fluorescently labelled proteins (e.g. Hepler & Hush, 1996). On the other hand, where the dynamism of F-actin network is not the main object of study, or if the cells of interest are deep inside a large organ or piece of tissue and are not easily accessible for the above mentioned techniques, it may be preferable to work with tissues that were fixed and further processed. Again, fluorescence is the

visualisation method of choice because of its high sensitivity and spatial resolution. The two most common approaches for actin filament staining are through the use of: 1) fluorescently labelled phalloidin, a toxin that has high affinity towards F-actin (affinity cytochemistry), and 2) anti-actin antibodies followed by a fluorescently labelled secondary antibody (indirect immuno-fluorescence).

Despite its simplicity, the phalloidin technique is not without problems. In some cell types, the phalloidin binding sites may be masked with other actin-binding proteins (e.g. Ao & Lehrer, 1995; Jiang et al., 1997; Nishida et al., 1987, see also Blancaflor & Hasenstein, this volume). Furthermore, there are often problems with the high background fluorescence and rapid fading of the signal (La Claire, 1989; McCurdy & Gunning, 1990). The use of antibodies is therefore preferable.

This chapter deals with immunofluorescence detection of F-actin in cells of plant tissue that has been fixed, embedded in wax and sectioned on a microtome. The procedure has four stages:

1. Fixation
2. Tissue embedding and sectioning
3. Immunostaining
4. Microscopy

## 1.1 Fixation

The aim of fixation is to kill the cell rapidly and to immobilise proteins in the same state and location as they were *in vivo*. Chemical fixatives commonly employ aldehydes as crosslinking agents. Formaldehyde is preferred for immunofluorescence at the light microscope level. It penetrates the tissue quickly but has only limited crosslinking capabilities. Glutaraldehyde is used less often. It is a stronger crosslinker, but penetrates slowly and causes strong tissue autofluorescence which hinders its use for immunofluorescence unless special precautions are taken. Moreover, glutaraldehyde induces immediately synthesis of callose in plant cells (Hughes & Gunning, 1980). There is a concern that the slowness of chemical fixation leaves time for abnormal re-arrangements of the cytoplasm and cytoskeleton (Doris & Steer, 1996; He & Wetzstein, 1995; Mersey & McCully, 1978). This is especially critical in the case of higher plant cells encased within robust cellulosic cell walls. Moreover, F-actin is traditionally considered to be extremely sensitive to aldehydes (Doris & Steer, 1996; Parthasarathy et al., 1985; Seagull et al., 1987; Traas et al., 1987). To stabilise F-actin, Sonobe and Shibaoka (1989) introduced treatment with MBS (n-maleimidobenzoic acid N-hydroxysuc-cinimide ester) prior to formaldehyde fixation. However, our and other authors' data indicate that fine F-actin network can be preserved with formaldehyde fixation. In our

material (*Zea mays* and *Arabidopsis thaliana*) MBS did not improve F-actin preservation. Just the opposite, incubation with MBS (>15 min) actually prevented immunolabelling of phragmoplasts (Vitha et al., 1997).

One of the alternatives to chemical fixation is rapid freezing followed by freeze-substitution (further referred to as cryofixation; e.g. Baskin et al., 1995; Roy et al., 1997). Cryofixation is fast-acting, thus preventing pre-fixation artefacts, which is an advantage for immunolocalization of epitopes that do not survive chemical fixation. Maneta-Peyret et al. (1999) also found cryofixation to be superior for EM immunocytochemistry of lipids. Cryofixation, however, may not work well for larger objects because the relatively slow cooling rates inside the tissue can cause ice-crystal damage. Also, since the proteins in cryofixed cells are not crosslinked, redistribution of some small proteins may occur. As emphasised by Melan and Sluder (1992), unless the fixative completely immobilises soluble proteins, further sample preparations and differential extraction can lead to artefactual redistribution and/or extraction of the antigens. All this is not an issue for electron microscopy where the embedding resin is not removed prior to immunolabelling. However, procedures for light microscopy usually include de-embedding and rehydration in order to make tissue epitopes accessible for antibodies. False localization is obviously not a problem with F-actin networks and the same localization patterns were seen in roots that were either cryofixed or formaldehyde fixed and then embedded in Steedman's wax (Vitha et al., 2000). However, researchers should be aware of this potential for localization artefacts if other proteins are being simultaneously immunolocalised in the same tissue. Various fixatives and their mode of action are discussed in depth by Pearse (1991). With respect of actin immunofluorescence, it is well known that fine meshworks are extremely difficult to visualize. Unless they are effectively cross-linked and/or bundled, their fine nature makes them sensitive towards fixation and permeabilization (Small et al., 1999). In contrast, robust bundles of actin filaments are more resistant even towards effective fixation and heavy permeabilization. For instance, dense meshworks at growing apices of root hair tips can be visualized using mild permeabilization (Brown et al., 1999) but not after heavy permeabilization (Miller et al., 1999).

## 1.2 Tissue processing

The purpose of this step is to enable penetration of antibodies and also to allow observation of tissue structure and the fluorescence signal with minimum background. The tissue that has been fixed can be either immunostained in toto, or macerated, hand-sectioned or vibratome sectioned, or as described in this chapter, embedded in wax and sectioned on a microtome. Embedding and sectioning allows study of individual cells in the

context of the surrounding tissue, and in the case of root tips, for example, to observe the succession of developmental stages. The choice of embedding medium is critical for successful immunostaining. Paraffin wax, the classical embedding medium of histology and anatomy, is unsuitable here because its relatively high melting-point destroys F-actin and renders many other proteins non-immunoreactive. Methacrylate-based resins can be used at low temperatures and polymerised by UV light. Although they provide good structural preservation of the tissue, the preservation of antigenicity may not be ideal and application of organic solvents to remove resin from tissue sections compromises antigenicity. Unless de-embedding steps are included, only the surface of the sections is available for immunostaining, and the signal is weak. Moreover, it is not possible to cut sections more than several micrometers thick and special equipment is needed, such as ultramicrotome or retracting microtome, in order to section resin blocks with glass knives. Unlike paraffin or Steedman's wax, resin sections do not form ribbons; serial sectioning is thus less convenient and more time consuming.

Steedman (1957) introduced a new, ribbon-forming, sectioning medium – low melting-point polyester wax (today also known as Steedman's wax). This wax has a melting point of 35-37°C, is soluble in ethanol, and has sectioning properties very similar to paraffin wax. Some of the technical conveniences of Steedman's wax compared to paraffin were noted by Sidman et al. (1961): „Sections of polyester wax fragment less. Hard tissues ... commonly split away from a paraffin ribbon, but not from one of polyester wax. Polyester ribbons do not generate static electricity.“ Although Steedman's wax never replaced paraffin for routine histology, its suitability for immunocyto-chemistry had been noticed early on (Baird, 1967; Sidman et al., 1961) and it has been used for detection of various antigens, including cytoskeletal, secretory and membrane-associated proteins (Oke & Suarez-Quian, 1993), BrDU-labeled DNA (Hanazono et al., 1990), as well as for *in situ* hybridization (Schneitz et al., 1998; Shuttlesworth & Mills, 1995). In plant tissues, Steedman's wax has proved suitable for the immuno-fluorescence detection of microtubules ( Brown et al., 1989; Baluška et al., 1992; Timmers et al., 1999), actin filaments (Baluška et al., 1997; Vitha et al., 1997, 2000, this volume), plasma membrane $H^+$-ATPases (Jahn et al., 1998), PSTAIR proteins (Mews et al., 1996), cyclins (Mews et al., 1997, 2000), arabinogalactan-protein (AGP) epitopes (Šamaj et al., 1999, 2000), calreticulin and HDEL proteins (Baluška et al., 1999), profilins (von Witsch et al., 1998), and cadherin- and catenin-like proteins (Baluška et al., 1999). Importantly, we routinely immunolocalize these and other antigens with the same procedure as for F-actin.

## 1.3 Immunostaining

After the microtome sections, which are attached to glass slides, have been dewaxed and rehydrated, antibodies have direct access to their antigens. Indirect methods, using a primary antibody that is then detected with labelled secondary antibodies, are preferred because of their high sensitivity.

### 1.3.1 Antibodies

We use the monoclonal C-4 anti-actin antibody (Lessard, 1988) from ICN Pharmaceuticals (Costa Mesa, CA, USA) for staining of all classes of F-actin in various plant species, including *Zea mays*, *Arabidopsis thaliana*, *Lepidium sativum, Oryza sativa* and *Medicago sativa*. Secondary antibodies conjugated with various fluorochromes are available from many suppliers, FITC conjugates being the most common.

### 1.3.2 Controls

Proper controls are essential in order to show that the immuno-fluorescence signal reflects localization of the target protein. The controls include:

a) Both antibodies omitted: This reveals the autofluorescence of the tissue.
b) Primary antibody omitted: In combination with the previous control, this control indicates non-specific binding of the secondary antibody.
c) Use of pre-immune serum (ideally from the same animal used to raise the primary antibody) or normal immunoglobulin, at the same dilution or protein concentration as the primary antibody: This control is similar to b), but also checks for non-specific binding of the primary antibody. Immunoglobulins often bind to plastids and starch granules.
d) Competition: Primary antibody is pre-incubated for 30 min with the target protein before it is applied to the sections. This control should not give any significant fluorescent staining. It is used to check for non-specific binding of the primary antibody.
e) Use of non-related primary antibody: This is expected to produce different localization pattern, e.g. anti-tubulin antibody, and is therefore a check for the distribution of the antigen in question.

### 1.3.3 Autofluorescence

Many tissues exhibit autofluorescence and this is associated especially with their cell walls and chloroplasts. Glutaraldehyde fixatives are known to induce autofluorescence and induce callose (Hughes & Gunning, 1980).

Therefore, they should be avoided, or special steps should be taken to quench the induced fluorescence, e.g. by treatment with $NaBH_4$ or with Schiff's reagent followed by reduction with $NaBH_4$ (Tagliaferro et al., 1997).

Several steps can be taken to minimise natural autofluorescence. Choice of fluorescence filters which closely match the excitation and emission spectra of the fluorochrome. Since autofluorescence is usually of a broad spectrum, this improves the signal-to-noise ratio. We have good experience with FITC-conjugated antibodies viewed with filter set containing a bandpass emission filter at 535 ± 20 nm (e.g. XF100 filter set, Omega Optical, Inc., Brattleboro, VT , USA). The added bonus is that such filter can be used for viewing GFP in fresh tissue since it eliminates red autofluorescence of chloroplasts. If FITC-conjugated secondary antibodies are used, labelled sections may be stained with Toluidine Blue (see Table 3) to diminish the autofluorescence. The sections should not be overstained, because the specific immunofluorescence signal could be quenched as well. Note that Toluidine Blue staining may not be used with some multi-color filter sets as it emits strong reddish fluorescence.

### 1.3.4 Non-specific staining

Similar to autofluorescence can be the effects of non-specific staining resulting from too high a concentration of either the primary or secondary antibodies and/or insufficient washing. The antibody dilutions given in this protocol (see Methods section 3.3) serve as a starting point. Optimal dilutions should be determined for a given material by pilot experiments with several dilutions of the primary and secondary antibodies. A comparison of control slides where the first or both antibodies were omitted will indicate whether the secondary antibody binds in a non-specific manner. If that is the case, the antibody can be pre-absorbed with tissue powder from corresponding plant material (see Section 2.2). Non-specific binding of antibodies is also reduced by pre-incubating slides with a blocking solution (SB – see section 2.3, containing 0.5–3% BSA or non-immune serum from the same species as used to raise the secondary antibody) and by diluting antibodies in the blocking solution. To remove any aggregates that may have formed during storage, microcentrifuge the diluted secondary antibody before use for 5 min at 4°C at maximum speed. A mild detergent such as 0.1% v/v Tween 20 increases the efficiency of washing, following the incubation with antibodies.

## 1.4 Fluorescence Microscopy

Because the tissue has been sectioned into relatively thin slices, standard epifluorescence microscopy can be used for rapid screening and

photography. When working with thicker sections and when three-dimensional arrays of F-actin are to be documented, confocal microscopy or computational deconvolution microscopy may be used. Discussion of these techniques is, however, outside the scope of this chapter. The choice of excitation and emission wavelengths is dictated by the fluorochrome used. Longpass emission (barrier) filters give brighter fluorescence signal, but for multiple labelling and/or in case of strong autofluorescence, bandpass barrier filters should be used (see Section 1.3.3). High quality fluorescence filters can be obtained, e.g., from Omega Optical (http://www.omegafilters.com). A database of excitation and emission spectra of many fluorochromes has been made available on-line by BioRad (http://fluorescence.bio-rad.com).

# 2. MATERIALS

## 2.1 Steedman's wax

Steedman's wax can be purchased ready to use under the name of 'Polyester wax' from Ted Pella (Redding, CA, USA) or Electron Microscopy Sciences (Fort Washington, PA, USA), or it can be easily and less expensively mixed in the laboratory. Among the different suppliers of the individual components, we were best satisfied with those obtained from Aldrich (Milwaukee, WI, USA).

To prepare the Steedman's wax from the individual components, melt 900 g polyethylene glycol 400 distearate (Aldrich, cat. # 30,541-3) and 100 g 1-hexadecanol (Aldrich cat. # 25,874-1) in a large beaker in an incubator at 65 °C. When completely melted, stir the wax very thoroughly for several minutes, using a stirring bar. Pour the wax into a tray lined with aluminium foil and leave at room temperature to harden. Alternatively, pour the wax in 50 ml plastic conical tubes and close. This prevents contamination by dust and other particles which can cause problems during sectioning. The wax can be stored at room temperature indefinitely. Do not use a microwave oven to melt the wax for embedding as it seems to affect its sectioning properties. For embedding, melt only the necessary amount of wax in an Erlenmeyer flask at 37°C. If using a water bath to melt the wax, ensure that the container is closed to keep out moisture.

## 2.2 Tissue powder

Use the same tissue type as the one under investigation (e.g. leaves, flowers). Fix the tissue and dehydrate through an ethanol series (see Sections 3.1 and 3.2), air-dry and then grind with a pestle and mortar in liquid nitrogen. The resulting powder can be stored at –20°C. To pre-absorb the

secondary antibody, add 1.5 ml diluted antibody to 0.2 ml tissue powder in a microcentrifuge tube, vortex and incubate at 4°C for 24 h. Vortex again, spin for 5 min at 4°C at maximum speed and use the supernatant. This powder (after Schneitz et al., 1998) works better than the acetone powders often recommended by immunocytochemical handbooks (Tanya Wagner, personal communication).

## 2.3 Buffers and solutions

a) **Stabilising buffer (SB)**: This is the same buffer as used in protocols for tubulin immunofluorescence (e.g. Brown et al., 1989), hence the name. The buffer consists of 50 mM PIPES, 5 mM $MgSO_4$ and 5 mM EGTA. PIPES-Na salt is soluble in water, whereas if the 'free acid' PIPES is used, KOH pellets must be added in order that the PIPES will be dissolved. Finally, adjust the pH to 6.9 with KOH or $H_2SO_4$. Store at 4°C for up to several weeks.
b) **Formaldehyde fixative**: Suspend paraformaldehyde powder (e.g. Sigma, St. Louis, MO, USA; Catalogue No. P-6148) in SB to 3.7% w/v. Heat in a water bath at 65°C and mix occasionally until it dissolves completely. Allow the solution to cool. Prepare fresh before use.
c) **PBS**: 0.14 M NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, pH 7.3. It is convenient to prepare 10× concentrated solution that can be autoclaved or preserved by 3.0 mM $NaN_3$. Caution: $NaN_3$ is toxic.
d) **1% BSA/SB stock**: 1% w/v Bovine serum albumin (BSA, preferably fatty acid free) in SB. Store 1 ml aliquots at –20ºC.
e) **Primary antibody**: Monoclonal anti-actin C-4 antibody (ICN) diluted 1:200 in 1% BSA/SB stock. Mix fresh before use.
f) **Secondary antibody**: Anti-mouse IgG-FITC conjugate (e.g. Sigma, Catalogue No. F-5262) diluted 1:100 in 1% BSA/SB stock. Mix fresh before use, spin 5 min at 15 000 rpm at 4°C to remove any aggregates. If pre-absorption with tissue powder is necessary, see Section 2.2.
g) **DAPI stock**: 4,6-diamidino-2-phenylidone (Sigma, Catalogue No. D-9542) 0.1 mg/ml in water, store at 4°C in the dark.
h) **Toluidine Blue solution**: 0.01% w/v Toluidine Blue O in PBS.

*Table 1*. Fixation, dehydration and embedding in Steedman's wax

| Treatment | Duration |
|---|---|
| Fixation with an initial vacuum infiltration | 1 h |
| SB wash | 2 x 10 min |
| PBS wash | 2 x 10 min |
| 30, 50, 70, 90% ethanol/PBS, 97% ethanol | 30 min each at 4°C |
| 0.1% Toluidine Blue in ethanol | 10 min |
| 97% ethanol wash | 10 min |
| Move samples to 37°C incubator | Allow samples to warm up |

| Treatment | Duration |
|---|---|
| Wax + ethanol 1+1 | Overnight |
| 100% Wax | 2 x 1 h |

**Anti-fade mounting medium with DAPI**: Use either commercially available anti-fade mountants, such as ProLong, SlowFade (Molecular Probes, Eugene, OR, USA) or Vectashield (Vector Labs, Burlingame, CA, USA), and add DAPI stock to 1 μg/ml. Alternatively, prepare the medium as follows. Dissolve 50 mg p-phenylendiamine in 5 ml phosphate-saline (0.01 M phosphate buffer pH 7.4, 0.15 M NaCl) and adjust the pH to 8.0 with carbonate/bicarbonate buffer (consists of 4 ml 0.2 M $Na_2CO_3$ + 46 ml 0.2 M $NaHCO_3$, pH 9.2). To this solution, add 45 ml glycerol and 0.5 ml of DAPI stock. Mix thoroughly. Aliquot into 1.5 ml microcentrifuge tubes and store in darknes at –20°C. Good for a year or until it turns dark.

# 3. METHODS

## 3.1 Fixation, dehydration and embedding

Cut pieces of tissue into glass vials containing the fixative. Vacuum infiltrate in a desiccator for 10 min or until no air bubbles come out of the tissue. Leaves are often more difficult to infiltrate (see Technical Note 3). Slowly raise the pressure back to normal and incubate for an additional time to complete the 1 h fixation. Wash with SB and PBS (see Table 1). Change all solutions using a glass Pasteur pipette with a rubber suction bulb. For small objects, such as suspension culture cells, it is advantageous to enclose the pipette tip in a fine nylon mesh (pore size of 50 μm or smaller) to prevent samples being sucked into the pipette and lost.

Either analytical or technical grade (97%) ethanol can be used for tissue dehydration; it is not necessary to use dried (100%) ethanol. Dehydration should be performed at 4°C to minimize possible protein extraction. To be able to see the samples better, the dehydrated tissue is stained with Toluidine Blue (about 0.1% in 97% ethanol). Afterwards, samples are moved to 37°C and all further steps are performed at this temperature. The duration of the steps depends on specimen size. The times given here are used for embedding maize root tips (about 7 mm long). Increase the times for larger specimens. For small seedlings and suspension culture cells, the times may be shortened by 50%. See also Technical Note 4.

To make the wax+ethanol mixture, simply add molten (37°C) wax to the vials containing samples in ethanol. At the next step, just exchange the mixture for a pure wax. For the final stage of embedding, use a warm plate (37°C); it is very important not to overheat the samples. If no warm plate with a fine temperature control is avaliable, work quickly at room temperature on a bench. Pre-warm a small Petri dish and the silicon embedding molds to 37°C. Fill the mold cavities with molten wax. Pour the samples from the glass vial into the pre-warmed Petri dish and transfer

samples into the mold cavities. For embedding large pieces of tissue, Petri dishes or Peel-A-Way® disposable histology molds may be used.

Samples can be picked directly from the vial, but sometimes the specimens stick together or are tangled (as with *Arabidopsis* seedlings) and are hard to separate from each other.

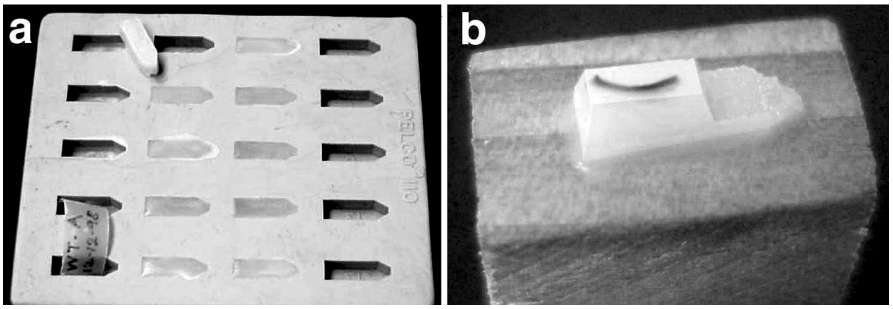


*Figure 1.* Wax blocks with embedded specimens in the embedding mold (a), and after mounting and trimming for sectioning (b).

Add more wax to the embedding molds so that the surface is convex. The wax will shrink somewhat upon solidification. Move the embedding mold away from the warm plate, leave at room temperature to solidify overnight (Fig. 1a). Blocks can then be used for sectioning immediately after hardening (the wax fully hardens 12 — 24 h after embedding) or can be stored in a dry place (avoid refrigerators for their humidity) at room temperature for several months (even for years).

## 3.2 Sectioning

Mount the wax blocks on wooden stubs. The top of a new stub should be first soaked with molten wax. Warm a razor blade briefly in a flame and lay it on top of the wooden block. Place the wax block on the blade and, as its base starts to melt, slide it off the blade on the wood. Do not use too much heat otherwise the whole wax block will melt. Avoid touching the top of the wax block. Allow the wax to harden at room temperature for about 30 min, then trim the sides of the block around the specimen with a razor blade, creating a pyramidal shape with straight, parallel edges (Fig. 1b).

Clamp the block into the specimen holder of a rotary microtome. Make sure the top face of the wax block is aligned with the knife. High cutting speeds (about 4 cuts per second) give better sections in our hands, but other authors prefer cutting at very low speed. As soon the wax block starts cutting and a ribbon of sections begins to form, pick up the end of the ribbon with a fine-tipped paint brush and lift the ribbon up so that it does not slide and crumple upon the knife surface (Fig. 2a).

Ribbons as long as 30 cm can easily be obtained. Use a second paint brush to detach the ribbon from the knife edge and transfer it on a clean woodboard, cardboard or sheet of paper (see Technical Note 5). Cut the ribbon into shorter pieces with a razor blade and place them on a glass slide.

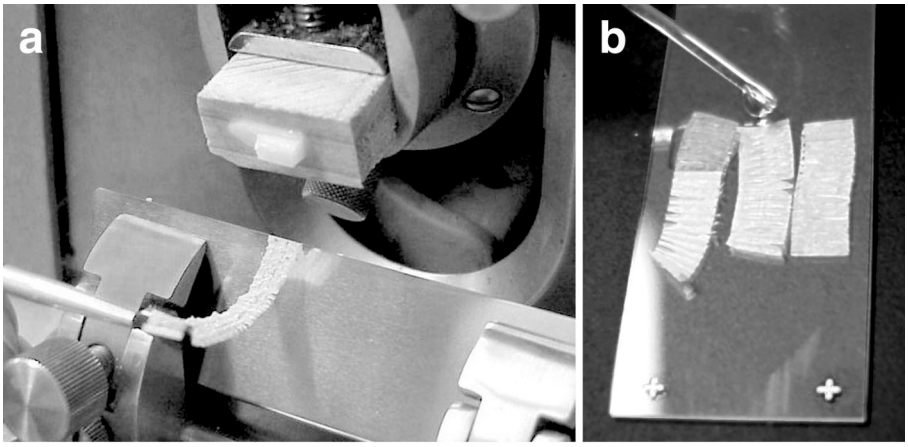


*Figure 2.* Sectioning and stretching of sections upon a slide. a) The end of the ribbon is being held by a paint brush during sectioning. b) A drop of water is added to the top of the ribbons on a glass slide, excess water is then removed from the lower end.

Note that sections will have been compressed during sectioning but will expand by 30–50% at the next step. Holding the slide with one end raised about 45°, add a small drop of water (at room temperature) to the upper end of the ribbons (Fig. 2b). The ribbons start to expand immediately. As soon as the water reaches the bottom of the ribbon, blot excess fluid with a piece of filter paper. Keep slides at an angle and let dry at room temperature for several hours or overnight. Slides can then be used for immunostaining or stored at 4°C for later use.

Sections sometimes do not stick well to slides and are lost during the immunostaining procedure. Several improvements of the initial protocol were accordingly proposed (see Technical Notes 1 and 2). For our routine work with sections of root tips, leaves and shoot apices, the standard protocol was satisfactory, when using slides coated with Mayer's glycerol-albumen or poly-L-lysine, or with Superfrost Plus® charged slides.

## 3.3 Immunostaining

Most steps are done in Coplin staining jars. Slides are transferred from jar to jar with forceps. Technical grade ethanol (97%) is sufficient for dewaxing, but it was noticed that if it contains acetone above 1%, the tissue-

antigenicity vanishes and autofluorescence of the tissue is somewhat increased.

Take the rehydrated slide and wipe excess fluid from around the sections. Apply 100 µl of the diluted primary antibody and rock the slide forth and back to distribute the antibody evenly over all tissue sections. Cover the sections gently with a piece of Parafilm® (American National Can, Chicago, IL, USA).

*Table 2.* Dewaxing and rehydration of sections. The steps indicated with * are optional, see Technical Note 6.

| Treatment | Duration |
|---|---|
| 97 % ethanol | 3 × 10 min (Thick sections may need longer) |
| 90% ethanol/PBS | 10 min |
| 50 % ethanol/PBS | 10 min |
| PBS | 10 min |
| SB* | 30 min |
| 100% methanol chilled to – 20°C * | 10 min at –20°C |
| SB | 90 min |

This will prevent the antibody from evaporating and also maintain a uniform layer of solution on the sections. Place the slides in a humid chamber (e.g., in a large Petri dish lined with wet filter paper), and incubate at room temperature. Both signal intensity and F-actin preservation in *Zea mays* are improved if incubation is done at room temperature rather than at 37°C (Vitha et al., 1997), but in *Arabidopsis*, 0.5 h at 37°C gives somewhat better results. The immunostaining procedure is outlined in Table 3.

*Table 3.* F-Actin immunostaining procedure. For steps indicated with *, see Technical Note 7.

| Treatment | Duration |
|---|---|
| Primary antibody* | 1 h at room temperature in a humid chamber |
| 0.1% w/v Tween 20 in SB* | 2 × 10 min |
| SB* | 10 min |
| Secondary antibody* | 1 h at room temperature, in a humid chamber, in the dark |
| 0.1% w/v Tween 20 in PBS | 10 min |
| PBS | 10 min |
| Toluidine Blue 0.01% in PBS | 10 min |
| PBS | 10 min |

After the last wash, the labeled slides are ready for mounting. Put a drop (20–30 µl) of the anti-fade mounting medium on the sections, rock the slide forth and back in order to distribute the medium evenly, and cover with a coverslip. Blot excess fluid with filter paper and seal with nail polish. The slides can be stored at +4°C for few days and at –20°C for 3–4 weeks, after which time the fluorescence will have faded too much.

Examples of F-actin immunofluorescence in shoot and root tissues are shown in Figures 3 and 4.

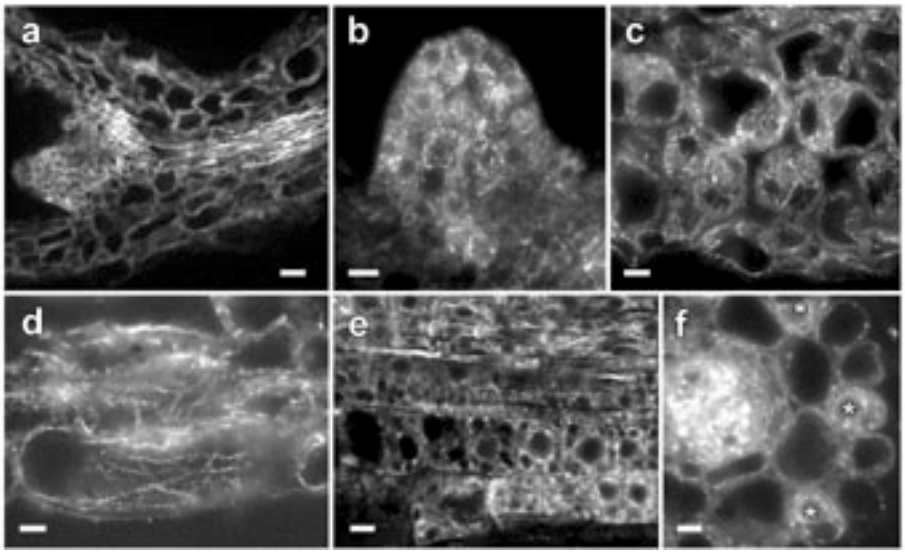


*Figure 3.* Actin immunofluorescence in six days-old Arabidopsis seedlings in longitudinal (a-e) and cross sections (f). Bars = 50 μm (a) or 10 μm (b-f). a) General view of a hypocotyl, apical meristem and basal parts of cotyledons. Note the intense F-actin fluorescence in the meristem and stele. b) Higher magnification view of the meristem from a. c) Cotyledon, paradermal section. d) Epidermis of the hypocotyl. e) Longitudinal section showing F-actin in epidermis, cortex and stele of the root elongation zone. f) Cross section of the root elongation zone. Note the difference in actin immunofluorescence between atrichoblasts and trichoblasts (asterisks) in the epidermis.

## TECHNICAL NOTES

1. To improve section adherence for *in situ* hybridisation, Shuttlesworth and Mills (1995) developed the following protocol: 0.5 g bacteriological grade bovine gelatin (EM Science, Gibbstown, NJ, USA) is dissolved in 80 ml of deionized water to which is added 0.5 ml of Hipure liquid gelatin (Norland Products, North Brunswick, NJ, USA). After mixing the two gelatins and cooling the solutions, 0.1 g of chrome alum ($CrK(SO_4)_2 \cdot 12H_2O$) dissolved in 20 ml of deionized water is added. Thoroughly pre-cleaned microscope slides are then subbed with this solution. A small drop (about 1–2 μl) of the subbing mixture is put onto a slide and, using gloved finger, the solution is rubbed thoroughly over the slide. Allow to dry. Once the wax sections are attached to the slide and dried overnight, they can be stored at 4°C until used. Dewax the tissue in two changes of 100% ethanol for 10 min each, air-dry for 15 min. Again treat with fresh 100% ethanol, air dry 15 min, rehydrate twice in 90% ethanol and twice in 70% ethanol.

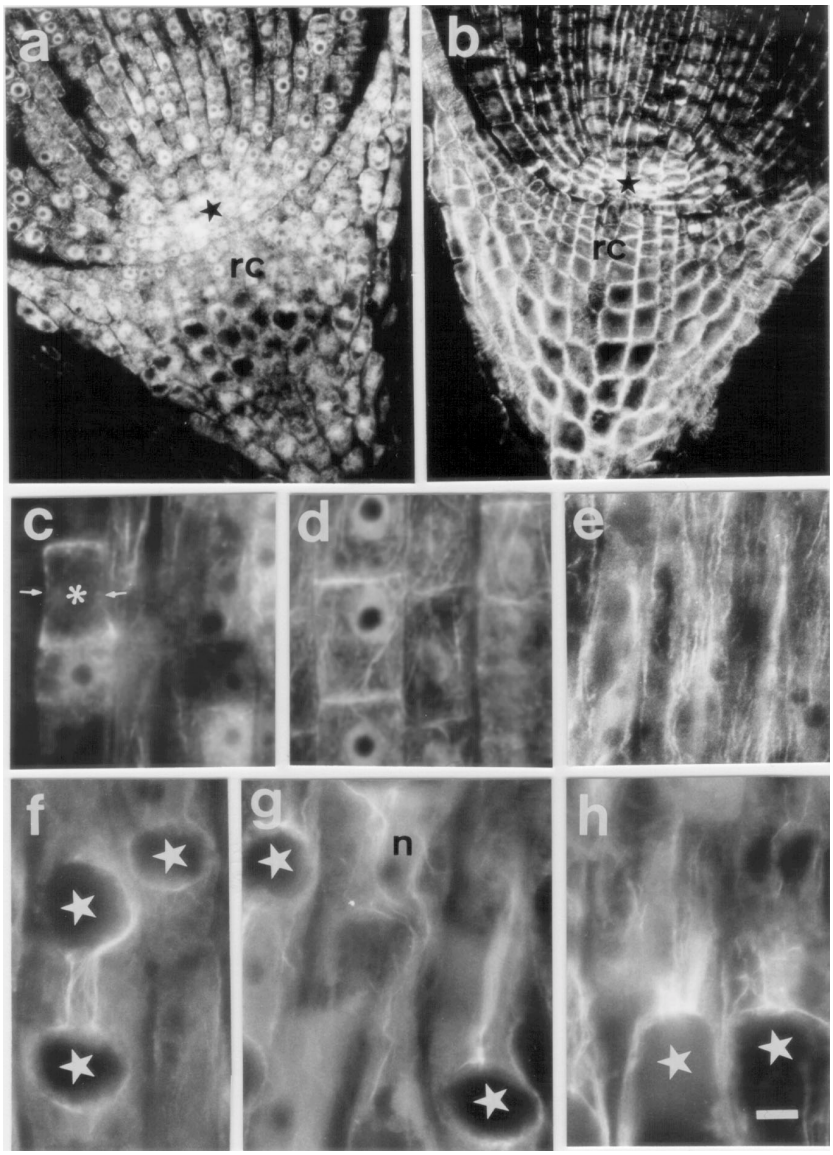


*Figure 4.* Actin in root cells of *Oryza sativa* (a,c,d,e), and *Vicia faba* (f,g,h). a) Root apex of rice shows accumulation of actin in quiescent centre cells (star) and depletion of actin in central cells of the root cap (rc). b) The same object but labeled with the anti-tubulin antibody. Note that the central cells lack endoplasmic microtubules bur are equipped with abundant cortical microtubules. c) Dividing cell (asterisk) in the rice root apex showing actin depletion at cell periphery domains marked by pre-prophase band (arrows). d) Cells of the transition zone accumulate actin at cross-walls and assemble perinulclear AF bundles. e) Elongating root cells with longitudinal AF cables. f,g,h). Root cells of *Vicia faba* show accumulation of actin at peripheries of vacuoles (stars) which organize longitudinal AF cables. n = nucleus. Bar = 30 μm (a,b) or 10μm (c-h).

2. Section adherence to slides for immunohistochemistry and *in situ* hybridization of plant material can be also improved simply by 15 min of air-drying after dewaxing in ethanol (Richardson & Dym, 1994) without any special coating of the slides (using SuperFrost Plus® charged slides; Tanya Wagner, personal communication). After air drying, continue with the standard protocol: rehydration in 90%, 50% ethanol, etc.
3. Leaves are difficult to infiltrate with the fixative, since they float on the surface and the cuticle is water-repellent. For such specimens, vacuum infiltration in a syringe can be performed: take a 10-ml syringe, remove the piston, stop the outlet of the syringe with a gloved finger and pour in several ml of the fixative with pieces of tissue. Insert the piston and invert the syringe so that the outlet is upwards. Purge all air and excess fixative from the syringe, leaving only about 2-3 ml liquid. Stop the outlet with the thumb and pull the piston back. This will create a vacuum. Many small bubbles appear on the surface of the leaves, as the air trapped inside the issue expands and escapes. Shake the syringe vigorously, still holding the piston back and keeping the thumb on the outlet. The goal is to shake the bubbles off the leaf surface so that the air does not return when the syringe is pressurised again. Samples will often get stuck on the syringe walls. Check this occasionally and keep shaking. When all specimens are in the fixative, release the piston. The pressure inside reverts to normal and fixative is pushed inside the tissue, replacing the air that escaped. Leaves which are infiltrated with the fixative turn darker green and are semi-translucent. Repeat the infiltration 2–3 times. Finally, remove the piston and transfer the samples into fresh fixative and fix for the desired time. It is still a good idea to put them under vacuum in a desiccator for 10 min or so, to complete the infiltration.
4. For small objects, such as suspension cultures and organs of *Arabidopsis* seedlings, a faster dehydration/embedding protocol may be used with dehydration steps of 15 min each. Warm the samples in ethanol to 37°C and add wax without mixing. Wax settles to the bottom, while the samples float upwards. Once the specimens have sunk (after about 1 h), remove the upper ethanol layer and part of the wax, and add more wax. When the samples sink again, change the old wax for fresh wax and after 30 min transfer to embedding molds and let harden at room temperature.
5. For cutting the ribbon of sections, a paper, cardboard or wooden surface is preferred, as the wax does not stick as much as it does to a plastic or metal surface.
6. Treatment of sections with cold methanol and the preceding wash with SB are optional. F-actin immunofluorescence of maize root sections is stronger without these steps, but background staining is higher (Vitha et al., 1997). In *Arabidopsis* seedlings and *Medicago* suspension culture cells, the immunostaining was satisfactory without the methanol step.
7. PBS can be used instead of SB for diluting antibodies and washing, but SB gives slightly better preservation of F-actin and stronger fluorescence labelling. The use of detergent (Tween 20) for washing helps reduce background staining, but in tissues with older, highly vacuolated cells, the cytoplasm is sometimes washed away and lost.

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