Immunofluorescence Detection of F-actin on Low Melting Point Wax Sections from Plant Tissues

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SUMMARY We developed a simple and reliable technique for immunofluorescence detection of F-actin on microtome sections of plant tissues. For the first time, large numbers of plant cells from various tissues that pass through their developmental stages could be consistently visualized on one section from plant organs. 

- Maleimidobenzoic acid N-hydroxysuccinimide ester-pretreated and formalin-fixed segments of plant roots and shoots were embedded in low melting point ester wax at 37°C and sectioned on a microtome. After dewaxing and rehydration, microfilaments were visualized by indirect immunofluorescence technique with a monoclonal anti-actin antibody. The technique has been successfully used for visualization of tissue- and development-specific F-actin arrays in cells of Zea mays and Lepidium sativum root tips and of maize stem nodes. (J Histochem Cytochem 45:89–95, 1997)

KEY WORDS Fixation Actin Cytoskeleton Immunofluorescence Maize root

Study of the actin cytoskeleton is essential for understanding many aspects of cell growth and development. Antibodies (e.g., McCurdy et al. 1988) or heavy meromyosin (Palevitz et al. 1974) have been successfully employed to detect plant actin. The use of phallootoxins (Twinkel et al. 1989; Wehland et al. 1980), i.e., rhodamine-labeled phalloidin (RLP), however, is by far the most common means of F-actin visualization in plant cells (e.g., Walker and Sack 1995; Seagull et al. 1987; Traas et al. 1987). Nevertheless, several uncertainties persist with the RLP technique. It is not known whether different isoforms of plant actin exhibit the same affinity to phalloidin. Furthermore, phalloidin applied to unfixed cells can lead to artificial stabilization and elongation of microfilaments (Andersland and Parthasarathy 1992; Cooper 1987).

Immunocytochemical techniques permit the visualization of specific isoform of actin. This is of great interest to plant biologists because of the high divergence in multigene families that encode plant actins (Baird and Meagher 1987). M CLEAN et al. (1990) demonstrated tissue specific localization of λ-actin and κ-actin in soybean roots. In addition to the detection of actin isoforms in fixed cells, immunolabeling provides more stable images and less background signal than the RLP procedure. However, both of these techniques were commonly successful only with isolated plant cells (e.g., Meijer and Simmonds 1989; White and Sack 1990), epidermal peels (Cho and Wick 1991), or hand sections of fresh tissue (Koropp and Volkmann 1994).

To study the actin cytoskeleton in plant tissues, a suitable embedding technique is needed to view the cells in their spatial context and to relate the cytoskeleton arrangement to the differentiation state of the cell. Investigators face several difficulties in this situation. The actin cytoskeleton was reported to be very sensitive to formaldehyde (FA) fixation (Parthasarathy et al. 1985), and the use of crosslinker n-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) was recommended to protect microfilaments (Sonobe and Shibaoka 1989). Paraffin embedding does not preserve fine microfilaments, at least in plant tissues (M CLEAN et al. 1990), probably because of the high temperatures used during embedment. Cryosectioning (Kobayashi et al. 1994) is labor-intensive and usually results in poor structural preservation of the plant tissue as well as of the actin cytoskeleton. Embedding in methacrylate resins (Baskin et al. 1992) provides...
good structure, but the tissue antigenicity does not appear to be optimal.

In our previous work on microtubules (Baluška et al. 1992, 1995) we had success with embedding of plant tissues in low melting point ester wax (Steedman’s wax) (Brown et al. 1989). This embedding medium allowed convenient handling and sectioning and the tissue antigenicity was also excellently preserved. We present a modification of this procedure that enabled us to visualize various F-actin arrays on sections taken from intact root and stem tissues of Zea mays and Lepidium sativum roots and incubated with a monoclonal antibody (MAb) raised against animal actin.

Materials and Methods

Seven-mm-long root tip segments of 3-day-old Zea mays L. cv. Alarik seedlings were used to test different variants of the procedure. Stem nodes from 6-week-old Z. mays L. cv. Alarik and 7-mm-long root tips of 2-day-old L. sativum seedlings were utilized to verify the optimized procedure.

Objects were optionally vacuum infiltrated with 100 μM MBS (100 mM stock solution in DMSO) in stabilizing buffer (SB; 50 mM PIPES, 5 mM MgSO₄, 5 mM EGTA; pH set to 6.9 using KOH pellets) for 15-60 min and then transferred to fixative: (a) 3.7% w/v FA in SB; (b) 1.5% w/v FA in SB; (c) 3.7% w/v FA in SB containing 10% v/v DM SO; (d) 0.5% w/v ZnCl₂ in SB. The fixation with a, b, and c was done at room temperature (RT) for 30 min to 72 hr. Fixation with d and a was performed for 10 min at RT, then for 1 min in a microwave oven, which increased the temperature to 60°C, and then the samples were transferred to the same fixative at RT for 10 min. After three washes of 10 min each in SB, and two washes of 15 min each in PBS (0.14 M NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄, 5 mM KH₂PO₄, 3.0 mM NaH₂PO₄; pH 7.3), the specimens were dehydrated in a graded ethanol/PBS series (30, 50, 70, 90, 97, 100% ethanol), each step for 30 min. For dehydration and embedding, analytical grade ethanol was used. The embedding medium was prepared in advance by melting 900 g polyethylene glycol 400 distearate at 60°C and adding 100 g of 1-hexadecanol (both from Aldrich; Milwaukee, WI) and stirring for several hours. The wax was then stored at RT. Embedding was done at 35–37°C, first for 30 min in 100% ethanol, then in graded wax/ethanol series (1:1, 1:2, 1:4, 1:8, 1:16 v/v) followed by three changes of pure wax, each for 1–2 hr. Objects were then put into embedding molds and left to polymerize overnight at RT.

Ribbons of 7-μm sections were placed on poly-l-lysine-coated slides and stretched by addition of a small drop of H₂O to one end of the ribbon. The excess water was soaked away from the opposite end of the ribbon by filter paper. Slides were allowed to dry overnight at RT. For dewaxing and rehydration of sections, technical grade ethanol (Roti-so); Carl Roth, Karlsruhe, Germany) was employed containing 5% v/v aceton and 1% v/v methylmethylektonet. After de-waxing three times for 10 min in ethanol and rehydration in ethanol/PBS for 10 min each step (90%, 50%, 20% v/v, PBS alone), the sections were finally left for 30 min in SB. Optionally, the slides were then dipped in absolute methanol precooled to −20°C or at RT. The 100-ml staining jar with methanol and slides was then placed in a −20°C freezer for 10 min. Methanol treatment was followed by a 30-min SB wash. On some sections, hemi cellulase digestion and Triton treatment were performed to improve the penetration of the antibodies. Sections were incubated for 20 min with 1% w/v crude hemicellulase (Sigma Chemical; St Louis, MO) in SB containing 0.5 mM EGTA, 0.4 M mannitol, 1% Triton X-100, and 0.3 mM phenylmethylsulfonyl fluoride. Sections were washed first with SB for 20 min, then in 1% Triton X-100 in SB for 10 min, and finally in SB for 20 min.

Sections were incubated with a mouse anti-actin MAb, clone C4 (Lessard 1988), purchased from ICN Pharmaceuticals (Costa Mesa, CA) diluted 1:200 or 1:400 in PBS, containing 0.1% w/v bovine serum albumin and 0.1% w/v NaN₃. Two types of negative controls were performed: (a) the primary antibody was omitted or (b) IgG from normal mouse serum was used instead of the MAb at a corresponding protein concentration. Incubation was done in a moist chamber for 90 min at RT or at 37°C. Slides were then washed twice for 10 min in SB and incubated for 90 min with secondary antibody, goat anti-mouse IgG–FITC conjugate (Sigma) diluted 1:100 in the same way as the first antibody. Incubation was performed at the same temperature as well as with the first antibody (RT or 37°C) in darkness. Slides were then washed for 10 min in PBS. The nuclei were fluorescently stained with 4,6-diamidino-2-phenylindole (DAPI, 1 μM in PBS) for 10 min, followed by a 10-min wash in PBS. Sections were then stained for 10 min in 0.01% w/v Toluidine blue dissolved in PBS to suppress the autofluorescence of the cell walls (Brown et al. 1989). After washing for 10 min in PBS, the sections were mounted under a coverslip using p-phenylenediamine anti-fade mountant (Krenik et al. 1989).

Detection of F-actin by RLP (Molecular Probes; Eugene, OR) was performed as described by Tewinkel et al. (1989) on sections after dewaxing and/or optional treatment with cold methanol (see above).

Fluorescence was examined with an Axiosvert 405 M inverted microscope (Zeiss; Oberkochen, Germany) equipped with epifluorescence and appropriate filter sets. Photographs were taken on Kodak T-max film 400 ASA.

The quantitative evaluation of the effect of fixatives used was performed by calculating percent of cells with actin microfilaments preserved. Five photomicrographs corresponding to that of Figure 1E were assessed for each variant. One-way analysis of variance and Newman–Keuls multiple comparisons were performed using WinKS statistical software (TexaSoft; Cedar Hill, TX).

Results

The immunofluorescence procedure enabled us to visualize actin microfilaments in cells of various developmental stages in the plant tissues studied. Table 1 shows the effect of various fixatives after MBS pre-treatment. Neither FA fixative containing 10% DM SO nor FA with microwave treatment preserved microfilaments. The best results were obtained with FA in SB at room temperature and with zinc chloride with mi-
crowave irradiation (Table 1). Zinc chloride-fixed roots gave stronger staining in the root cap and pro-
meristem compared to the FA-fixed material. In both variants, however, the staining pattern in root cap
cells was not filamentous (Figures 1C and 1D). FA was preferred in further experiments, because actin
microfilaments appeared to be more intact. MBS-pre-
treated (15 min) specimens gave identical results after
30 min or 72 hr of FA fixation. No gross differences
were found among FA- or zinc-fixed specimens when
MBS pretreatment ranged from 15 to 60 min. The
only exception was labeling of the phragmoplasts,
which was successful only if MBS treatment did not
exceed 15 min. Omission of MBS pretreatment had no
dramatic effect when it was followed by FA fixation
(60 min; data not shown). Zinc fixation without MBS
pretreatment led to significant damage of microfila-
ments.

Hemicellulase digestion and Triton X-100 extrac-
tion caused fragmentation or even total destruction of
microfilaments. Incubation with antibodies at RT led
to better preservation of F-actin than incubation at
37°C.

Methanol treatment was not necessary for good re-
results. Without the use of methanol the signal was
much stronger, but the background fluorescence was
higher as well. Best results were obtained when sec-
tions were submerged in methanol at RT and placed
for 10 min in a −20°C freezer.

First antibody diluted 1:200 gave optimal results.
Controls in which the sections were incubated in the
presence of preimmune IgG (Figure 1A) or in which
the primary anti-actin MAb was omitted did not show
any staining above faint diffuse background. The opti-
mized procedure is summarized in Table 2 and its re-
sults are shown in Figures 1 and 2. Actin microfila-
ments were more abundant in cells of the stele than of
the cortex (Figures 1E and 2B). Only faint signal was
observed in cells of the quiescent center (Figure 1B).

Discussion

The procedure introduced here allows, for the first
time, easy and sensitive detection of extensive net-
works and fine bundles of actin filaments on serial mi-
crotome sections of plant tissues incubated with an
anti-actin MAb. The embedding medium, low melting
point (35°C) ester wax, allowed gentle embedding at
low temperature (35–37°C), which is only about 15°C
higher than the cultivation temperatures common for
most plants. The preservation of antigenicity in this
medium is excellent, allowing short incubation times.
The physical characteristics of the medium are similar
to those of paraffin, and it is easy to obtain ribbons
with serial sections. Because of the low melting point
of the wax, in hot weather it might be necessary to
perform the microtomy in a cold room and to cool the
knife in a refrigerator. The use of a rotary microtome
and high cutting speeds is recommended for best re-
results. Sections 4–20 μm thick can be routinely ob-
tained, which enables one to adjust the section thick-
ness to the average cell size for a given tissue.

Table 1  Fixation variants tested for actin immunofluorescence in Zea mays root tips

<table>
<thead>
<tr>
<th>Variant</th>
<th>Fixative used</th>
<th>% positive</th>
<th>SD</th>
<th>n^2</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>FA 1.5% in SB, 1 hr at RT</td>
<td>97.4</td>
<td>2.9</td>
<td>303</td>
<td>Only weak labeling in meristem and cap. In cap cells, the signal is diffuse</td>
</tr>
<tr>
<td>b</td>
<td>FA 3.7% in SB, 1 hr at RT</td>
<td>97.0</td>
<td>1.1</td>
<td>558</td>
<td>As in a</td>
</tr>
<tr>
<td>c</td>
<td>FA 3.7% in SB, 5' at RT + 1' MW + 10' at RT</td>
<td>44.4</td>
<td>23.4</td>
<td>207</td>
<td>Many microfilaments fragmented</td>
</tr>
<tr>
<td>d</td>
<td>FA 3.7% + DMSO 10% in SB, 1 hr at RT</td>
<td>60.2</td>
<td>21.3</td>
<td>275</td>
<td>Many microfilaments fragmented</td>
</tr>
<tr>
<td>e</td>
<td>ZnCl_2 0.5% in SB, 5' at RT + 1' MW + 10' at RT</td>
<td>98.3</td>
<td>1.3</td>
<td>509</td>
<td>Similar to a, strong signal even in meristem and the cap</td>
</tr>
</tbody>
</table>

Analysis of variance result p<0.001

Fixation variant | a | b | c | d | e |
<table>
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</table>

* Unless indicated otherwise, all steps were performed as given in Table 2. Analysis of variance and multiple comparisons test results are shown at the bottom of the table.

^ Percent of cells with microfilaments detected.

^ Total number of cells assessed.

SD, standard deviation; FA, formaldehyde; SB, stabilizing buffer, RT, room temperature; MW, microwave treatment; DMSO, dimethylsulfoxide; p, probability value; *, significant difference (α=0.05) between variants.
Figure 1  Immunofluorescence of actin microfilaments in maize root cells. Procedure as described in Table 2 except for D, in which ZnCl₂ fixation was used (see Materials and Methods). (A) Negative control with normal mouse IgG instead of the primary antibody. (B) The root tip. F-actin fluorescence is weak or absent in cells of the quiescent center (asterisk) and of the root cap (rc). (C) Diffuse, weak fluorescence in cells of the root cap of FA-fixed roots. (D) Zinc-fixed root cap cells exhibit stronger and less diffuse actin immunofluorescence than the FA-fixed ones. (E) F-actin is more abundant in stele (lower left corner) than in cells of the cortex. Arrowheads indicate mitotic cells; arrow indicates phragmoplast labeling in a late mitotic cell. (F) Perinuclear conical “cages” of F-actin bundles in elongating cells of the stele. Note curling of actin bundles during transition of cells into their rapid elongation. Cells of endodermis (en) and pericycle (pc), not rapidly elongating yet, display relatively straight actin bundles. Cells undergoing rapid elongation have a curled appearance of the actin bundles (asterisk). Bars = 20 μm.
Table 2  Optimized immunofluorescence procedure for F-actin visualization

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration (min)</th>
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</thead>
<tbody>
<tr>
<td>MBS</td>
<td>15</td>
</tr>
<tr>
<td>1.5% FA in SB</td>
<td>60</td>
</tr>
<tr>
<td>SB</td>
<td>3 x 10</td>
</tr>
<tr>
<td>PBS</td>
<td>2 x 15</td>
</tr>
<tr>
<td>Dehydration, embedding, sectioning, dewaxing, rehydration</td>
<td>See Materials and Methods</td>
</tr>
<tr>
<td>SB</td>
<td>30</td>
</tr>
<tr>
<td>Methanol, −20°C</td>
<td>10</td>
</tr>
<tr>
<td>SB</td>
<td>30</td>
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<tr>
<td>1st antibody 1:200</td>
<td>90</td>
</tr>
<tr>
<td>SB</td>
<td>2 x 10</td>
</tr>
<tr>
<td>2nd antibody 1:100</td>
<td>90</td>
</tr>
<tr>
<td>PBS</td>
<td>10</td>
</tr>
<tr>
<td>DAPI</td>
<td>10</td>
</tr>
<tr>
<td>PBS</td>
<td>10</td>
</tr>
<tr>
<td>Toluidine blue</td>
<td>10</td>
</tr>
<tr>
<td>PBS</td>
<td>10</td>
</tr>
<tr>
<td>Mounting in an anti-fade mountant</td>
<td></td>
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</table>

*All steps were performed at room temperature unless indicated otherwise. MBS, n-maleimidobenzoic acid N-hydroxysuccinimide ester; FA, formaldehyde; SB, stabilizing buffer; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylidone.

The conclusion that the staining patterns observed are due to presence of actin is based on the following. (a) The antibody used (C4) was shown to bind to an antigenic determinant highly conserved among the actins (Lessard 1988). (b) Cho and Wick (1991) found the same staining patterns when they compared C4 (ICN Pharmaceuticals) and N 350 (Amersham; Arlington Heights, IL) antibodies and rhodamine-phalloidin staining in rye. (c) The intracellular arrangement of actin visualized in the presented procedure by the C4 antibody is similar to that detected by other antibodies or rhodamine-phalloidin in plant cells and tissues (M ejer and Simmonds 1989; Vaughan and Vaughn 1987). (d) The negative controls with normal IgG instead of the anti-actin M Ab did not show any filamentous staining pattern (see Figure 1A). For immunoblotting with C4 and N 350 antibodies, see Koropp and Volkman (1994).

The positive effect of M BS pretreatment on F-actin preservation is in agreement with the data of Sonobe and Shibaoka (1989). Nevertheless, the omission of M BS did not lead to substantially worse results in our material. On the other hand, it appears that different microfilament arrays are affected differently by M BS. This is illustrated by the inability to detect phragmoplasts if M BS pretreatment exceeds 15 min. More data from other plant species and organs are needed to evaluate the effect of M BS in the procedure. At present, the use of M BS is recommended. Zinc chloride fixation gave a stronger signal in the promeristem and root cap, where the use of FA fixation led to only a weak signal. This is in accord with the original report of Beckstead (1994), who found zinc fixatives superior to FA for preserving antigenicity in paraffin-embedded animal tissues. However, the effect of zinc chloride on the plant tissue has not been thoroughly characterized. It is not known how rapidly the cells are killed and the biochemical processes stopped by the zinc chloride fixation used in our experiments. Microwave irradiation raised the temperature very quickly to 60°C (within 1 min) and extensive postfixation changes therefore appear to be unlikely. The possibility of such artifacts, however, cannot be completely ruled out. We therefore adhered to FA fixatives, which are routinely used in many microscopic procedures.

Intensive labeling of actin microfilaments could be achieved without treating the sections in methanol. This may appear to be in contrast with the findings of Cho and Wick (1991), who concluded that anti-actin antibodies react only with actin treated with organic solvent, such as acetone or methanol. It should be stressed, however, that the ethanol used for dewaxing our sections was of technical grade (chosen for its low cost) and contained 5% v/v acetone (see Materials and Methods).

Surprisingly, incubation with antibodies at RT gave remarkably better results (both in intensity and in F-actin preservation) than at 37°C. It follows that the microfilament arrays in the sectioned material, although it has been fixed, are extremely sensitive to high temperature and are still subject to damage by incubation conditions. Actin microfilaments were detected in all growing cells of the root proper, which indicates good penetration of the antibodies in 7-μm sections of the root tip. Differences in relative fluorescence intensities within the section (stele vs cortex) or absence of fluorescence (quiescent center) can be explained by differences in F-actin abundance rather than by differential penetration of antibodies. Given the size of the cells, all of them were probably cut by the microtome knife, and antibody penetration therefore does not appear to be a problem. These differences are also unlikely to be solely due to unequal preservation of microfilaments. As shown in Figure 2C, fine actin microfilaments are preserved in both stele and cortex cells.

The present method appears to be incompatible with detection of F-actin by RLP. Therefore, direct comparison of these two methods is not possible here. Although the RLP technique is often believed to provide better preservation of fine actin microfilaments, Schmit and Lambert (1987) obtained better resolution with immunofluorescence. Walker and Sack (1995) found less extensive staining of F-actin with anti-actin antibody than with RLP. The results of the former technique were explained as an artifact caused by fixation. It is possible, however, that polymerization of ac-
Figure 2  F-actin immunofluorescence in maize root tips. Procedure as described in Table 2. (A) Bundles of F-actin in early postmitotic, already heavily vacuolated meristem metaxylem cells. (B) Cross-section of the root. Immunofluorescence signal is stronger in rhizodermis and stele than in the root cortex. (C,D) Same as in B at higher magnification. Actin microfilaments are abundant along transverse cell walls (asterisks). Cross-sectioned F-actin bundles comprising the perinuclear "cages" are distinct, especially in endodermis and pericycle cells (arrows in C). (E) In cells undergoing mitosis, short actin microfilaments are concentrated close to transverse walls (arrows). No actin is detected in the central part of the cell, forming distinct actin depletion zones (asterisks). (F) DAPI fluorescence of DNA in the same section as in E. Bars = 20 μm.
tin in the presence of RLP might have contributed to these differences.

The procedure presented here is a contribution to the tools available for the study of actin microfilaments, because it allows actin filaments to be visualized in sections from intact plant tissues. In this way, developmental changes in the cytoskeleton can be documented.

Acknowledgments

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