

Magdalena Szechyńska-Hebda · Maria Wędzony ·  
Ewa Dubas · Henk Kieft · André van Lammeren

## Visualisation of microtubules and actin filaments in fixed BY-2 suspension cells using an optimised whole mount immunolabelling protocol

Received: 5 July 2005 / Revised: 2 September 2005 / Accepted: 31 October 2005 / Published online: 10 March 2006  
© Springer-Verlag 2005

**Abstract** Excellent visualisation of microtubules and actin filaments was obtained in fixed tobacco BY-2 suspension cells after optimising a protocol for whole mount immunolabelling. The procedure is based on modification of fixation, cell wall digestion, dimethyl sulfoxide (DMSO) treatment, post fixation, and blocking. The most critical aspects of successful preservation and visualization of cytoskeletal elements appeared to be: a two-step fixation with paraformaldehyde and glutaraldehyde before enzymatic cell wall digestion and a post fixation with aldehydes thereafter. The method allows the improved visualization of the organisation of the microtubular and actin filament arrays during the successive stages of cell division and at interphase. Although we present the application of our protocols for cytoskeleton labelling, the excellent results show the potential of using this method for the analysis of various proteins and molecules in plant cells.

**Keywords** Actin filaments · Cell division · Cytoskeleton · Immunocytochemistry · Microtubules · Tobacco BY-2 cells

**Electronic Supplementary Material** Supplementary material is available for this article at  
<http://dx.doi.org/10.1007/s00299-005-0089-y>

Communicated by H. Lörz

M. Szechyńska-Hebda · E. Dubas · H. Kieft ·  
A. van Lammeren (✉)  
Laboratory for Plant Cell Biology, Wageningen University,  
Arboretumlaan 4,  
6703 BD Wageningen, The Netherlands  
e-mail: andre.vanlammeren@wur.nl  
Tel.: +31-317-484318  
Fax: +31-317-485005

M. Wędzony  
Institute of Plant Physiology Polish Academy of Sciences,  
ul. Niezapominajek 21,  
30-239 Kraków, Poland

*Present address:*  
M. Szechyńska-Hebda  
Institute of Plant Physiology Polish Academy of Sciences,  
ul. Niezapominajek 21,  
30-239 Kraków, Poland

**Abbreviations** AF: Actin filament · BSA: Bovine serum albumin · BY-2 cells: Tobacco Bright Yellow-2 cells · DMSO: Dimethyl sulfoxide · GFP: Green fluorescent protein · MBD: Microtubule binding domain of the microtubule associated protein 4 · MBS ester: 3-Maleimidobenzoyl-*N*-hydroxysuccinimide ester · MSB: Microtubule stabilizing buffer · MT: Microtubule · PFA: Paraformaldehyde

### Introduction

Plant biology is currently experiencing the development of easy and repeatable techniques to localize proteins and other molecules in cells. Microtubules (MTs) in living cells can be visualized by introducing fluorescently labelled proteins; e.g. by injection of fluorescently labelled tubulin (Hepler et al. 1993), or by expressing a chimeric protein consisting of green fluorescent protein (GFP) fused to the microtubule binding domain (MBD) of the microtubule-associated protein 4 (MAP4) (Marc et al. 1998). Indirect immunolabelling of MTs is an alternative when GFP techniques with live cells are not possible, or not the first or only choice (Van Lammeren et al. 2004). Fluorescent dyes are attached to general antibodies that specifically bind to antibodies raised against specific antigens (usually proteins) present in the biological material. The immunocytochemical labelling with fluorescent dyes permits specific light microscopic detection of MTs in animal cells (Osborn and Weber 1982) but in plant cells the cell walls are barriers for antibodies. Sections of cells or tissues are used to circumvent the impermeability of plant cell wall for antibodies but they do provide limited information about the three-dimensional localization of the MTs in the cell (e.g. Van Lammeren et al. 1985; Hause et al. 1993). Because cells and tissues are three-dimensional objects, and the spatial organisation of their components are of importance for their function, whole mount immunolocalisation is preferred and can be achieved by enzymatic digestion of the cell wall (Lloyd et al. 1979) or freeze shattering (Wasteneys et al. 1997). The whole mount approach is particularly useful for

visualizing structural elements such as microtubules and actin filaments, which is the aim of this article.

The sensitivity of protein detection by immunolabelling depends upon the retention of antigenicity during tissue processing (Craig and Goodchild 1982). Epitopes, should be recognized by the antibody but can be destroyed by various factors such as temperature extremes, fixatives, or the organic solvents commonly used in fixation procedures. Moreover, the pH of various solutions used in the labelling procedure may alter the charge properties and, hence, the binding properties of antibodies to antigens. Therefore, the success of an immunolabelling protocol greatly depends on the reactivity and quality of the reagents employed as well as on the labelling conditions.

Microtubules and actin filaments (AFs) are dynamic structures and play integral roles in the architecture of cells (Volkman and Baluška 1999; Staiger et al. 2000; Wasteneys 2002) and in the processes of cell division, cell expansion, intracellular transport and morphogenesis in plants (Straight and Field 2000; Verma 2001; Stoeckel and Takeda 2002; Lloyd and Chan 2004). The MT cytoskeleton is present in four distinct arrays: (1) The pre-prophase band (PPB), marking the future division site, (2) the mitotic apparatus, segregating the chromosomes, (3) the phragmoplast, involved in producing the cell plate, and (4) the interphase cortical MT array, involved in cellulose-microfibril deposition in the cell wall (Giddings and Staehelin 1991; Goddard et al. 1994). Actin filaments, although not as well characterised as MTs, are also a ubiquitous component of the plant cytoskeleton (Hepler et al. 1993; Kumagai et al. 2001). Actin filaments have been observed in association with the PPB and phragmoplast of dividing cells as well as with the cell cortex and throughout the cytoplasm of interphase cells (Collings and Emons 1999), in spindles during division (e.g. Van Lammeren et al. 1989) and in the phragmoplast (Valster et al. 1997). To gain insight in the structural organisation and the function of the microtubular and actin cytoskeleton, BY-2 suspension cells are frequently used because they have a short cell cycle and the events of the cell cycle progression from the M phase to G<sub>1</sub> phase can be well monitored microscopically (Nagata et al. 1992; Hasezawa et al. 1997; Kumagai et al. 2001; Stoeckel and Takeda 2002). As such, the BY-2 cells serve as a useful and accurate model for plant cytoskeleton studies.

In this paper, we describe methods leading to an improved preservation and visualization of cytoskeletal elements in tobacco BY-2 cells analysed at successive stages of the cell cycle.

## Material and methods

### Cell cultures

Long-term tobacco Bright Yellow-2 suspension cultures (BY-2) were routinely cultured in a standard BY-2 growth medium, containing Murashige and Skoog macro and micro-salts (Murashige and Skoog 1962) supplemented with 3% sucrose, 100 mg/l myo-inositol,

200 mg/l KH<sub>2</sub>PO<sub>4</sub>, 1 mg/l thiamine and 0.2 µg/l 2,4-dichlorophenoxyacetic acid (Nagata et al. 1992; Nagata and Kumagai 1999). Suspension cultures were diluted at weekly intervals (1 ml of old culture in 50 ml of fresh medium) in 250 ml Erlenmeyer flasks. The cell suspension was agitated on a rotary shaker at 100 rpm and maintained at 25°C and in the dark (Vos et al. 2004). To improve synchronization, cultures were kept for 2 weeks without subculture, followed by dilution of 1 ml of old culture in 50 ml of fresh medium. Undifferentiated and dividing cells were counted 2 days after that subculture (a mitotic index of up to 40% was observed), while differentiated non-dividing cells were observed after 10 days of culture. Samples of 0.5 ml of suspension were used for whole mount procedures.

### Basic immunolabelling protocol

The protocol for whole mount immunolabelling was based on the procedure of Friml et al. (2003). Samples of BY-2 cell suspension (0.5 ml) were taken at 2–4 days or 10 days after subculture. Cells were collected on a 20 µm nylon sieve, or by 1 min centrifugation at 500 rpm at room temperature (RT), and fixed immediately in 4% paraformaldehyde (PFA) in microtubule stabilizing buffer (MSB: 50 mM 1,4-piperazinediethane sulfonic acid (PIPES, Merck), 5 mM EGTA (Serva), 5 mM MgSO<sub>4</sub>, pH 7.0, adjusted with KOH) for 1 h at RT. Samples were rinsed with MSB/0.1% Triton (Sigma) (5 × 10 min) and with deionised water (5 × 10 min). Cell walls were digested with 2% Driselase in MSB/0.1% Triton for 25 min at RT, washed with MSB/0.1% Triton (5 × 10 min), and then incubated in 10% Dimethyl Sulfoxide (DMSO) with 3% NP-40 (both from Sigma) in MSB for 50 min. After another washing in MSB/0.1% Triton (5 × 10 min), the cells were incubated in 2% acetylated BSA in MSB for 30 min at 32°C to prevent unspecific binding of antibodies. Samples were incubated in MSB with primary antibodies (either monoclonal anti α-tubulin (Clone DM1A, Molecular Probes, dilution 1:200), or monoclonal anti actin (Molecular Probes, dilution 1:10) overnight at 4°C in the presence of 3% acetylated BSA. After washing with MSB/0.1% Triton (8 × 10 min), the cells were incubated with secondary antibody raised in goat against mouse (GaM/IgG) conjugated with Alexa 488 (Molecular Probes, dilution 1:100) in 3% BSA/MSB for 2 h at 32°C. Finally, the samples were washed (5 × 10 min) with MSB/0.1% Triton, and with sterile deionised water (5 × 10 min). Samples were stored at 4°C in PBS (4% NaCl, 0.1% KCl, 0.1% KH<sub>2</sub>PO<sub>4</sub>, 0.7% Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O) or in water, both supplemented with 0.02% NaN<sub>3</sub> to prevent bacterial contamination.

### Modified immunofluorescence protocol

#### Fixation process

Instead of a single fixation with 4% PFA, samples were pre-fixed (without centrifugation or collection on a sieve) by adding an equal volume of 4% PFA in MSB (final PFA

concentration 2%). After 25 min, cells were collected by centrifugation, or with a sieve, and then fixed in 4% PFA in MSB for 35 min at RT. Alternatively, 0.1 and 1% glutaraldehyde were added to the prefixation and fixation solutions, respectively. Samples were rinsed with MSB/0.1% Triton (Sigma) (5×10 min) and with deionised water (5×10 min).

#### *Enzymatic digestion*

Enzymes were dissolved in MSB buffer supplemented with 0.1% Triton, sonicated 2×10 min, centrifuged for 8 min at 1,000 rpm, and filtered through a paper filter. Samples were digested with 2% Driselase or with 1% Cellulase R10 + 0.5% Pectolyase Y-23 (all enzymes from Sigma) for 10, 15, 25 and 30 min at RT, and washed with MSB/0.1% Triton (5×10 min).

#### *DMSO treatment*

The influence of DMSO and NP-40 treatment on the labelling procedure was tested by incubating cells with 10% DMSO and 3% NP-40 in MSB for 0, 10 and 50 min. Samples were washed in MSB/0.1% Triton (3×10 min).

#### *Post-fixation*

Preservation of the cyto-architecture was markedly improved by a fixation step after the enzymatic digestion of the cell walls. Post-fixation was performed with 4% PFA, or with a mixture of 4% PFA with 0.1 or with 1% glutaraldehyde in MSB for 15 min. Samples were washed in MSB/0.1% Triton (3×10 min).

#### *Blocking*

Samples were treated with freshly prepared 1 mg/ml NaBH<sub>4</sub> in MSB for 5 min to reduce autofluorescence caused by free aldehydes from the glutaraldehyde fixation, and then incubated with 2% acetylated BSA in MSB for 30 min at 32°C.

#### *Antibody incubation*

Cells were incubated with primary antibody (anti actin, dilution 1:10 or anti  $\alpha$ -tubulin, dilution 1:200) and 3% acetylated BSA in MSB: overnight at 4°C, overnight at RT, or overnight at 32°C. When samples were incubated at RT and 32°C, 0.02% NaN<sub>3</sub> was added to the incubation solution.

#### *Labelling of actin filaments*

Generally, the steps for immunolabelling of actin filaments were the same, except for the fixation procedure (prefixation, fixation and post-fixation) where 10  $\mu$ M 3-

Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS ester) was added.

#### *Microscopic observation*

Immunofluorescence images were collected with a CELL MAP IC Bio-Rad (Microscience Division, Hemel Hempstead, UK) confocal laser-scanning microscope, coupled to a Nikon Eclipse TE 2000-S inverted microscope. Images were collected by Kalman averaging of four full scans. Three-dimensional images and z-projections of the cells were obtained by collecting series of approximately 30–60 optical sections in the Z-axis, each section 0.5–1  $\mu$ m thick. Images were acquired and processed using software programs including Lasersharpe 2000 software (Microscience Division, Hemel Hempstead, UK), Confocal assistant 4.02 (written by Todd Clark Brelje) and Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA).

## **Results**

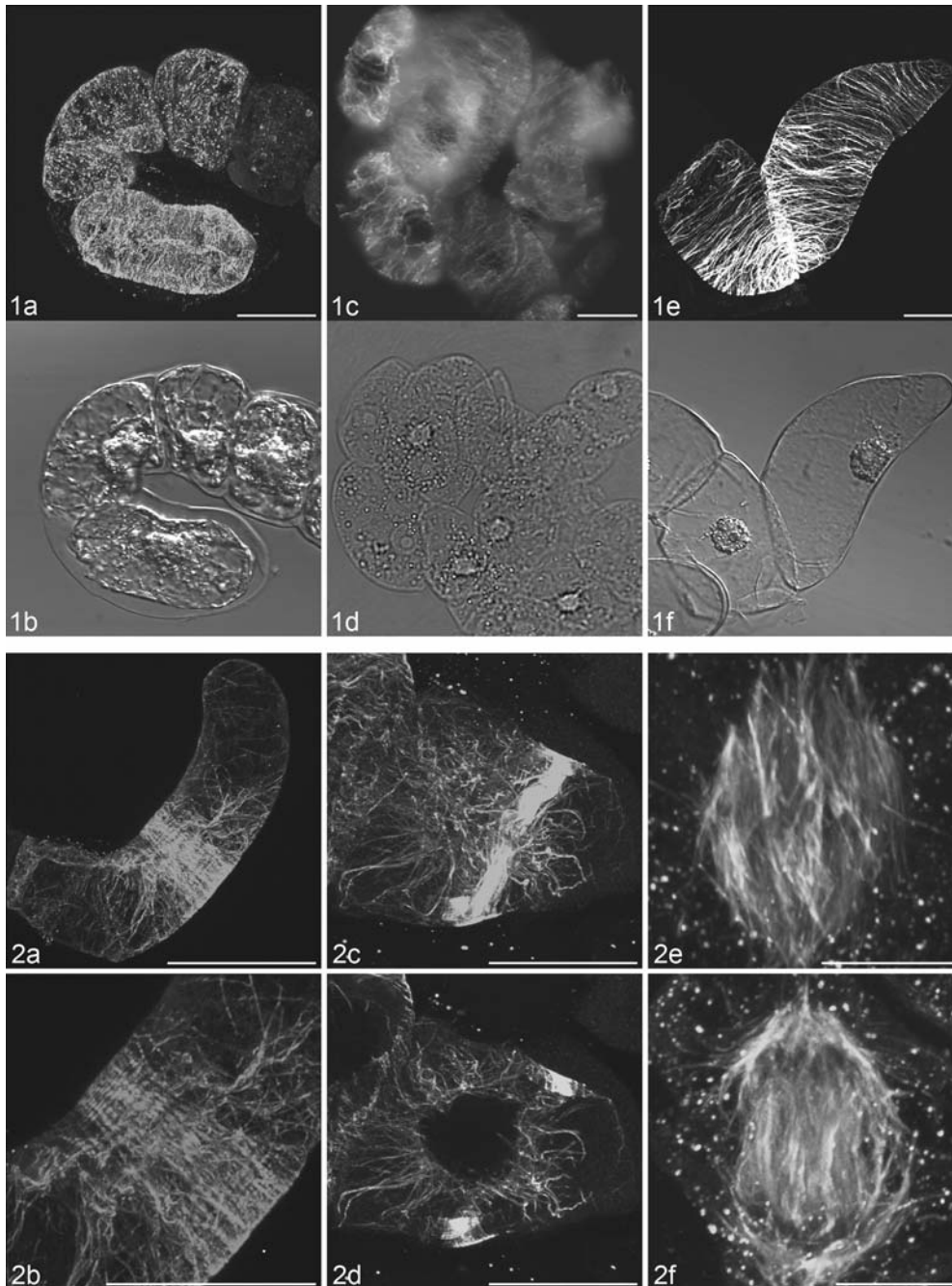
A standard whole mount immunolabelling procedure consists of five steps: fixation, cell wall permeation, blocking with a blocking agent, incubation with primary and secondary antibodies, and signal detection. Here the fixation consisted of a three-step fixation, two steps before partial cell wall digestion, and a post fixation step thereafter.

#### *Fixation process*

A single fixation with 4% PFA was not sufficient for the proper preservation of MTs and cyto-architecture in whole mount BY-2 cells (Fig. 1a and b). Microtubules were fluorescent but they were wrinkled and the cells had changed shapes; there was plasmolysis and cytoplasmic strands had disappeared as compared to untreated cells. After prefixation with 2% PFA followed by fixation with 4% PFA, MTs were not well preserved yet, but plasmolysis was reduced, and cytosol and transvacuolar strands were intact (Fig. 1c and d). In order to further improve the preservation of both the three-dimensional structure of the cells and the stability of the MT cytoskeleton, the effects of additional 0.1 and 1% glutaraldehyde were examined. Whereas addition of 0.1% glutaraldehyde was not effective, excellent fluorescent signal and well preserved cyto-architecture and cell shapes were obtained with 1% glutaraldehyde (Figs. 1e,f and 4c,d).

#### *Cell wall digestion*

Cell walls were partially digested to become permeable for antibodies. Cell walls were digested with either 2% Driselase, or with 1% Cellulase R10 + 0.5% Pectolyase Y-23. Enzyme composition had less influence on digestion effectiveness than the time of incubation. Best immunolabelling



**Fig. 1** Effects of fixation procedures on cell morphology and visualization of the microtubular (MT) cytoskeleton in tobacco BY-2 cells. Results of a one-step fixation procedure with PFA alone (**a, b**) and of two two-step fixation procedures with either PFA alone (**c, d**) or with PFA supplemented with glutaraldehyde (**e, f**). **a, b**: 4% PFA fixation, **c, d**: 2% PFA pre-fixation, followed by 4% PFA fixation, **e, f**: 2% PFA + 0.5% GA pre-fixation, followed by 4% PFA + 1% GA fixation. Immuno-fluorescence images **a, c** and **e** show the microtubule organisation in BY-2 cells in combination with bright field images depicting the organisation of the cytoplasm (**b, d** and **f**). Note the improvement of quality from **a** to **e**, and from **b** to **f**. **a** and **b** show cells with plasmolysis and distorted shapes of the MTs; **c** and **d** have no plasmolysis but still distorted MTs; **e** and **f** show well pre-

served cells with undisturbed arrays of cortical MTs. Bars: 25  $\mu$ m. **Fig. 2** Distribution of cortical microtubules during the formation of the preprophase band from early (**a, b**) to late stage (**c, d**), and during spindle formation (**e, f**) in tobacco BY-2 cells, visualized with the optimised immunolabelling protocol including post fixation. Images are projections of sequential optical sections at 1  $\mu$ m intervals. Part **a** of this figure gives an overview of a cell with a PPB at an early stage of formation, with a detail in **b**. Full projection of PPB in **c** at a later stage of formation. Microtubules outside the PPB radiate from the nuclear surface and are well visible in **d** which is a part of the projection of **c**. **e** and **f** show a mitotic spindle at metaphase and anaphase, respectively. Bars **a-d**: 25  $\mu$ m; **e, f**: 10  $\mu$ m

results for interphase cell wall digestion were obtained after incubation for 15–20 min and for dividing cells about 10–15 min. Whereas insufficient digestion (less than 10 min) resulted in low label intensity, over-digestion (25–30 min) lead to changed cell shapes and reduced fluorescence. Enzyme solutions were cleaned by centrifugation and filtration because enzyme granules were hard to remove and lead to continued cell wall digestion during the successive steps of the labelling procedure.

### DMSO treatment

After partial cell wall digestion, cells were incubated in MSB with 10% DMSO and 3% NP-40 to further improve the accessibility for antibodies. Comparing 10 and 50 min treatments, the longest incubation time provided the best results. Labelled MTs were obtained after a 50 min DMSO treatment, weak signals after incubation for 10 min, and no signal at all when DMSO treatment was omitted.

### Post-fixation process

Cytoskeletal elements showed Brownian movement after the two-step fixation, the DMSO/NP-40 treatment and the labelling procedure when no further fixation was done. As this is an indication of a limited fixation, we applied an additional fixation with 4% PFA and 1% glutaraldehyde, immediately after DMSO treatment. A 15-min post fixation immobilized MTs, resulting in a markedly improved preservation of the configuration of the microtubular cytoskeleton (e.g. see Fig. 4c and d).

### Observations on the cytoskeleton

Application of the optimised protocol enabled excellent visualization of cytoskeletal configurations during cell division and interphase.

### Cell division

Reorganisation of the interphase cortical microtubule cytoskeleton at the beginning of cell division lead to the formation of the preprophase band (PPB) (Fig. 2a–d). The PPB is positioned in the cell cortex, on the site at which the future cell plate will fuse with the parent cell wall. As chromatin condensation proceeded, transverse cortical MTs concentrated in a very wide band (Fig. 2a and b), that progressively narrowed (Fig. 2c and d). Individual MTs could not be distinguished in the narrow band. This stage was followed by increased fluorescence in the two spindle pole regions marking the formation of the perinuclear spindle apparatus (data not shown). During the progression from prometaphase to metaphase, the mitotic apparatus became increasingly distinct and MTs were restricted only to the spindle (Fig. 2e and f). At late anaphase the phragmoplast

developed and two cylinders of parallel MTs developed in the zone between the two sets of chromosomes (Fig. 3a–f). While the initial shape of the phragmoplast is a cylindrical or barrel-like structure (Fig. 3a and b and S1), centrifugal expansion leads to the formation of a ring-shaped phragmoplast (Fig. 3c–f and S2,3). This is caused by MT breakdown in the center and formation of MTs along the edge of the growing cell plate. Microtubules disappeared in the central region when the phragmoplast microtubule array occupied approximately from two-thirds to three-fourth of the cell diameter. During late cytokinesis, short MTs with a random orientation were observed at the two poles of the expanding phragmoplast (Fig. 3e and f, S3).

### Cortical microtubules and actin filaments

Cortical MTs were undetectable through mitosis and cytokinesis. In interphase cells just after division, they were observed in cytoplasmic strands and in the cell cortex. Whereas the MTs did not show preferential orientation initially (Fig. 4a), they attained an orientation perpendicular to the long axis of the cell (Fig. 4b). In elongating or elongated cells their orientation was predominantly transverse to the cell axis (Fig. 4c and d). The cortical microtubules were observed as individual, fluorescent strands, with such a high resolution that it was possible to recognize sites where they formed pairs, where they separated from each other and where they had their outer ends (Fig. 4d arrow-heads).

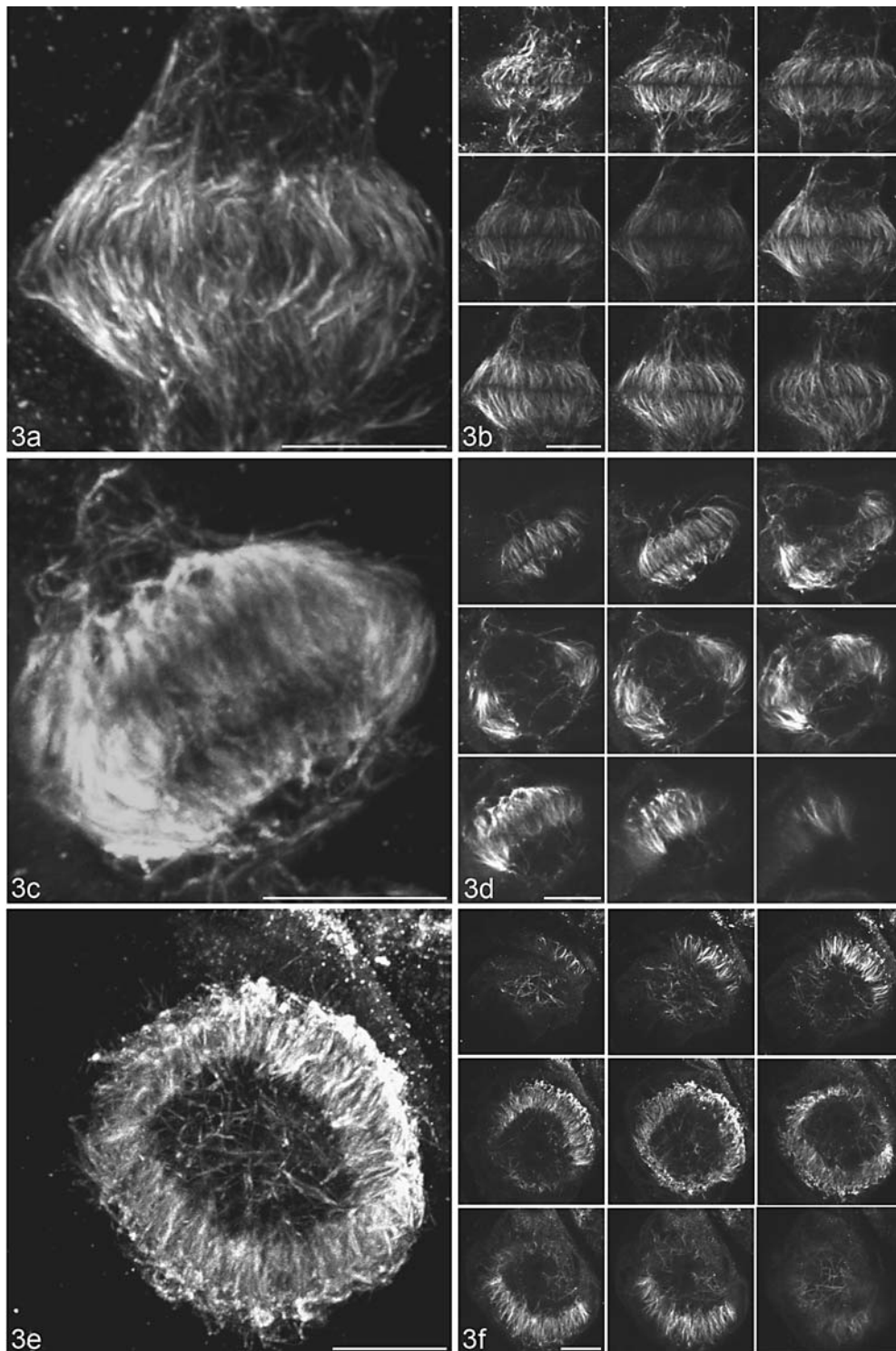
Actin filaments were observed in cortical cytoplasm of interphase cells (Fig. 5a and b). Although not as well preserved as MTs, they were well labelled and arranged perpendicular to the long axis of the cell. In addition, AFs were found in cytoplasmic strands and in the perinuclear region. Only a limited number of cells was labelled (approximately 1%) which is likely caused by loss of integrity antigenicity of the AFs.

---

## Discussion

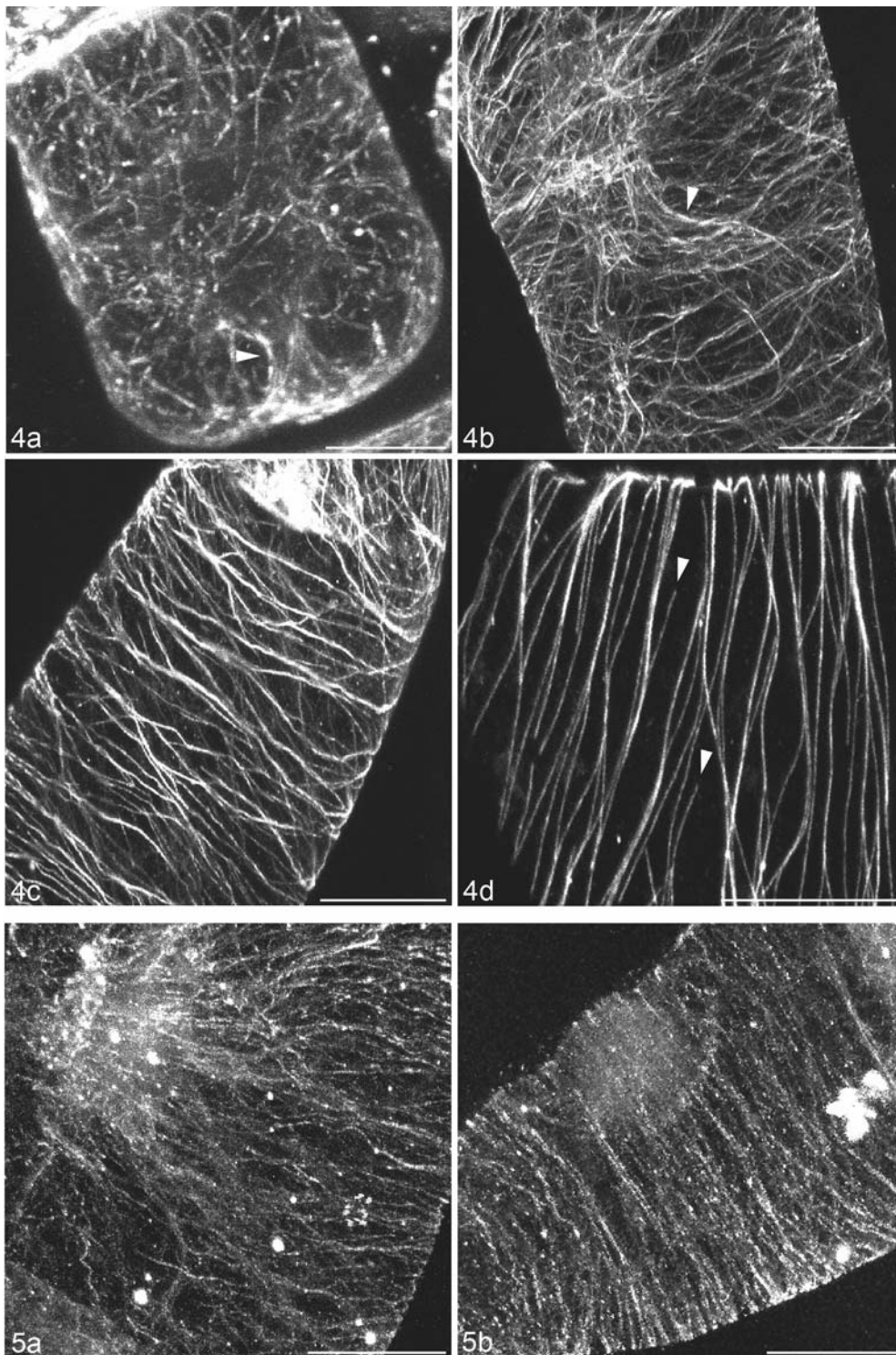
Although chimeric proteins containing GFP are often used for the visualization of cytoskeletal elements in live cells to study the dynamic properties of the cytoskeleton, comparative studies still show that the application of immunocytochemical procedures are of importance (Sieberer et al. 2002) and in case transformation is recalcitrant or impossible, they could be the only choice and should therefore be optimised where possible.

Whole mount immunolabelling is to be preferred over embedding the cells and tissues in various matrices and the sectioning of the embedded material, which is more laborious and can change the antigenic properties of proteins (Goodbody and Lloyd 1994; Friml et al. 2003). Additionally, it preserves the three-dimensional organisation of the cytoskeletal configurations in the investigated cells. Although the method is limited by the inability to observe dynamics in living cells, these dynamics can be deduced by fixing and preparing cells at closely spaced developmental stages.



**Fig. 3** Distribution of microtubules during phragmoplast formation in tobacco BY-2 cells visualized with the optimised immunolabelling protocol—including post fixation—from early (**a**, **b**) to late stage (**e**, **f**). **a**, **c** and **e** are projections, and **b**, **d** and **f** sequential optical sections at  $2\ \mu\text{m}$  intervals. Note that in the early phase microtubules run to the cell plate region throughout the phragmoplast, although with lower numbers in the central zone than in the periphery (series in **b**, see also

S1). From **d** it is clear that the number of microtubules in the central zone decreased but did not disappear completely (see also S2). **e** and **f** show a phragmoplast at  $90^\circ$  tilting. Note that not all MTs are in the phragmoplast cylinder but some are at the caps of the cylinder at the two sides of the phragmoplast structure shown in the sequential images of **f** (see also S3). Bars:  $10\ \mu\text{m}$



**Fig. 4** Cortical microtubule (MT) configurations in young (**a**) to old, elongated tobacco BY-2 cells (**d**) visualized with the optimised immunolabelling protocol—including post fixation. Images are projections of sequential optical sections at 1  $\mu\text{m}$  intervals. Note that cytoplasmic MTs only occur in a and b and that cortical MTs attain an orientation more and more transversal to the long axis of the cell. **c** is a projection of a Z-series of a whole cell and **d** is a projection of a Z-series of the cortical cytoplasm alone. Note in **d** that single MTs

can be seen as single fluorescent lines. They sometimes come close to neighbouring MTs and sometimes separate from them. Free ends of single MTs are in the focus plane (arrow-heads). Bars: 25  $\mu\text{m}$ .

**Fig. 5** Cortical actin filaments in tobacco BY-2 cells visualized with optimised immunolabelling protocol—including post fixation. Note the parallel orientation of the actin filaments. Images are projections of sequential optical sections at 1  $\mu\text{m}$  intervals. Bars: 25  $\mu\text{m}$

Originally, the detection method of Friml et al. (2003) was developed for the detection of mRNAs and proteins in *Arabidopsis thaliana*, *Petroselinum crispum* and *Nicotiana tabacum* seedlings using automated whole mount localisation techniques. Its application to BY-2 cells was hindered by several problems resulting from poor permeability of reagents through the cell wall and the cell membrane, a high level of fluorescence background and trembling of cytoskeletal elements. However, modifications to the basic protocol lead to immunolabelling results with excellent resolution and sensitivity. In the fixation protocol a prefixation of 25 min, a fixation of 35 min, and a post fixation of 15 min provided excellent results. The partial digestion of the BY-2 cell walls was successful with pectolyase and cellulase or with driselase. Because of the cell wall properties of interphase cells and dividing cells differ, a longer time was preferable for interphase cells (15–20 min) than for dividing cells (10–15 min). Following cell wall digestion, the plasma membrane of plant cells has to be permeabilized as well with detergents such as Triton X or saponin (Goodbody and Lloyd 1994; Fletcher et al. 2000; Schwindling et al. 2002). In the present experiments with BY-2 cells, incubation with 10% DMSO and 3% NP-40 for 50 min was necessary to enable antibodies to enter the cells.

The labelling of MTs was superior to the labelling of AFs. Because the accessibility for tubulin and actin epitopes was the same, it is suggested that, despite the application of MBS ester, the preservation of the AFs or their epitopes was inferior. It was indeed reported that AFs are very difficult to preserve especially at the ultrastructural level (Hepler et al. 1993; Staehelin and Hepler 1996; Volkmann and Baluška 1999). Thus, further protocol development is necessary. Alternatively, cells can be microinjected with fluorescently labelled phalloidine. This method is successfully applied to study AFs role in plant cells (Cleary et al. 1992; Valster et al. 1997).

Microtubules were visualized in detail during the successive stages of cell division, as well as during interphase. The structural organisation during prophase, metaphase and anaphase clearly demonstrates the change of microtubules from a PPB and perinuclear organisation at prophase (Fig. 2c and d) to a spindle organisation at metaphase and anaphase (Fig. 2e and f), to a phragmoplast organisation at telophase (Fig. 3). Post mitotic reorganisation of the cortical MT cytoskeleton is shown in detail at high resolution in which individual MTs were clearly identified (Fig. 4b–d).

Alternatives for whole mount immuno labelling of BY-2 cell MTs are amongst others microinjection of fluorescently labelled tubulin (Schwindling et al. 2002) or GFP expression studies (Ludin and Matus 1998; Granger and Cyr 2000; Vos et al. 2004). However, the data presented here not only aim to be used for this model cell system and for tubulin but for other cell types and proteins as well.

**Acknowledgements** The participation of M. Szechyńska-Hebda, E. Dubas and M. Wędzony was supported by the 5th Framework Programme project CROPSTRESS contract number QLK5-CT-2002-30424.

## References

- Cleary AL, Gunning BES, Wasteneys GO, Hepler PK (1992) Microtubule and F-actin dynamics at the division site in living *Tradescantia* stamen hair cells. *J Cell Sci* 103:977–988
- Collings DA, Emons AMC (1999) Microtubule and actin filament organization during a central division in potato suspension culture cells. *Protoplasma* 207:158–168
- Craig S, Goodchild DJ (1982) Post-embedding immunolabelling. Some effects of tissue preparation on the antigenicity of plant proteins. *Eur J Cell Biol* 28(2):251–225
- Fletcher LM, Welsh GI, Oatey PB, Tavaré JM (2000) Role of microtubule cytoskeleton in GLUT4 vesicle trafficking and in the regulation of insulin-stimulated glucose uptake. *Biochem J* 352:267–276 <http://dx.doi.org/10.1042/0264-6021%3A3520267>
- Friml J, Benkova E, Mayer U, Palme K, Muster G (2003) Automated whole mount localization techniques for plant seedlings. *Plant J* 34:115–124 <http://dx.doi.org/10.1046/j.1365-313X.2003.01705.x>
- Giddings TH, Staehelin LA (1991) Microtubule mediated control of microtubule deposition: a re-examination of the hypothesis. In: Lloyd CW (ed) *The cytoskeletal basis of plant growth and form*. Academic Press Inc., San Diego, California, pp 85–100
- Goddard RH, Wick SM, Silflow CD, Snustad DP (1994) Microtubule components of the plant cell cytoskeleton. *Plant Physiol* 104:1–6
- Goodbody KC, Lloyd CW (1994) Immunofluorescence techniques for analysis of cytoskeleton. In: Harris N, Oparka KJ (eds). *Plant Cell Biology: a practical approach*. IRC Press, Oxford, UK, pp 221–243
- Granger CL, Cyr RJ (2000) Microtubule reorganization in tobacco BY-2 cells stably expressing GFP-MBD. *Planta* 210:502–509 <http://dx.doi.org/10.1007/s004250050037>
- Hasezawa S, Kumagai F, Nagata T (1997) Sites of microtubule reorganization in tobacco BY-2 cells during cell-cycle progression. *Protoplasma* 198:202–209 <http://dx.doi.org/10.1007/BF01287569>
- Hause B, Hause G, Pechan P, van Lammeren AAM (1993) Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* L. *Cell Biol Int* 17:153–168 <http://dx.doi.org/10.1006/cbir.1993.1052>
- Hepler PK, Cleary AL, Gunning BES, Wadsworth P, Wasteneys GO, Zhang DH (1993) Cytoskeletal dynamics in living plant cells. *Cell Biol Int* 17:127–142 <http://dx.doi.org/10.1006/cbir.1993.1050>
- Kumagai F, Yoneda A, Tomida T, Sano T, Nagata T, Hasezawa S (2001) Fate of nascent microtubules organized at the M/G<sub>1</sub> interface, as visualized by synchronized tobacco BY-2 cells stably expressing GFP-tubulin: time sequence observations of the reorganization of cortical microtubules in living plant cells. *Plant Cell Phys* 42:723–732 <http://dx.doi.org/10.1093/pcp/pce091>
- Lloyd CW, Slabas AR, Powell AJ, MacDonald G, Badley RA (1979) Cytoplasmic microtubules of higher plant cells visualised with anti-tubulin antibodies. *Nature (Lond)* 279:239–241
- Lloyd CW, Chan J (2004) Microtubules and the shape of plants to come. *Nat Rev Mol Cell Biol* 5:13–22 <http://dx.doi.org/10.1038/nrm1277>
- Ludin B, Matus A (1998) GFP illuminates the cytoskeleton. *Trends Cell Biol* 8:72–77 [http://dx.doi.org/10.1016/S0962-8924\(97\)01169-0](http://dx.doi.org/10.1016/S0962-8924(97)01169-0)
- Marc J, Granger CL, Brincat J, Fisher DD, Kao T, McCubbin AG, Cyr RJ (1998) A GFP-MAP4 reporter gene for visualizing cortical microtubule rearrangements in living epidermal cells. *Plant Cell* 10:1927–1940 <http://dx.doi.org/10.1105/tpc.10.11.1927>
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Nagata T, Kumagai F (1999) Plant cell biology through the window of the highly synchronized tobacco BY-2 cell line. *Meth Cell Sci* 21:123–127 <http://dx.doi.org/10.1023/A:1009832822096>



- Nagata T, Nemoto Y, Hasezawa S (1992) Tobacco BY-2 cell line as the "HeLa" cell in the cell biology of higher plants. *Int Rev Cyt* 132:1–30
- Osborn M, Weber K (1982) Immunofluorescence and immunocytochemical procedure with affinity purified antibodies: tubulin-containing structures. *Methods Cell Biol* 47:121–131
- Schwindling SL, Faust M, Montenarh M (2002) Determination of the mitotic index by microinjection of fluorescently labeled tubulin. *Eur J Cell Biol* 81:169–174
- Sieberer BJ, Timmers ACJ, Lhuissier FGP, Emons AMC (2002) Endoplasmic microtubules configure the subapical cytoplasm and are required for fast growth of *Medicago truncatula* root hairs. *Plant Phys* 130:977–988 <http://dx.doi.org/10.1104/pp.004267>
- Staehelein LA, Hepler PK (1996) Cytokinesis in higher plants. *Cell* 84:821–824 [http://dx.doi.org/10.1016/S0092-8674\(00\)81060-0](http://dx.doi.org/10.1016/S0092-8674(00)81060-0)
- Staiger CJ, Baluška F, Volkmann D, Barlow P (eds) (2000) Actin: a dynamic framework for multiple plant cell functions. Dordrecht: Kluwer Academic Publishers
- Stoeckel H, Takeda K (2002) Plasmalemmal voltage-activated K<sup>+</sup> currents in protoplasts from tobacco BY-2 cells: possible regulation by actin microfilaments? *Protoplasma* 220:79–87 <http://dx.doi.org/10.1007/s00709-002-0028-5>
- Straight AF, Field CM (2000) Microtubules, membranes and cytokinesis. *Curr Biol* 10:R760–R770
- Valster AH, Pierson ES, Valenta R, Hepler PK, Emons AMC (1997) Probing the plant actin cytoskeleton during cytokinesis and interphase by profilin microinjection. *Plant Cell* 9:1815–1824 <http://dx.doi.org/10.1105/tpc.9.10.1815>
- Van Lammeren AAM, Bednara J, Willemse MTM (1989) Organization of the actin cytoskeleton during pollen development in *Gasteria verrucosa* (Mill.) H Duval. *Planta* 178:531–539 <http://dx.doi.org/10.1007/BF00963823>
- Van Lammeren AAM, Keijzer CJ, Willemse MTM, Kieft H (1985) Structure and function of the microtubular cytoskeleton during pollen development in *Gasteria verrucosa* (Mill.) H Duval. *Planta* 165:1–11 <http://dx.doi.org/10.1007/BF00392205>
- Van Lammeren AAM, Wędzony M, Dubas E, Szechyńska-Hebda M (2004) Fluorescent microscopy to study plant response to stress. In: Filek M, et al. (eds) Analytical methods in plant stress biology. Krakow, Poland, pp 133–142
- Verma DPS (2001) Cytokinesis and building of the cell plate in plants. *Ann Rev Plant Physiol Plant Mol Biol* 52:751–784 <http://dx.doi.org/10.1146/annurev.arplant.52.1.751>
- Volkmann D, Baluška F (1999) Actin cytoskeleton in plants: from transport networks to signalling networks. *Microsc Res Tech* 47:135–154 [http://dx.doi.org/10.1002/\(SICI\)1097-0029\(19991015\)47:2<135::AID-JEMT6>3.0.CO;2-1](http://dx.doi.org/10.1002/(SICI)1097-0029(19991015)47:2<135::AID-JEMT6>3.0.CO;2-1)
- Vos JW, Dogterom M, Emons AMC (2004) Microtubules become more dynamic but not shorter during preprophase band formation: a possible "Search-and-Capture" mechanism for microtubule translocation. *Cell Motil Cytoskeleton* 57:246–258 <http://dx.doi.org/10.1002/cm.10169>
- Wasteneys OG, Willingale-Theune J, Menzel D (1997) Freeze shattering: a simple and effective method for permeabilizing higher plant cell walls. *J Microsc* 188:51–61 <http://dx.doi.org/10.1046/j.1365-2818.1977.2390796.x>
- Wasteneys GO (2002) Microtubule organization in the green kingdom: chaos or self order? *J Cell Sci* 115:1345–1354