

Original Article

A Comparison of Techniques for Localizing Actin and Tubulin in Hyphae of *Saprolegnia ferax*¹

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We have evaluated protocols for immunofluorescence (IF) staining of the potentially interacting actin filaments (F-actin) and microtubules in hyphae of *Saprolegnia ferax*, using rhodamine-phalloidin (RP) and freeze-substitution electron microscopy (FSEM), respectively, as standards for their distribution. *Saprolegnia* has four distinguishable cortical F-actin populations with characteristic organizations and RP- and actin-IF-staining affinities, all of which could be labeled with both probes after some protocols. Other protocols stained only some of the populations. Cortical F-actin was always more reproducibly and sharply stained with RP than IF, indicating that the former is the probe of choice for F-actin in these cells. Although no single IF protocol revealed all of the F-actin and microtubule populations, showing the

potential need to optimize protocols for specific antibodies, simultaneous localization was readily achieved by dual labeling with RP and tubulin IF. Tubulin IF patterns differed from FSEM: mitotic spindles were revealed but not the more abundant prophase microtubule arrays, and the cytoplasmic microtubules were subapically displaced and bundled into long cables. These cables, which apparently linked nuclei, indicate a previously undetected involvement in nuclear spacing. The tubulin antibody successfully used for IF failed to recognize any proteins in immunoblots, indicating that immunoblots may not always be a useful indicator of success with IF. (*J Histochem Cytochem* 42:523-530, 1994)
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Introduction

Tip-growing cells from taxonomically diverse organisms have many features in common (Heath, 1990a). These include polarized cytoskeletons containing F-actin, involved in cell extension and cytoplasmic migration (Heath, 1990b; Steer, 1990), and microtubules, required for mitosis and organelle positioning (Derksen and Emons, 1990). It is likely that these two components interact in some of their roles, since in other systems their distributions or roles are partly congruent or interdependent (Palmer et al., 1992; Derksen and Emons, 1990; McKerracher and Heath, 1987). These interactions are likely to be mediated by diverse actin-binding proteins (ABPs) (Hartwig and Kwiatkowski, 1991; Stossel et al., 1985) and microtubule-associated proteins (MAPs) (Olmsted, 1986,1991). However, our understanding of these interactions is incomplete because F-actin and microtubules are rarely visualized simultaneously in a single cell. Before we can understand the assembly and functions of the cytoskeleton, we need techniques that simultaneously provide faithful preservation and high-resolution visualization of all components. Electron microscopy (EM), although having the

potential to provide the best resolution, is notoriously poor for preserving F-actin, is very time-consuming if whole cell distributions are desired, and has yet to address the associated proteins. Immunofluorescence (IF) techniques can deal with a wide range of proteins at the whole-cell level with useful resolution but are not without problems. IF protocols can induce structural rearrangements of many cell components (Kaminskyj et al., 1992; Melan and Sluder, 1992) and give misleading impressions of structural continuity (Williamson, 1991). To deal with these problems, it is necessary to use a model in which the organization of the major cytoskeletal elements is known from IF-independent means and then to develop IF protocols which faithfully reveal that organization. Hyphae of the oomycete *Saprolegnia ferax* are a rare example of such a model among tip-growing cells. They contain delicate and complex F-actin arrays which have been described in detail using rhodamine-labeled phalloidin (RP) (Jackson and Heath, 1990a,1993; Heath, 1987,1988). Although RP is not without its uncertainties (Haarer et al., 1990; Tang et al., 1989), it is the only current alternative to IF that avoids the known uncertainties of EM for F-actin preservation. Similarly, the microtubule complement of the hyphae has been described in quantitative detail using freeze-substitution (FS) EM (Heath and Kaminskyj, 1989), the best currently available technology for high-resolution analysis of microtubules. Therefore, we have a single cell type in which the organization of the two major cytoskeletal components is known in detail and in which we can evaluate IF protocols for their labeling fidelity. This report

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describes such protocols, shows their limitations, and indicates previously unknown functional properties of the cytoskeleton in these cells.

Materials and Methods

Cultures. *Saprolegnia ferax* (Gruith.) Thuret (ATCC #36051) was grown in a medium designated OM (Gay et al., 1971), and transferred and anchored to coverslips as described in Kaminskyj et al. (1992). To protect against damage during IF preparations, the colony was enmeshed in fibrin (Forer, 1972) with 5 μ l fibrinogen and 1 μ l thrombin, so that hyphal movement was reduced during solution changes.

Buffers. Solutions were prepared in (a) 60 mM PIPES (piperazine-*N,N'*-bis-[2-ethanesulfonic acid]), Na salt (Calbiochem; San Diego, CA) pH 7, alone, or (b) 60 mM PIPES, pH 7, containing 2 mM EGTA [ethylene glycol-bis-[β -amino ethyl ether] *N,N'*-tetra acetic acid (Sigma; St Louis, MO)], 2 mM MgCl₂, 10 μ g/ml aprotinin (Boehringer Mannheim; Pointe Claire-Dorval, PQ, Canada), 1 mM phenylmethylsulfonylfluoride (Eastman Kodak; Rochester, NY), 137 mM NaCl, and 268 μ M KCl, referred to as "saline." For RP staining after aldehyde fixation, both a and b gave qualitatively similar results. Otherwise, better results were achieved with saline.

Fixation. Living colonies were fixed or treated on coverslips with 1 ml of solution (Kaminskyj et al., 1992). We compared aldehyde fixation, MBS (m-maleimidobenzoyl *N*-hydroxy succinimide ester) (Molecular Probes; Eugene OR) cross-linking, FS, and cold acetone or methanol fixation, as primary stabilization methods.

Aldehyde fixation was for 5–30 min in 2–12% formaldehyde (freshly prepared from paraformaldehyde) (JBS; Pointe Claire-Dorval, PQ, Canada) in PIPES, or 4% formaldehyde plus 1% glutaraldehyde in PIPES.

MBS cross-linking followed the method of Sonobe and Shibaoka (1989). Living hyphae were treated with 100 μ M to saturated (~500 μ M) MBS, 0.1–1% DMSO (dimethyl sulfoxide) (Fisher; Fairlawn, NJ), and 0.05% Triton (Triton-X100) (JT Baker; Phillipsburg, NJ) in PIPES or saline. Staining followed directly, after subsequent fixation in 6% formaldehyde in PIPES, or after fixation/extraction in 15% acetone prepared in 6% formaldehyde in PIPES or saline. Alternatively, 100 μ M MBS and 0.1% DMSO were added to 6% formaldehyde in PIPES or saline or to 4% formaldehyde plus 1% glutaraldehyde in saline, and used as primary fixative for 5–30 min.

For FS, colonies growing on dialysis tubing were plunge-frozen in liquid propane at -185°C , and substituted in anhydrous acetone, in acetone containing 3% formaldehyde, or in acetone containing 2% formaldehyde plus 1% glutaraldehyde (from 37% and 70% aqueous stocks, respectively). Colonies were rehydrated in 4% formaldehyde in saline and transferred to coverslips.

Cold solvent fixation of colonies growing on coverslips used immersion in -20°C acetone or methanol for 20 min, followed by rehydration in either 6% formaldehyde in saline or in saline alone.

Staining. Permeabilization treatments were 1 ml of (a) 0.01–0.2% Triton in PIPES or saline for 2–20 min, (b) 10–35% acetone prepared in 6% formaldehyde in PIPES or saline for 2–25 min, or (c) 100% acetone or ethanol at -20°C for 1–20 min. Dual labeling by RP and actin IF of cortical filamentous arrays required treatment b. Formaldehyde/MBS-fixed hyphae required higher concentrations of acetone during extraction for dual labeling of cortical filamentous arrays than did formaldehyde-fixed ones.

Hyphal walls were degraded for 5 min in 50 μ l of 10 mg/ml Driselase (Kyowa Hakko Kogyo; Tokyo Japan) in MES (2-[*N*-morpholino] ethanesulfonic acid [Sigma]) buffer, pH 5.8, containing 10 mg/ml bovine serum albumin (BSA) and 50 μ g/ml leupeptin (Sigma) (Temperli et al., 1990). Non-specific binding was blocked with 5% (v/v) egg albumin plus 2.5% (w/v) skim milk powder (Carnation; Toronto, ON, Canada) for 20 min.

Antibody labeling employed 20 μ l of (a) N350 monoclonal mouse anti-chicken gizzard actin IgM (Amersham; Chicago, IL) diluted 1:100–1:250, (b) polyclonal rabbit anti-chicken back muscle actin (ICN; Lisle, IL) diluted 1:100, or (c) monoclonal mouse anti-chick brain α -tubulin IgG, diluted 1:5000–1:10,000 (Cedarlane; Hornby, ON, Canada). Antibodies were diluted in saline containing 1% (v/v) egg albumin and 0.5% (w/v) skim milk powder. Primary incubations were for 45–60 min in the dark at room temperature. RP staining before the primary antibody or after the rinses following the secondary antibody, both at 5 μ g/ml, gave similar results to the simultaneous staining. After incubation in primary antibody, colonies were rinsed several times in saline and then incubated in FITC-conjugated sheep anti-mouse Ig or donkey anti-rabbit Ig (Amersham), diluted 1:10, for 45–60 min in the dark. Finally, colonies were rinsed again several times in saline and mounted in Citifluor (Marivac; Halifax, NS, Canada).

RP staining followed the procedure of Heath (1987). Fixed colonies were stained for 5–10 min in 5 μ l of 5 μ g/ml RP in PIPES or saline in the dark, rinsed, and mounted in Citifluor. After antibody labeling, nuclei were stained with mithramycin (Pfizer; Pointe Claire-Dorval, PQ, Canada) (Heath, 1980). Hyphae were incubated for 5 min in 10 μ l of 100 μ g/ml mithramycin in PIPES buffer containing 15 mM MgCl₂, rinsed in buffer without MgCl₂, and mounted in Citifluor.

Microscopy. Hyphae were examined with a $\times 100$, NA 1.32 objective and differential interference contrast (DIC) optics. Epifluorescence filter sets for rhodamine (G2), fluorescein [B1, modified by replacing the barrier filter with 515–545-nm bandpass filter (Omega Optical; Brattleboro VT) to reduce rhodamine interference] and mithramycin (V2) were supplied by Leica (Willowdale, ON, Canada). Some RP emission occurred between 515–545 nm (Haugland, 1992) and was detectable with the FITC filter. However, the areas of congruence between RP and FITC images were not attributable to this bleed-through because the RP-stained actin cap was not discernible in the apex of a tubulin IF dual stained hypha (Figures 12a, 12b, 13a, 13b, 14a, and 14b), and with actin dual labeling, many regions of strong RP staining did not show in the FITC images (e.g., Figures 10 and 11). Images were recorded on 35-mm film or enhanced with a Hamamatsu DVS-3000 image analyzer (Photonic Microscopy; Oak Brook, IL), recorded on videotape, and printed with a video printer.

Hyphae were also examined with a $\times 60$ NA 1.40 phase-contrast objective and confocal laser scanning microscopy (CLSM), using a MRC600 scanner (BioRad; Mississauga, ON, Canada), krypton-argon laser, K1 (FITC) and K2 (rhodamine) filter sets, and COMOS software. Images were recorded on optical disk and printed with a video printer.

Electrophoresis and Immunoblotting. Colonies were grown on dialysis tubing on solid OM. Hyphae were collected and frozen in liquid nitrogen (~10 sec total), ground in a mortar at -80°C , lyophilized, and stored at -80°C until use. For electrophoresis, samples were re-suspended in 100 μ C SDS-sample buffer (10 μ l sample buffer/mg lyophilized *Saprolegnia*), boiled for 3 min, and chilled in ice-water. Sample buffer contained 2.2% SDS (Aldrich, Milwaukee, WI), 55 mM dithiothreitol (BRL; Gaithersburg, MD), and 69 mM Tris, pH 6.8 (Fisher). Samples were centrifuged at 20,000 \times g for 20 min at 4°C . The supernatant contained 6 μ g protein/ μ l (Lowry et al., 1971) using BSA as a standard. Glycerol (10% v/v) and bromophenol blue (0.001% w/v; BDH) were added to samples before electrophoresis. Proteins (80–160 μ g/lane) were separated by SDS-PAGE (Laemmli, 1970), transferred to nitrocellulose (Towbin et al., 1979), and stained with 0.2% Ponceau S (Allied Chemical; Morristown, NJ) in 3 mM trichloroacetic acid (Fisher) to visualize the lanes. Blocking was in TBS-T [10 mM Tris, pH 7.5, containing 150 mM NaCl and 0.5% Tween 20 (Fisher)] containing 5% milk powder, for 45 min at 23°C , during which the stain was eluted.

Primary antisera were diluted 1:1000 (N350 anti-actin), 1:500 (tubulin), or 1:100 (ICN anti-actin) in TBS containing 0.5% milk powder; incubations were 1–2 hr at 23°C in a moist box on a rotary table. Washes were

in TBS-T. Appropriate peroxidase-conjugated secondary antibodies were used at 1:200; incubations were 1–2 hr. Washes after peroxidase-conjugated antibodies were in TBS without Tween. Peroxidase was developed with H₂O₂ and 4-chloro-1-naphthol (Sigma).

Results

F-actin Staining

We evaluated 76 protocols for their effect on RP or actin IF staining. Of these, Table 1 summarizes the 19 most informative treatments: those illustrated, those giving RP/actin IF dual staining, and those distinguishing features of tubulin IF. Treatments are grouped into 13 major types, with letter designations for reference in the text.

Growing *Saprolegnia* hyphae contain four cortical populations of F-actin (Figure 1): an apical cap of fibrils which often stained so intensely that the individual fibrils were not resolvable, a transition zone of fibrils in the tapering, near-apical region which were readily resolvable, and subapical cables with interspersed plaques (Figure 1) plus diffuse F-actin, which permeates the central cytoplasm (Jackson and Heath, 1990b,1993). This central F-actin was seldom easily characterized and therefore has not been included in our analysis. The actin cap was previously (Heath, 1987,1988; Jackson and Heath, 1990a) defined as including all the cortical arrays in the apex, above the most-apical plaque. However, the cap and transi-

tion zone are affected differently by certain treatments (Table 1: C, D, H, K, M) and therefore are described separately.

Initially we used the simpler RP staining to evaluate treatments (Table 1). The previously used 4% formaldehyde fixative (Heath, 1987) was increased to 6% to compensate for dilution by the carryover of medium on the coverslips compared with dialysis membrane cultures. The addition of 100 μ M MBS and the use of saline rather than PIPES improved this fixation by increasing the stability of the staining to at least 20 days while showing the same patterns (Figure 1). Diverse detergent and solvent treatments, previously indicated in the literature to be necessary for IF and applied either during or after fixation, generally reduced the quality of RP staining, and dual labeling with actin-IF generally further degraded the actin images. Nevertheless, treatments F (Figures 6–11) and H revealed all F-actin populations with both RP and IF staining. Other protocols were selective for only some of the populations. Even the best IF staining was inferior to RP, since cortical filamentous populations were less well preserved and there was considerable variation in the intensity of the cortical staining, both along single hyphae and between neighboring hyphae. In contrast, cortical RP patterns were highly reproducible. Variations in actin IF intensity were seen after protocols that did not bind RP, such as treatment I, so modifications for dual staining were unlikely to be the cause of variation in actin IF intensity in the dual stained preparations.

Rapid freezing/freezing-substitution, shown to be a superior fix-

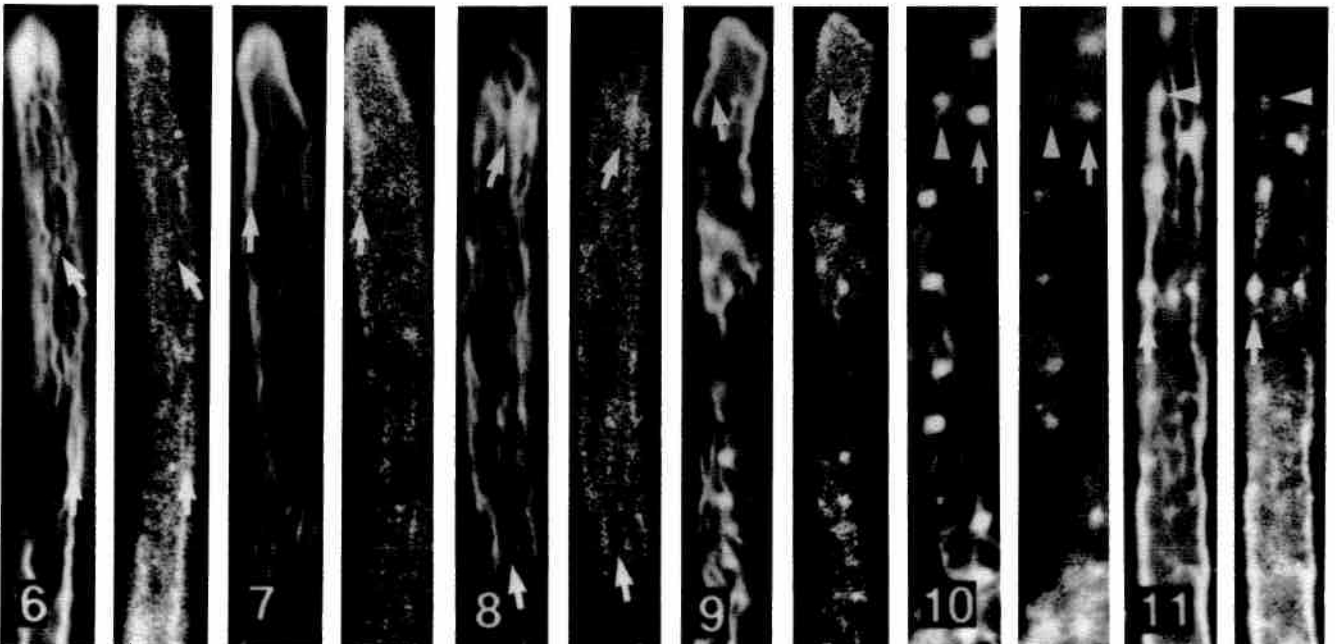
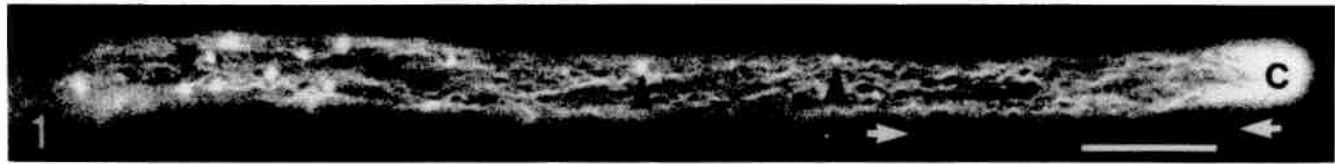
Table 1. Effect of preparation on RP^a, actin IF, and tubulin IF staining^b

Treatment	Actin populations									Microtubules		
	Caps		Transition		Cables		Plaques		Cytopl	Spindle	Figure	
	RP	RP/IF	RP	RP/IF	RP	RP/IF	RP	RP/IF				
A. Form6 + MBS100 + Sal	3	-/-	3	-/-	3	-/-	3	-/-	-	-	1	
B. TX0.05 + PIPES → Form6 + PIPES	1	1/1	1	1/1	1	1/0	0	0/0	Absent	Present	-	
C. MBS100 + TX(0.05,0.2) + Sal	2	-/-	3	-/-	3	-/-	1	-/-	-	-	2,3	
D. MBS100 + TX0.05 + Sal → Ace15 + Form6 + PIPES	-	2/?	-	1/1	-	1/1	-	1/1	- ^c	- ^c	-	
E. MBS100 + TX0.05 + Sal → Form(4,6) + PIPES	3	2/1	3	2/1	3	2/1	1	0/0	-	-	-	
F. MBS100 + TX0.05 + Sal → Ace15 + Form6 + Sal	-	2/1	-	2/2	-	2/1	-	2/2	- ^c	- ^c	6–11	
G. MBS500 + TX0.05 + Sal	3	-/-	3	-/-	2	-/-	2	-/-	-	-	4	
H. MBS500 + TX0.05 + Sal → Ace15 + Form6 + Sal	-	2/1	-	3/1	-	3/1	-	2/1	- ^c	- ^c	-	
I. Freeze-substitution	0	0/?	0	0/?	0	0/1	0	0/1	- ^c	- ^c	5	
J. Form6 + PIPES → TX(0.01, 0.05,0.1) + PIPES	2	1/0	2	1/0	2	1/0	1	0/0	Present	Present	12,16–18	
K. Form6 + PIPES → Ace15 + Form6 + PIPES → TX0.05 + Sal	-	2/1	-	1/1	-	2/0	-	2/2	Absent	Absent	-	
L. Form4 + Glut1 + PIPES → TX (0.01,0.05,0.1) + PIPES	3	1/0	2	0/0	2	1/0	2	1/0	Present	Present	15	
M. Form4 + Glut1 + MBS100 + Sal → TX0.05 + Sal	-	3/0	-	2/0	-	2/0	-	3/0	Present	Present	13,14	

^a Actin staining scored on reproducibility, detail, and intensity: 0, absent; 1, poor; 2, adequate; 3, good; ?, unable to assess. Microtubules had no treatment-related variation; scored as present or absent. For both: -, not determined. Treatments were +, combined; →, consecutive. Bracketed treatments gave similar results. Numbers are percentages except for MBS, which is in μ M.

^b Abbreviations: Ace, acetone; cytopl, cytoplasmic; Form, formaldehyde; Glut, glutaraldehyde; IF, immunofluorescence; RP, rhodamine-phalloidin; Sal, saline; TX, Triton X-100.

^c All tested treatments (some not listed) employing solvents prevented tubulin IF; therefore, these specific treatments were not tested for this feature.



ation protocol for cell preservation at the EM level (Hoch, 1991), failed to give any RP staining patterns and only poorly preserved plaques and cables after actin IF (Figure 5; Table 1: I). Cold solvent treatment, commonly used in IF protocols (Bullock and Petrusz, 1982) with or without post-fixation in formaldehyde, did not reveal any cortical F-actin populations; only faint, central actin IF staining was seen.

Mono- (all illustrations) and polyclonal anti-actins gave equally bright and indistinguishable IF patterns, which is somewhat surprising since, on immunoblots, the monoclonal antibody showed the expected single band at ~42 KD, whereas the polyclonal anti-actin recognized additional higher-weight bands (data not shown).

Tubulin Staining

The distribution of microtubules was examined with tubulin IF and compared with the pattern known from FS EM (Heath and Kaminsky, 1989) to determine if the IF protocols that worked for actin were also acceptable for microtubules. We did not find any protocol that gave IF staining of all actin populations as well as microtubules (Table 1). However, hyphae could be dual labeled with RP and tubulin IF (Figures 12–14; Table 1: J, L, M), then showing the expected cortical RP patterns, cytoplasmic microtubules, and mitotic spindles. Cytoplasmic microtubules formed anastomosing cables of various lengths and thicknesses (Figures 12–16). Cables showed no treatment-related variation in appearance.

Cables sometimes appeared to connect nuclei (Figures 15 and 16). Cables were rare ahead of the most apical nucleus (range 0–3, 33 hyphae) and abundant thereafter (3–9 per optical section, 43 hyphae). This increase in the population of microtubules behind the first nucleus is comparable to that seen in FSEM-prepared hyphae (Heath and Kaminsky, 1989). The nucleus population was displaced subapically (from about 13 μm in FS to 21–24 μm in the formaldehyde or formaldehyde/glutaraldehyde fixatives employed for tubulin IF) (Kaminsky et al., 1992), and the associated cytoplasmic microtubules were also displaced, as seen in Figure 17.

Mitotic spindles were short (Figures 15, 16, and 18) or, less frequently, extended (Figure 17). Fluorescence intensity profiles along short spindles showed their bipolar organization (Figure 18). Prophase kinetochore microtubule arrays (Heath et al., 1984; Heath and Rethoret, 1981) were only rarely detected (Figure 17).

Unlike actin IF, all tubulin IF was abolished by acetone treat-

ments (Table 1: K). Furthermore, cytoplasmic microtubule staining was more labile than that of the spindles because pre-fixation with Triton abolished the former but not the latter (Table 1: B).

Immunoblotting with the tubulin antibody failed to reveal any positive bands, even when used at a 200-fold excess to that used for IF (data not shown).

Discussion

We have developed actin IF protocols that reveal patterns of F-actin similar to those previously observed with RP in these hyphae (Heath, 1990b). All cortical F-actin populations could be dual stained with RP and actin IF, showing that neither probe is restricted to a unique subpopulation. Therefore, actin IF has neither revealed any new populations of F-actin nor failed to reveal any previously known populations. We conclude that the previously described RP-staining populations are likely to represent the total F-actin complement of the hyphae of *Saprolegnia*. There are reports to the contrary in other systems, i.e., that RP is sometimes a less faithful indicator of actin distribution than actin-IF (Haarer et al., 1990; Tang et al., 1989), but those studies used different protocols for RP and actin IF localization; in *Saprolegnia*, staining affinity of the F-actin arrays is related to treatment (Table 1).

The conclusion that previous studies have faithfully revealed the F-actin complement in *Saprolegnia* is especially important for the peripheral plaques, which are easily induced or labile. For example, the concentration of MBS (Table 1: C vs G) in the MBS protocol, or the use of saline rather than PIPES, determined in their presence, appearance, or abundance. Because we have preserved plaques with diverse actin IF protocols, including FS, as well as with RP, it does seem most likely that they are real, even if their precise cellular equivalents remain unclear (reviewed in Heath and Harold, 1992; Heath, 1990b; see also Harold and Harold, 1992; Bourett and Howard, 1991 for recent observations on this topic). However, we cannot rule out some artifactual concentration, even in FS, during the substitution phase. Similarly, because the RP patterns are all seen in fixed material, we cannot be sure that they present a true in vivo organization of F-actin. However, recent studies on RP-stained living hyphae and the effect of fixation on RP patterns suggest that they are indeed real in vivo arrays (Jackson and Heath, 1993).

Figures 1–11. RP- and actin IF-stained actin patterns in *Saprolegnia* hyphae. Original magnification $\times 1700$. Bar = 10 μm .

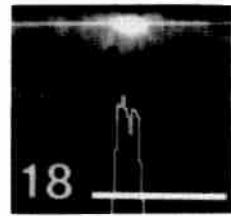
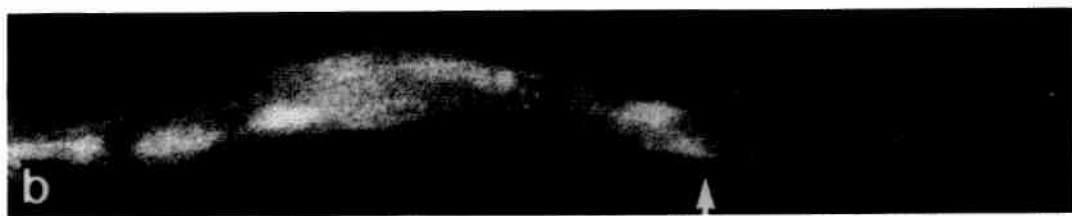
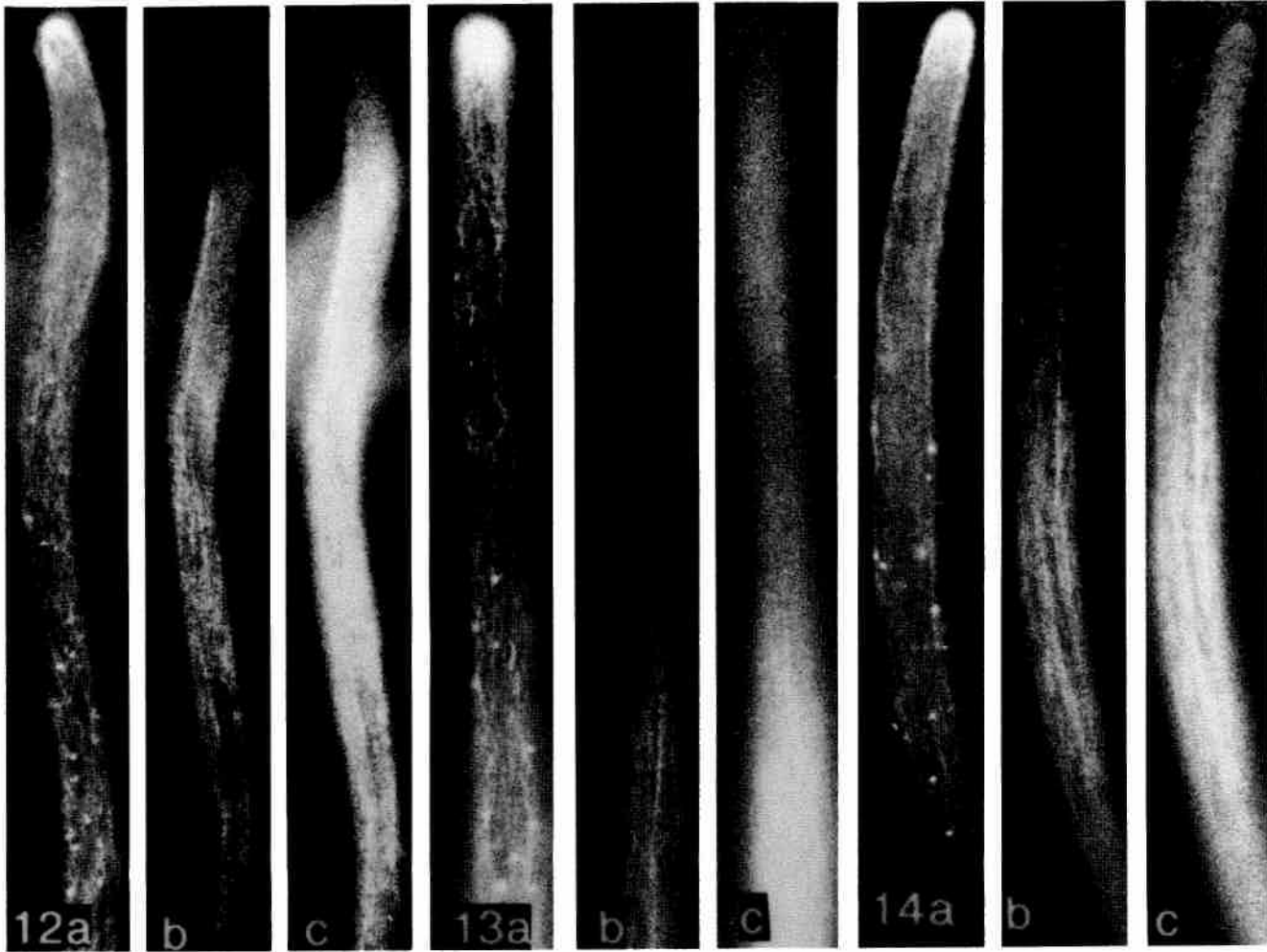
Figure 1. Cortical RP-staining populations in a *Saprolegnia* hyphal tip after treatment A, showing the cap (c), transition zone (between the white arrows), cables (arrows), and plaques (arrowheads).

Figures 2 and 3. RP staining after treatment C. Plaques were seldom seen with this protocol, especially in the first 100 μm , but when present were similar to those seen in Figure 1. Figure 2. Hyphal tip showing the cap, transition zone and cables. Figure 3. Subapical region showing cables and plaques.

Figure 4. RP staining after treatment G. Plaques were generally coarser than treatment C, but the length of the transition zone was similar (cf. Figure 1).

Figure 5. Actin IF staining after treatment I showing cables (arrows) and plaques (arrowheads) in a subapical region.

Figures 6–11. Pairs of CLSM images of dual-stained cortical actin arrays after treatment F, using RP (left) and actin IF (right). The shallow depth of focus possible with CLSM gives the misleading impression that these hyphae are narrower than those in Figures 1–5, since only a very thin, peripheral optical section is shown, without out-of-focus flare; cables and plaques are similar in size to those in Figures 1–5. The actin IF images are grainier than the RP in each pair, probably due both to larger IF probe size (~225 nm) and the higher confocal scanner gain necessitated by the less bright images. Examples of dual staining are indicated by pairs of arrows. RP staining without associated actin IF is indicated by arrowheads in Figures 10 and 11.



From a technical perspective, comparison of the actin IF and RP results suggests that, in general, RP is the technique of choice for studies of F-actin in these cells. The patterns are finer and sharper, there is less interhyphal variability with RP than with actin IF, and the processing protocols are simpler, shorter, and cheaper. Furthermore, only with RP can we obtain simultaneous labeling of all known F-actin and microtubule populations. Our development of protocols that stabilize staining for simultaneous visualization of actin and microtubules permits analysis of their probable interactions and their associated ABPs and MAPs in this system.

It is salutary to note that many of the RP and actin IF protocols tested failed to preserve or reveal some of the known F-actin arrays, even when the differences were apparently minor compared with superior protocols. These variations were derived from procedures used in the literature, indicating the need for caution in concluding that all functionally significant actin arrays are necessarily revealed by single preparation method.

Although our best IF protocols are adequate for the known F-actin localizations, there were significant disparities between FSEM and IF microtubule patterns. For example, cytoplasmic microtubules appeared as cables with IF, which contrasts with the short and unlinked arrays seen with FSEM (Heath and Kaminskyj, 1989). These cables were also displaced subapically with their associated nuclei. Furthermore, the abundant prophase kinetochore microtubule arrays (Heath et al., 1984; Heath and Rethoret, 1981) were seldom detected. These prophase microtubule arrays are present for 80% of the nuclear cycle (Heath and Rethoret, 1981) but were observed much less frequently than mitotic spindles. Conversely, mitotic spindles occurred at the expected frequency. In the apical 75 μm of hyphae observed with IF, there were 2.2 ± 0.2 ($n = 26$) spindles, compared with 9.2 ± 0.6 ($n = 33$) nuclei previously recorded (Kaminskyj et al., 1992). Mitotic spindles occupy 20% of the nuclear cycle (Heath and Rethoret, 1981), thus predicting 20% of the $9.2 = 1.8$ spindles per tip, a number not significantly different ($p > 0.05$, t -test) from that observed. This differential detection of spindles versus prophase microtubules is consistent with the resistance of spindles to Triton pre-treatment, a process that also failed to reveal the cytoplasmic microtubules (Table 1: B). We conclude that tubulin IF does not faithfully describe all of the microtubule arrays. This suggests that the positional stability of the cortical F-actin system is greater than that of the microtubule system, probably from its presumed association with the cell membrane (Heath, 1990b). The separation of the F-actin system from the microtubule system implied in their differential preservation

features mirrors their functional separability deduced from experimental manipulations in *Basidiobolus* hyphae (McKerracher and Heath, 1986).

From a functional perspective, the treatments summarized in Table 1 produce population-selective differences in F-actin staining, showing that the different populations have different properties. It is unlikely that these differences are due to multiple actin isoforms. The oomycetes *Phytophthora megasperma* (Dudler, 1990) and *Achlya* (Bhattacharya et al., 1992), and so probably *Saprolegnia*, have single-copy actin genes lacking introns. However, *Phytophthora infestans*, unique among reports for oomycetes and fungi, has a second actin gene (Unkles et al., 1991), which leaves open the possibility of different isoforms. Variations are also unlikely to be due to cell environment because all populations are adjacent, and probably interconnected, in a common cytoplasm. The differential staining responses may relate to selective removal of ABPs. ABPs are likely to influence RP vs anti-actin staining in different ways: RP binds to specific amino acid residues in a conserved region of actin, probably on adjacent monomers (Vandekerckhove et al., 1985); the N350 epitope is likely to be elsewhere, because it binds to actin monomers on immunoblots as well as the polymer with IF.

Our results also indicate previously unsuspected information on the functional organization of the cytoplasmic microtubules. The cables shown by tubulin IF are likely due to visual overlap of separate, antibody-decorated microtubules (Williamson, 1991; Seagull, 1986), formaldehyde-induced cytoplasmic translocations, and hyphal shrinkage (Kaminskyj et al., 1992). However, they also appear to interconnect nuclei, with which they are subapically translocated, implying that dispersed cytoplasmic microtubules (Heath and Kaminskyj, 1989) may be functionally interconnected and mediate nucleus spacing, also suggested for *Phytophthora* (Hyde, 1991). Consistent with this, normal spacing is disrupted by the microtubule inhibitor nocodazole (data not shown).

We conclude that the organization of microtubules is not fully and accurately revealed by any IF protocol we have tested; RP and actin IF both recognize all F-actin populations but the former gives superior results. In addition, the absence of tubulin IF (at least in *Saprolegnia* with DM1A antibody) after solvent treatments that were needed for actin IF of some populations shows that the procedures must be optimized for specific protein/antibody combinations, a point that may limit dual-labeling studies. The absence of positive staining in our tubulin immunoblots shows that immunoblotting may not be a reliable predictor of IF results. These observations

Figures 12–18. Tubulin IF-stained MTs, RP-stained actin, and mithramycin-stained nuclei in *Saprolegnia* hyphae. Original magnification $\times 1700$. Bar = 10 μm .

Figures 12–14. (a) RP and (b,c) tubulin IF patterns after treatments J (Figure 12) and M (Figures 13 and 14). RP patterns extended into the extreme apex of the hypha, but tubulin IF patterns were subapically displaced compared to FSEM by greater (Figure 13) or lesser (Figures 12 and 14) degrees. To show that the lack of tubulin IF cables in the apex was not from underexposure, the tubulin IF patterns (b) are shown at a longer exposure to reveal the hyphal apex (c).

Figure 15. Tubulin IF and nuclei after treatment L, showing their relationship. (a) Tubulin IF; (b) mithramycin-stained nuclei. Cytoplasmic microtubules, which appear to interconnect nuclei, are more abundant in the nucleus-containing region. Arrow indicates the most-apical nucleus in b, with the corresponding microtubule pattern in a.

Figures 16–18. Tubulin IF patterns after treatment J. Figure 16. Short ($\sim 3 \mu\text{m}$) spindles (arrows) with associated cytoplasmic microtubules. Spindles are coincident with the position of nuclei in the corresponding DIC image, not shown. Figure 17. Extended ($\sim 6 \mu\text{m}$) spindle and possible prophase kinetochore microtubule array (arrow). Figure 18. Fluorescence intensity profile generated by the image analysis system, along the axis (line shown) of a short spindle demonstrates its bipolar organization.

show the need to exercise care in drawing conclusions from IF protocols alone and to base analyses on more than one preparative method.

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