chapter twenty four

Actin fluorescent staining in the filamentous brown alga Ectocarpus siliculosus

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24.1 Introduction

Brown algae (Phaeophyceae) are a very interesting group of macroalgae that acquired a complex multicellularity independently of land plants or animals (Bogaert et al. 2013). In the past 10 years, genetic and molecular

studies of fundamental processes governing brown algal development have benefited from the selection of *Ectocarpus siliculosus* as a new model species for this group, because of its very simple filamentous body plan and of the easiness of its cultivation (Peters et al. 2004; Charrier et al. 2008; Le Bail and Charrier 2013).

A major component involved in brown algal morphogenesis is the cytoskeleton, consisting of microtubules (MTs) and actin filaments (AFs). The role of the cytoskeleton in the brown algal morphogenesis has repeatedly been underlined in several studies, particularly after the development of immunofluorescence techniques (Katsaros et al. 2006, for literature).

Contrary to higher plants, brown algae bear a unique and complex cell wall, which caused problems in the localization of cytoskeleton, when protocols described for plant cells were followed. Therefore, it became necessary to develop modified protocols suitable for brown algae (Coelho et al. 2012a).

As in land plants (Viridiplantae), fungi and animals (Opistokonts), AFs are thought to play a very important role in cell and tissue development in brown algae. Previous works showed their importance in the polarization processes in fucoid zygotes (Hable et al. 2003; Bisgrove and Kropf 2004; Hable and Kropf 2005; Bogaert et al. 2013), branching in filamentous gametophytes (Varvarigos et al. 2004), cytokinesis (Karyophyllis et al. 2000a; Bisgrove and Kropf 2004; Nagasato et al. 2010), and growth and morphogenesis of tip-growing apical cell of *Sphacelaria rigidula* (Karyophyllis et al. 2000b; Katsaros et al. 2002, 2003, 2006).

In some of these studies, the fine structure of AF networks was determined by immunolabeling. AF networks can display various organizations, and to understand their role in cellular processes such as polarization, intracellular trafficking or growth, knowledge of its 3D organization within the cell is required (Chebli et al. 2013).

When the transformation is possible, an actin-binding protein probe labeled with GFP can be used to reveal the actin organization in living cells (Wilsen et al. 2006; Meijer et al. 2014), but this technique is not available in brown algae yet. Immunolocalization on fixed material represents a good alternative to GFP tagging, with the additional advantage of revealing the majority of the AF structures present in a cell in a single experiment, contrary to *in vivo* GFP-tagged probe that can only recognize some specific AF configurations (Wilsen et al. 2006).

As AFs are very delicate structures, moreover sensitive to the fixation procedure (Katsaros et al. 2006), a modified protocol has been developed for brown algal cells. The detailed steps of this alternative protocol using Rhodamine–Phalloidin labeling are described in this chapter. It has proven to be successful in *Sphacelaria* and *Dictyota*, but the version given has been adapted for *Ectocarpus siliculosus*, the model brown algal

species for which protocols for immunolabeling of cytoplasmic proteins has already been established (Coelho et al. 2012a, b).

24.2 State of the art

Actin fluorescent staining has been tried in all the major plant lineages, including land plants (Traas et al. 1987; Kakimoto and Shibaoka 1987a; Sonobe and Shibaoka 1989; Doris and Steer 1996; Lovy-Wheeler et al. 2005), fungi (Heath et al. 2000), microalgae (Harper et al. 1992; Pflügl-Haill et al. 2000; Hawkins et al. 2003), and macroalgae (Garbary et al. 1992; Katsaros et al. 2006). A fluorescent probe conjugated to phalloidin, a toxic bicyclic peptide first extracted from the fungus *Amanita phalloides* (Barden et al. 1987), is used to label AFs, although several anti-actin antibodies are also used.

Most of AF staining techniques on plant cells were first established for terrestrial plants (Perdue and Parthasarathy 1985; Kakimoto and Shibaoka 1987a, b; Parthasarathy 1987; Traas et al. 1987; Sonobe and Shibaoka 1989; Heslop-Harrison and Heslop-Harrison 1991; Doris and Steer 1996; Wasteneys et al. 1997; Vitha et al. 2000; Lovy-Wheeler et al. 2005; Dyachok et al. 2010). One of the major inputs from these works on land plant immunocytochemistry was the use of *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (commonly abbreviated as MBS) as an actin-stabilizing reagent added before the fixation steps (Sonobe and Shibaoka 1989; Doris and Steer 1996). Another finding was the necessity to partially digest the cell wall (CW) to allow the fluorescent probe to get access to the cytoplasm. In this case, the enzymes used were cellulases and pectolyases (Lloyd et al. 1979; Kakimoto and Shibaoka 1987b; Sonobe and Shibaoka 1989; Vitha et al. 2000; Szechyńska-Hebda et al. 2006; Dyachok et al. 2010).

In green macroalgae, very few studies of AF organization were conducted, except in the Characean algae (*Chara*, *Nitella*) and some coenocytic algae such as *Acetabularia* and *Caulerpa*. As in land plants, the fixation procedure was commonly carried out with classical aldehyde compounds, but sometimes it could be omitted, and AFs were labeled directly *in vivo* (Tewinkel et al. 1989; Sampson and Pickett-Heaps 2001). Curiously, the CW digestion step was omitted in most studies. In some cases, simple cell permeabilization appeared sufficient (Tewinkel et al. 1989; Braun and Wasteneys 1998; Sampson and Pickett-Heaps 2001), whereas in others, methods to get through the CW barrier were used (e.g., freeze-chattering of rhizoid and protonemata of characean algae; Braun and Wasteneys 1998). Moreover, in the giant coenocytic green alga *Acetabularia*, the CW showed to be insensitive to enzymatic treatments (Sawitzky et al. 1996), and for this species and other giant coenocytic algae, AF staining was conducted on microdissected cells (Menzel 1987) or cytoplasm extruded from its cell wall (La Claire II 1989; Mine et al. 2001).

AF staining procedure was also developed for red macroalgae in the 1990s (McDonald et al. 1993). Red macroalgae possess a complex CW (Garbary et al. 1992; Popper et al. 2011) and so several enzyme cocktails have been used, including cellulase alone (Reis et al. 2013), a mix of cellulase and snail gut extract (McDonald et al. 1993), or β-glucuronidase (Garbary et al. 1992; Garbary and McDonald 1996). However, the CW digestion step had to be adapted to each species (McDonald et al. 1993), sometimes leading to damaged cell ultrastructures, which raises some doubts about the observed AF organization. As in green macroalgae, some studies reported successful AF labeling in red macroalgae without CW digestion steps (Kim and Kim 1999; Kim et al. 2001; Wilson et al. 2003).

On brown macroalgae (*Phaeophyceae*), few AF staining studies have been conducted until now, and most of them were only focused on fucoid zygotes during polarization and germination (Brawley and Robinson 1985; Kropf et al. 1989; Alessa and Kropf 1999; Hable et al. 2003). In the earliest studies, protocols did not include any CW digestion step, but only cell permeabilization using compounds such as saponin or Triton X-100 after fixation (Brawley and Robinson 1985; Kropf et al. 1989; Alessa and Kropf 1999). However, fucoid zygotes are free naked cells, directly in contact with the external medium, in which the cell wall is just beginning to form (Bisgrove and Kropf 2001). Therefore, this simple procedure would not be as effective for other cell types, especially for cells encased in a tissue. Moreover, *Phaeophyceae* have a very complex cell wall, the composition of which strongly differs from that of land plants or other macroalgae. As mentioned in the introduction, land plants and brown algae differ strongly in their CW composition and structure, cellulose, hemicellulose, arabinogalactan, and callose being the only polymers that the two groups have in common (Popper et al. 2011; Deniaud-Bouët et al. 2014; Hervé et al. 2016). The two major components of the brown algal cell wall are alginates and sulfated fucans that are specific to this class of macroalgae (Mabeau and Kloareg 1987; Popper et al. 2011). Brown algal cell wall also contains proteins, halogenated and sulfated phenolic molecules (named phlorotannins), and halides (Deniaud-Bouët et al. 2014). As a consequence, the digestion steps necessary to permeabilize the cell must be adapted to these particular molecules.

In 2000, Karyophyllis and colleagues adapted several protocols from previous studies on pollen tubes (Sonobe and Shibaoka 1989) and red algae (Garbary et al. 1992) to improve AF staining on the apical cells of *Sphacelaria rigidula* (Karyophyllis et al. 2000a, b). This protocol improved AF staining by introducing MBS as an AF-stabilizing agent before chemical fixation. In addition, CW is digested by a complex mixture of enzymes, including cellulase, β-glucuronidase, and sulfatase (both from abalone acetone powder), pectinase, hemicellulase (both from macerozyme

product, Onozuka R-10, Yakult), laminarase, and xylanase (both from driselase product, Sigma) (Karyophyllis et al. 2000a). Using Rhodamine–Phalloidin or anti-actin antibody to tag AFs and with some modifications, this new protocol allowed the visualization of the fine AF organization in several species and cell types of *Phaeophyceae*, including apical and other vegetative cells of *Sphacelaria rigidula* (Karyophyllis et al. 2000b), and *Dictyota dichotoma* (Katsaros et al. 2002), polarizing cells of *Macrocystis pyrifera* gametophytes (Varvarigos et al. 2004, 2007) and again in fucoid zygotes (Hable et al. 2003; Bisgrove and Kropf 2004). Recently, it has been successfully applied to *Ectocarpus* sporophyte cells, and the different steps are described in this chapter.

24.3 Materials

24.3.1 Chemicals

- DMSO 100%.
- Glycerol 100%.
- Cell-wall digesting enzymes:
    - Cellulase (Onozuka R-10, Yakult).
    - Hemicellulase (H2125, Sigma).
    - Driselase (D8037-1G, Sigma).
    - Macerozyme (Onozuka R-10, Yakult).
    - Pectinase (P2401, Sigma).
    - Alginate lyase (A1603, Sigma).
- *m*-maleimido benzoic acid *N*-hydroxy succinimide ester (MBS, M2786, Sigma).
- Natural or artificial seawater (ASW) (to wash free-floating filaments before prefixation. *Must be autoclaved and kept sterile, if possible*).
- Paraformaldehyde (PFA, powder).
- *p*-phenylenediamine (PDA).
- Poly-L-lysine (1 mg mL^{-1}).
- Rhodamine–Phalloidin (Sigma or Biotium) or AlexaFluor568–Phalloidin (in general, AlexaFluor568 is more suited for plant material than Rhodamine–Phalloidin).
- Triton X-100.

24.3.2 Solutions and recipes

24.3.2.1 Preparation of Rhodamine–Phalloidin (R415) or AlexaFluor568–Phalloidin (A12380, life technology)

Dilute the content of a whole tube (300 U) into 1.5 mL of methanol, following the manufacturer's directions. The final concentration is about 6.6 μM (=200 U mL^{-1}). Store at −20°C in the dark.

The instructions for the preparation of buffers and other solutions are indicated in Tables 24.1 through 24.10.

24.3.2.2 Preparation of buffers

Table 24.1 Microtubule stabilizing buffer (MTB) modified for actin

Composition	Final concentration	Quantity to add (for 1 L final)
Pipes	50 mM	15.12 g
EGTA	5 mM	1.318 g
$MgSO_4$	5 mM	1.902 g
KCl	25 mM	1.86 g
NaCl	4% (w/v)	40 g
PVP (Polyvinylpyrrolidone 25)	2.5% (w/v)	25 g
DTT ((−)-1,4-dithio-L-threitol)	1 mM	0.154 g
Pure water	–	qsp 1 L

Note: Adjust pH to 7.4. Store at 4°C.

Table 24.2 Phosphate-buffered saline (PBS)

Composition	Final concentration	Quantity to add (for 1 L final)
NaCl	137 mM	8.01 g
KCl	0.7 mM	0.052 g
Na_2HPO_4	5.1 mM	0.72 g
KH_2PO_4	1.7 mM	0.22 g
Distilled water	–	qsp 1 L

Note: Adjust pH to 7.4. Store at 4°C.

Table 24.3 Stock solution of MBS (*m*-maleimido benzoic acid *N*-hydroxy succinimide ester)

Composition	Final concentration	Quantity to add (for 1 mL final)
MT-buffer	–	980 µL
DMSO	2%	20 µL
MBS	100 mM	0.0943 g

Note: Store in the dark at −20°C. As the product is better preserved as a powder (at −20°C), prepare preferably a small volume of stock solution just before a series of experiments.

Table 24.4 Actin-stabilization solution

Composition	Final concentration	Quantity to add (for 1 mL final)
MTB	–	975 µL
Triton X-100	0.2%	2 µL
DMSO	2%	20 µL
MBS	300 µM	3 µL of a stock solution at 100 mM

Note: Prepare fresh solution just before use.

Table 24.5 Fixation solution

Composition	Final concentration	Quantity to add (for 10 mL final)
MT buffer	–	10 mL
PFA (Paraformaldehyde)	4%	0.4 g

Note: Distribute in 1 mL aliquots and store at −20°C.

Table 24.6 Cell wall lysis buffer

Composition	Final concentration	Quantity to add (for 2 mL final)
PBS:MTB 1:1	–	1946 µL
Triton X-100 (pure)	0.2% (v:v)	4 µL
Rhodamine – (or AlexaFluor568) – Phalloidin	0.17 µM	50 µL of stock solution at 6.6 µM
Cellulase	2% (w:v)	40 mg
Hemicellulase	2% (w:v)	40 mg
Driselase	1% (w:v)	20 mg
Macerozyme	1% (w:v)	20 mg
Pectinase	0.5% (w:v)	10 mg

Note: Just before use, prepare the lysis buffer (with PBS, MTB, Triton X-100, and Rh-Ph). Stir and dissolve the enzymes in it, and centrifuge for 5 min to eliminate the precipitate. Adjust the pH to 5.5 (this is important for the activity of enzymes). The given composition of the cell wall digestion solution is only indicative and must be adjusted for each type of material. We also recommend trying using alginate–lyases (concentration 1%–2% w:v), because alginate is a common cell wall compound in brown algae.

24.3.2.3 Other solutions

Table 24.7 Extraction solution

Composition	Final concentration	Quantity to add (for 1 mL final)
DMSO	5%	50 μL
Triton X-100	3%	30 μL
PBS	–	920 μL

Note: Prepare fresh solution just before use.

Table 24.8 Actin-staining solution

Composition	Final concentration	Quantity to add (for 300 μL final)
Rhodamine–Phalloidin	0.33 μM	15 μL of stock solution at 6.6 μM
PBS:MTB	–	285 μL

Note: Rhodamine–Phalloidin can be replaced by AlexaFluor568–Phalloidin (A12380, Life Technology), that is thought to give better results on plant cells. In both cases, prepare the staining solution just before use. Store on the ice and protect from light.

Table 24.9 DNA staining solution

Composition	Concentration	Quantity to add (for 10 mL final)
PBS	–	9.9 mL
Hoechst 33258 (94403, Sigma)	10 μg mL^{-1}	100 μL of a 1 mg mL^{-1} stock solution

Note: Store at 4°C.

Table 24.10 Mounting solution

Composition	Concentration	Quantity to add (for 1 mL final)
Glycerol	33.3%	800 μL
PBS	66.6%	400 μL
PDA (*p*-phenylenediamine, P6001, Sigma)	0.2% (w:v)	0.02 g

Note: Store at 4°C, in the dark. The solution gets darker with time.

24.3.3 Equipment

1.54 mL Eppendorf® tubes.
15- and 50 mL plastic tubes.
Fine forceps.
Glass coverslips (sterilized, if possible).
Microscope slides (sterilized, if possible).
Parafilm®.
Pipettes and tips (10/100/1000 µL range).
Plastic or glass Petri dishes.
Plastic Pasteur pipette.

24.4 Experimental procedures

24.4.1 Preparation of poly-L-lysine-coated coverslips

This is for free-floating filaments. They must be prepared at least one day before the staining experiment (*Note 1*).

1. Soak coverslips in nitric acid for at least several hours.
2. Wash the coverslips with distilled water, then with acetone, and again with water. Dispose them one-by-one on a paper towel, and let them dry until all drops of water have disappeared.
3. Using a cotton bud spread a solution of 1 mg mL^{-1} poly-L-lysine on the whole surface of coverslips.
4. Let the coverslips dry at room temperature (RT). The poly-L-lysine solution is thick and dries very slowly, so it is better to prepare the coverslips at least one day in advance. Drying step can be speeded up by putting the coverslips under a chemical hood.

24.4.2 Preparation of Ectocarpus *samples*

24.4.2.1 Filaments grown on coverslips

The preparation of *Ectocarpus* algal material is described in Chapter 23 by Rabillé et al.

1. On the bottom of the lid of an empty Petri dish, place a round piece of Whatman paper (that must cover all the Petri dish's bottom surface). Press a small piece of cotton wool on the rim of the lid. Add a small volume of water on the Whatman paper and the piece of cotton wool to have enough humidity. Remove any exceeding water. Finally, dispose a large piece of Parafilm® on the Whatman paper (*Note 2*).

2. Using forceps, takeoff the coverslips with the filaments grown on them from the bottom of the culture dish. Wipe the coverslips' bottom and edge with a paper towel.
3. Put the coverslips on the surface of the Parafilm® with the filaments upside. For all the subsequent steps, dispose a small volume of each solution on the surface of the coverslips and close the moist chamber for incubation.

24.4.2.2 Free-floating filaments

1. Gently crap or takeoff the filaments from the medium with forceps, and stack them in a pile. Quickly wash the pile of filaments by gently shaking it in clean sea water.
2. Put a dense pile of filaments into an Eppendorf tube. The following steps up to the enzyme digestion will take place in the tube. When changing the liquids by a pipette, be careful of not losing or damaging the filaments (*Note 3*).
3. After the cell wall lysis step, spread the filaments on the surface of poly-L-lysine-coated coverslips (or slides) with forceps. Separate every tuft of filaments from one another (*Note 4*).
4. Let some medium evaporate, keeping the coverslips damp. The rest of the protocol is then proceeded as for filaments that are directly grown on coverslips. Alternatively, the procedure can be continued in Eppendorf tube.

24.4.3 Actin staining procedure

1. Incubate the filaments with the actin-stabilization solution (300 μM MBS, 2% DMSO, 0.1% Triton X-100 in MTB, pH 7,4) for 30 min in dark at room temperature (RT) (*Note 5*).
2. Wash 3 × 10 min with MTB, at RT (this step might be omitted, *Note 6*).
3. Fix the filament with 4% PFA in MTB for 40 min at RT (*Note 5*).
4. Wash 3 × 10 min in PBS:MTB 1:1 (v:v), at RT.
5. Incubate the filaments with the cell wall lysis solution (2% cellulase, 2% hemicellulase, 1% driselase, 1% macerozyme, 0.5% pectinase, 0.2% Triton X-100, 0.17 μM Rhodamine–Phalloidin, pH 5.5) for 15–20 min in dark, at RT.
6. Wash 3 × 10 min in PBS:MTB 1:1, in the dark at RT (*Note 7*).
7. Optional: Incubate the filaments in the extraction solution (2% DMSO in MT buffer) for 10 min, in the dark, RT (*Note 5*).
8. Wash 3 × 10 min in PBS:MTB 1:1, in the dark at RT.
9. Incubate the filaments in actin-staining solution (0.66 μM Rh–Ph in PBS:MT 1:1) in the dark for at least 1 h or overnight at 4°C or at RT (*Note 5*).
10. Wash 3 × 10 min in PBS, in the dark at RT.

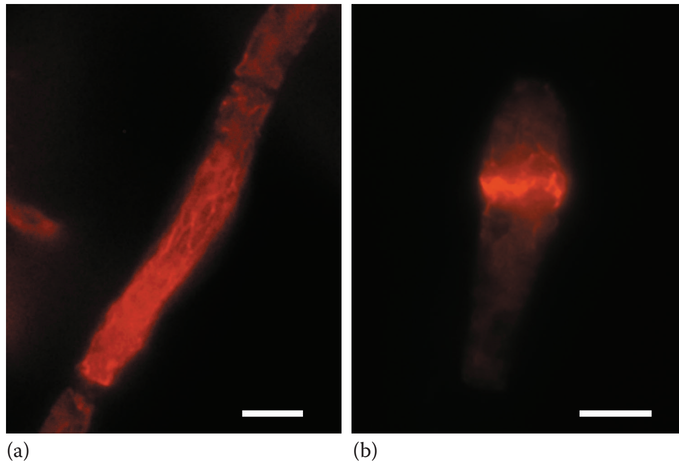

Figure 24.1 Organization of actin cytoskeleton in vegetative cells of *Ectocarpus* filaments labeled with Rhodamine–Phalloidin, as seen under epifluorescence microscopy. Long bundles of actin filaments are observed in intermediate (elongated) cell during interphase (a) and during cytokinesis (b), in which actin filaments are densely packed at the cytokinetic diaphragm plane. Scale bars represent 10 µm on each picture.

11. Stain the nucleus in 10 µg mL^{-1} Hoechst 33258 in PBS for 10 min, in the dark at RT.
12. Wash 3 × 10 min in PBS, in the dark at RT.
13. Mount in 0.2% phenylenediamine in glycerol:PBS 2:1. The slides are now ready to be observed under the fluorescent microscope. Two examples of actin-staining pattern observed in *Ectocarpus* sporophyte vegetative cells are shown in Figure 24.1.

24.5 Notes

Note 1: Alternatively, slides can be used instead of coverslips.

Note 2: By turning over the Petri dish on its lid, you create a moist chamber in which the coverslips will be incubated. The moisture avoids the evaporation of the medium deposited on the surface of the coverslips, which is important especially for long incubation times. The Parafilm® is hydrophobic and prevents the medium to flow over the surface of the coverslips.

Note 3: Avoid sucking filaments into the pipette tip when removing the medium from the tube while manipulating as gently as possible. If needed, slowly centrifuge the tube to take the filaments at the bottom of the tube. Avoid centrifuging filaments until they have been fixed.

Note 4: Do not pressure the filaments with the forceps, just spread them around very gently, because *Ectocarpus* filaments are easily damageable. Do not leave large piles of entangled filaments, which prevent proper staining of the filament, subsequently interfering with the observation. If needed, spread the filaments under a dissecting microscope using a fine needle.

Note 5: Incubation duration might require adjustments, depending on the amount and nature of the treated material.

Note 6: The first washing step can be omitted. Alternatively, remove half quantity of the first solution above and complete with the solution of PFA (8%) without washing step.

Note 7: Spread free-floating filaments on poly-L-lysine-coated coverslips (or slides) as described in Section 24.4.1. The rest of the protocol is identical for both types of sample.

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