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Actin-Dynamics in Plant Cells: The Function of Actin Perturbing Substances Jasplakinolide, Chondramides, Phalloidin, Cytochalasins, and Latrunculins

Andreas Holzinger and Kathrin Blaas

Abstract

This chapter will give an overview of the most common F-actin perturbing substances, that are used to study actin dynamics in living plant cells in studies on morphogenesis, motility, organelle movement or when apoptosis has to be induced. These substances can be divided into two major subclasses – F-actin stabilizing and polymerizing substances like jasplakinolide, chondramides and F-actin severing compounds like cytochalasins and latrunculins. Jasplakinolide was originally isolated from a marine sponge, and can now be synthesized and has become commercially available, which is responsible for its wide distribution as membrane permeable F-actin stabilizing and polymerizing agent, which may even have anti-cancer activities.

Cytochalasins, derived from fungi show an F-actin severing function and many derivatives are commercially available (A, B, C, D, E, H, J), also making it a widely used compound for F-actin disruption. The same can be stated for latrunculins (A, B), derived from red sea sponges, however the mode of action is different by binding to G-actin and inhibiting incorporation into the filament. In the case of swinholide a stable complex with actin dimers is formed resulting also in severing of F-actin.

For influencing F-actin dynamics in plant cells only membrane permeable drugs are useful in a broad range. We however introduce also the phallotoxins and synthetic derivatives, as they are widely used to visualize F-actin in fixed cells. A particular uptake mechanism has been shown for hepatocytes, but has also been described in siphonal giant algae. In the present chapter the focus is set on F-actin dynamics in plant cells where alterations in cytoplasmic streaming can be particularly well studied; however methods by fluorescence applications including phalloidin- and antibody staining as well as immunofluorescence-localization of the inhibitor drugs are given.

Keywords

Actin filaments; Chondramides; Cytochalasins; Depsipeptide; Jasplakinolide; Latrunculin; Phalloidin; Phallotoxin; Swinholide

1 Introduction

Filamentous Actin (F-actin), as a major structural cytoskeletal component has been targeted by inhibitor drugs over decades and the importance of an undisturbed turnover has been established as prerequisite for living functions in plant cells. Yet, a comprehensive overview of the different categories of F-actin perturbing substances is still missing. We will here give a short overview on (A) actin stabilizing and polymerizing substances like jasplakinolide,

chondramide and phalloidin, actin depolymerizing substances like cytochalasin, latrunculin and swinholide (Fig. 1).

The actin system is involved in different cellular processes like exo- and endocytosis, organelle motions and maintenance of organelle distribution, motility, cell division and cytoplasmic streaming. Several substances are known to influence these processes by altering intracellular actin organization and have been used extensively for cell biological research (1). While the mechanism of action is different for the described substances, the effect on the cells might be quite similar – a disruption of the actin related functions.

The present article does not cover drugs or inhibitors acting only indirectly on F-actin polymerization (i.e. ATP-synthase blockers like ATPase-blockers *N*-ethylmaleimide (NEM) and 2,3-butanedione monoxime (BDM) (2)) or inhibitors interacting with actin binding proteins like the Arp2/3 komplex (i.e. wiskostatin, (3)).

1.1 F-Actin stabilizing and polymerizing substances

Actin stabilization is generated mostly by three different compounds: phalloidin (4, 5), jasplakinolide (6, 7) and chondramide (8, 9). While all three substances have the capacities to stabilize F-actin, phalloidin is not membrane permeable, while jasplakinolide and chondramides readily enter cells (10, 11); Therefore phalloidin is used in cell biological research mainly for visualization of F-actin after fluorescence labeling of the compound in fixed tissues (12). In contrast, the commercially available jasplakinolide can be used for F-actin stabilization in living cells due to its membrane permeability (13).

1.1.1 Jasplakinolide—The common feature of these actin stabilizing substances is a cyclic depsipeptide (Fig. 1), which is a polymeric compound containing both, amino acids and hydroxy acids, joined by peptide and ester bounds. Chemically jasplakinolide is a cyclo-depsipeptide containing a tripeptide moiety linked to a polyketide chain (14, 15), (Fig. 1). Jasplakinolide is also called jaspamide (15, 16, 17, 18), For jasplakinolide also F-actin polymerizing capacities were described (19, 20, 21, 22, 23).

Originally jasplakinolide was isolated from the marine sponge *Jaspis* sp. collected at Fiji or the Palau islands (14, 16). The sponge was authenticated as *Jaspis johnstoni* (15), however taxonomic difficulties were pointed out (24) and the compound can also be isolated from other sponge genera (24, 25). Detailed procedures for jasplakinolide extraction can be found in (26).

Different approaches were made to synthesize this substance (27, 28) or generate nonpeptide mimetics (29), finally Jasplakinolide became commercially available from Molecular Probes in the late 90s. An enantioselective total synthesis of (+)-jasplakinolide has been described recently (30).

The biological activities of jasplakinolide are described as anthelmintic, antifungal, insecticidal and selective antimicrobial (14, 16, 31). *In vitro* investigations have elucidated the actin polymerizing- and stabilizing capacities of jasplakinolide (7, 19, 23).

In plant systems the unicellular green alga *Micrasterias* (20), green algae *Acetabularia*, *Pseudobryopsis* and *Nitella* (11), fucoid (brown algal) Zygotes of *Silvetia compressa* and *Pelvetia compressa* (32), *Allium* bulb scale cells and *Sinapis* root hairs (11), tobacco BY-2 cells (33), *Lilium* pollen tubes (34), *Papaver rhoeas* pollen tubes (35) have been tested for jasplakinolide reaction.

Jasplakinolide causes tremendous effect already visible at the light microscopic level by malformation of cells after recovery in the development of *Micrasteris* (20) (Fig. 2 a). Inhibition or retardation of cell development occurs; during recovery from drug treatment the cells develop a malformed pattern (20). In pollen tubes the normal bidirectional cytoplasmic streaming is altered, instead a rotary streaming is observed in the swollen apex (34). Moreover altering F-actin levels or dynamics by jasplakinolide plays a functional role in initiating programmed cell death in *Papaver* pollen, triggering a caspase-3-like activity (35). Polarity establishment is severely changed in fucoid zygotes (32). When applied during mitosis, binucleated cells are generated as a consequence of jasplakinolide treatment (20).

Jasplakinolide specifically targets F-actin, other cytoskeletal components like desmin or β -tubulin were not influenced by jasplakinolide (36), microtubule dependent processes are not affected (37) and the distribution of microtubules and microtubule dependent processes were not altered in green algae (20).

1.1.2 Chondramides—Another group of substances with actin stabilizing and polymerizing capacities are chondramides (9, 38). These depsipeptides were originally obtained from myxobacteria of *Chondromyces crocatus* (8, 39). Chemically they are very similar to jasplakinolide, but contain instead of an 19-membered ring an 18-membered macrocyclic ring (40). Until to date they are underrepresented due to their limited availability. However, synthetic chondramides were produced recently (40), and may become commercially available. In future, chondramides may also be used for cancer therapy (41, 42).

1.2 Actin depolymerizing substances

A prerequisite for an F-actin perturbing drug is its membrane permeability. Cytochalasins as well as latrunculins represent the most widely used F-actin sequestering drugs (43).

1.2.1 Cytochalasins—Cytochalasins have been shown to act on cytoplasmic streaming in plant cells (44, 45, 46, 47). A vast number of different cytochalasins (A, B, C, D, E, H, J) have been described, with different inhibitory concentrations and chemical properties, occasionally termed cytochalasanes (48). It is generally accepted that the cytochalasins slow the rate of filament polymerization by inhibiting the rate of elongation (49); This action is caused by the high affinity binding of cytochalasins to the barbed (plus end) of F-actin, thus the monomer addition is prevented by a ‘capping’ mechanism (43, 49). Due to the prevention of G-actin incorporation into the filament, a net-depolymerization is provoked (43). While the main target is the F-actin cytoskeleton, for cytochalasins A and B also inhibit monosaccharide transport across the plasma membrane (5).

Cytochalasins are fungal metabolites, independently discovered and isolated from distinct fungal species (cytochalasin A and B derived from *Helminthosporium dematioideum*; cytochalasin C and D from *Metarhizium anisopliae*) by Aldridge et al. (50). Previously, Rothweiler and Tamm (51) termed the substance ‘phomin’ as it was detected in *Phomas* sp. (*Fungi imperfecti*, strain S 298). Chemically cytochalasins are a diverse group of polyketide–amino acid hybrid metabolites (48).

Various cytochalasins have a wide range of biological activities, some of which not directly related to actin binding. Some cytochalasins (e.g. cytochalasin B) interfere with the monosaccharide transport systems by binding to high-affinity sites on glucose-transporter proteins and may interfere with hormones.

1.2.2 Latrunculins—Latrunculins (Fig. 1) provide another useful drug for the study of actin polymerization with a complementary mode of action when compared to cytochalasin (52). The main mechanism of latrunculin action is in an interaction with actin monomers (G-actin) in order to avoid actin polymerization (52). It has been shown by an in vitro assay that latrunculins bind to purified G-actin resulting in a nonpolymerizable 1:1 complex (6). Latrunculin A is a toxin purified from the red sea sponge *Latrunculia magnifica* (52, 53). It has been used in a variety of plant cells inhibiting pollen germination and pollen tube growth (47) and induced plant dwarfism (54). Both Latrunculins arrested cytoplasmic streaming after disrupting the subcortical actin bundles (46). In a mesophyll cell of the high alpine plant *Oxyria digyna* latrunculin B arrested cytoplasmic streaming (Fig. 2b) (55). Latrunculin B has a diminished binding capacity when compared to latrunculin A (6).

1.2.3 Swinholide—Swinholide A (Fig. 1) was isolated from the marine sponge *Theonella swinhoei*, collected from the Red Sea (6, 56). It is membrane permeable and acting as an F-actin disrupting toxin that stabilizes actin dimers and thus severs F-actin. Chemically it consists of a 44-carbon ring dimeric dilactone macrolide with a 2-fold axis of symmetry (57, 58). The effects of swinholide A on the actin cytoskeleton and cell morphology are similar to latrunculins rather than of cytochalasins (57).

1.3 Visualization of inhibitory effects

The function of these drugs can be either studied by arresting or inhibiting the cytoplasmic streaming (Fig. 2b) (55). The velocity of cytoplasmic streaming can provide useful insights in the function of the different drugs (46).

Visualization of the changes in F-actin is usually performed by phalloidin staining (46). It has been shown in the charophyte green alga *Nitella pseudolabellata*, that thick F-actin bundles may not appear influenced by e.g. cytochalasin A and H (Fig. 3), despite that the cytoplasmic streaming is arrested. After jasplakinolide or chondramide treatment an altered F-actin distribution was found, characterized by a patchy appearance of cortical actin (59), the formation of actin dots (60) or a disruption of the actin cytoskeleton (11, 36).

As competitive binding inhibition of phalloidin to F-actin by jasplakinolide is described (19), and decreased FITC-phalloidin labeling is noted as a consequence of jasplakinolide treatment in MDCK cells (61), the problems visualizing jasplakinolide effects by

fluorescently labeled phalloidin are obvious. Moreover, phalloidin and jasplakinolide have the same effect when applied to living *Dictyostelium* cells, namely the formation of actin aggregates (22).

In order to avoid the difficulties mentioned, only electron microscopy appears to be a sufficient technique for detection of jasplakinolide effects on F-actin (20, 62).

At electron micrographs tangential sections of F-actin can easily be recognized by the filament diameter of about 4-5 nm. In organisms like *Micrasterias*, where normally almost no bundling of F-actin occurs, and thus F-actin is hardly found at electron microscopical images, alterations causing extensive bundling of actin are easily detected (20) (Fig. 3). Also immunodetection of the altered F-actin system after jasplakinolide treatment is successful (59) and allows a comprehensive picture to be drawn in combination with other techniques.

Herein different procedures shall be described for the detection of actin filament aggregates generated by application of jasplakinolide. Due to the advantages of electron microscopy and/or immunomethods, these will be mentioned more in detail, however different protocols for phalloidin stainings will be given as they have been used for some organisms in combination with jasplakinolide treatment (22, 59, 61).

2 Materials

2.1 Cell cultures

1. *Micrasterias denticulata* Bréb. (unicellular green algae) are used for light microscopy and electron microscopy (20) and immuno-electron microscopy since alterations in the cell shape caused by drug treatments are easily detected. The cells are grown in culture medium under semisterile conditions in a light/dark regime of 14/10 h at 20°C.
2. Characean green algae (*Chara* sp., *Nitella* sp.), fresh collected or culture grown material
3. *Acetabularia acetabulum*;
4. Stably transformed *Arabidopsis thaliana* expressing a fusion construct of GFP with the actin binding domain 2 (ABD2) of the plant actin-binding protein fimbrin (63, 64).

2.2 Chemicals, Buffers, Solutions

2.2.1 Actin Perturbing Drugs and solvents

1. Jasplakinolide, Mol. Wt. 709.68, (Molecular Weight 709.67, J4580-100UG Sigma-Aldrich). 100 µg diluted upon arrival in methanol to achieve 10 mM stock solution and kept refrigerated at -20°C (see Note 1).
2. Chondramides (while the original isolate from myxobacteria (8, 39) is not commercially available, synthetic condramide A analogous have been produced, see ref. 40).

3. Cytochalasins (all available from Sigma-Aldrich): cytochalasin A: M. Wt. 477.59, C6637-1MG; cytochalasin B: M. Wt. 479.61, C6762-1MG; cytochalasin C: M. Wt. 507.62, C30382-1MG; cytochalasin D: M. Wt. 507.62, C8273-1MG; cytochalasin E: M. Wt. 495.56, C2149-1MG; cytochalasin H: M. Wt. 493.63, C0889-5MG; cytochalasin J: M. Wt. 451.60 (currently not available).
4. Latrunculin (A, B): LAT A: M. Wt. 421.55, L5163-100UG (Sigma-Aldrich); LAT B: M. Wt. 395.51, L5288-1MG (Sigma-Aldrich).
5. Swinholide A, M. Wt. 1,389.87, S9810-10UG (Sigma-Aldrich).
6. Phalloidin M. Wt. 788.87, P2141-1MG (Sigma-Aldrich).
7. Methanol reagent.
8. DMSO (dimethyl sulfoxide) reagent.

2.2.2 Chemicals, Buffers and Solutions for Electron Microscopy

1. Cacodylic buffer: 50 mM cacodylic acid sodium salt, pH 7.2.
2. Cacodylate-glutaraldehyde: 50 mM cacodylic buffer, 1% glutaraldehyde, pH 7.2.
3. Cacodylate-osmium: 50 mM cacodylic acid buffer, 1% osmiumtetroxide, 0.8%, potassium hexacyanoferrate III.
4. Phosphate buffer-fixative: 25 mM sodium hydrogen phosphate, 25 mM potassium dihydrogen phosphate, 1% glutaraldehyde, 1% osmiumtetroxide, pH 6.2.
5. Calcium chloride.
6. 2% Aqueous uranyl acetate dihydrate.
7. Ethanol solutions: 15%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%.
8. 1,2- Propylene oxide reagent.
9. Propylene oxide-ethanol mixture: 1,2- Propylene oxide and ethanol (1:1 v/v).
10. A suitable embedding resin: Embed 812, Araldite 502, Spurr's resin or others.
11. Propylene oxide-embedding resin mixture: Mixture of 1,2 Propylene oxide and embedding resin (1:1 v/v).
12. Formvar 1595 E.
13. Chloroform reagent.
14. Lead citrate.

2.2.3 Chemicals, Buffers and Solutions for Immunoelectron Microscopy

1. Lecithin (10%) dissolved in chloroform.
2. Liquid nitrogen.
3. Acetone-tannic acid: acetone containing 0.1% tannic acid (v/v).
4. Acetone-osmium: acetone containing 2% osmiumtetroxide and 0.05% uranyl acetate.
5. LR white resin (London resin company, medium grade).
6. TBS: Tris buffered saline, 50 mM, pH 7.5.
7. Blocking solution: TBS containing 1% BSA (fraction V), 1% acetylated BSA, 0.1% Tween-20.
8. 10% bovine calf fetal serum.

Primary Antisera/Antibodies, e.g. monoclonal anti actin N 350 monoclonal anti actin (Amersham).
9. 10 nm colloidal gold conjugated, secondary antibody appropriate for the primary antibody.

2.2.4 Chemicals, Buffers and Solutions for Fluorescence Microscopy

1. MT-stabilizing buffer (MTSB): 50 mM PIPES buffer, 50 mM MgSO₄, 50 mM EGTA, pH 6.9
2. Dimethylsulfoxide (DMSO) reagent.
3. Paraformaldehyde fixative: 4% paraformaldehyde and cytospin (Cytospin 3 preparation systems, Shandon Scientific Ltd. Cheshire, UK).
4. 50 μ M ammonium chloride.
5. PBS: Phosphate buffered saline: 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium hydrogen phosphate, 1.4 mM potassium dihydrogen phosphate.
6. Triton solution: 0.1% Triton X-100 in PBS.
7. 2% bovine serum albumin (BSA).
8. Anti actin monoclonal antibody N 350 (Amersham) (see Note 2).
9. Anti actin monoclonal antibody (clone C4).
10. Phalloidin: 0.4 mg/mL FITC (or alexa) conjugated phalloidin Alexa phalloidin (Molecular Probes, Leiden, The Netherlands; prepare from a 6.6 mM stock solution in methanol) at a concentration of 0.16 mM.
11. Phenylenediamine.
12. Texas red conjugated antitmouse IgM antibody (Jackson Immuno Research).

13. Steedman's wax: PEG 400 distearate and 1-hexadecanol mixed in proportions 9:1 (w/w) (68).
14. Isotonic perfusion solution: 200mM sucrose, 70mM KCl, 4.5mM MgCl₂, 5mM ethyleneglycoltetraacetic acid (EGTA), 1.48 mM CaCl₂, 10mM piperazine-N, N'-bis (2-ethanesulfonic acid) (PIPES, pH7.0).

3 Methods

3.1 Treatment and Recovery Experiments

1. Incubate cells (from the above mentioned plant cell types or tissue see Subheading 2.1) in 1 mL of culture medium containing respective inhibitory drug concentrations for time periods of 0.5, 1, 2, 3, 4, 24, 48 h and observe the effect on cytoplasmic streaming (Fig. 2, see Note 3).
2. Incubate cells in 1 mL of culture medium containing the respective drug (see **step 1**), wash several times with culture medium and allow developing in culture medium for up to 24 h. This is regarded as 'recovery experiment'.
3. Investigate the cells treated as in **steps 2.** and **3.** under a regular light microscope or fix for electron microscopy (see Subheadings 3.2.1).

3.2 Visualization of F-actin after drug treatment

3.2.1 Chemical Fixation/Electron Microscopy of Algal Cells after Holzinger and Meindl (20)—The procedure described herein has first been used for the unicellular green alga *Micrasterias* by (65).

1. Fix treated cells, cells allowed to recover in nutrient solution and untreated control cells in cacodylate-gutaraldehyde for 15 min (see Note 4).
2. Wash cells 3 times 5 min each in cacodylic buffer.
3. Postfix cells in cacodylate-osmium for 2 h.
4. Wash cells, as in **step 2**, 3 times 5 min each in distilled water.
5. Incubate cells in 2% uranyl acetate for 2 h (see Note 5).
6. Wash cells, as in **step 2**, 3 times 5 min each in distilled water.
7. Dehydrate the cells in increasing concentrations of cold ethanol (see Note 6), 15 min per concentration:
15%, 30%, 40%, 50%, 70%, 80%, 90%, 95%, 100%. Keep in 100% for 30 min.
8. Transfer the cells to the propylene oxide-ethanol mixture, allow to equilibrate for 10 min, transfer to propylene oxide.
9. Transfer the cells to the propylene oxide-embedding resin mixture (see Note 7).

10. Rotate the cells in this mixture for 48 h in order to allow the propylene oxide to evaporate and a sufficient penetration of the cells with the resin to be achieved.
11. Transfer the cells to freshly prepared resin in aluminum dishes and orientate them by the use of eyelashes connected to holders.
12. Incubate in an exsikkator for 4 days.
13. Polymerize the resin for 24 h at 60°C.
14. Select cells and section at an ultramicrotome.
15. Collect sections on formvar coated copper grids (*see* Note 8).
16. Counterstain the sections with 2% uranyl acetate and Reynold's lead citrate (*see* Note 9).
17. Investigate and photograph at a transmission electron microscope at 60-80 kV.

3.2.2 Immuno-electron Microscopy—The following fixation technique has been carried out for *Micrasterias* for the first time by Meindl et al. (66). The recipe for actin detection by means of immunogoldlocalization is based on (67).

1. Prepare gold or aluminum specimen holders with bed depths of 100 - 300 µm by dipping in lecithin.
2. Collect treated cells to be transferred to specimen holders. Large cells like *Micrasterias* (200 µm in diameter) can be easily collected under a stereo microscope by wrapping with cotton fibers.
3. Fix treated cells by high pressure freeze fixation by the use of a Hyperbaric freeze device (formally Balzers HPM 010, later taken over by BAL-TEC; then produced by ABRA Fluid AG, Widnau, Switzerland; currently available as "High Pressure Freezing Machine HPM 010" by Boeckeler Instruments Inc., Tucson/AZ, USA), for other methods (*see* Note 10).
4. Collect and store samples under liquid nitrogen.
5. Transfer samples to a freeze substitution device (Reichert-Jung, cs auto or LEICA EM AFS, Leica Microsysteme GmbH, Vienna, Austria).
6. Substitute samples at -80°C for 24 h in acetone-tannic acid solution.
7. Wash several times with acetone.
8. Substitute samples at -80°C for 24 h in acetone-osmium solution (*see* Note 11).
9. Allow samples to reach -30°C within 5 h.
10. Continue substitution at -30°C for 10 h.

11. Allow samples to reach room temperature (20°C) within 5 h.
12. Remove osmium tetroxide/uranyl acetate solution and rinse several times with acetone.
13. Change acetone for ethanol by several rinses.
14. Transfer samples to LR white in aluminum dishes and cover with cellophane foil.
15. Allow samples to infiltrate in an exsikkator for 24 h.
16. Polymerize under UV light at room temperature for 24 h.
17. Prepare sections for electron microscopy (compare 3.2.1) and transfer to formvar coated gold- or gilded copper grids.
18. Incubate in blocking solution for 30 min up to 1 h. A blocking step with 10% bovine calf fetal serum may be applied if blocking is not sufficient.
19. Transfer into primary antibody against actin. Purified antibody may be diluted in blocking solution. Incubation should last for 1.5 h at room temperature or for up to 24 h at 4°C.
20. Wash 4 times 15 min in TBS by transferring grids to 50 µL droplets of TBS.
21. Incubate in 10 nm-gold (*see* Note 12) labeled secondary antibody diluted in blocking solution (*see* Note 13).
22. Wash by rinsing with TBS, followed by incubation in droplets of TBS for 2 min, followed by a brief rinse with distilled water.
23. Counterstain with uranyl acetate and lead citrate if necessary (*see* Subheading 3.2.1.).
24. Investigate at a transmission electron microscope at 80 kV.

3.2.3 Immunofluorescence Microscopy of Latrunculin B treated Maize Roots after Baluska et al. (54, 68)

1. Grow maize root tips for 48 h.
2. Cut apical segments (6-8 mm) and transfer into 4.6 ml MTSB buffer mixed with 0.5 ml of DMSO for 15 min.
3. Fix for 1 h in MTSB/DMSO solution containing 4% paraformaldehyde.
4. Dehydrate in increasing ethanol series in PBS.
5. Embed in Steedman's wax by stepwise infiltration in proportions 2:1, 1:1 and 1:2 (v/v) for 2 h each step at 37°C.
6. Infiltrate under vacuum three changes in pure wax.
7. Polymerize wax at room temperature.

8. Prepare longitudinal sections with a thickness between 4 and 8 μm .
9. Mount sections on poly-L-lysine coated slides.
10. Dewax the sections in ethanol.
11. Rehydrate in ethanol/PBS series and incubate in MTSB for 45 min.
12. Digest cell walls in 1% hemicellulose dissolved in 0.5 MEGTA, 0.4 M mannitol, 1% Triton X-100, 0.3 mM phenylmethanesulphonyl fluoride (dissolved in MTSB).
13. Incubate in blocking solution (2 % BSA) for 30 min.
14. Incubate in anti actin monoclonal anti-actin antibody (clone C4, ICN, Costa Mesa, CA) for 2 h at room temperature.
15. Rinse 3 times 15 min in MTSB.
16. Incubate in FITC-conjugated anti-mouse IgGs (diluted 1:100 -1:200) for 1 h at room temperature.
17. Remove secondary antibody and mount in phenylenediamine.
18. Examine at a Confocal Laser scanning microscope at excitation with an argon laser at 488 nm.

3.2.4 Perfusion of *Nitella* intermodal cells for FITC-phalloidin staining of F-actin after (46) (Fig. 3; see Note 14)

1. Place an intermodal cell on the cover slip bottom of a perfusion chamber and press into vacuum grease lines; place small reservoirs with grooves over the grease and firmly press down without damaging the cells.
2. Cover the central portion of the cell between the reservoirs with silicon fluid to avoid evaporation
3. Bath the ends of the cells with isotonic perfusion solution.
4. Reduce the turgor pressure of the cells in hypertonic solution.
5. Cut the ends with small scissors and allow the perfusion solution to enter the cells.
6. After 1 min replace the perfusion solution by an F-actin staining solution containing Alexa-phalloidin.
7. After 20 min examine at the CLSM (see section 3.2.3)

4 Notes

1. Jasplakinolide is also dissolvable in DMSO (31).
2. The choice of the anti actin antibody is very important. The epitope of the N 350 (a mouse monoclonal IgM antibody directed against chicken gizzard actin) binding site is obviously not changed by jasplakinolide (11),

whereas binding sites for other actin antibodies (eg. clone C4 – a mouse monoclonal IgG antibody directed against chicken gizzard actin, ICN) seem to be altered substantially which prevents visualization of F-actin after jasplakinolide treatment (11). This antibody may be used without problems in combination with latrunculine B treatment (54).

3. The concentrations of the inhibitory drugs may vary drastically. For jasplakinolide was found to be effective at 1.5 μM to 3 μM in producing F-actin aggregates for *Micrasterias denticulate* (20). Concentrations above 250 μM of jasplakinolide were lethal. The concentrations of jasplakinolide needed for other cells have to be found empirically. Variations in the literature range from as low as 100 nM to 1 μM , 2.5 μM to 10 μM (11, 32, 59, 69). However they should be in the same range as described herein, since the effective concentrations correlate with data obtained from the literature (22, 31, 61). Latrunculins had a different lowest effective concentrations in *Micrasterias denticulata*: chondramide A 20 μM , chondramide B 15 μM , chondramide C 5 μM , chondramide D 10 μM (38). In contrast in human tumor cell lines, 3-85 nM chondramide effectively inhibited proliferation (9). For cytochalasins (46) found streaming-arresting concentration in *Nitella pseudoflabellata* between 1 and 200 μM . The lowest concentrations to arrest streaming were at 1 μM in cytochalasins A and E, 30 μM in cytochalasin H, 60 μM cytochalasin D and 200-220 μM in cytochalasins C, J, B. These authors distinguish between streaming arresting (i.e. fully arresting streaming within 1 h, Fig. 3) and streaming inhibiting (i.e. reducing streaming velocity by 15-80% within 1 h) concentrations. In another green alga *Xanthidium armatum*, cytochalasin D arrested cytoplasmic streaming at concentrations below 10 μM .

In contrast, latrunculins have lower effective concentrations; Latruncunlin B may cause severe effects even at extremely low concentrations in the pM to nM range (45). Several authors use it in higher concentration in the μM range (10 μM (54); 5 μM , 10 μM (55). Moreover, a combined treatment with latrunculins and cytochalasins, rapidly arrested cytoplasmic streaming even at concentrations that had only mild effects on the streaming rate when used separately (46). Swinholide has not been extensively applied to plant cells, only IC 50 values for human nasopharynx cancer cells have been determined at 6 nM (70).

4. The fixation procedure is performed in a "Balzers Fixomat" - a device not commercially available anymore - consisting of sintered glass suction filters connected via a valve to a pump station, allowing to remove solutions by underpressure while cells remain in the filter. Moreover the temperature can be adjusted via a cooling bath. Alternatively the cells can be fixed in glass dishes, ideally in the shape of a hemisphere. In this case it is best to remove only the solutions with a pipette and not to transfer the cells.

For other organisms more specialized methods might be necessary - for example embedding of the specimens in agarose prior to the fixation procedure.

5. During incubation in uranyl acetate solution it is necessary to keep the cells in darkness, by covering the filter or dish with aluminum foil.
6. During the whole procedure of dehydration the temperature should be kept at 6°C. At the steps 50% and 70% ethanol temperature might be lowered to 4°C. In case of fixation in round glass dishes they should be kept over ice. At the step of 100% ethanol the temperature should be increased to room temperature.
7. Very good results are obtained with a 1:1 mixture of glycid ether 100 (a substance equivalent to Epon 812) and MNA. Prior to use DMP must be added (5 droplets to 10 ml of resin). However a mixture of Embed-812/ Araldite 502/DDSA (DMP-30 or BDMA must be added prior to use) will also give adequate results. When different hardness has to be achieved, Spurr's resin may be the best choice: As several components of the original mixture are not deliverable any more, we now use the kit "Agar Low Viscosity Resin (LV)" by Agar Scientific Ltd. Essex CM24 8DA, England.
8. Coating of grids with formvar may not be necessary for all objects. We use the following procedure: A light microscopic slide is cleaned with lens tissue, tipped into 0.3 % formvar dissolved in chloroform. The resulting formvar film is cut at the edges of the slide and allowed to float on distilled water. Grids are placed on the film which is then removed with parafilm.
9. The time periods needed for counterstaining depend on the staining already achieved during the fixation/staining procedure and are typically 5-30 min for uranyl acetate and 1-5 min for Reynold's lead citrate.
10. For large cells like *Micrasterias* (about 200 µm in diameter) high pressure freezing has been found the only appropriate technique for freeze fixation. Alternatively to the HPM 010, also the Leica EMPACT high pressure freezer (Leica Microsysteme GmbH, Vienna, Austria) gives reasonable results. However for small and less vacuolated cells plunge freezing which might be achieved with a relative simple equipment, will also give reasonable results.
11. Osmium tetroxide dissolves well in acetone. Uranylacetat is best to be sonicated to dissolve it. For other organisms it might be appropriate to substitute without osmium tetroxide and uranyl acetate.
12. Gold particles with 10 nm diameter are used commonly. The usage of smaller gold particles might enhance the accuracy of the detected locus.

13. The actual dilution factor has to be found empirically for each system, but it should be in the range of 1:50 – 1:200.
14. As mentioned in the introduction, the use of fluorescently labeled phalloidin is problematic due to competitive binding with jasplakinolide (19, 23). It has been reported that simultaneous addition of jasplakinolide and FITC-phalloidin (1:1) did not result in F-actin labeling, whereas addition of the inactive analogue jasplakinolide B and FITC-phalloidin resulted in staining as strong as in controls (11).

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Jasplakinolide **Phalloidin** **Chondramide** **Cytochalasin A** **Cytochalasin D** **Latrunculin A** **Latrunculin B** **Swinholide A**

Fig. 1. Molecular structures of a selection of F-actin stabilizing/polymerizing drugs (jasplakinolide, phalloidin, chondramide) and F-actin disrupting drugs (cytochalasin A, cytochalasin D, latrunculin A, latrunculin B, swinholide A).

Fig. 2.

Light microscopy of F-actin perturbation. **a** Effect of jasplakinolide treatment on developing *Micrasterias denticulata* cell (Subheading 3.1.) young developmental stage treated with 3 μ M jasplakinolide for 0.5 h and allowed to recover for 3 h in nutrient solution, young semi-cell exhibits complete loss of normal cell pattern (arrow), chloroplast (chl). **b** Effect of 5 μ M latrunculin B on *Oxyria digyna* mesophyll cell; cytoplasmic streaming is inhibited, between the chloroplasts (chl) granular cytoplasmic portions are visible. Bars: **a** 20 μ m, **b** 5 μ m. **a** reprinted from reference (20) with permission. 'Copyright Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc'. **b** reprinted from reference (55) with permission of 'G. Thieme Verlag KG, Stuttgart.'

Fig. 3.

Subcortical F-actin bundles in intermodal cells of *Nitella pseudoflabellata* treated with 1 μM cytochalasin A, 1 d, b cytochalasin H 50 μM 2 d. Despite these concentrations lead to an arresting of cytoplasmic streaming, the F-actin bundles are clearly visible after phalloidin staining with the perfusion method (*see* Subheading 3.2.4) clear cytoplasm. Reprinted from reference (46) with permission of Oxford University Press.

Fig. 4. Detail of the ultrastructure of *Micrasterias denticulata* cell treated with 3 μ M jasplakinolide for 2 h and fixed for electron microscopy according to Subheading 3.2.1., dense accumulations of F-actin are visible in the cytoplasm. Bar = 0.2 μ m. Reprinted from reference (20) with permission 'Copyright Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.'