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„Electron tomography of actin networks in plastic embedded tissue culture cells “

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1. Introduction

1.1 The actin cytoskeleton

Cells have a complex three dimensional structure which is mainly formed by their cytoskeleton. The term “cytoskeleton”, introduced by Wintrebert in the 1930s [reviewed in Frixione, 2000], is nowadays adapted for the description of the entirety of filamentous structures in cells, which were already observed in the early days of light microscopy [Heidenhain, 1899]. This comprises microtubules, actin filaments, on which I want to focus in here, as well as intermediate filaments and their respective associated proteins [Steinmetz *et al.* 1997]. To call these structures “skeleton” is misleading, since all of them are highly dynamic.
Actin, the most abundant cytoskeletal protein [Amos and Amos, 1991], was firstly isolated by Straub in 1942 from rabbit skeletal muscle [Kabsch and Vandekerckhove, 1992]. Decoration of filamentous structures with heavy meromyosin delivered a strong evidence for the appearance of actin also in non-muscle cells [Ishikawa *et al.*, 1969; Pollard *et al.*, 1970]. The protein by itself is highly conserved in eukaryotes [Steinmetz *et al.*, 1997] and plays an important role in different biological processes including muscle contraction, cell migration, cytokinesis and intracellular transport, respectively [reviewed in Pollard and Cooper, 2009]. Beside of its localisation in the cytoplasm it can also be found in the nucleus [Vartiainen, 2008; Weston *et al.*, 2012]. Monomeric actin (=globular actin, G-actin) can self-assemble to stable polarized helical filaments (=filamentous actin; F-actin) under physiological conditions [Pollard and Cooper, 2009; Otterbein *et al.*, 2001, Fujii *et al.*, 2010], also called microfilaments, which have a diameter of 5 – 8 nm [Pollard *et al.* 1969, Spooner *et al*. 1971]. In order to accomplish the diverse functions mentioned above, actin can be arranged in several different ways (Fig. 1).

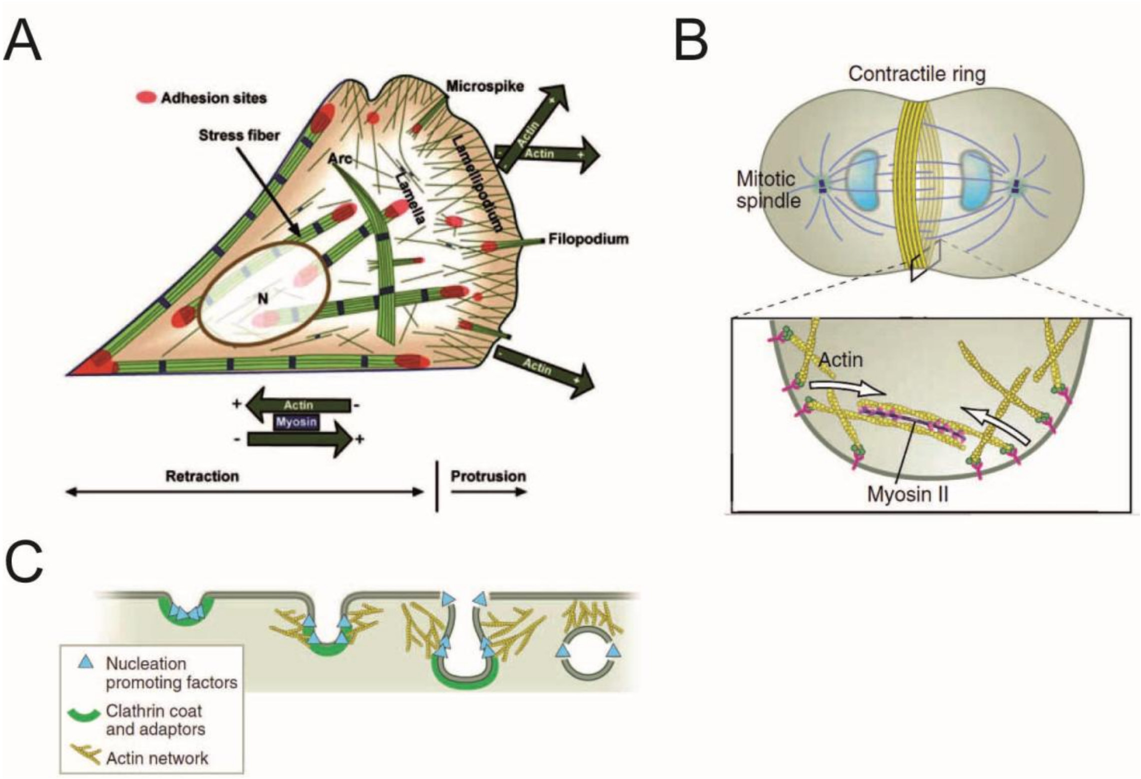
A
Adhesion sites
Stress fiber
Microspike
Arc
Lamella
Lamellipodium
Filopodium
N
Actin
Actin
Actin
Actin
Myosin
Retraction
Protrusion
B
Contractile ring
Mitotic spindle
Actin
Myosin II
C
Nucleation promoting factors
Clathrin coat and adaptors
Actin network

Figure 1 Arrangements of filamentous actin in cells. (A) Actin cytoskeleton of a polarized fibroblast, showing an actin filament meshwork in the lamellipodium, associated radial bundles in filopodia and microspikes and contractile bundles of actomyosin at the cell rear (stress fibers). Sometimes also arc-shaped bundles are found in the area behind the lamellipodium, termed lamella. Adhesion sites are indicated as red circles [adapted from Small and Rottner, 2010]. (B) A contractile ring of acto-myosin enables constriction during cytokinesis. (C) Invagination of plasma membrane is facilitated by patches of F-actin [(B) and (C) adapted from Pollard and Cooper (2009).

Crystal structures revealed that G-actin consists of 4 subdomains [Kabsch *et al.* 1990; McLaughlin *et al.* 1993; Schutt *et al.* 1993; Robinson *et al.* 1999; Otterbein *et al.* 2001]. Actin can bind to adenosine triphosphate (ATP). Following polymerization ATP can be hydrolysed to adenosine diphosphate (ADP) [de la Cruz and Pollard, 2001], leading to a rearrangement in subdomain 2, whereby a flexible loop (ATP-bound state) folds into an ordered α-helix (ADP-bound state; Fig. 2), which most likely interferes with monomer-monomer-interaction, leading to a weakening of filament stability and subsequent depolymerization [Graceffa and Dominguez, 2003].

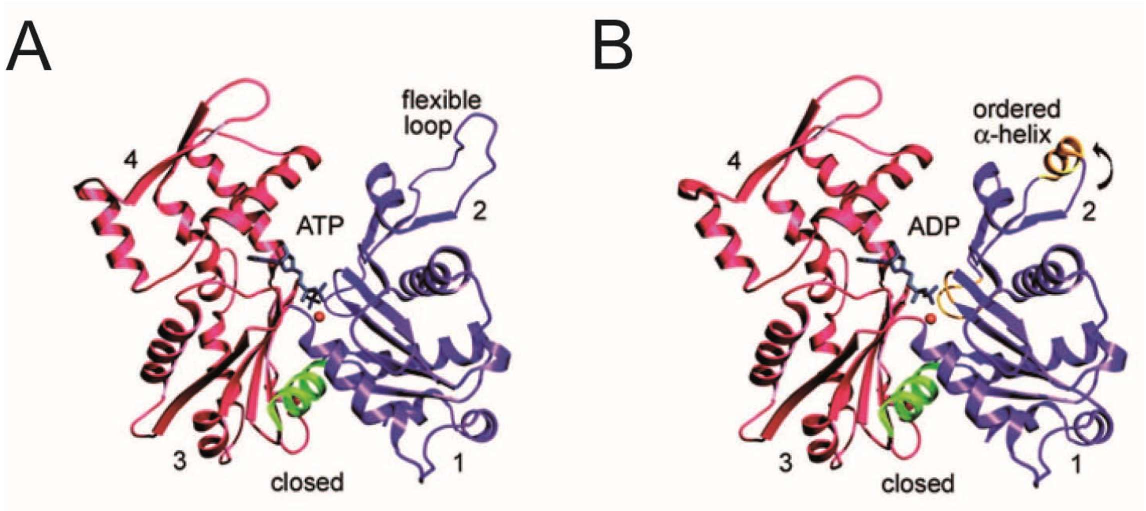

Figure 2 Crystal structures of G-actin in ATP-bound (A) and ADP-bound state (B). Both structures reveal the organization of G-actin into 4 subdomains. Upon ATP hydrolyzation the flexible loop in subdomain 2 folds into a ordered α-helix [adapted from Graceffa and Dominguez, 2003]

De novo filament formation starts with nucleation of a seed containing three associated actin monomers. Polymerization is energetically unfavourable until this stage is reached [Winder and Ayscough, 2005]. The polymerization reaction depends on the availability of free G-actin. The concentration has to reach a certain limit which is the so called critical concentration. [Amos and Amos, 1991]. Since the critical concentration of monomeric actin is different at both ends of the filament, one can distinguish between a fast growing (plus-) and a slow growing (minus-) end. As a result a particular filament can grow on one end, whereas it shrinks on the other, this process is known as “treadmilling” [reviewed in Neuhaus *et al.* 1983].

By addition of heavy meromyosin (myosin-S1), the polarity of the filaments can be visualized [Huxley, 1963; Steinmetz *et al.* 1997]. F-actin binds myosin-S1 stoichiometrically *in situ* and *in vivo*, resulting in the formation of an “arrowhead like” structure with a pointed minus- and a barbed plus-end (Fig. 3) [Moore *et al.*, 1970; Neuhaus *et al.*, 1983; Steinmetz *et al.* 1997].

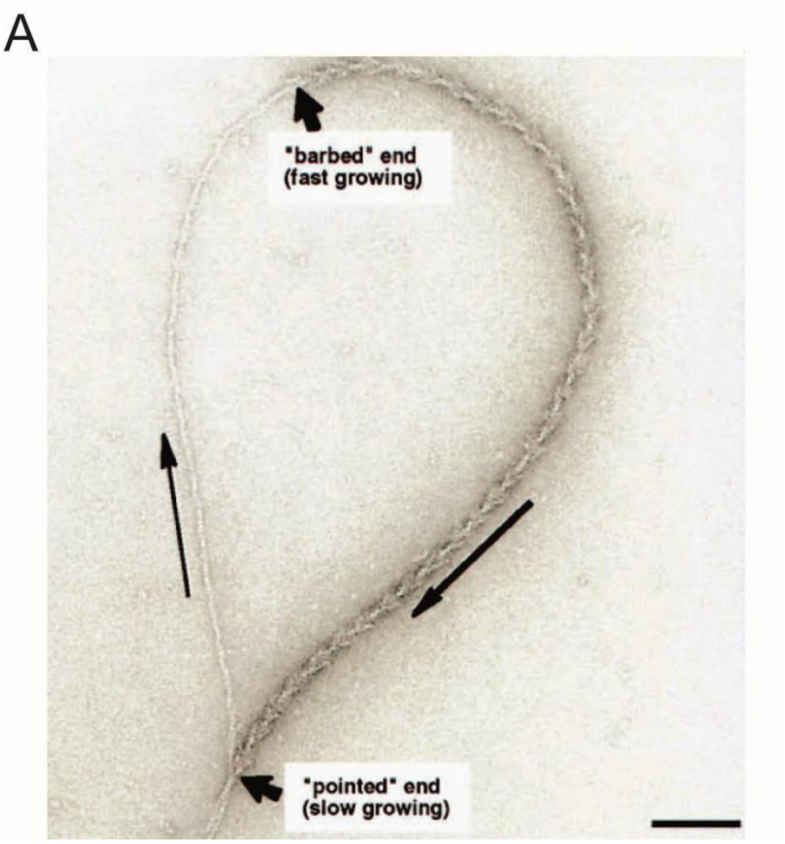

Figure 3 Decoration of F-actin with myosin-S1. Decoration results in “arrow-like” structure with a pointed (slow growing) and a barbed (fast growing) end, revealing the polarized structure of F-actin; scale bar: 100 nm [adapted from Steinmetz *et al.*, 1997].

To facilitate its versatile functions, actin interacts with numerous other proteins. The motor proteins of the actin cytoskeleton belong to the family of the myosins. These generate force by the hydrolyzation of ATP and are involved in muscle contraction and intracellular transport [Amos and Amos, 1991; Winder and Ayscough, 2005]. In addition myosin and actin form contractile fibers, the so called stress fibers, in non-muscle cells (Fig. 1 A), which enable cell migration (see below) [Small and Rottner, 2010]. Nucleation is one of the limiting steps of filament assembly. The heptameric actin related protein 2/3 (Arp2/3) -complex is known to be the main nucleator of actin filaments. It promotes both, *de novo* polymerisation as well as induction of branches on pre-existing filaments [Pollard and Borisy, 2003]. Beside the Arp2/3 complex, there exist two additional classes of nucleators, class 2 which consists of formins and class 3 proteins like Spire, Cordon bleu (Cobl) and Leiomodin (Lmod) [reviewed in Chesarone and Goode, 2010].

To circumvent continuous polymerisation filaments can be capped by capping proteins like CapZ and gelsolin. Elongation of filaments is also controlled by the actin elongation factors formins and Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP), [reviewed in Chesarone and Goode, 2010]. An additional negative regulator of polymerization is thymosin-β4. It binds and sequesters monomeric actin, providing an available pool of G-Actin [Pollard and Borisy, 2003]. The antagonist of this process is profilin, which also binds to actin monomers and replaces thymosin and shuttles ATP-actin to filament plus-ends [Pollard and Borisy, 2003].

Depolymerization can be induced by association of actin depolymerization factor (ADF)/cofillin to the filaments [Pollard and Borisy, 2003]. Stress fibres in non-muscle cells and actin fibers in striated muscles are protected from depolymerization by tropomyosin [Pollard and Borisy, 2003]. Apart from proteins regulating filament assembly actin also interacts with other network organizing proteins, enabling both the interaction of actin filaments among themselves as well as the connection with other cellular elements. Other examples of actin interactors are fascin, α-actinin and Filamin which can bundle F-actin [Pollard and Borisy, 2003]. Beside its function as F-actin cross-linker, Filamin can also attach the actin network to β-integrin, thus releasing talin from adhesion sites, resulting in an inactivation of integrins [Kiema *et al.*, 2006; Ithychanda *et al.*, 2009]. The organization of the actin cytoskeleton is also controlled by small GTPases of the Rho-family, like Cdc42, which have a strong influence on cell migration (see below).

Natural toxins can interfere with actin dynamics. A prominent example is phalloidin, the toxin of the mushroom *Amanita phalloides.* Phalloidin binds to actin filaments and thereby stabilizes them [Cooper, 1987]. Phalloidin is also used in cell biological approaches as a fluorescently tagged marker for F-actin in fluorescence microscopy. In addition it can be used for preparations of actin cytoskeletons in electron microscopy (EM) [Faulstich *et al.*, 1988; Small *et al.*, 1981; Small *et al.*, 1999]. Another common drug interfering with the actin cytoskeleton is lantrunculin, which sequesters actin monomers and thus prevents polymerization [Morton *et al.*, 2000].

1.2 Cell migration or crawling with actin

Migration of cells is an essential process during the whole life of an organism, in both healthy as well as in pathological conditions. It is indispensable during tissue formation in embryogenesis and wound healing. For example, upon muscle rupture, satellite cells start to proliferate and migrate to the injured point where they fuse with the muscle fiber for repair [Koller *et al.*, 2001].

Classically cell migration is a cycle of repeating steps: (1) extending the leading edge, (2) adhesion formation, (3) retraction of the cell rear accompanied with (4) the removal of old adhesion sites [Raftopoulou and Hall, 2003; Pollard and Borisy, 2003]. This so far reflects the situation of cells in a two-dimensional context, but leukocytes in a three-dimensional (3d) collagen matrix can even migrate without integrins [Lämmermann *et al*., 2008], implicating that formation of adhesions is not a prerequisite of cell migration *in situ*. However most of the knowledge gained on this process, has been acquired through studies of cells crawling over a plane surface. Under these conditions migration is initiated by extensions of the membrane including thin (0,1 – 0,3 µm) sheet like lamellipodia, firstly described by Abercrombie, and finger-like rods, named filopodia [Abercrombie 1980; Small *et al.*, 2002; Small and Rottner 2010; Vinzenz *et al.*, 2012]. Both structures are based on actin filaments. Whereas lamellipodia also contain branched filaments, the filopodia, enriched by the F-actin bundling factor fascin, contain bundles of actin filaments (Fig. 1A) [Pollard and Borisy, 2003, Nemethova *et al.*, 2008; Small and Rottner 2010]. In addition also microspikes can be found at the leading edge, these can be described as actin bundles, which in contrast to the filopodia do not extend beyond the edge of the Lamellipodium (Fig. 1A) [Small *et al.*, 2002]. The term ruffle is used for lamellipodia that detach from the substrate and move backwards [Abercrombie, 1971; Mitchinson and Cramer, 1996]. Membrane protrusion is driven by pushing forces generated by actin polymerization, with insertion of monomer at the lamellipodium tip [Pollard and Borisy, 2003]. Lamellipodia maintain a constant width, since polymerization at the front is balanced by depolymerization at the rear in a treadmilling mode [Small *et al.*, 2002]. The mode of force production and actin network generation was explained by Pollard and Borisy (2003) in their “dendritic nucleation model”. The basis of this model was the observation that Arp2/3 can induce branching (see above) and that the peripheral area of the lamellipodia are highly enriched in branched networks [Svitkina and Borisy, 1999]. From this they concluded, that filaments have to be short and stiff to push [Pollard and Borisy, 2003]. But in a correlating live-cell and electron tomography (ET) study the proposed enrichment of short, branched filaments at lamellipodia tips could not be observed [Vinzenz *et al.* 2012]. An explanation for this is the method used by Svitkina and Borisy, since hints for the production of artifacts in the steps involved were already evident (see below) [Resch *et al.* 2002a]. Thus, ideas about the basis of force generation by actin polymerization need to be revised.

Besides the described arrangements of actin at the cell front, additional contractile bundles of actin and myosin, termed stress fibers, can be found at the cell rear, which enable retraction of the trailing cell body as well as cell adhesion (Fig. 1A) [Small, 1988; Small and Rottner 2010].

As mentioned before, actin rearrangements during cell migration underlie the control by Rho GTPases [reviewed in Ridley, 2001; Raftopoulou and Hall, 2003]. Rac is described to activate proteins of the Scar/WAVE family and Cdc42 activates WASp as well as N-WASp, leading to Arp2/3 complex activation, followed by actin polymerization (see above) [Raftopoulou and Hall, 2003]. Signaling through Cdc42 induces the formation of filopodia, Rac activation lamellipodia [Small *et al*., 1999; Small and Rottner 2010], although both pathways stimulate Arp2/3. Stress fibers can be induced through Rho by interaction with ROCK and mDia [Ridley, 2001].

Taken together cell migration is facilitated by two features of the actin cytoskeleton, polymerization for membrane protrusions and contraction by interaction with myosin to retract the trailing cell body.

1.3 Actin network preservation for electron microscopy

Starting with the pioneering work of Keith R. Porter, Albert Claude and Ernest F. Fullam in 1945, electron microscopy (EM) has developed to an important tool in cell biology. Although the coexistence of different filamentous systems was revealed by immunofluorescence microscopy [Small, 1988], EM is the only method which allows to resolve densely packed arrays of filaments. And despite the fact, that a recently developed fluorescence microscopy method allows imaging of singular actin fibers by breaking the limits of diffraction [Xu *et al.*, 2012], EM still remains the method of choice as it is even possible to resolve secondary structures of molecules with its application [Fujii *et al.*, 2010].
A limitation of EM is sample thickness [Lučić *et al.*, 2013]. Approaches using sectioning of embedded tissue culture cells [Abercrombie *et al.*, 1971] resulted in distortions of actin structures (Fig. 4) [Small *et al.*, 2008]. As a result, most of our knowledge about actin cytoskeletal ultrastructure is restricted to thin peripheral areas of cells, gained by the investigation of whole mount preparations.

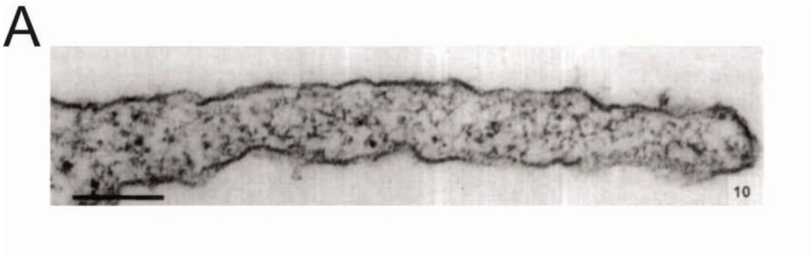

Figure 4 Vertical longitudinal section of a lamellipodium. A loss of ultrastructural arrangements of F-actin can be detected; scale bar: 200 nm [adapted from Abercrombie *et al.*, 1971].

For whole-mount procedures cells are grown directly on EM grids coated with a support film, e. g. a formvar film [Small 1988]. The cells have to be extracted with detergents like Triton to expose the cytoskeleton prior to fixation [Small *et al.*, 1999]. Chemical fixation can lead to distortion of the actin network order [Small *et al.*, 2002]. For example, the appearance of a “microtrabecular lattice” has been reported as “the cytoplasmic ground substance” [Wolosewick and Porter, 1979]. But Small *et al.* (1981) could show that the appearance of this “microtrabecular lattice” is due to the use of osmium tetroxide (OsO_4) and the dehydration steps applied in the preceding study. OsO_4, commonly used as a fixative in EM, was already shown to destroy filamentous structures *in vitro* in 1979 by Maupin-Szamier and Pollard. The harmful effects of OsO_4 can be reduced by addition of tannic acid (TA) [Maupin and Pollard, 1983]. TA was initially described in 1972 as a fixative, suitable for microtubules and intermediate filaments [Futaesaku *et al.*, 1972]. The challenge of actin preservation begins already with the choice of the right buffer conditions, as Ca^{2+} can induce F-actin depolymerization and should be chelated with EGTA [Small 1988]. Inclusion of glutaraldehyde (GA) into the lysis medium also resulted in improvement of structure [Hoglund *et al.*, 1980; Small, 1988].

Several different approaches for the visualization of the actin cytoskeleton, including negative staining, quick-freeze deep etching and critical point drying, have been used [reviewed in Small, 1988; Small *et al.*, 1999 Small *et al.*, 2008]. During negative staining cytoskeletons are dried in heavy metal salts after extraction and fixation [Small *et al.*, 1999]. Uranyl acetate (UA) delivers a strong contrast, but has a tendency to produce distortions during drying, which can be decreased by the alternative use of neutral stains such as sodium silicotungstate [Small, 1988], which however producing lower contrast. Negative staining is easy to apply, but also induces compression. Furthermore, the contrast can be variable within the same specimen [Small, 1988]. An advantage is that it can be combined with electron tomography (ET; see below) investigations, and thus deliver an overview of the 3D arrangement of the actin cytoskeleton in the leading edge [Vinzenz *et al*. 2012]. Inclusion of phalloidin into the preparation steps improves structural preservation [Small *et al.*, 1981]. I

The quick-freeze deep etch method is peformed as follows. Samples are vitreously frozen, e. g. by slamming on a cooled metal block, and then transferred into a vacuum in which the ice sublimes at low temperature and exposes the upper layers. These are than coated with platinum and the metal replica is floated off and observed by EM [Small *et al.*, 1999]. This method was used, for instance by Heuser and Kirschner (1980) for cytoskeletons of fibroblasts. The filament density in specimens prepared in this way was comparable with the results from negative staining, but filaments in lamellipodia were shorter and highly connected to each other (Fig. 5A, B) [Small, 1988].

During critical point drying, samples are dried under high pressure in Carbon dioxide, allowing the transition from liquid to gas above the critical point [Small *et al.*, 1999]. Before this, the specimen has to be dehydrated in organic solvents [Small, 1988]. Svitkina *et al*. (1995) established an improved critical point drying procedure, including a treatment with TA and UA following fixation with GA (Fig. 5C). With this method a region of highly branched networks at the leading edge was identified [Svitkina and Borisy, 1999], which was the basis for the “dendritic nucleation model” (see above). However, it was shown that this improved method leads to aggregation and induces branching of filaments [Resch *et al.*, 2002a]. In addition, it was proposed that some filament structures at the leading edge were just broken filaments [Resch *et al.*, 2002a].

With respect to this, also the recent results obtained by this method, regarding the structural organization of actin cytoskeleton in the context with clathrin-mediated endocytosis [Collins *et al.*, 2011] are questionable and need further investigations.

Beside the methods described here, several advances in cryo-EM were made. It was shown that cytoskeletons embedded in vitreous ice can be visualized by this method [Resch *et al.*, 2002b]. Furthermore Medalia *et al.* (2002) could deliver the first cryo-ET reconstructions of the actin cytoskeleton of cells being frozen without any further treatment.

In summary, several methods have been used more or less successfully to visualize the actin network. However, until now a fully comprehensive visualization of this network has not been achieved. New methods have to be developed, to answer several open questions, e. g. the structural organization of the actin cytoskeleton in thicker cell compartments.

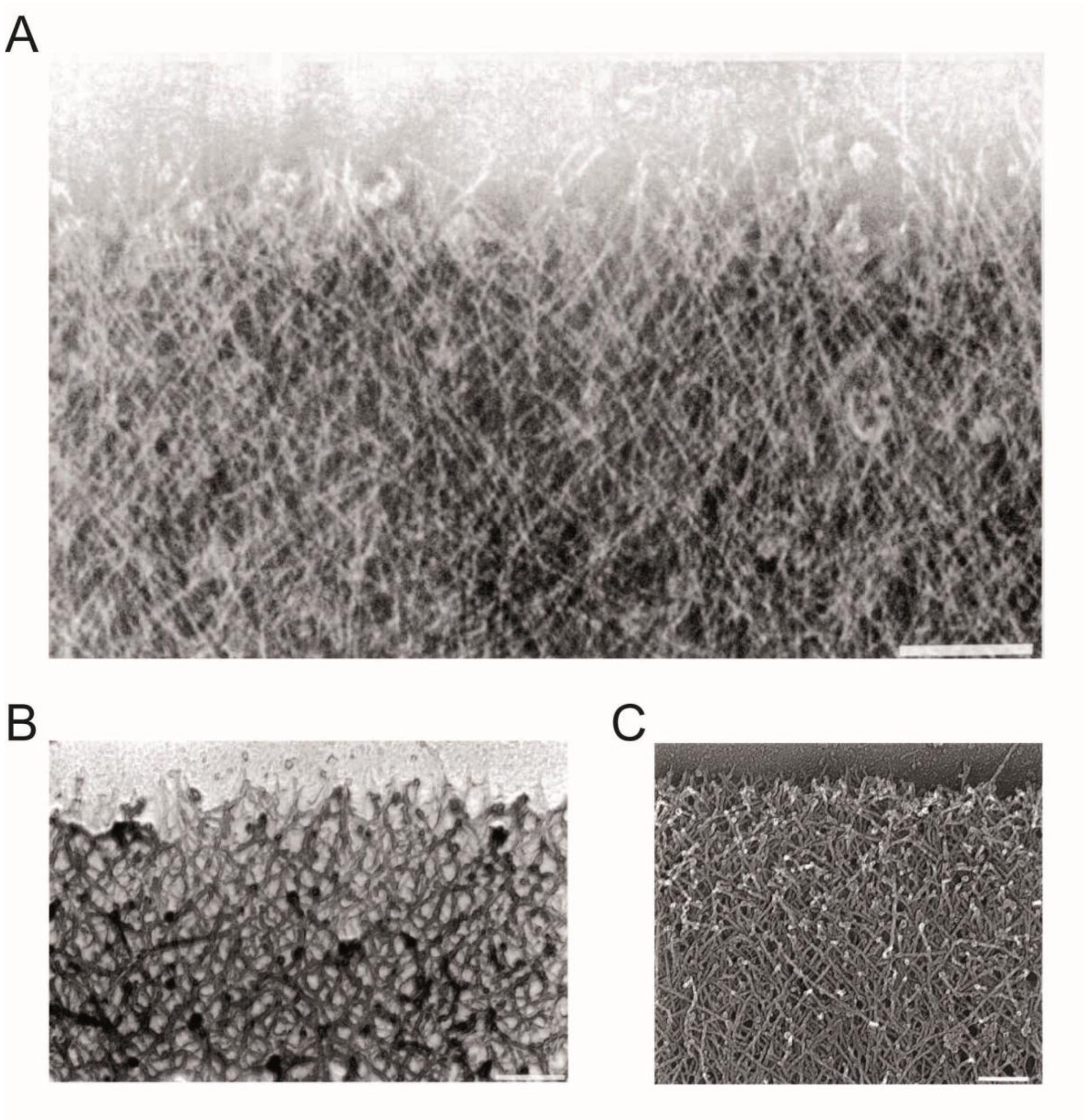

Figure 5 Electron micrographs of lamellipodia prepared by (A) negative staining, (B) deep-etch freeze drying and (C) the optimized critical point of Svitkina *et al.* (2005). (A) Continuous thin filaments are obtained by negative staining (B) deep-etch freeze drying resulted in short and highly connected filaments compared to (A). (C) Filament appearance is similar as those in (B) a brushed border containing branched short filaments is found at the leading edge?; (A, B, C): scale bars: 200 nm [adapted from Small *et al.* 1999]

1.4 High pressure freezing, freeze substitution and electron tomography

In this study, I applied a combination of high-pressure freezing (HPF) and automated freeze-substitution (AFS) for actin cytoskeleton as well as membrane preservation of plastic embedded tissue culture cells, suitable for ET investigations.

HPF uses high pressure (~2000 bar) to freeze the specimen [McDonald and Auer, 2006]. It enables vitreous freezing [Lučić *et al.*, 2013] and thus fast immobilization of molecules inside the sample. For this purpose, liquid nitrogen was used as cooling and pressure medium. The samples were placed into a holding device, filled with hexadecene to prevent a collapse of structure due to air contamination.

During subsequent freeze-substitution (FS), initially described by Simpson (1941), the vitreous frozen water in the specimen is replaced at low temperatures with an organic solvent, e. g. acetone based FS medium [Giddings, 2003]. Beside the solvents, FS media contain also chemical fixatives such as GA or OsO_4 [Hawes *et al.*, 2006]. An improvement of membrane contrast is observed by addition of TA [Giddings, 2002]. FS typically starts with a temperature around -90°C [Sobol *et al.*, 2010] which is followed by slow heating up to room temperature. This enables cross-linking through the fixatives included in the FS medium. Following AFS, samples were embedded in Epon, sectioned and investigated by ET.

ET enables the 3d reconstruction of the sample [Sali *et al.*, 2003]. For this approach a transmission electron microscope with a distinct feature is required, namely a specimen holder, which allows sample tilting perpendicular to the electron beam [Frank, 2006]. 2d images can be taken at certain angles and these can be reconstructed in a computer to generate a tomogram [Medalia *et al.*, 2002].

1.5 Aim of this study

The actin cytoskeleton is essential for diverse cellular processes including cell migration and vesicular transport. To understand the functions of these processes, visualization of the ultrastructural details is indispensable. Several EM approaches have shown that conventional chemical fixation and plastic embedding can lead to distortions of actin filament networks. Thus, our knowledge concerning the actin cytoskeleton is restricted to the peripheral regions of cells processed without embedding that are thin enough to be penetrated by the electron beam. In addition, cells have to be extracted with detergent to expose their cytoskeleton.

As a result of these limitations, we lack structural informations of actin filaments interacting with the plasma membrane or vesicles and of the arrangements of actin in thicker compartments, for instance of cells migrating in a 3d environment. To this end, I wanted to develop a new plastic sectioning approach, allowing both the preservation of membranous compartments and actin network organization. This approach should be suitable for ET, enabling the investigation of 3d organization of these structures. Ultrastructural information already gained by ET of negatively stained or vitreously frozen lamellipodia served thereby as reference for the degree of preservation.

2. Material & Methods

2.1 Cell culture

NIH/3T3 cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM; SIGMA) supplemented with 10% fetal bovine serum (FBS; SIGMA), 2 mM L-glutamine (SIGMA), 1 mM sodium-pyruvate (GIBCO) and 1% penicillin/streptomycin (pen/strep; SIGMA) as adherent cultures in 25 cm^2 tissue culture flasks (Thermo Scientific) at 37°C in a humid environment in the presence of 5% CO_2 in a *HERA cell 150* (Thermo Scientific) incubator.

2.2 High-pressure freezing

NIH/3T3 cells were seeded on sapphire discs (Engineering Office M. Wohlwend GmbH) coated with 0,01% Poly-L-Lysine (SIGMA) and grown until they attached (1-2 hours (h)) in the medium described above. After attachment cells were serum starved by incubation in Dulbecco's Modified Eagle's Medium (DMEM; SIGMA) supplemented with 2 mM L-glutamine (SIGMA), 1 mM sodium-pyruvate (GIBCO) and 1% penicillin/streptomycin (pen/strep; SIGMA) to induce cell spreading. Cells were either (1) frozen directly or (2) extracted using lysis medium containing Triton X-100 (AppliChem; concentrations as indicated in the result section) and 0,25% GA (Electron Microscopy Sciences) in cytoskeleton buffer (10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$, pH 6,1) for 1 minute (min) at room temperature (RT). Freezing was done as follows: The sapphire discs were placed with the cells facing upwards on a holder with a polished surface (no.:479; Engineering Office M. Wohlwend GmbH) and covered with a second holder bearing a concave surface (0,025 mm deep, no.:289; Engineering Office M. Wohlwend GmbH). Hexadecene was used as a cryoprotectant. Freezing was done in *HPF Compact 01* (Office M. Wohlwend GmbH). Specimens were stored in liquid nitrogen until FS.

2.3 Freeze-substitution

The AFS-device *Leica EM AFS2* (Leica) was used for FS. Frozen specimens were transferred into a cryo tube containing FS medium comprising 0,5% GA (Electron Microscopy Sciences) and 0,2% TA (Mallinckrodt) in anhydrous Acetone (Merck) pre-cooled to -90°C in the AFS-device. After incubation for 36 h specimens were either (1) warmed up to -25°C in steps of 5°C/h, incubated at this temperature for 12 hours and further warmed up to +25°C in steps of 5°C/h or (2) warmed up to -24°C in steps of 5°C/h, washed 3x with pre-cooled (-24°C) anhydrous acetone (Merck) and transferred to a second pre-cooled (-24°C) FS medium containing 0,5 % UA (Electron Microscopy Sciences) in anhydrous acetone (Merck) and warmed up to +25°C in steps of 5°C/h after 16 h incubation. After FS specimens were embedded in epon (agar 100 resin; Agar Scientific).

2.4 Chemical fixation

For chemical fixation NIH/3T3 cells seeded on sapphire discs (Engineering Office M. Wohlwend GmbH) coated with 0,01% Poly-L-Lysine (SIGMA), were allowed to attach and subsequently serum starved (see above). Cells were extracted with lysis medium containing 1% Triton X-100 (AppliChem) and 0,25% GA (Electron Microscopy Sciences) in cytoskeleton buffer (10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$, pH 6,1) for 1 min and fixed with 2% GA and 0,2 % TA for 30 min at RT either (1) dehydrated in a graded series of acetone (50%, 75%, 90%, 2x anhydrous acetone; incubation: 5 min in each concentration on a shaker at RT) or (2) high-pressure frozen (see above) and freeze-substituted in anhydrous acetone (Merck) according to protocol (1) described above. Specimens were than embedded in epon (agar 100 resin; Agar Scientific).

2.5 Embedding

Specimens prepared by the methods described were washed 3x with anhydrous acetone for 5 min and infiltrated first with a 2:1 anhydrous acetone (Merck)/epoxy resin (agar 100 resin; Agar Scientific) mixture for 15 min on a shaker at RT, followed by a 1:2 anhydrous acetone (Merck)/epoxy resin (agar 100 resin; Agar Scientific) mixture for 45 – 60 min on a shaker at RT. Finally, infiltration with the epoxy resin (agar 100 resin; Agar Scientific) alone followed (60 min). The resin was polymerized at 60°C for 48 h.

2.6 Sectioning

Prior to sectioning sapphire discs were flipped away with the edge of a razorblade after short cooling (~1 second) the epon block. Blocks were trimmed to pyramid at an *Ultracut E* microtome (Reichert-Jung) equipped with a glass knife, without touching the specimen surface. Sections of 200 nm were cut with an *Ultra 45°* diamond knife (DIATOME) parallel to the substrate. Sections were allowed to spread in the knife boat filled with ddH_2O and were picked up with 100 mesh copper/palladium grid (Agar Scientific) supported with a formvar film. After drying at RT, sections were post-stained with 2% aqueous UA for 60 min and after rinsing with deionised water Reynolds lead citrate [Reynolds, 1963] for 5 min. Sections were rinsed with deionised water again and dried at RT. Grids were fixed upright in a holding device and incubated with colloidal gold (10 nm; British BioCell International), allowing attachment of the gold particles on both grid sides. After rinsing with deionised water, sections were dried at RT.

2.7 Electron microscopy

For EM/ET a *FEI Tecnai F30 Helium Polara* TEM was used. The microscope was cooled to -190°C, operated at 300kV and was equipped with *UltraScan 4000* CCD camera (Gatan). Tilt series were acquired using *SerialEM 3.x.* and the average tilt range was -60° to +60° images with a magnification of 23.000x. Images were acquired in 1°steps.

With the gold particles as fiducials for alignments, re-projections from the tilt series utilizing IMOD software (available as freeware: http://bio3d.colorado.edu/imod/). Tomograms comprised typical a Z-stack of 65-130 sections of about 0,9 nm each. IMOD was also used for model generation.

3. Results

3.1 Sectioning

For sectioning the diamond knife had to be aligned perfectly to collect the first section of the specimen. The first section should contain the thin peripheral lamellipodal region of cells, which allows to estimate the degree of preservation of the fragile actin network (Fig. 6). Specimens showing typical lamellipodia regions, like in Fig. 6, were used for ET.

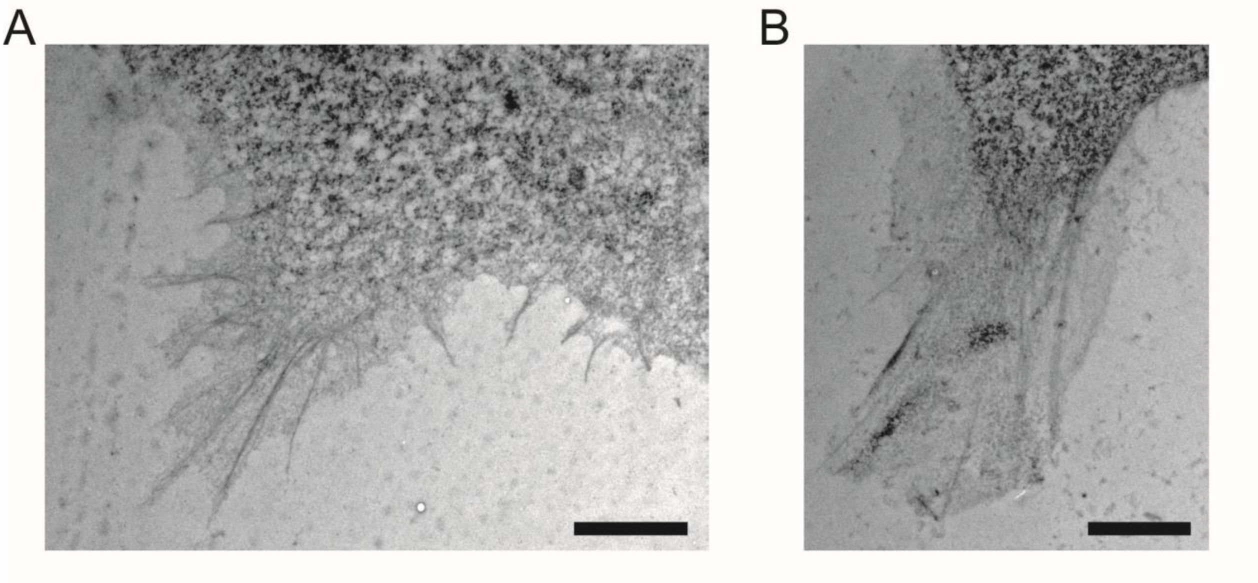

Figure 6 Low magnification electron micrographs as sectioning controls. (A) and (B) show lamellipodal regions of NIH/3T3 sectioned parallel to the substrate, cells were extracted with 0,1 %Triton, prior to freezing and FS (protocol (1)); *Scale bars: 5 µm*

3.2 Structure preservation of unextracted cells

Freezing cells without prior extraction and FS substitution in acetone supplemented with 0,5% GA and 0,2% TA allowed the preservation of membranous, like mitochondria, vesicles and the plasma membrane as well as structural elements, like microtubules (Fig. 7 A, B and 8). Bundles of F-actin appeared as relatively straight dark lines. Their extensions into filopodia could be observed despite the reduced contrast, which was due to the high cytosolic background (Fig. 8). The plasma membrane of the cell appeared as 2 black dense lines separated by about 7 nm (Fig 8 B, inset). Analysis of the electron tomograms of mitochondria revealed two membranes, the outer and the inner with the typical cristae (Fig 7 A, B). Modelling of the structures gave an insight into the degree of three dimensionality of the section, as well as the level of preservation (Fig. 7 C).

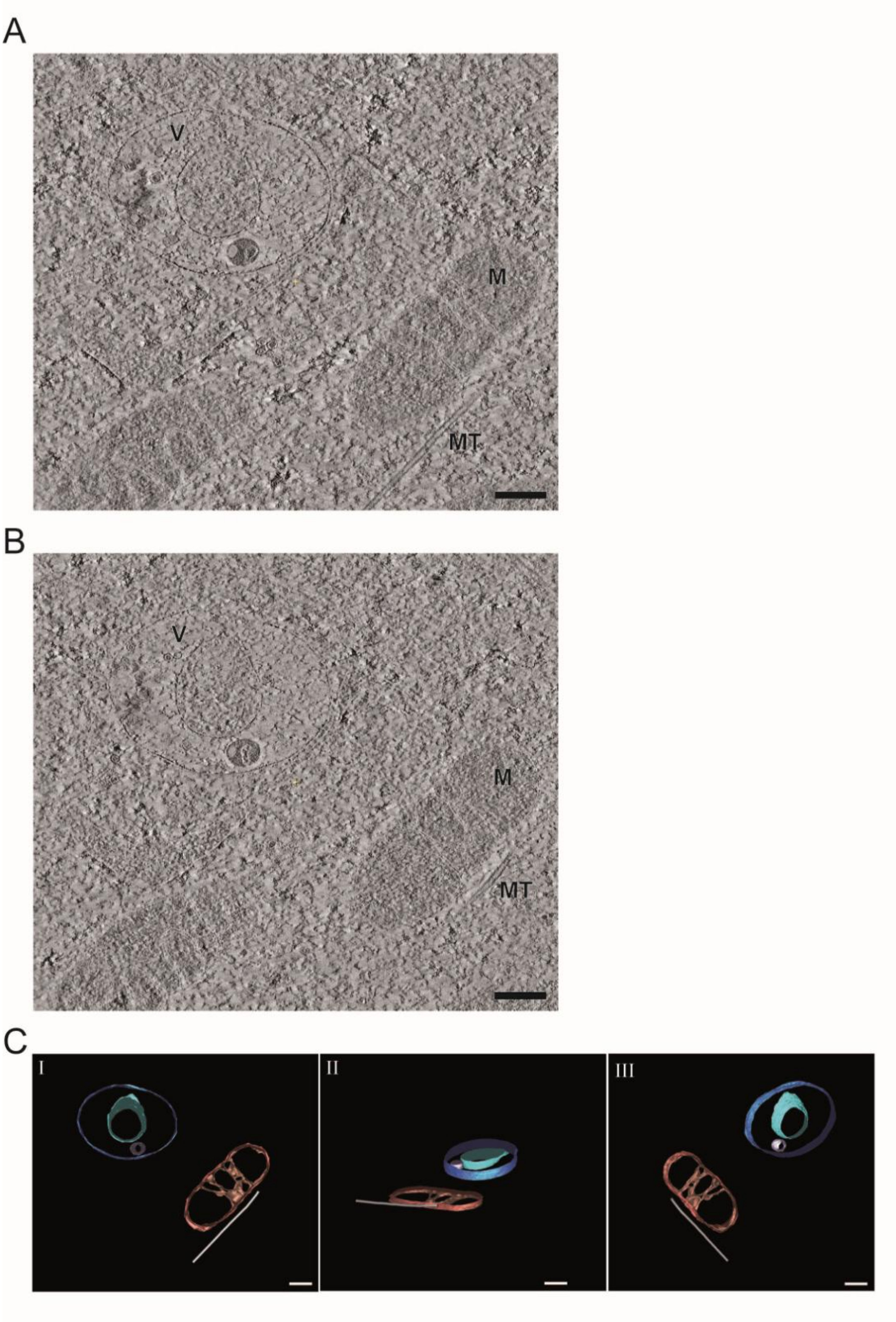

Figure 7 Overall preservation of unextracted cells. (A) and (B) 10 sections each of a tomogram in different z-positions, frozen without extraction and freeze-substituted in GA/TA. Structures are indicated with letters: mitochondria (M), microtubule (MT), vesicle (V). (C) shows a model of the tomogram in (A) and (B) turned in different angles: I front view, II side view, III back view; *Scale bars: 200 nm*

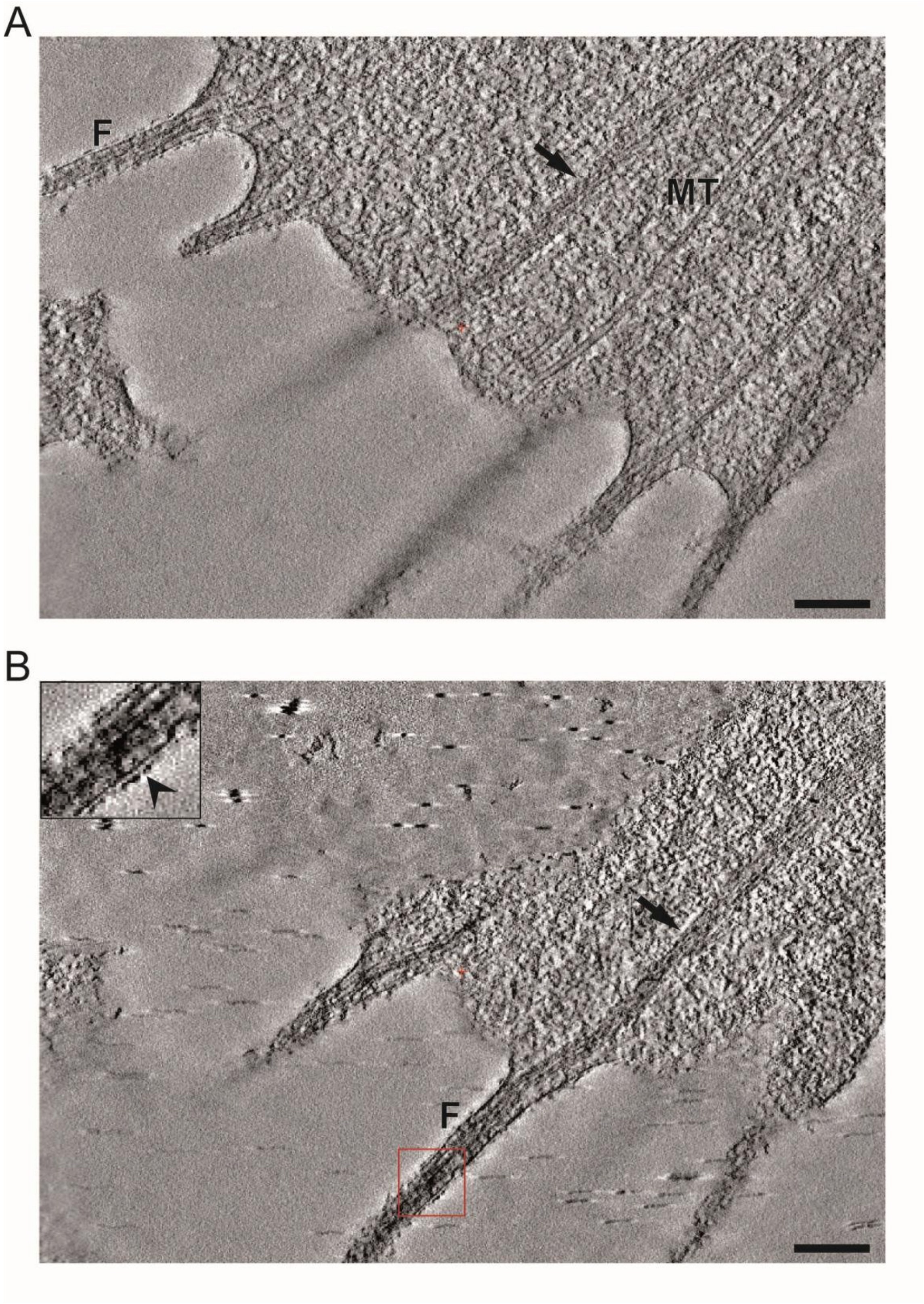

Figure 8 Preservation of F-actin in unextracted cells. (A) and (B) 10 sections each of a tomogram in different z-positions, frozen without extraction and freeze-substituted in GA/TA. The tomogram shows extended filopodia (F) containing bundles of F-actin (arrow) and a microtubule (MT). Inset in (B) shows a magnification of the area indicated with the red rectangle. The plasma membrane appears as 2 dark lines separated by a bright area; *scale bars: 200 nm*

3.3 Extraction enhances contrast of F-actin

As seen above, actin filaments in cells frozen and freeze-substituted without any further treatment showed a reduced contrast due to the high cytosolic background. With the aim to enhance the contrast by reduction of this background, several extraction procedures were tried. Extraction of cells with 0,5% Triton X-100 before freezing and FS led to a total loss of plasma membrane as well as an enhancement of F-actin contrast (Fig. 9). Actin filaments appeared straight and well contrasted; also branch sites were visible.

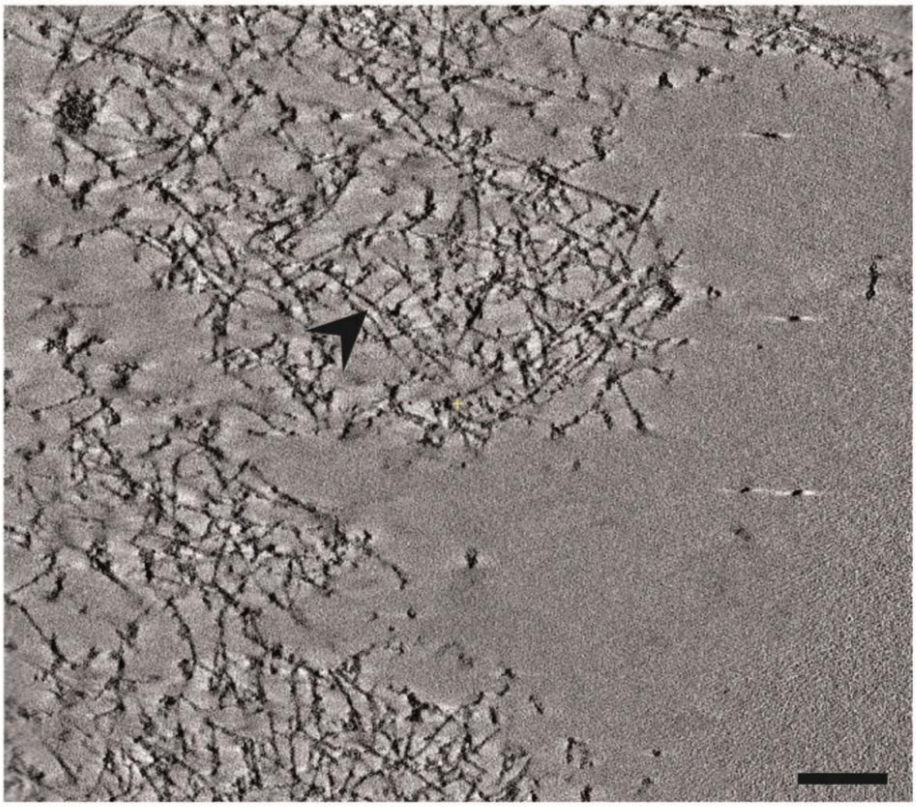

Figure 6 Preservation of F-actin in totally extracted NIH/3T3 cells. 15 sections of a tomogram. Specimens were frozen after extraction with 0,5% Triton X-100 in lysis medium and freeze-substituted in GA/TA. The tomogram was recorded in a lamellipodial region; the plasma membrane is totally lost; the contrast of F-actin is enhanced. Arrowhead points on a branch side; *scale bar: 200 nm*

Pre-treatment of cells with 0,1% Triton X-100 facilitated both, preservation of actin cytoskeleton and residues of the plasma membrane (Fig. 10).

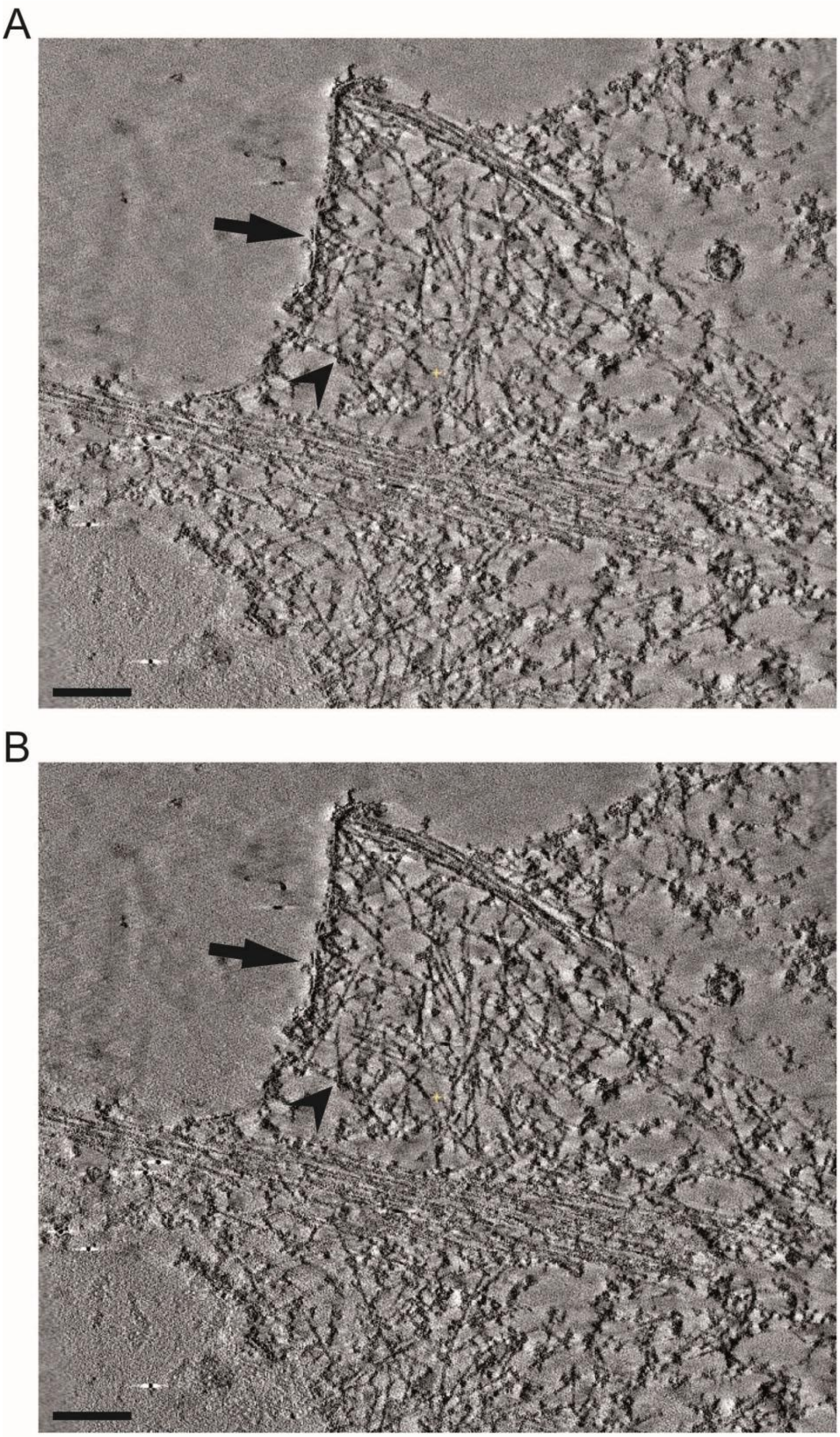

Figure 7 Preservation of F-actin in partially extracted cells. (A) and (B) stacks of 15 sections each of a tomogram at different z-positions, Specimens were frozen after extraction in lysis medium consisting 0,1% Triton X-100 and freeze-substituted in GA/TA. The tomogram was recorded in a lamellipodial region. Actin filaments appear straight and well contrasted. Arrowhead indicates a branch site; arrow, the plasma membrane residue; *scale bars: 200 nm*

In addition, clathrin coated vesicles connected to stress fibers and clathrin cages could be visualized (Fig. 11).

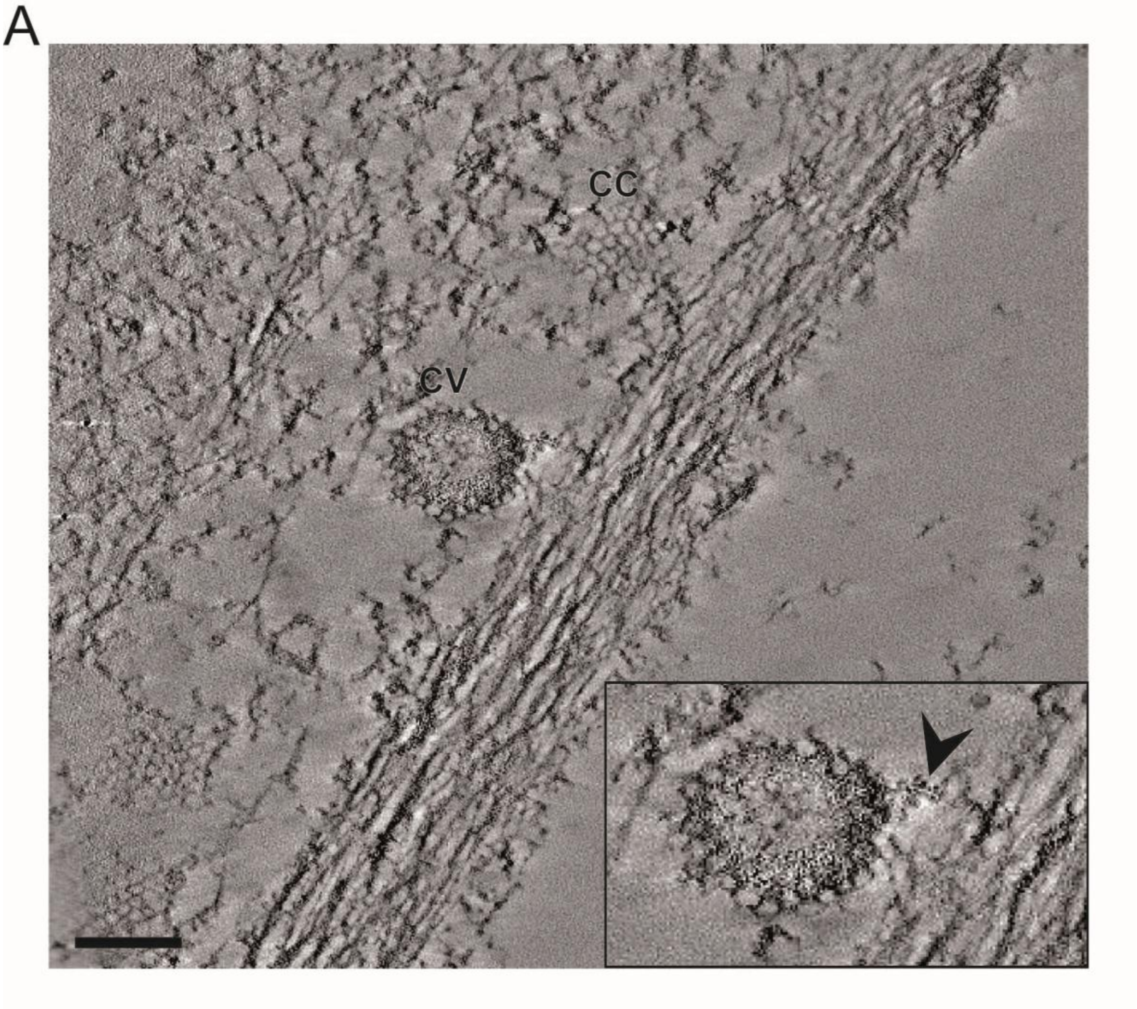

Figure 8 Preservation of clathrin coated vesicles in extracted NIH/3T3 cells. 20 sections of a tomogram, processed as in Fig. 10. The tomogram was recorded in a thicker area in the cell center, showing a clathrin coated vesicle (CV) attached to a stress fiber and clathrin cages (CC). Inset shows a magnification of the CV; arrowhead marks the putative connection to the stress fiber; *scale bar: 200 nm*

3.4 Uranyl acetate induces thickening of F-actin

With the aim to further enhance the contrast; UA was included in the FS process (according to protocol (2)). Actin filaments in specimens treated in that way appeared thicker and aggregated in contrast to cells, without exposure to UA (Fig. 12). In addition the network order seemed distorted and filaments were curved.

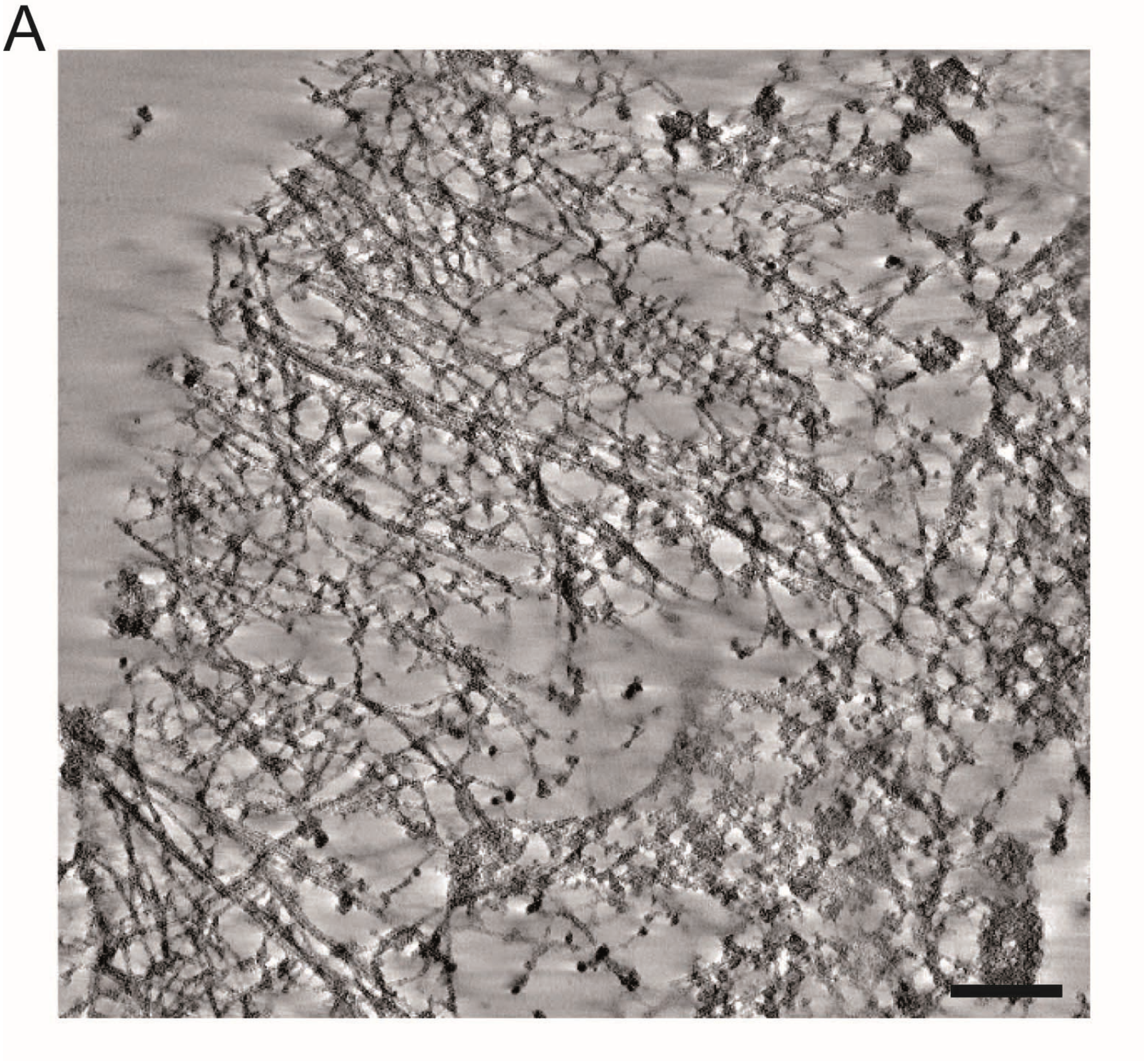

Figure 9 Inclusion of UA into FS induces distortions in actin network order. Stack of 15 sections of a tomogram, frozen after extraction with 0,1% Triton X-100 in lysis medium and freeze-substituted in GA/TA followed by UA (FS-protocol (2)). The tomogram was recorded in a lamellipodial region; the plasma membrane is totally lost. Actin filaments appear aggregated and distorted; *scale bar: 200 nm*

3.5 High-pressure freezing and freeze substitution are essential for structural preservation of actin filaments

In order to prove if freezing followed by FS is needed for structure preservation of actin, specimens were chemically fixed with a TA containing fixative and dehydrated in graded series of anhydrous acetone (Fig. 13 A) or frozen and dehydrated during FS in anhydrous acetone (Fig. 13 B) prior to embedding in epon. This treatment led to complete loss in actin network structure in the case of specimens dehydrated at RT. Samples dehydrated at low temperature during FS showed better preservation, but filaments appeared aggregated and their 3d organization was totally distorted.

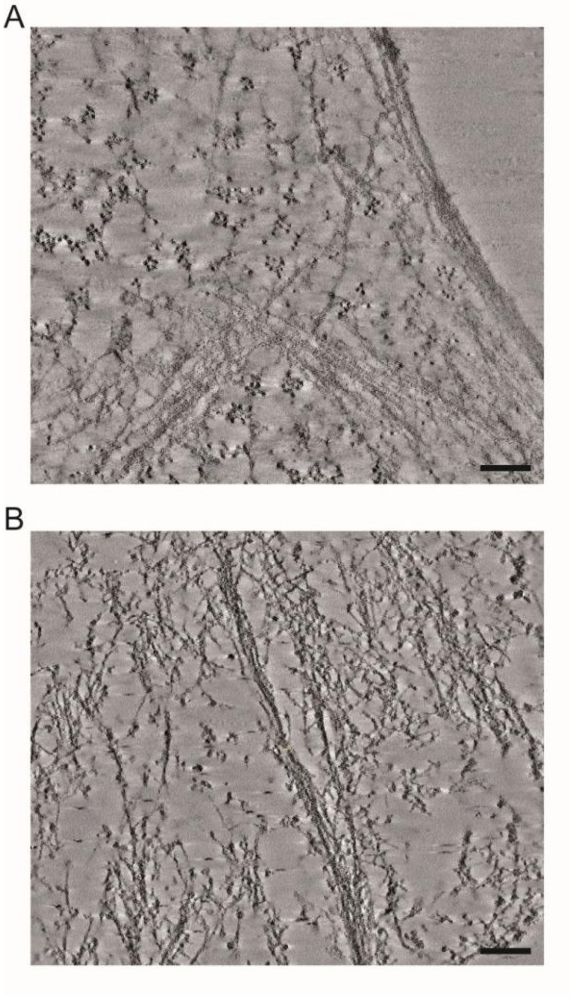

Figure 10 Chemical fixation followed by dehydration led to distortions of the actin cytoskeleton (A) and (B) stacks of 15 sections each of a tomogram at different z-positions, NIH/3T3 cells were chemically fixed, dehydrated at RT (A) or frozen and dehydrated during FS. The tomograms were recorded in peripheral cell regions; *scale bars: 200 nm*

4. Discussion

4.1 Comparison of the applied approaches

Actin is an essential component of several processes in cells, including migration and intracellular transport [reviewed in Pollard and Cooper, 2009]. To understand its function in these processes, a detailed knowledge of its 3d network organization is crucial. To achieve this, several approaches have been applied. It has been shown that whole mount preparations of extracted cells in which membranes are removed by detergent best reveal the organization of actin filaments in thin regions of the cytoplasm [reviewed in Small, 1988]. As a result, we lack structural information regarding actin-membrane interactions as well as of actin in thick cell compartments. In this study I wanted to develop a new method for actin preservation suitable for ET investigations. To overcome the specimen thickness problem existing in EM, I decided to go for a plastic sectioning approach. Different sectioning methods were already tried by others [Abercrombie *et al.*, 1971; Yamada et al., 1971], but none of these showed a satisfying structural preservation of all F-actin populations, especially in the lamellipodium. For fixation I used a combination of HPF and FS. Standard FS-media commonly contain GA or OsO_4 [Hawes *et al.*, 2006; McDonald and Auer, 2006]. But OsO_4 was shown to destroy actin filaments [Maupin-Szamier and Pollard, 1979; Small *et al.*, 1981]. Therefore, I developed a new FS-medium. Best results were obtained by the use of a FS-medium containing 0,5% GA and 0,2% TA in acetone, which by coincidence resembles the medium used by Giddings to visualize membranes (2003). TA is described to stabilize actin filaments [Begg *et al.,* 1978; Maupin and Pollard, 1983] and to preserve and stain membranes [Maupin and Pollard, 1983; Giddings, 2003]. Applying this medium, I could obtain improved 3d preservation of the plasma membrane and several intracellular structures including actin filaments. Unfortunately, these filaments revealed a reduced contrast due to high cytosolic background. To overcome this problem, I tried several extraction approaches prior to freezing. Extraction of cells followed by cryo-fixation was used by Resch *et al.* (2002b) who included GA into the lysis medium to improve structural preservation [Small, 1988]. Treating cells with 0,5% Triton X-100 totally extracted the cell membranes and revealed a well preserved actin cytoskeleton. I found that reduction of the Triton X-100 concentration to 0,1 % enabled in addition the partial preservation of the plasma membrane. Branch sides could also be observed with this method, but a high branch density at the leading edge as described by Svitkina and Borisy (1999) could not be observed, consistent with the findings of Vinzenz *et al.* (2012). This supports the idea that short filaments in branched arrays are not concentrated at the lamellipodium front as depicted in the “dendritic nucleation model”. To further enhance the contrast in the sections another variation was introduced into the FS-protocol. The specimens were transferred into a second FS-medium containing UA in acetone following washing in acetone and preliminary FS substitution in GA/TA. UA is commonly used for freeze-substitution, contrasting of sections and negative staining [Small; 1988; McDonald and Auer, 2006]. Inclusion of UA into the FS procedure resulted in a thickening or aggregation of filaments. These distortions could arise from: 1) additional dehydration during the washing steps and the prolonged FS procedure or 2) aggregation in TA and UA [Small *et al.*, 1981; Small, 1988]. The problem of additional dehydration could possibly be avoided by the addition of phosphotungstic acid, since it is described to improve the ultrastructure [Sakai *et al.*, 2005].

To test whether the freezing and FS are essential for structural preservation, two additional approaches were used. Cells were extracted followed by Fixation with GA and TA. Concentrations of these substances were chosen according to Svitkina and Borisy (1998). The specimens were then either dehydrated in a graded acetone series at RT or high-pressure frozen and dehydrated by applying FS. Specimens obtained by the RT-dehydration method revealed a total loss in structure, most likely due to dehydration [Small *et al.*, 1981; Small, 1988]. Dehydration during FS marginally improved structure preservation. This indicates that the low temperature reduces the harmful effects of dehydration and that HPF together with FS are essential during the specimen preparation.

In conclusion, I evaluated several approaches for preparing tissue culture cells with respect to the resulting structural preservation of actin filaments and the plasma membrane after plastic embedding (summarized in Tab. 1). The best results were obtained by extracting the cells with lysis medium containing 0,1 %

Triton X-100 and 0,25 % GA in cytoskeleton buffer followed by HPF and FS in GA/TA.

Table 1 Summary of the results obtained by the applied approaches

Extraction	Fixation	Preservation		Contrast	
		F-actin	Plasma Membrane	F-actin	Plasma Membrane
-	HPF, FS (GA/TA)	+	+	-	+
0,5% Triton X-100	HPF, FS (GA/TA)	+	-	+	-
0,1% Triton X-100	HPF, FS (GA/TA)	+	+	+	+
0,1% Triton X-100	HPF, FS (GA/TA/UA)	-	+	+	+
1% Triton X-100	Chemical Fixation + RT-dehydration	-	-	-	-
1% Triton X-100	Chemical Fixation + HPF, FS (acetone)	-	-	-	-

4.2 Additional note on structural preservation

As described above the best results were obtained by extraction with low Triton X-100 concentrations prior to HPF and FS in a GA/TA containing medium. Besides F-actin and the plasma membrane also clathrin-coated vesicles, located in central cell compartments could be preserved and visualized in that way. An improvement of their preservation and staining was previously observed in plastic sections treated with GA/TA by Maupin and Pollard, (1983), but not with the quality of preservation achieved in the present study and without 3D information.

The 3d appearance of the vesicles and the clathrin cages localized nearby were comparable to those provided by quick freeze deep etching (Fig. 14) [Heuser and Anderson, 1989], but the clathrin coat as well as the surrounding appeared more fine. In addition, the filament density was higher. A recent EM study also addressed the structural organization of actin interacting with clathrin coated vesicles [Collins *et al.*, 2011]. Here, the improved critical point drying method described in Svitkina *et al*. (1995) was used. It would be of interest to systematically compare the structural preservation of clathrin coated vesicles interacting with actin obtained by the method developed here and the method used by Collins *et al.*, (2011).

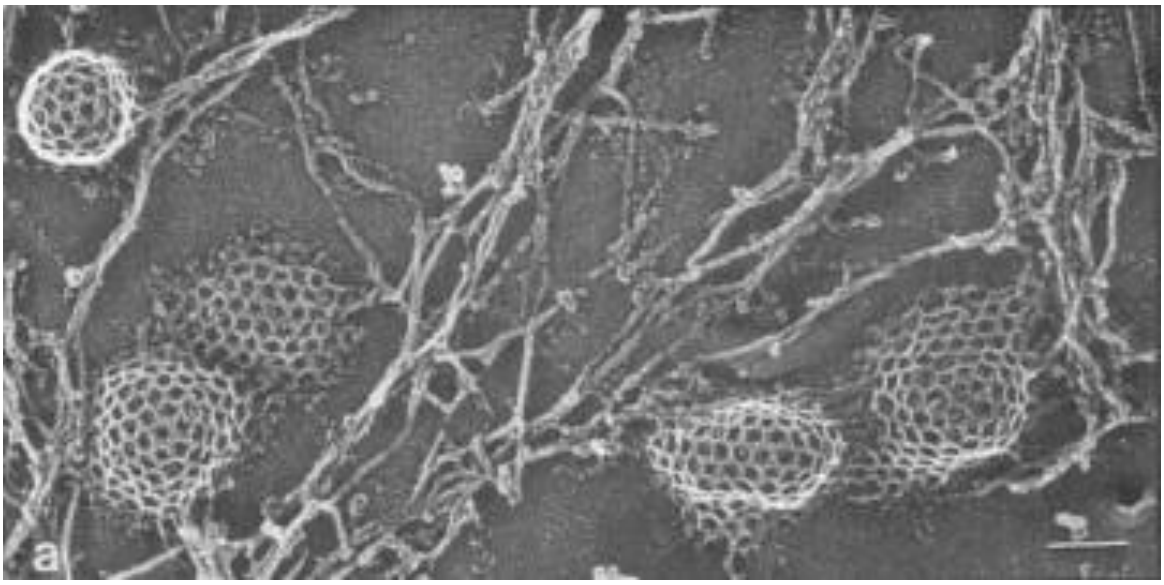

Figure 11 Clathrin coated vesicles and cages in a chick fibroblast. Sample was prepared with the quick freeze deep etching method [adapted from Heuser and Anderson, 1989]; *scale bar: 100 nm*

4.3 Limitations

Beside the advantages described so far, the developed method revealed also some drawbacks. The whole process is relatively complicated and time consuming in comparison to e.g. negative staining. Furthermore, no quality control could be done in between the single steps. The most challenging part is the sectioning of the specimens. This needs a lot of experience, but it should be possible to dissect whole cells by serial sectioning, allowing a 3d reconstruction. Another error-prone step is freezing and FS. After freezing specimens have to be transferred fast into liquid nitrogen and subsequently into the FS-medium without warming them. Extensive training and routine is needed to apply this method with high reproducibility.

4.4 Outlook

The procedure described here enables for the first time the simultaneous preservation of actin cytoskeleton as well as plasma membrane of plastic embedded tissue culture cells. In combination with other results, obtained for example by negative staining or cryo-ET, it can provide new insights into versatile functions of the actin cytoskeleton. Furthermore, this method can be the starting point for the development of a method with the aim to investigate the structural organization of cells migrating in a 3d context.

Appendix

Abstracts

Deutsch

Konventionelle Fixierung und Plastikeinbettung von Zellen für die Elektronenmikroskopie führt zur Zerstörung von Actin Netzwerken migrierender Zellen. Das sowie die limitierte Penetrationstiefe in der Transmissionselektronenmikroskopie haben die Analyse des Actin Zytoskeletts auf die dünnen peripheren Lamellipodien von Zellen in Monoschichtkulturen beschränkt, welche zusätzlich mit Detergenzien extrahiert werden müssen um das Zytoskelett frei zu legen. Daraus resultierend fehlen uns Strukturinformationen von Zellen die sich in einer 3d Matrix bewegen, über die räumlichen Interaktionen von Actin Filamenten mit der Plasmamembran sowie, über die strukturelle Organisation von zentralen Zellbereichen. Aus diesem Grund habe ich eine neue Methode zur Herstellung von in Plastik eingebetteten Schnittpräparaten entwickelt. Hierbei wurden Zellen in Monoschichtkulturen zunächst mit einer niedrigen Triton X-100 Konzentration extrahiert und nach Hochdruckgefrieren in einem Aceton basierendem Medium, welches Tanninsäure und Glutaraldehyd enthielt, gefriersubstituiert. Dies ermöglichte eine simultane Erhaltung der Membran und des Actin Zytoskeletts für elektronentomographische Untersuchungen.

English

Conventional fixation and plastic embedding of tissue culture cells for electron microscopy leads to destruction of actin filament networks of migrating cells. This situation, together with limitations set by the penetration depth in transmission electron microscopy, has restricted analysis of actin network architecture to the thin peripheral lamellipodia of whole cells in monolayer culture that have been extracted with detergent to expose the cytoskeleton. As a result, we lack structural information on cells moving in 3d matrices as well as on the spatial relationship of actin filaments with the plasma membrane and structural organization of central cell compartments. To this end, I developed a new plastic sectioning approach, in which monolayer tissue culture cells were extracted with a low Triton X-100 concentration prior to high-pressure freezing followed by freeze-substitution in an acetone based freeze-substitution medium containing tannic acid and glutaraldehyde. This approach enabled simultaneous structural preservation of membranous compartments together with the actin cytoskeleton for electron tomography investigations.

Curriculum vitae

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Education

Since Oct. 2011	Master study of Molecular Biology at University of Vienna (Austria)
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Tables

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Abbreviations

2d	two-dimensional
3d	three-dimensional
ADP	adenosine diphosphate
AFS	automated freeze-substitution
Arp2/3	actin related protein 2/3
ATP	adenosine triphosphate
Cobl	Cordon bleu
DMEM	Dulbecco's Modified Eagle's Medium
Ena/VASP	Enabled/vasodilator-stimulated phosphoprotein
EM	electron microscopy
ET	electron tomography
F-actin	filamentous actin/actin filaments
FBS	fetal bovine serum
FS	freeze-substitution
GA	glutaraldehyde
G-actin	globular actin/monomeric actin
h	hours
HPF	high-pressure freezing
Lmod	Leiomodin
min	minutes
OsO_4	osmium tetroxide
pen/strep	penicillin/streptomycin
RT	room temperature
TA	tannic acid
TEM	transmission electron microscopy
UA	uranyl acetate

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