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Dynamics and organization of actin-labelled granules as a rapid transport mode of actin cytoskeleton components in Foraminifera

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Abstract. Recent advances in fluorescent imaging facilitate actualistic studies on organisms used for palaeoceanographic reconstructions. Observations of cytoskeleton organization and dynamics in living foraminifera foster understanding of morphogenetic and biomineralization principles. This paper describes the organisation of a foraminiferal actin cytoskeleton using *in vivo* staining based on fluorescent SiR-actin. Surprisingly, the most distinctive feature in the organisation of actin in Foraminifera is the prevalence of actin-labelled granules (ALGs) within pseudopodial structures. Fluorescent signal obtained from granules dominate over dispersed signal from the actin meshwork. Actin-labelled granules are small (around 1 µm in diameter) actin-rich organelles demonstrating a wide range of motility behaviours from almost stationary oscillating around certain points to exhibiting rapid motion. These structures are present both in Globothalamea (*Amphistegina*, *Ammonia*) and Tubothalamea (*Quinqueloculina)*. They are found to be active in all kinds of pseudopodial ectoplasmic structures, including granuloreticulopodia, globopodia, and lamellipodia, as well as within the endoplasm itself. Two hypotheses regarding their function are proposed: (1) They are involved in endocytosis and intracellular transport of different kinds of cargo; (2) They transport prefabricated and/or recycled actin fibres to the sites where they are needed. These hypothesis are not mutually exclusive. The first hypothesis is based on the presence of similar actin structures in fungi, fungi-like protists and some plant cells. The later hypothesis is based on the assumption that actin granules are analogous to tubulin paracrystals responsible for efficient transport of tubulin. Actin patches transported in that manner are most likely involved in maintaining shape, rapid reorganization, and elasticity of pseudopodial structures, as well as in adhesion to the substrate. Finally, our comparative studies suggest that a large proportion of actin-labelled granules probably represent fibrillar vesicles and elliptical fuzzy coated vesicles often identified in TEM images. Correlative fluorescent electron microscopic observations are proposed to verify this interpretation.

1 Introduction

Since Foraminifera were firstly recognized by science in the beginning of 19th century, thanks to works of d'Orbigny (Lipps et al., 2011), they became subject of extensive studies. Most Foraminifera species create shells (tests) that have great potential

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for preservation in the fossil record, they are important primarily for Earth Sciences. Application of foraminiferal research includes among others biostratigraphy, paleoclimatology, paleo-environmental studies and oil and gas exploration. As a consequence, morphology, geochemical composition and evolution of their tests are much better understood than their biology. However, to properly understand fossils, it is essential to take into account the physiology of the living organisms. Recognition of this problem together with advances in research methods has led to an increasing number of studies concerning ultrastructure of foraminiferal cytoplasm and its role in biomineralisation (e.g. Spero 1988; de Nooijer et al., 2009; Tyszka et al., 2019).

Cytoplasm in Foraminifera can be divided into two parts/sections: ectoplasm (outside the test) and endoplasm (inside the test) (e.g., Boltovskoy and Wright, 2013). They differ not only in location relative to the test, but also in composition and appearance under the light microscope: endoplasm is much thicker and usually is coloured even in the non-symbiotic species, ectoplasm is less dense and transparent. In addition, many organelles such as nucleus, ribosomes, Golgi apparatus are reported to occur only in endoplasm (Bowser and Travis, 1991). Most prominent ectoplasmic structures in Foraminifera are pseudopods, which have characteristic granular appearance, distinguishing Foraminifera from amoeba such as *Gromia* (Cavalier-Smith et al., 2018). This versatile network of branching pseudopods is involved in motility (Kitazato, 1988), feeding, construction of the test and responding to environmental stimuli (Goldstein, 1999). As granuloreticulopodia are typically the outermost part of foraminiferal cell these structures must fulfil a crucial role in that process. The presence of granuloreticulopodia is the most fundamental morphological feature of Foraminifera and it must have appeared very early in the evolutionary history of this group (Pawlowski et al., 2003). Foraminifera probably owe much of their evolutionary success to this versatile structure.

Despite numerous studies concerning structure and function of granuloreticulopodia, many aspects of their organization and physiology are still unclear. The most striking reticulopodial features are fine granules that exhibit various behaviours. Granules are moving rapidly along threads of pseudopods and even along a single thread they exhibit movement in both directions (Jahn and Rinaldi, 1959; Kitazato, 1988). There are numerous different categories of granules including food particles (phagosomes), defecation vacuoles, mitochondria, dense bodies, clathrin-coated vesicles, elliptical vesicles (Bowser and Travis, 1991). Granuloreticulopodia are not the only forms of exoplasmic (pseudopodial) structures present in Foraminifera. Pseudopodial structures are also represented by lamellipodia (*sensu* Travis et al., 1983; Tyszka et al., 2019), globopodia and frothy pseudopodia (*sensu* Tyszka et al., 2019). All these pseudopodial structures are highly functional that is well expressed by their different morphologies and temporal organization linked to life strategies and behaviours.

Previous studies have shown that pseudopodial structures in Foraminifera depend on cytoskeleton organization that includes microtubules (built from tubulin proteins) and actin filaments (Travis et al., 1983; Koonce et al., 1986b; Tyszka et al., 2019). Latest investigations on morphogenesis of foraminiferal shells revealed that chamber formation and biomineralization are directly supported by actin meshworks and closely associated with microtubular networks (Tyszka et al., 2019). The same study also reported granularity of actin detected under fluorescent light of live actin stained foraminifera. This active, bi-directional granular organisation of actin was observed in all types of pseudopodial structures, including reticulopodia, as well as globopodia and lamellipodia during chamber formation of *Amphistegina lessonii* d'Orbigny. Motile granules followed relatively straight and often anastomosing tracks (Tyszka et al., 2019, movies S1-S6). However, the authors neither focused on this aspect of actin organisation nor on its dynamics. Structural and functional relationships between actin meshworks and their association with actin granularity have never been described nor interpreted.

This paper is an attempt to fill the gap in our knowledge on actin organisation and dynamics in Foraminifera. Therefore, main objectives of this study include:

(a) live fluorescent labelling of actin within ectoplasmic (pseudopodial) structures during various behavioural and/or physiological activities;
(b) live fluorescent co-labelling of mitochondria to check selectivity of granules labelling via testing the possibility of mitochondrial co-localization with actin-labelled structures;
(c) identification and detailed description of the actin cytoskeleton organisation in Foraminifera with particular focus on its granularity by means of fluorescence imaging;
(d) assessment of unspecific labelling risk in order to evaluate reliability of staining results;
(e) comparative analysis of published images of cytoplasmic foraminiferal ultrastructure observed in Transmission Electron Microscope (TEM). The main aim is to identify granular structures on TEM images that may correspond to ALGs;
(f) interpretation and discussion of working hypotheses regarding functionality of ALGs and its evolutionary consequences. This will take into account physiological role of similar actin structures identified and described so far in other organisms.

2 Materials and Methods

2.1 Foraminiferal culture

The experiments were performed on various species of Foraminifera, such as *Amphistegina lessonii*, *Ammonia sp.*, *Quiqueloculina sp.*. Specimens of *A. lessonii* were collected from the coral aquarium in Burgers' Zoo in Arnhem (the Netherlands). This aquarium contains diverse population of corals and other organisms from Indo-Pacific, among them there are fifty species of benthic foraminifera including *Amphistegina lessonii* (Ernst et al., 2011). Samples of sediment with living foraminifera were transferred to the Alfred Wegener Institute (AWI) in Bremerhaven (Germany) and the Institute of Geological Sciences of the Polish Academy of Sciences (ING PAN) in Kraków (Poland), where cultures were established in 10 l aquaria immediately after delivery. Samples containing *Quiqueloculina sp.* were collected in the oceanarium as a part of the Africarium in the Zoo Wrocław (Poland) and transported to the ING PAN in Kraków, where they were cultured in 50 l aquaria. Cultures of *A. lessonii* were kept in 12:12 light:dark cycles and natural sea water (salinity of 34). Samples of mud with *Ammonia sp.* were collected from tidal flats in Dorum (Lower Saxony, Germany), transported to ING PAN (Kraków) and stored in 0.25-0.5 l bottles with natural sea water (salinity of 34) in thermostatic cabinet (12:12 light:dark cycle;8 °C).

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We employed two slightly different methods of preparation of samples for observation during experiments in Bremerhaven (AWI) and in Kraków (ING PAN). At the AWI we picked juveniles from asexually reproduced clones from *A. lessonii* individuals that had climbed the glass walls of the aquaria. The juvenile individuals were picked using a paint brush and transferred into sterile imaging Petri dish (ibidi® polymer coverslip bottom) containing 2 ml of clean culture medium (up to ten individuals per dish). After one dark phase of a light:dark cycle, when individuals attached to the coverslip bottom, they were examined under a binocular looking for pseudopodial activity and chamber formation.

At the ING PAN, adult individuals of *A. lessonii*, *Ammonia sp. Quiqueloculina sp.* were picked from the culture aquaria or bottles and cleaned with fine paint brushes under the binocular to remove algae and grains of sediment covering the specimens. Then, they were transferred to glass bottom Petri dishes previously treated with hydrochloric acid over 16 hours containing 2 ml calcium free artificial sea water prepared as described in Bowser and Travis (2000). After acclimation to the calcium free sea water, when reticulopodia were extended and adhered to the bottom glass, specimens were stained and observed. In the AWI we conducted the observation mainly on chamber formation, while at the ING PAN, most investigations were focused on reticulopodia.

2.2 Staining procedure

In our experiment we focused on staining F-actin with SiR actin but also used Mitotracker Green to stain mitochondria and calcein AM for staining cytoplasm. At the AWI we added stock solution of probes prepared according to manufacturers' instruction) directly to the imaging Petri dish with living specimens of *A. lessonii* to a final concentration of 1 µM. At ING PAN the final concentration of SiRactin was 0.5 µM and Mitotracker Green was 1 µM. SiR actin is a cell-permeable, fluorogenic probe labelling F-actin, thus it is suitable for live-staining (Lukinavičius et al., 2014). As the absorption and emission parameters of the probe are overlapping with the autofluorescence of chlorophyll from endosymbionts it is not possible to use this probe to label F-actin within the endoplasm of *Amphistegina lessonii*.

2.3 Fluorescent microscopy

Images were obtained with a Leica SP5 inverted confocal microscope at the AWI and with a Zeiss Axio Observer Z.1. equipped with ApoTome.2. at the ING PAN in Cracow. ApoTome.2. is a device enabling removal of scattered light in fluorescent imaging. It takes between 3 to 15 images with different positions of a grid placed in the light path between fluorescent lamp and the sample. On the basis of those images, the dedicated ApoTome software calculates optical sections of the sample using a structured illumination principle to enhance signal/noise ratio of the image (Weigel, 2009). In case of living samples containing moving structures it may result in multiplication of some rapidly moving objects. Because foraminiferal ectoplasmic structures are highly dynamic, we choose to set up ApoTome.2. to take only three pictures per frame and use maximum light intensity to decrease exposure time. Despite of this, the most rapidly moving objects may appear tripled in some images.

3 Results

3.1 Identification of actin-labelled structures by fluorescent microscopy

Fluorescent SiR-actin labelling has revealed three considerably different patterns, i.e. (1) weak but non-uniform staining following all membranous surfaces of pseudopodial structures, (2) linear or ring-like structures showing intense fluorescence, and (3) small but strongly labelled granular structures that often exhibit very rapid dynamics (Fig. 1; Movie S1 in supplement). The term actin-labelled granules (ALGs) is introduced here for these small oval objects. Their size has been estimated to be approximately 1 µm. This is consistent with measurement of size of objects corresponding to ALGs seen in Nomarski contrast (DIC) images (Figs. 1, 2).

ALGs are present within: lamellipodia covering the foraminiferal tests (Fig. 3; Figs. S1-S2 in supplement) or any other structure they are attached to, finger-like rhizopodial structures, constructing outer protective envelopes of chamber formation sites (Fig. 4), reticulopodia during feeding and locomotion (Fig. S3 in supplement). They are also present in endoplasmic structures within the chambers, close to surfaces of internal walls of the test (Fig. 5). At first glance ALGs seem to show fast and random movements but actually they can display different "behavioural dynamics". All labelled structures observed under fluorescent light co-localize with pseudopodial structures and granular microstructures identified in the Nomarski contrast (DIC) or in the brightfield image. Figures 1-2 presents a lamellipodial structure attached to the glass surface with a weak, dispersed fluorescent signal of SiR-actin staining F-actin meshwork. Very fine brighter spots represent ALGs that co-localize with granules observed with DIC optics.

3.2 Testing selectivity of granules labelling: ALGs vs mitochondria

Direct comparative analysis of fluorescent vs DIC images indicates that actin-labelled granules do not overlap with all granules observed in DIC (Fig. S3 in supplement). It means that SiR-actin does not stain all the granules observed. Therefore, labelling of ectoplasmic granules is selective. In order to test ALGs relationships with selected, well-defined granules, mitochondria were chosen for double labelling experiments. Mitochondria were best candidates because they had frequently been recognized within the cytoplasm, including reticulopodia (e.g., Travis and Bowser, 1986; Hottinger, 2006; Nomaki et al., 2016; LeKieffre et al., 2018a). Mitochondria usually appear oval or kidney shaped in cross section with a length in the range of 0.5 to 1 µm, although they are sometimes larger and take various, even tubular shapes (LeKieffre et al., 2018a).

MitoTracker Green has been applied in living specimens of *A. lessonii* following the procedure described above (Material and Methods). This probe selectively accumulates in the mitochondrial matrix by covalent binding to mitochondrial proteins (Presley et al., 2003). Results of replicated live experiments do not show co-localization of ALGs and mitochondria stained by MitoTracker Green (Fig. 3; Fig. S3 and Movie S2 in supplement). Therefore, they indicate that generally mitochondria and SiR-actin labelled granules form two non-overlapping categories.

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3.3 Dynamics of actin-labelled granules

The dynamics of the ALGs should be described separately in pseudopodia and in a globopodium during chamber formation. The dynamics (velocity and overall pattern of movement) may differ according to location in the cell. Not all of the ALGs have the same pattern of movement. At first glance their movement may appear chaotic, but closer analysis reveals some general patterns.

For the sake of simplicity, particular threads of granuloreticulopodia may be considered as one-dimensional structures that constrain possible directions of the movement: they can move along the thread of reticulopodia either inward or outward. Indeed bidirectional movement along a single thread is commonly observed (Figs. S4-S5 in supplement), however in case of thick pseudopodial treads there may by a spatial separation: in the core of pseudopodium ALGs moves towards the cell body, while in the cortex they travel in the opposite direction (Movie S3 in supplement). Usually one direction is dominant: when reticulopodia are formed, outward (centrifugal) transport is more common: during retraction of reticulopodia inward (centripetal) movement is prevalent. During extension of a newly formed very fine thread of pseudopodium, there usually is a single ALG at the tip of this tread (Fig. 6). Sometimes clusters of granules moving together with the same speed along a pseudopodium may be identified. As the granuloreticulopodia themselves are very dynamic structures, it is not always possible to measure displacement within them due to lack of constant frame of reference. Another problem is that ALGs can be so abundant in reticulopodia that they may be extremely difficult to track. It is possible to track individual ALGs only in stable and not very dense reticulopodial threads (Figs. S4-S5 in supplement). Because these conditions are rarely met it is hard to unequivocally determine the maximum and mean speed of granules in reticulopodia. Nevertheless, we recorded velocities of ALGs in reticulopodia up to 9 µm/s.

Lamellipodia overlying the test form two-dimensional sheets, resulting in a more complex pattern of displacement of ALG than the one observed in granuloreticulopodia. There are areas dominated by directional protoplasm streaming that contrast to areas showing less organised behaviour. Accordingly, actin granules can be divided into several categories based on pattern of demonstrated movement: (1) stationary or almost stationary ALGs that oscillate within very narrow space; (2) ALGs showing saltatory movements as described in Travis and Bowser (1991); (3) ALGs exhibiting extremely rapid movement that can be observed for up to a few seconds. Moreover, in some areas actin granules may move along a single line but with different speeds and in different directions. Their interactions not always result in visible changes in their dynamics. They may pass some stationary granules with no significant interaction observed.

4 Interpretation and Discussion

4.1 Assessment of unspecific fluorescent labelling risk

All microscopy techniques are associated with risk of capturing some artefacts instead of imaging target structures. In case of fluorescent microscopy the greatest danger is unspecific labelling or autofluorescence. It may be caused by using a too high

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concentration of the probe or too much excitation intensity of emission measurement sensitivity. Another problem might be that the probe may binds to other chemical compounds in the cell, which structure mimics the target structure. Most fluorescent probes were developed and tested to study mammalian cells, therefore, the risk of unspecific fluorescent labelling, especially applied to Foraminifera, should be addressed to avoid confusion and over-interpretation of the results.

The first argument for reliability of actin live staining using SiR-actin is the fact that attachment sites of pseudopodia to the substratum often demonstrate strong fluorescent signals (Fig. 1) as predicted from the essential role of actin for adhesion (Bowser et al., 1988). Secondly, as mentioned above, granular actin structures are visible on images of fixed reticulopodia stained with phalloidin (see Koonce et al., 1986a, fig. 3C; 1986b fig. 1F). It could be speculated that ALGs might serve as part of a defence strategy that could remove and dispose toxic compounds introduced into the cell. If this is true we would expect that vesicles containing those probes would be transported outward. As they are often moving bi-directionally (both in- and outwards, see Figs. S4, S5 in supplement), this hypothesis is not convincing. Actually, ALGs' inward movement is observed when a pseudopodial structure is being withdrawn which seems to indicate relocation of labelled actin into the endoplasm. Such observations support actin specific labelling and a functional response of ALGs during withdrawal of pseudopodial structures.

Another issue is the risk of interference of a probe with the physiology of actin itself, it may for instance cause an artificial polymerisation of F-actin (Melak et al., 2017). In that case it would disturb morphogenesis and biomineralization of new chambers which has not been observed (Tyszka et al., 2019). Moreover, if SiR-actin causes polymerisation of F-actin, live actin staining should have a visible impact on organisation and motility of pseudopodia. Such effects have not been reported.

4.2 Previous studies on actin in Foraminifera using fluorescent labelling

The most commonly used method of fluorescent labelling of the actin cytoskeleton is phalloidin staining (Melak et al. 2017). Its utility is limited mostly to staining fixed cells. Phalloidin staining was previously employed to study actin cytoskeleton in reticulopodia of a few species of foraminifera, i.e. mainly *Reticulomyxa filosa* (Koonce et al., 1986a, Koonce et al., 1986b) and *Allogromia* sp. (Bowser et al., 1988). Actin staining of *R. filosa* showed cable-like structures concentrated in the cortex of reticulopodia as a dominant pattern of actin organisation in reticulopodia. Along those structures there are visible granular actin structures in images of the cited publication, which presence has not been discussed or mentioned by the authors of this publication (Koonce et al., 1986a, fig. 3C; Koonce et al., 1986b, fig. 1F). In *Allogromia* sp. actin cytoskeleton has a different organisation depending on the area of the reticulopodium: in proximal parts of pseudopodia it is seen as thick linear fibres, in more distal region flattened on the glass it is visible only in a few foci; in the most peripheral areas actin staining is absent (Bowser et al., 1988, fig. 1C, 2C, 3C). Those foci probably correspond to actin-labelled granules as shown in our paper.

Although Figure 1 demonstrates an actin-labelled linear structure and Figure 4d presents indistinct SiR-actin-labelled fibres, clear cable-like structures are almost missing in our study in comparison to previous publications that may be a result of different staining procedures. This is due to the fact that every probe may have affinity to different epitopes of F-actin, therefore, may not label equally all of different F-actin-containing structures. Effectiveness of staining F-actin using different

probes was comported by Lemieux et al. (2014). Authors of this paper reported that different probes did not stain all of subsets of F-actin equally. Effectiveness of staining of F actin depends of location of actin filaments within the cell. Even though this analysis does not include SiR-actin, this problem may also apply to this probe. Moreover, interaction between probes and F-actin may lead to stabilisation or enhanced polymerisation of F-actin due to its structural similarities to Jasplakinolide (Melak et al., 2017). On the other hand, cell fixation procedures may stabilise dynamic structures or create some artefacts.
Previous studies concerning dynamics of granules in Foraminifera were conducted mostly on *Allogromia* and *Astramina*, maximal speed of granules within reticulopodia was reported to be approximately 25 µm/s but most of them has speed below 10 µm/s (Travis and Bowser, 1991). Velocities of ALGs falls within this range. Average speed of granules in foraminiferal pseudopodia reported by Kitazato (1988) is 13 µm/s, what is comparable to our measurement of 9 µm/s.

4.3 Main hypothesis regarding the physiology of actin granules

Actin granules described in this paper appear to be one of the main forms of actin cytoskeleton organisation in external cytoplasm (ectoplasm) of foraminifera. As they are ubiquitous in pseudopodia during feeding behaviour and in globopodia during chamber formation, they probably serve an important physiological role. At present, it is difficult to determine their function, however, there are a few hypothesis that could be proposed based on two sources of data.
As mentioned above there are two possible explanation of their role: (1) ALGs mediate transportation of various types of cargo; (2) ALGs are involved in transport of prefabricated or recycled actin fibres. The following paragraphs are dedicated to the discussion of these hypotheses. Firstly, we will discuss the relation of actin granules in foraminifera to similar structures described in other organisms. There are actin patches known from some fungi and fungi-like protists. Secondly, we compare actin granules to different ultrastructures known mostly from TEM images of foraminifera. We will focus on organelles or structures which function is questionable e.g. fibrillar vesicles (LeKieffre et al., 2018a; Goldstein and Richardson, 2018), and elliptical fuzzy-coated vesicles also called Motility Organizing Vesicles (Travis and Bowser, 1991).

4.4 Comparison of actin structures in other organisms

Structures similar to actin-labelled granules described in Foraminifera have been found in other organisms. They are present in water moulds: *Saprolegnia ferax* (Geitmann and Emons, 2000), *Phytophthora infestans* (Meijer et al., 2014), as well as in yeast *Saccharomyces cerevisiae* (Moseley et al., 2006; Rodal et al., 2005; Waddle et al., 1996; Winter et al., 1997), where they are abundant in high numbers in buds. They are referred to as cortical actin patches in budding yeast and *S. ferax* (Geitmann and Emons, 2000) or actin plaques in *P. infestans* (Meijer et al., 2014). In those organisms they occur alongside different actin structures such as actin cables or rings.
Fluorescent images of *Saprolegnia ferax* (Geitmann and Emons, 2000) indicate that actin patches have a globular shape and diameters of approx. 0,5 µm. In yeast they appear to have a similar size. Therefore their size is comparable to actin-labelled granules in Foraminifera. Maximal velocity of actin patches observed in yeast is 1.9 µm per second (Waddle et al., 1996), thus it is significantly lower than the velocity of actin granules in foraminifera. Cortical actin patches are most likely involved in

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endocytosis (Moseley et al., 2006) and cell growth (Geitmann and Emons, 2000). For instance in budding yeast actin patches are present during budding within the daughter cell.

In foraminifera ALGs appear in large numbers in the course of chamber formation, as well as within reticulopodia, which are known for their ability for rapid extension and retraction. Formation of a globopodium and reticulopodia in Foraminifera and budding in yeasts require quick expansion of the cytoplasm and may share similar mechanisms facilitating those processes. Assembling actin filaments may generate a physical force that can be used to impose a pressure requisite for expansion of new protoplasm (Mogilner and Oster, 2003).

4.5 Comparison of actin-labelled granules to organelles identified in TEM images of foraminifera

Transmission electron microscopy (TEM) represents a principal method of investigation of cell ultrastructure. However, TEM images alone do not provide information on the chemical composition of certain structures. In contrast to the classical TEM methodology, fluorescent labelling sometimes gives detailed insight into chemical compositions of certain areas of the cell but in much lower resolution. Thus, combing those two approaches is essential to unravel the ultrastructure and function of cell components. Hence, for a better understanding of the role of actin granules in foraminiferal cells, it is important to find the corresponding structures on TEM images.

4.5.1 Fibrillar vesicles

Fibrillar vesicles (FV) are best candidates for the corresponding structures that represent ALGs under TEM. They are present in many different species of benthic foraminifera relatively abundant in various parts of their cytoplasm (Hottinger, 2006; LeKieffre et al., 2018a; Jauffrais et al., 2018; Koho et al. 2018). Their size range (up to ~1000 nm in diameter) and vesicular, globular shape (LeKieffre et al., 2018a; Goldstein and Richardson, 2018) correspond to ALGs (Figs. 2-3, 5-6; Figs. S3-S5 in supplement). Fibrillar vesicles appear to be separated from the cytosol by a lipid membrane (Figs. 7a, 8). Membranes enveloping the fibrillar vesicles may not cover the entire vesicle. It may form characteristic open vase-shaped structure (Goldstein and Richardson, 2018).

Although the chemical composition of FV is uncertain we can assume from a high content of nitrogen (LeKieffre et al., 2018b) that they likely contain proteins. Internal material contained within FV appears to have a specific 3D net-like nanostructure. Most fibres are oriented along the long axis of the FV, but they are not perfectly parallel. They form a network of cross-linked and branching fibres, spreading in two dominant directions and forming recurrent angles. This organisation pattern resembles the actin meshwork observed by cryoelectron tomography in *Dictyostelium* (Medalia et al., 2002) or in nanotomography of lamellipodium in keratocyte of zebrafish (Mueller et al., 2017), as well as in many other eukaryotic organisms (Fig. 7e). Similarity in the spatial pattern of fibres inside FV to the actin meshwork leads to the conclusion that FVs contain a network of actin filament (Fig. 7). Similar but less organised structures of cross linked fibres form actin meshwork in pseudopod of *Allogromia* (Bowser et al., 1988; Koury et al., 1985).

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It is also not clear how FVs are formed, LeKieffre et al. (2018a), however, proposed that they are produced according to a model of forming of Golgi Vesicles published by Anderson and Lee (1991). This model assumes that they originate in the *trans* surface of Golgi apparatus, thus translation of the protein inside those vesicles must occur in the endoplasmic reticulum. This seems to be inconsistent with our hypothesis that fibrillar material consists of prefabricated actin filaments, as actin is a cytoplasmic protein, thus its translation takes place on ribosomes in the cytosol and not in the endoplasmic reticulum (ER). However assuming that FVs are formed by enclosing fibrillar material produced in the cytosol by thickened cistern of Golgi apparatus may resolve this issue. Moreover the last assumption agrees with finding by Goldstein and Richardson (2018) that the membrane may not cover the entire vesicle.

4.5.1 Fibrillar system of planktic foraminifera

It is worthwhile to mention that Anderson and Bé (1976) described in planktic foraminifera another subcellular structure called the fibrillar system or the fibrillary bodies (acording to Hemleben et al., 1989; Schiebel and Hemleben, 2017). Spero (1988) presented this system contained proteins involved in construction of a protective envelope during chamber formation in *Orbulina universa*. However, it is not clear, whether these structures representstructurally and functionally analogous organelles to FVs. Spero (1988, - see figs. 4e, f, 5d) documented under TEM vesicles that resemble FVs and are associated with the “primary organic membrane” during chamber formation. In fact, “fibrillar” as a descriptive term seems to describe different filamentous structures at different spatial scales. Fibrillar vesicles show a fibrillar internal ultrastructure, in contrast to the fibrillar system that represent massive “massive fibrous deposits” constructed from individual tubular structures called fibrillar units (see Spero, 1988). Therefore, the fibrillar system is often tubular that contrast to granular (vesicular) appearance of FVs and ALGs. Nevertheless, Hemleben et al. (1989) note that fibrillar bodies originate in the cytoplasm inside the test as small vacuoles filled with densely packed fibrous material and they typically enlarge and expand as they are transferred outside the test. However, in rhizopodia of *Orbulina universa*, there may be found small vacuoles resembling FVs, e.g. object described as a vesicle containing adhesive substance in fig. 3.5(6) in Schiebel and Hemleben (2017). More comparative studies are needed to reveal whether FVs in benthic species are somehow homologous to the fibrillar system in planktic ones.

4.5.2 Elliptical fuzzy-coated vesicles

Elliptical fuzzy-coated vesicles are additional ultrastructural components that may correspond to ALGs. These vesicles are structures unique to Foraminifera. They include elongated structures that aretypically approximately 300 nm in length identified in TEM images of reticulopodia (Travis and Bowser, 1991; Koury et al., 1985). Elliptical fuzzy-coated vesicles , consist of a membrane coated with an unknown material having a characteristic fibrillar appearance. They are reported to be involved in regulation of motility, thus, the term Motility Organizing Vesicles was coined to describe those structures (Travis and Bowser, 1991). Material coating these organelles shows characteristic fuzzy appearance that might resemble actin mesh.

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4.6 Functional implications, evolutionary consequences, and future research prospect

Actin-labelled granules (ALGs) are highly dynamic structures that are abundant in foraminiferal ectoplasm (Figs. 1-10). They are small organelles involved in the physiology of granuloreticulopodia and other types of pseudopods, some of them directly involved in morphogenesis of new chambers and biomineralization of the wall (see Tyszka et al., 2019). As they are ubiquitous in the cells of many species of both globothalamean and tubothalamean foraminifera (sensu Pawlowski et al., 2013), they have most likely evolved very early during evolution of Foraminifera. Interpretation of their function must take into account studies of foraminiferal ultrastructure based on TEM. Nevertheless, it is very likely that they correspond to fibrillar vesicles or fuzzy coated vesicles observed in much higher resolution in TEM (Figs. 12). More studies are needed to corroborate or refute this hypothesis, particularly applying correlative light and electron microscopy as a crucial method to solve this puzzle.

The second question that should to be addressed regards the presence of analogue structures in other eukaryotic organisms. Indeed in some fungi or fungi-like protists similar actin structures have been identified in many previous studies (Geitmann and Emons, 2000; Meijer et al., 2014; Moseley et al., 2006; Rodal et al., 2005; Waddle et al., 1996; Winter et al., 1997). It is too early to state whether all these structures serve the same physiological function and share the same evolutionary origin. However, there are some facts suggesting that this actually may be the case. Firstly, all of them have similar size and tend to be concentrated in a cortical layer of protoplasm just under the plasma membrane. Moreover, all the cells that contain them have the ability to rapidly expand the volume of protoplasm and actin networks/patches may be involved in generating the force needed in this process. Investigation of the molecular basis of actin cytoskeleton regulation in broad phylogenetic context is required to address this issue.

Our working hypothesis is that ALGs most likely play a crucial role in intracellular transport, that may be two-fold: (1) they may be involved in transport of various cargo inward (endocytosis) or outward (exocytosis), and/or (2) they facilitate transfer of prefabricated actin filaments from endoplasm to the external parts of the foraminiferal cell. If the second hypothesis is correct, ALGs are fundamental for extension and adhesion of reticulopodia, as well as formation and shaping the glopopodium during chamber formation.

This model may solve the puzzle of efficient transport of proteins within extensive pseudopodial networks. In Foraminifera ribosomes are absent in the pseudopodial cytoplasm (Travis and Bowser, 1991) consequently protein synthesis is restricted to the endoplasm. Foraminifera must have mechanisms to efficiently transport proteins needed for the formation of extensive pseudopodial networks. This issue applies primarily to the transportation of cytoskeletal proteins that are in high demand within reticulopodia due to their critical role in morphogenesis and movement of this network. Simple diffusion of monomers of tubulin and assembly of MT on site may not be sufficient enough (Bowser and Travis, 2002). Hence it was proposed that foraminiferal cell use tubulin paracrystals as a storage of prefabricated MT (Travis and Bowser 1991). Here, we suggest an analogous mechanism for efficient actin transport in form of microfilaments. This mode of transport facilitates a rapid formation, restructuring, and retraction of actin meshwork.

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Such functional mechanisms employed for optimization of intracellular motility of building blocks, pseudopodial dynamics and their overall morphogenesis may be one of the main evolutionary adaptations specific to Foraminifera and possibly to related phylogenetic taxa included into the phyllum Retaria (see Cavalier-Smith et al., 2018). Similar granuloreticulopodial organization of pseudopods is known from Radiolaria (Anderson, 1976; Anderson, 2012). Radiolaria, also called Radiozoa are very likely a sister group of Foraminifera (Burki et al., 2010; Cavalier-Smith et al., 2018). It is not clear if all types of granules in ectoplasm of Radiozoa and Foraminifera are the same. It has been reported that granules in Radiolarian pseudopodia include mitochondria, digestive and defecation vacuoles, and osmophilic granules (Anderson, 2012).

Molecular phylogeny based on conservative actin gene sequences suggests that actin in Foraminifera evolved in higher rates than in most other Eukaryotes (Keeling, 2001). Moreover duplication of gene encoding actin has occurred early in evolution of a lineage containing Foraminifera resulting in the presence of two paralogs of that gene in many species (Falkowski et al., 2005). There is some evidence that this duplication is shared by the group Acatharea belonging to the Radiolaria (Burki et al., 2010). However in at least some Foraminifera actin genes have been duplicated many times forming extraordinarily diverse gene families as in *Reticulomyxa filosa*. It has been suggested that the diversification of actin genes was a key step in evolution of mechanisms of rapid transport between reticulopodia and the cell body (Glöckner et al. 2014).This leads to the conclusion that physiology, dynamics, organization and function of the actin cytoskeleton in Foraminifera may differ significantly from most other organisms. More studies are essential for the understanding of the physiological functions of the actin cytoskeleton, including:

(1) research regarding the expression of actin;

(2) identification of actin-binding proteins in Foraminifera;

(3) experiments on inhibition of actin formation during different behaviours (feeding, chamber formation, locomotion etc.);

(4) imaging of actin structures with more refining methods including correlative light- and electron microscopy or super-resolution confocal microscopy.

5 Conclusions

This paper demonstrates results of live fluorescent labelling of actin in Foraminifera with focus on ectoplasmic (pseudopodial) structures during various behavioural and physiological activities. Fluorescent labelling has revealed three considerably different SiR-actin-labelled patterns that include: (1) weak but not uniform staining following all membranous surfaces of pseudopodial structures (Figs.1, 2), (2) linear or ring-like structures showing intense fluorescence (Fig. 1), and (3) small, strongly labelled granular structures that often exhibit very rapid dynamics (Figs. 2-3, 5-6; Figs. S5-S5 and Movies S2-S3 in supplement).

The granular appearance is the principal characteristic of actin cytoskeleton in all studied foraminiferal taxa. Actin-labelled granules (ALGs) have been described as small (c. 1 μm in diameter), oval and dynamic objects that are numerous in pseudopodia, but present in endoplasm as well. Besides ALGs, actin cytoskeleton in foraminiferal pseudopodia may form a linear and ring-like structures (Fig. 1).

Co-labelling of mitochondria with Mitotracker Green and actin cytoskeleton with SiR-actin has been performed in order to verify whether ALGs overlap with mitochondria that tests selectivity of granules labelling. As presented images (Fig. 3; Fig S7 in supplement) indicate, there is very little co-localization between those two types of organelles, however, ALGs and mitochondria probably constitute majority of granules present in pseudopodia.

Detailed interpretation of obtained images has been given, including the risk that ALGs may a result of unspecific labelling. Presented arguments allow to exclude this possibility. Furthermore, relation of ALGs to similar structures found in other eukaryotes (mostly some fungi, fungi-like protists) has been discussed.

It has been proposed that a main function of ALGs in physiology of Foraminifer is facilitating transportation of different types of cargo, most likely including transport of prefabricated and/or recycled actin filaments themselves.

Finally, a question regarding correspondence of ALGs to objects known from published TEM images has been addressed. According to our presented hypothesis, most of ALGs correspond to fibrillar vesicles (see LeKieffre et al., 2018a; Goldstein and Richardson, 2018) and/or elliptical fuzzy-coated vesicles (Travis and Bowser, 1991). This is still a working hypothesis that should be verified by correlative TEM-fluorescence methods.

6 Information about the Supplement

The Supplement contains 3 movies and 5 additional figures showing different actin structures in Foraminifera and their dynamics.

Author contributions

Author contributions: J.G. designed research, performed research, analysed data, wrote the paper and prepared graphics; J.T. proposed and supervised research; U.B. and J.B., contributed new reagents/analytic tools; J.T. consulted interpretations; J.T., U.B., and J.B. corrected the text.

Competing interests

The authors declare that they have no conflict of interest.

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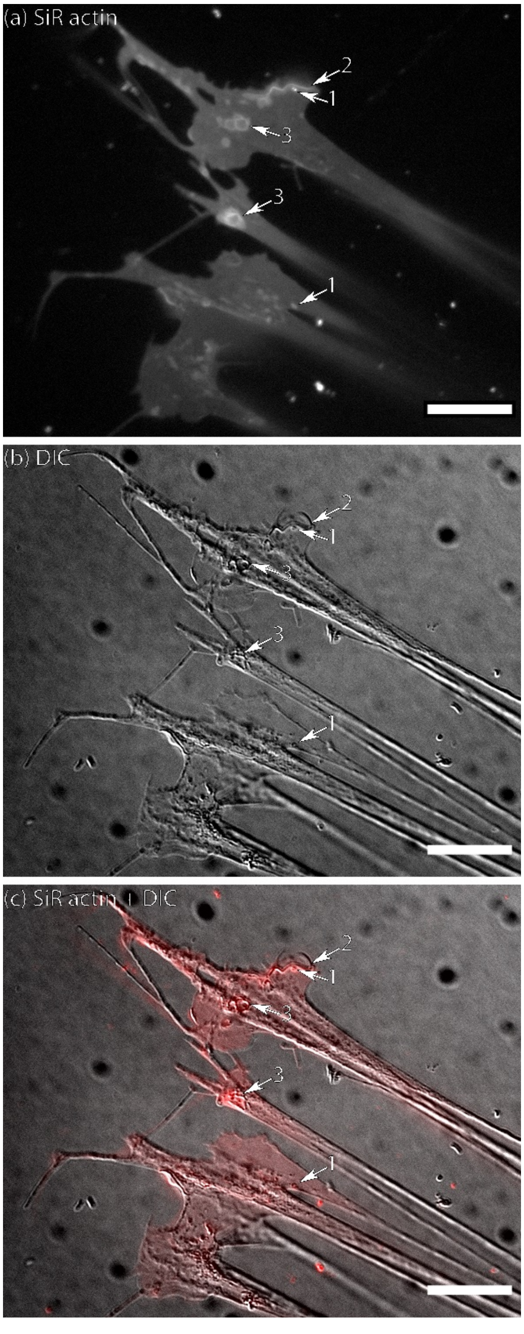

Figure 1: Frame from time lapse images showing flattened lamellipodia of living *Ammonia* sp. attached to glass: (a) fluorescence of actin-labelled structures, (b) DIC image of the same area, (c) merged image of fluorescence and DIC channels (since the reticulopodia were moving, the DIC image is slightly shifted in relation to fluorescent one). Numbers indicates: 1 – actin labelled granules (ALGs); 2 – linear actin-labelled structures; 3 – actin-labelled rings. Note weak but not uniform actin-labelling following all membranous surfaces of pseudopodial structures. The linear structure (2) was subsequently transformed into ring structure (see Movie S1 in supplement). Structures corresponding to ALG, actin-labelled ring and linear structures can be seen in DIC image. Images were obtained with *Zeiss Axio Observer Z.1*. Scale bar 20 µm.

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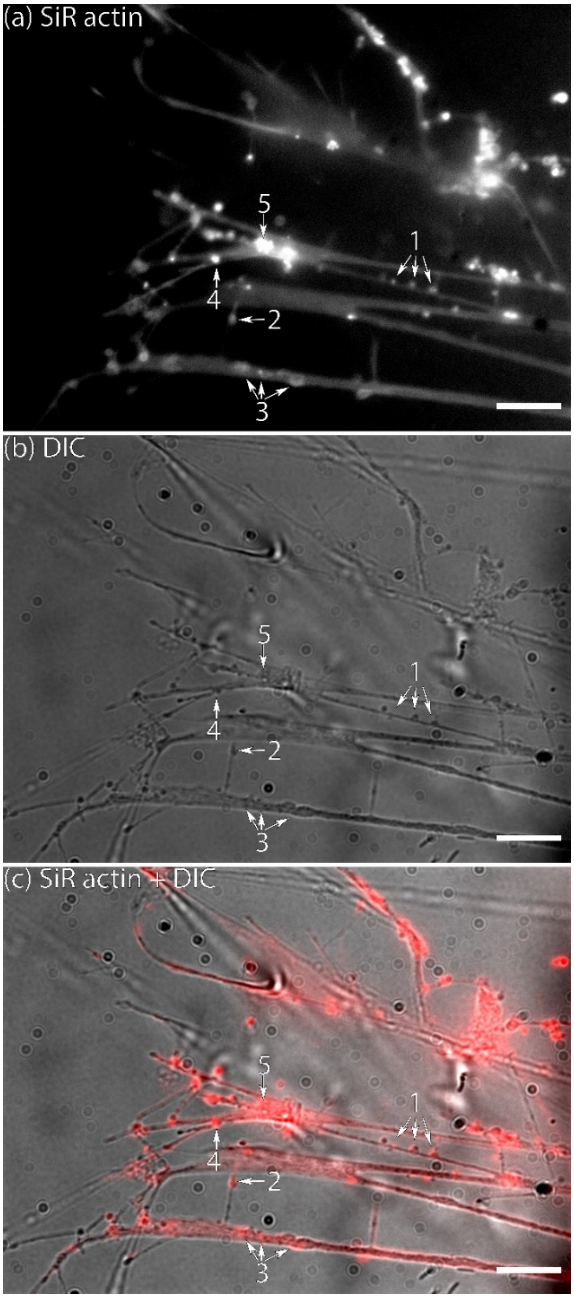

Figure 2: Frame from time lapse imaging showing pseudopodia of living *Ammonia sp*. attached to glass: (a) conventional fluorescence of actin-labelled structures, (b) DIC image of the same area, (c) merged image of fluorescence and DIC channels (since the reticulopodia were moving, the DIC image is slightly shifted in relation to fluorescent one). Weak but not uniform actin-labelling following all membranes can be seen in pseudopodia. Numbers indicates: 1 – group of tree actin-labelled granules (ALGs) transported along one thread of pseudopodia; 2 – actin in the tip of thin fillopodium; 3 – larger actin-labelled areas showing smudgy fluorescence weaker than in most ALGs; single 4 – ALG in bifurcation of retiulopodia; 5 – group of very bright densely packed ALGs in the thick reticulopodium. Images was obtained with *Zeiss Axio Observer Z.1*. Scale bar 10 µm.

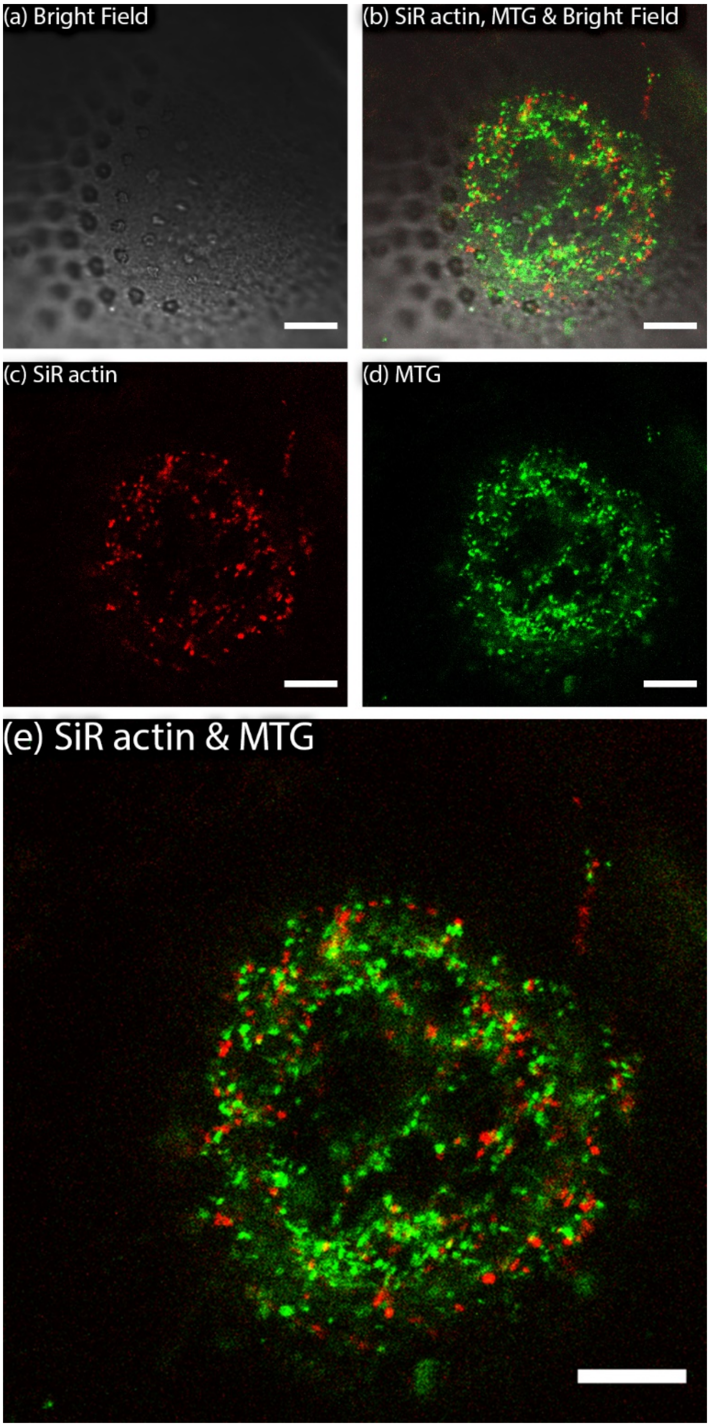

Figure 3: Single frame from time lapse showing actin-labelled granules (ALGs) and mitochondria in cross-section of a newly forming chamber in *Amphistegina lessonii* during biomineralization (pores are already visible in transmitted light). ALGs and mitochondria do not show co-localization. Images were obtained with Leica SP5 inverted confocal microscope. For the entire time lapse see Movie S2. Scale bar 10 µm.

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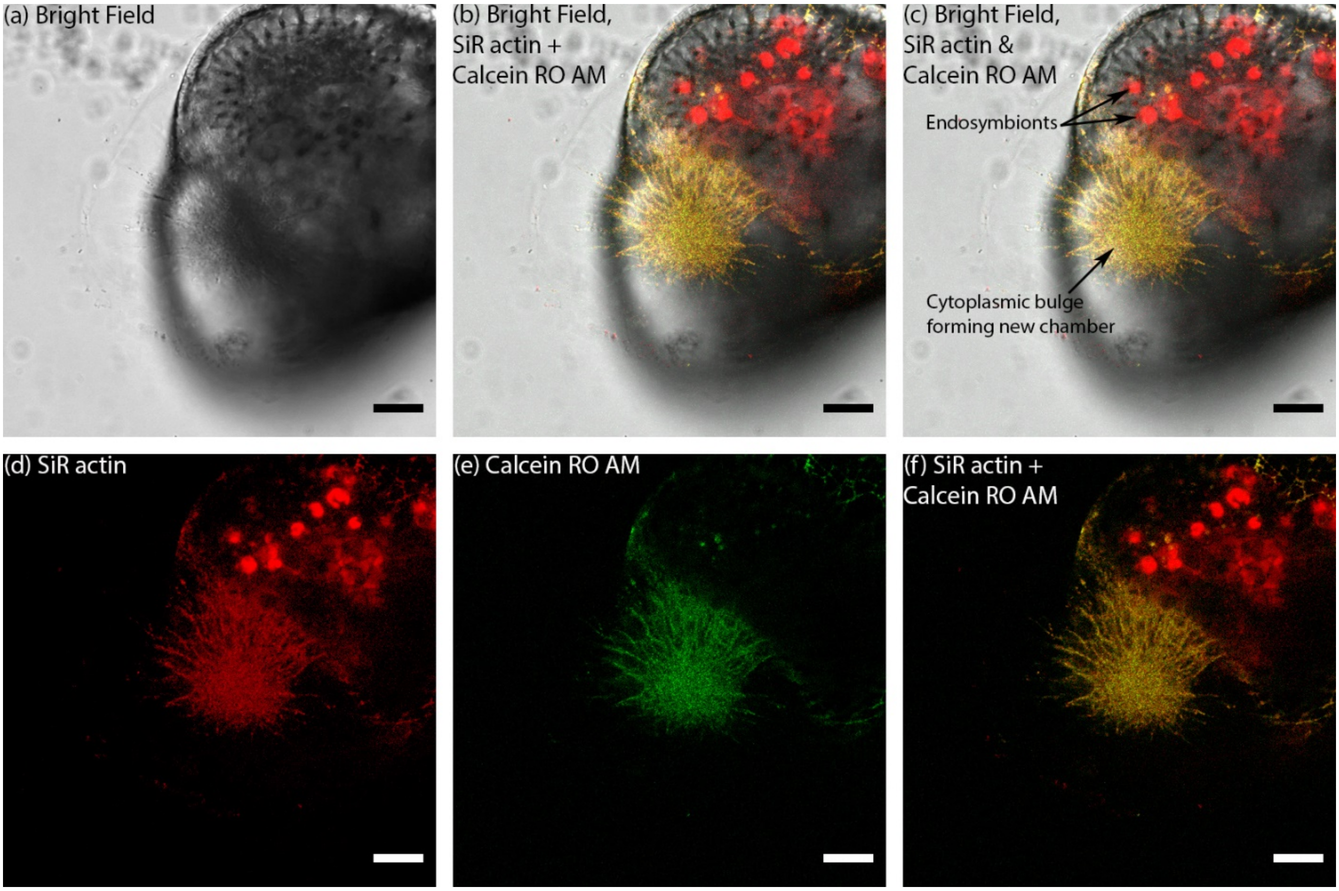

Figure 4: Organisation of actin within in finger-like structure preceding globopodium during chamber formation compared with localisation of cytoplasm stained with calcein red orange AM. Images was obtained with *Leica SP5* inverted confocal microscope. Scale bar 20 µm.

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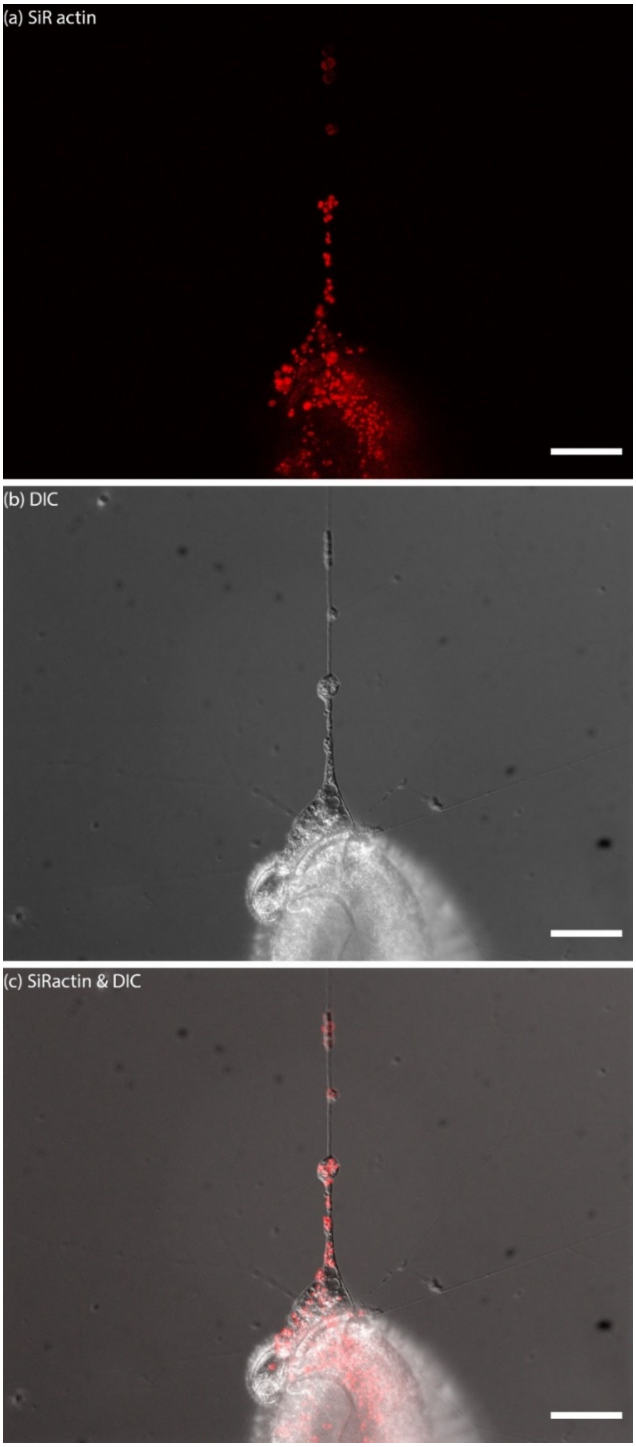

Figure 5: Actin-labelled granules (ALG) in pseudopodia and endoplasm of *Quinqueloculina sp.* Top image presents actin stained with SiR actin, middle image presents images obtained with DIC optics (inverted LUT), bottom image presents merged fluorescent and DIC channels. Images obtained with *Zeiss Axio Observer Z.1*. Scale bar 50 µm.

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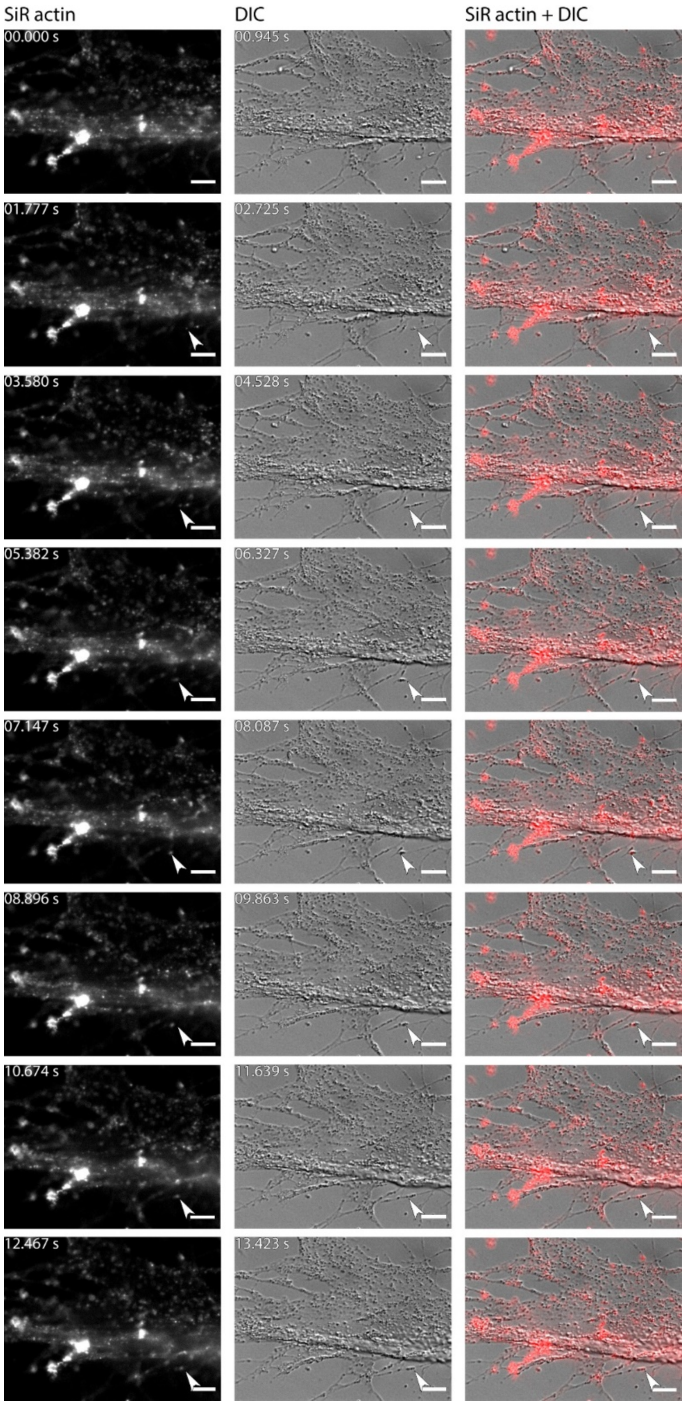

Figure 6: Dynamics of actin-labelled granules (ALG) in reticulopodia of *Amphistegina lessonii*. Eight frames of time lapse. Right column: actin stained with SiR actin; middle column: DIC; right column: overlay of fluorescent and DIC channels. Arrows indicate granule in the tip of one very fine thread of forming pseudopodium. Numbers in top right corner of each image of SiR actin and DIC channel indicate time of acquisition. Images obtained with *Zeiss Axio Observer Z.1*. Scale bar 10 µm.

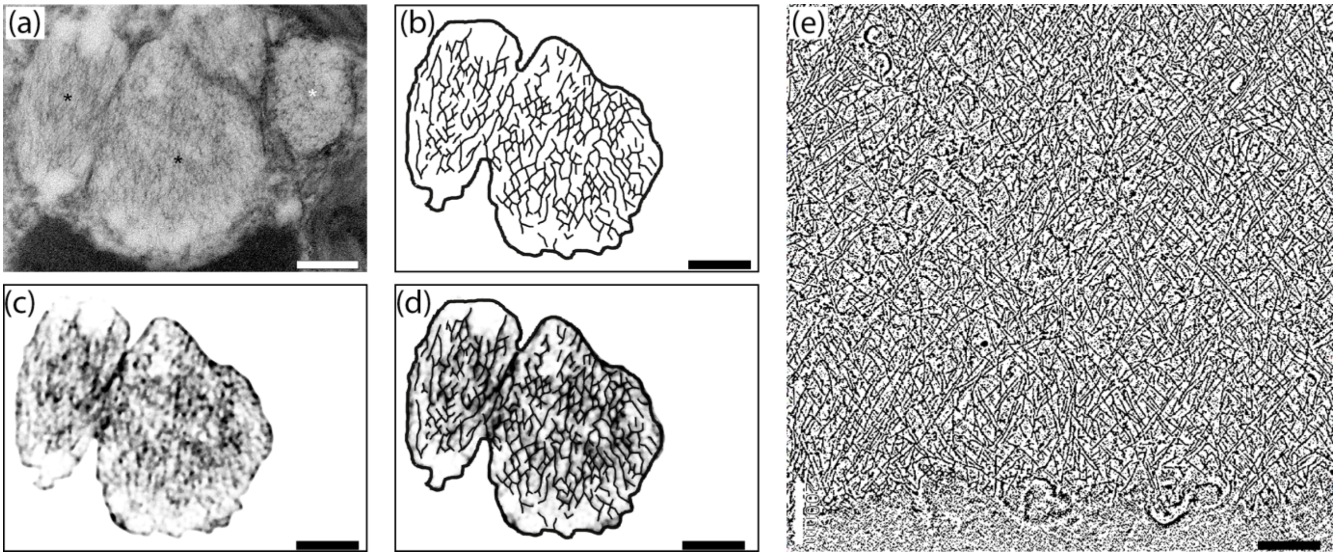

Figure 7: Comparison of internal nanostructure of Fibrillar Vesicles (a–d) to actin meshwork (e), Scale bar 200 nm. (a) TEM image of FV, reprinted from *Mar. Micropaleontol*, 138, LeKieffre et al., An overview of cellular ultrastructure in benthic foraminifera: New observations of rotalid species in the context of existing literature, 12-32, Copyright (2018a), with permission from Elsevier (fig.14). (b) Model of geometry of fibrillary structures inside FB based on image (a). (c) first step in drawing a model shown in (b) - fragment of image (a) with background removed and processed in FIJI software in order to make the geometry more apparent. (d) Overlay of image (c) with sketch of internal structure of FB drawn in CorelDraw. (e) structure of actin meshwork in lamellipodia based on nannotomogram reprinted from *Cell,* 171.1, Mueller et al., Load adaptation of lamellipodial actin networks, 188-200, Copyright (2017), with permission from Elsevier (fig. 4b, modified).

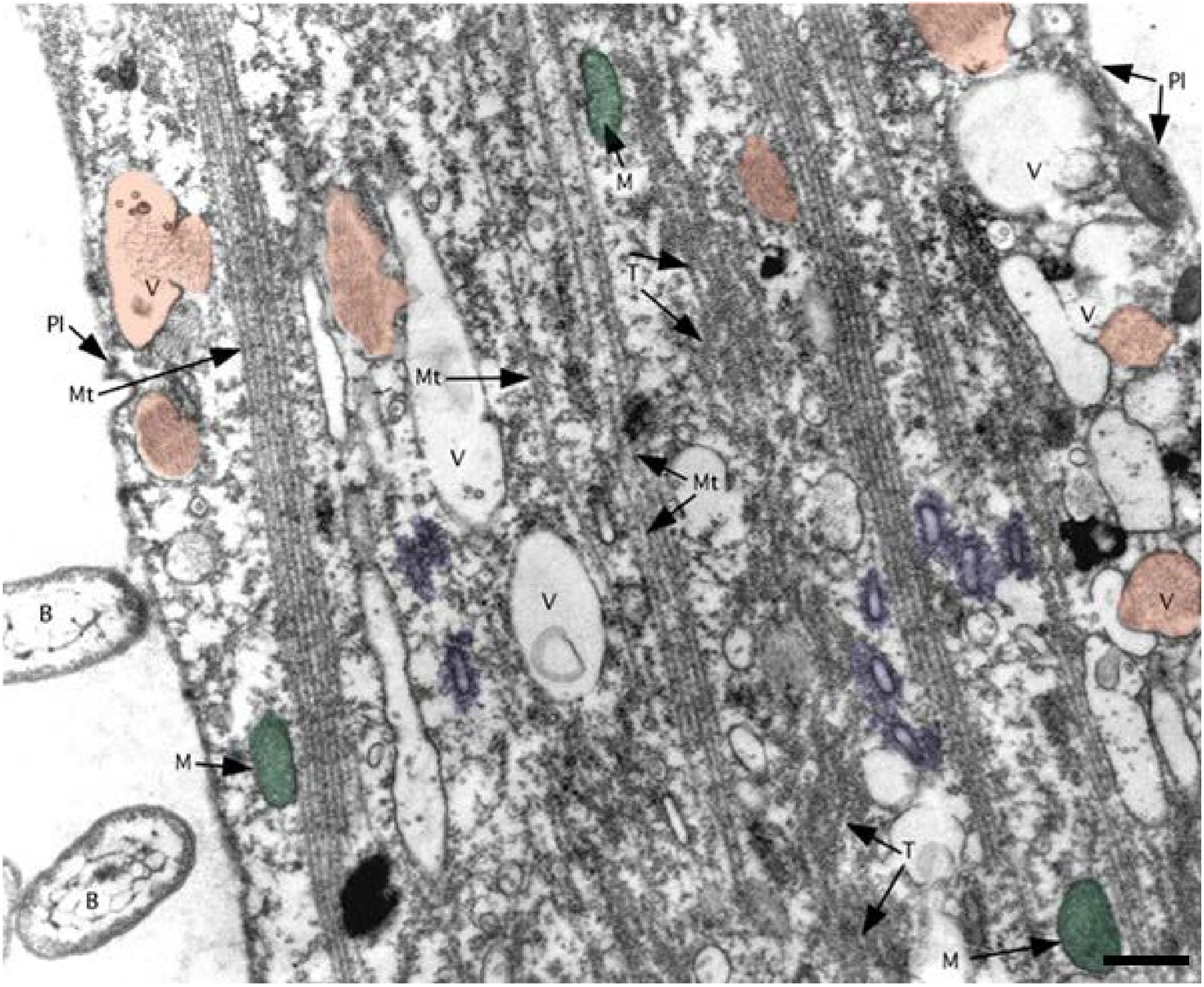

Figure 8. TEM image of ectoplasm of *Assilina ammonoides* (Gronovius) (modified from Hottinger, 2006, fig. 67 based on Creative Commons Attribution 2.5 License). Areas marked in pink indicates vesicles we interpret as fibrillar vesicels. Areas marked in violet indicates what we interpret as elliptical fuzzy-coated vesicles also called Motility Organizing Vesicles (MOVs). Green areas correspond to mitochondria. B: bacteria; M: mitochondria; Mt: microtubule; Pl: plasmalemma; T: tubulin paracrystals; V: vacuoles with or without fibrillar content. Scale = 500 nm.