Dear Prof. Kitazato,

We would like to express our gratitude for assessment and constructive reviews of the proposed manuscript 'Dynamics and organization of actin-labelled granules as a rapid transport mode of actin cytoskeleton components in Foraminifera' (BG-2019-182). In this document we would like to present our detailed response to reviewers' comments. The manuscript has been modified according to referees. The main changes include modification of the title of the manuscript, re-writing selected chapters, including Chapter 2 'Materials and Methods' and Chapter 4 'Interpretation and Discussion', as well as updating the results of measurement of the velocities of the granules we observed. We corrected typographic, punctuation, and stylistic errors throughout the entire manuscript. All these changes can be found in the marked-up version of the revised manuscript at the end of this document. We further made necessary changes to the supplementary materials as well. We hope all our efforts have clarified presentation of our latest scientific results.

Yours sincerely,
Jan Goleń

Response to referees' comments

Response to an interactive comment by Samuel Bowser (Referee) on "Dynamics and organization of actin-labelled granules as a rapid transport mode of actin cytoskeleton components in Foraminifera" by Jan Goleń et al.

Referee comments are given in italics

General response:

Dear Dr. Bowser,

Thank you very much for your critical review of our contribution. We greatly appreciate the time and effort to provide us with constructive feedback. We are glad to hear that our work represents a novel and interesting contribution as a logical extension of research on F-actin in foraminiferal reticulopodia done primarily in the 1980s and 1990s.

Possibly, the most novel aspect of our research is the introduction of live actin staining in foraminifera using the SiR-actin fluorescent probe. We therefore observe and describe the organization of F-actin in action that is undisturbed by fixation. Although this method was already partly presented in the study by Tyszka et al. (2019), our paper has a different objective focused on description of intriguing granular microstructures observed in a much smaller scale. This study is based on observations of various pseudopodial structures identified in very different taxa.

Besides many profits, a new method often comes with additional problems and questions. The fact that we demonstrate granularity as a main feature of the actin cytoskeleton in foraminifera is one of them. We are aware that a certain dose of skepticism is needed when such unexpected findings appear using a novel technique. However, we have observed the same consistent pattern across a wide range of species using various microscopes, including Leica, Olympus, and Zeiss in different laboratories.

Comment: The results presented are interesting, but the author's conclusions can only be considered hypothetical at this point.
Re: We agree that our conclusions can only be considered hypothetical at this point. We modified the manuscript significantly to stress this fact out. We hope that our contribution fosters further studies testing all alternative hypotheses.

Comment: Of paramount importance is the correlation of fluorescence light microscopy images with electron microscopy; the simple comparisons with published photographs used here are not at all convincing. The authors should be obligated to show directly what the staining patterns correspond to ultrastructurally. (There are many straightforward ways to do this.) To be more complete, it would also be desirable to illustrate motile events (granule motion, etc.) immediately prior to fixation for electron microscopy.
Re: We fully agree that testing the different scenarios and conclusively settle important questions on ultrastructural analogs of the observed staining pattern in Foraminifera is necessary and requires additional detailed studies, ideally using a TEM-fluorescence correlation microscopy. This is probably the most sophisticated and time-consuming type of experiments that needs to be carried out. Our intention is to run such correlative studies that is an excellent idea for a new collaborative project we would like to apply for. We should mention that this idea was already expressed in our conclusions, i.e. "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."

We do agree that it would be most desirable to document dynamics of granules immediately prior to fixation for TEM. This would be the best experimental scenario. However, it might be reasonable to avoid standard fixatives that tend to alter actin organization during fixation. We would like to test different fixation methods. Possibly, the most optimal would be to apply a high pressure freezing (e.g., cryfixation in propane) to avoid preparation artifacts. Our guess is that documentation of all replicated experiments would need another extensive and well-illustrated publication.

Comment: Critical controls, missing from the present study, include demonstrating that the observed SiR staining patterns are not caused by the action of jasplakinolide. The authors (and sales literature) suggest that they are not, but to examine this important issue experimentally the authors should fix the cells first and then stain for f-actin using SiR and fluorescent phalloidin; equivalent patterns using two independent f-actin probes in fixed cells would be much more convincing. An important allied question is: what is the effect of unlabeled jasplakinolide on f-actin distribution and reticulopodial motility?

Such information would help flesh out their study and provide important new information on the pharmacological disruption of foram cytoskeletal dynamics.

Re: We highly acknowledge all recommendations. We are currently planning additional experiments to address most of these points. We would like to publish them in the future. At this stage we present results of replicated experiments conducted over last three years. Our intention is to identify the problem, then to propose and discuss all working hypotheses. We made major changes to the proposed manuscript and supplementary material and include negative controls (comparison images of stained and unstained individuals of *Amphistegina lessonii*). All our experiments indicate necessity of further extensive and collaborative studies.

Referring to the comment on the action of jasplakinolide, we compare stained and unstained (control) individuals. In the corrected version of the manuscript we disused it more extensively. We did not observe any long term changes in the overall reticulopodial morphology nor in the dynamics after staining with SiR-actin (jasplakinolide-based probe). We have run replicated experiments indicating that SiR-actin (incl. jasplakinolide) does not disturb pseudopodial dynamics associated with chamber formation. Neither chamber morphogenesis nor biomineralization is modified. Our methodology follows staining technique described in Nature Chemistry or Nature Methods (Lukinavičius et al. 2013; 2014).

We would also like to stress that small dynamic objects stained with SiR-actin typically overlap with a subpopulation of well-defined granules visible in differential interference contrast (DIC) or in bright field images (see figs 1, 2, 5, 6 in the paper being reviewed). It seems to be clear that this type of granularity is immanent to the foraminiferal pseudopodial system. We don't think jasplakinolide induces formation of new granules. However, according to Melak et al. (2017), it is likely that untagged jasplakinolide induces F-actin assembly. Melak et al. (2017 on p. 527) suggest that "caution must be taken in live-cell imaging as SiR-actin might cause F-actin stabilization or induce actin polymerization owing to its structural similarities to Jasplakinolide", and later propose that "further studies are therefore needed to fully assess the advantages and possible limitations of SiR-actin over more established actin probes." We would like to take these points into account in future experiments and projects.

We would like to change the title of the paper to avoid interpretative connotations. We propose to change a title of our paper (under review) to "SiR-actin-labelled granules in Foraminifera: Pattern, dynamics, and hypotheses". Furthermore, we propose to modify the interpretative part of the text that should describe and discuss all working hypotheses. Our intention with this manuscript was to present possible hypotheses based on our and published data and to propose the best research strategy for designing future experiments.

We proposed alternative explanations for the observed Actin Labeled Granules (see Fig. S7 in the updated version of supplement). We describe three possible scenarios in which SiR-actin specifically labels F-actin within structures that have a granular appearance (Fig. S7A-C), and present possible artifacts caused by staining foraminiferal reticulopodia with SiR-actin (Fig. S7D-F). The most likely scenario assumes that SiR actin labels actin filaments inside vesicles separated from the rest of the protoplasm with a lipid membrane, possibly corresponding to Fibrillar Vesicles known form TEM ultrastructure studies (Fig. S7A). This hypothesis is discussed in detail in our manuscript. The second scenario assumes that actin filaments,

surrounding some membranous vesicles, are stained specifically with SiR-actin (Fig. S7B). These vesicles may contain different kinds of cargo and F-actin is assumed to play a role in endocytosis and/or in transport. Alternatively, they may represent elliptical fussy-coated vesicles described by Koonce et al. (1986), and involved in regulation of a motility of reticulopodia Travis and Bowser (1991). The third scenario is a combination of first two as it assumes that actin filaments are both inside and outside of vesicles (Fig. S7C).

Three additional scenarios assume that SiR-actin does not stain functional actin filaments in Actin Labeled Granules in foraminiferal pseudopods. These scenarios include unspecific labelling of proteins (or other complex molecules) different than actin, but mimicking a similar structure, inside (Fig. S7D) or outside (Fig. S7E) membranous vacuoles. The last scenario assumes that SiR-actin induces assemblage of actin filaments in specific regions of cytoplasm rich in G-actin (Fig. S7F) that follows comments by Melak et al. (2017). We will stress multiple scenarios in the final version of the manuscript and discus them more extensively.

As mentioned above, we will follow your valuable suggestion to monitor the movement of granules prior to fixation and perform correlative light-electron microscopy to validate, whether ALGs (or a subset of them) are indeed identical with Fibrillar Vesicles.

We further paln to conduct additional control experiments, including phalloidin and SiR-actin parallel staining and monitoring of Actin Labelled Granules in living specimen treaded with inhibitors of F-actin polymerization. We expect a high degree of overlap between the signal form phalloidin and SiR-actin but not necessarily 100% correlation due to bonding to different epitopes on the F-actin surface. On the other hand, the risk of fixation artifacts can never be discarded. Some granules/vesicles might react to fixation by fusing or dispersing. Another problem might be related to detergents used for permeabilization that might break or modify granules, as they may affect not only the cell membrane but also internal organelles, including Actin Labelled Granules, if our assumption is correct and they consist of densely packed actin filaments enveloped in lipid membrane).

We also agree that the impact of unlabeled jasplakinolide on the motility and morphology of reticolopodia and the F-actin distribution is worth testing in details. As far as we observe, labelled jasplakinolide (i.e. SiR actin) is not disturbing the movement of reticulopodia or chamber formation at all. Either with or without labelling, chamber formation works the same way. The density of granules (seen in transmitted light), as well as their speed are comparable. It is worth mentioning that it was shown for animal cells that the cytotoxic effect of labelled jasplakinolide is much lower than unlabelled (Lukinavičius et al. 2014). More important is the deteriorating impact of laser light, especially during longer experiments (overnight time lapses).

*Comment: A storage form of f-actin? Because actin is *highly* abundant in eukaryotic cells, it would be remarkable for it to be transported as oligomers or filaments, as suggested. To make the claim believable, the authors would have to provide evidence that g-actin concentrations in reticulopods are insufficient to support localized assembly. (There is a vast literature*

on g-actin transport or storage forms of g-actin complexed with assembly regulatory proteins, in neurons, sperm acrosomes, etc., that the authors can consult to guide their work.)

Re: We are aware that such a mode of transport would be remarkable in the eukaryotic system. However, this mode might represent an analog to tubulin paracrystals. With regard to the presence of G-actin in reticulopodia, we plan to perform experiments in near future to measure the G-actin/F-actin ratio in reticulopodia and to estimate if the G-actin content in reticulopodia is enough to explain the presence of the observed F-actin structures. We agree that G-actin is very abundant in eukaryotic cells, hence the proposed system of transporting prefabricated actin filament seems to be unusual. Foraminiferal cells, however, differ from most of other eukaryotic cells in their size and ability to rapidly extend pseudopods. The abundance of G-actin may be restricted to endoplasm. Reticulopodia, and especially their distal parts probably differ in that regards, as they do not contain ribosomes (Bowser and Travis, 1991). Actin among other structural components must be somehow transported to those places, simple diffusion may not be sufficient. It was mentioned in the referee's comment that some cells, such as neurons have systems of G-actin transport. Even though there are many analogies between axon growth and reticulopodia extension in foraminifera, and they share many physiological mechanisms, there are also some significant differences. The most prominent difference between neurons and foraminifera is the time scale of morphogenetic processes. Travis and Bowser (1991) state that foraminiferans extends pseudopods at speeds in excess of 1 µm/s […]. In contrast, neurite outgrowth from neurons cultured at 37ºC (albeit only a superficially similar process) occurs at approximately 10 µm/h. Similar growth rates of neurites are found in several other studies as well, e. g. for Xenopus a growth rate of 54.6+1.22 µm h^{-1} has been reported (Konopacki et al. 2016). Moreover, there is growing evidence that even in neurons there are some systems of transport and turnover of prefabricated elements including lipid membranes and numerous receptors (Vitriol and Zheng, 2012).

If transportation of actin cytoskeleton components from the endoplasm to reticulopodia in foraminifera occurs as proposed in our hypothetical model (in form of discrete portions most likely separated from the rest of the protoplasm with lipid membrane), it can easily be controlled by the foraminiferal cell. This may explain coordinated and directed movements displayed typically by pseudopodia. If our hypothesis is correct then the membrane covering the ALGs may also serve an important regulatory function requiring the co-transport of various additional membrane proteins (such is receptors or proteins involved in membrane fusion or mechanical properties).

Taking into account published data (Bowser et al. 1988), one may assume that actin and tubulin are two complementary parts of the system responsible for morphogenesis and support of the form of reticulopodia where they possibly serve two opposite functions: tubulin provides stiffness and actin is mainly responsible for adhesion, elasticity and the dynamic aspects of reticulopodia.

We cannot rule out the possibility that G-actin is transported within special vesicles and that SiR-actin induces the assemblage of actin filaments within them (Fig. S7F). What we call Actin Labeled Granules may actually be a transportation vesicle of a concentrated solution of G-actin. In that case jasplakinolide would just initiate assembly (polymerization) of F-actin. If this were true, it would definately be an interesting physiological property of Foraminifera. However, this hypothesis seems to be less likely than the hypothesis that ALGs primarily serve as a transportation and storage vehicle of prefabricated

F-actin, because Fibrillar Vesicles (FVs) known from TEM images may correspond to ALGs. FVs have a similar size and their internal structure is compatible with this hypothesis. An alternative hypothesis assumes that ALGs contain both prefabricated F-actin (at least oligomers) and some pool of G-actin.

In conclusion, all these presented hypotheses should be verified and tested by further extensive studies. We hope that our submitted and discussed contribution is a good motivation to carry on such complex studies.

As a final point, I question the "fit" for this study being published in *Biogeoscience*. It seems more suitable for a cell biology or protistology journal, where it will receive much more attention. This true that this paper seems more suitable for a cell biology or protistology journal. It might receive more attention. However, Foraminifera is a model group of organisms critical in Earth sciences. We would like to present our results in *Biogeosciences* because this journal perfectly links “bio-” with “geo-sciences”. This open access journal has published many studies investigating recent foraminifera or different physiological processes such as mechanisms of biomineralization. F-actin is indeed involved in biomineralization in Foraminifera, we have observed dynamic Actin Labeled Granules within globopodium and lamellipodium during chamber formation and biomineralization. We would like to stress this fact in the final version of our manuscript. Moreover, presence of Actin Labeled Granules is very unusual feature of this taxon may have a great evolutionary significance.

We have to admit that most cell biology journals neglect marine protists, being focused on model organisms, such as *Drosophila melanogaster*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Zea mays* or various cells of selected mammalian taxa. Furthermore, we believe that Creative Commons License offered by BG offers the best strategy to cross the BIO/GEO “demarcation line”.

Thank you very much for all valuable suggestions and constructive comments.

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Response to the interactive comment on "Dynamics and organization of actin-labelled granules as a rapid transport mode of actin cytoskeleton components in Foraminifera" by Dr. Takashi Toyofuku (a referee)

Referee comments are given in italics

Dear Dr. Toyofuku,

we would like to thank you for all your valuable comments and suggestions, which help us significantly improve quality of our manuscript. Below the entire review is pasted in italics and our responses are given in regular font and follow individual comments.

General comments

Comment: This study used a fluorescent probe "SiR-actin" that specifically stains actin filament. The authors describe whether or not it is actin with various potential pieces of evidence. Further, the authors recorded the behaviour of stained materials. In particular, they describe the movement of particles that are present on the pseudopodia. The particles were transported rapidly on the pseudopodia. The authors are assuming that it is a "packet of actin filament". It is hypothesized that it is one of the causes for the pseudopodia of foraminifers to be rapidly extendable and retractable. I like this series of observations and estimations from a general point of view. The content is complementary to Tyszka et al. (2019), which was previously published on ProNAS.

The results are presented in beautiful photomicrographs and are ambitious scientific manuscripts with new suggestions. I'm positive about this manuscript, but there are some points that I would like authors to improve for publication.

Re: Thank you very much for detailed review of our manuscript. We highly acknowledge your encouraging comments on the series of our observations on SiR-actin staining experiments. We especially appreciate constructive criticism focused on

methodological aspects of our studies. We are confident all the remarks and suggested changes will help us significantly improve the quality of our manuscript. We further deeply appreciate comments regarding quality of presented images.

Comment: They suggest that what is stained in SiR-actin were the membranous surfaces of pseudopodial structures, linear or ring-like structures, and small but strongly labelled granular structures. Then, they defined these small but strongly labelled granular structures as actin-labelled granules (ALGs). The behaviour of ALGs is partially documented.

Furthermore, since mitochondria are known to distribute on the pseudopodia, they distinguish ALGs from mitochondria by Mitotracker green. Mitochondria should be indicated by Mitotracker green and not by SiR-actin. Then, the authors deny the possibility that ALGs are mitochondria. That is the reason why particles stained with SiR-actin present on the pseudopodia are not mitochondria but actin.

In interpretation and argument, it is claimed that the materials stained with SiR-actin are actin, this would be over-interpretation. Since this point is the limit of the fluorescent staining method. Argue the certainty from the comparison of the current results with the previous studies, TEM, and the fact that the distribution with mitochondria does not overlap. I would like you to discuss this point clearly and collectively in section 4.1. The possibility of the existence of the structure observed in the TEM of the previous studies should be included in this paragraph. If the authors will not discuss reliability and robustness in 4.1, readers cannot consider the following argument.

Re: We would like to thank for this comment. We do agree that other possible scenarios explaining observed staining pattern must be explicitly described. To avoid risk of over-interpretation, we followed your comments and propose to re-write the discussion section in the final version of the manuscript. We present all possible scenarios. As pointed out in earlier comments by Dr. Samuel Bowser, we have already tried to address this issue in our response. We also add a figure illustrating different possible scenarios, explaining patterns of SiR-actin staining that we observe to the final version of supplementary materials (see Fig. S7 – in the corrected version of supplementary materials). To address both referee's concerns, we add this following paragraph to the section **4.1 Assessment of unspecific fluorescent labelling risk**:

"As the granular pattern of SiR-actin staining is unusual compared to other eukaryotes, it requires an extensive discussion of all possible scenarios (see Fig. S7). We can see three possible scenarios in which ALGs may represent real F-actin-containing structures that are labelled by SiR-actin probe (Fig. S6A-C in Supplement), and three additional possibilities that would reveal the observed patterns as artifacts (Fig. S6D-F in Supplement). The first and most likely scenario (Fig. S6A in Supplement) assumes that foraminifera possess granular structures filled with densely packed actin filaments that are specifically stained with SiR-actin. These structures possibly correspond to Fibrillar Vesicles known from TEM ultrastructure studies (see below in Section 4.5.1). According to the second scenario, labelled actin filaments surround some membranous vesicles (Fig. 1B in Supplement). These vesicles are possibly involved in transport and endocytosis and F-actin probably plays role in those processes. Alternatively, they may correspond to elliptical fussy-coated vesicles described by Koonce et al. (1986) regulating motility of reticulopodia (see below section 4.5.2 Elliptical fuzzy-coated vesicle). The third scenario assumes that actin filaments are located both inside and outside of some membrane-bound vesicles (Fig. S6C). Alternatively, the observed staining pattern may be explained as an artifact, if SiR-actin binds to another, unidentified, organic molecule that is different

from, and not associated with F-actin, either inside (Fig. S6D in Supplement) or outside (Fig.S6E in Supplement) of membranous vesicles. Lastly, SiR-actin may induce assemblage of actin filaments in the areas rich in G-actin (Fig. S6F in Supplement) as suggested by Melak et al. (2017)."

We noticed already in P7.L15-16 of original version our manuscript that "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)". We were also aware that hypothesis assuming that ALGs are real actin-rich granules corresponding to Fibrillar Vesicles is not sole possible scenario as we described it in our conclusions (P14.L13-15) as "a working hypothesis that should be verified by correlative TEM-fluorescence methods." All these aspects are elaborated more in the Interpretation and Discussion section.

Comment: Description of the methodology that can be reproduced experimentally is essential for the scientific paper. Basic information such as what you observed with the filter set is missing in this study. The authors need to improve the writing of methodology. This point is the most unacceptable problem in this manuscript.

Re: We especially appreciate all methodological comments, pointing out the lack of some information on hardware setups we used in our experiments. We will add all necessary information needed for reproduction of our results.

We present detailed point-by-point response to your specific comments and questions below.

Questions and Comments

P1. L26 Correlative fluorescent.... It is not done in this study. Is the description necessary in the abstract?

Re: Indeed it may be misleading to include this in the abstract as we did not run this type of experiments in the presented study. We left this out from the abstract in the final of the manuscript.

P3. L20 Describe the exact number of used species in this study.

Re: We re-written the beginning of this paragraph, so it would more clearly specify species used for this study:

'Experiments performed on 3 species of foraminifera *Amphistegina lessonii* d'Orbigny, *Ammonia* sp., *Quinqueloculina* sp. They belong to both main classes of multilocular foraminifera (first two species belong to Globothalamea and the third one to Tubothalamea). We have observed similar staining patterns in other species, such as *Calcarina sp*. and *Peneroplis sp.*'

P4. L14 How long? How about food material? What will be labelled by calcein-AM with calcium-free seawater?

Re:

We made major changes to the section 2.2 to answer these questions.

P4. L16 Categorize by purpose, not the institute.

Re: We change the categorization according to the referee's comment.

P4. L16 Refer to Ohno, Y., et al. "Cytological Observations of the Large Symbiotic Foraminifer Amphisorus kudakajimensis Using Calcein Acetoxymethyl Ester (vol 11, e0165844, 2016)." PLOS ONE 12.4 (2017).

Re: We added citation suggested by the referee and described in detail the fluorescent dye that we used.

P4. L17 remove ")"

Re: We corrected this typographic error.

P4. L19 Indicate the excitation and emission wavelengths of all probes.

Re: We summarised necessary information in a new table added to the final version of the supplementary materials (Table S1).

P4. L20 " it is not possible to use this probe to label F-actin within the endoplasm" The authors show the SiR-actin fluorescent in the cell (Fig. 4). Explain exactly. In fact, it is difficult to distinguish between signals and autofluorescent from chlorophyll.

Re: We rewrote this sentence to make it more accurate: 'Absorption and emission parameters of the probe are overlapping with the autofluorescence of chlorophyll from endosymbionts, thus, distinguishing between SiR-actin signal and autofluorescence emitted from the endoplasm of *Amphistegina lessonii* or other species hosting endosymbionts is difficult.'

P4. L22 The descriptions are not enough to reproduce the experiment. How did authors decide experimental/observation settings (e.g. excitations/emissions and exposure times) of each probe? The settings of conventional optical observation should be indicated, too. Were there some negative controls? What was the frequency of time-lapse imaging?

Re: We added necessary information on hardware settings in the Tables S2 and S3 in the supplementary materials and the following sentence to the manuscript:

'Hardware settings used for obtaining images are summarised in Tables S2 (exposure time, binning mode, objectives and Apotome mode in experiments conducted on Zeiss Axio Observer Z.1.), S3 (hardware settings in experiments conducted on Leica SP5 inverted confocal microscope), and S4 (filter sets used for different fluorescent dyes for experiments performed on Zeiss Axio Observer Z.1.) We optimised these settings using trial and error method.'

Moreover we added brief information on negative control in the manuscript '[c]omparison stained individual of *A. lessonii* to unstained control shows that SiR-actin fluorescent probe indeed stains endoplasmic structures in foraminifera (Fig. S6 in supplement)' and the picture comparison of stained and unstained individual of *A. lessonii* has been included in the supplementary materials. We also added the information regarding the interval between frames used for recordings of the time-lapse.

P4. L22 Indicate the setting of fluorescent cubes.

Re: We added Table S4 summarising the settings of fluorescent cubes to the supplementary material and we referred to this table in the manuscript.

P5. L5 Be sure to indicate whether each image is by laser confocal or by ApoTome.

Re: We added specific information to captions of images obtained by Zeiss Axio Observer Z.1 to distinguish conventional fluorescence images from optical sectionings made by ApoTome.

P5. L6 Rather than being confirmed to be actin, it is used in the sense of particles stained with SiR-actin. Is it not misleading?

Re: We have change the term to SiR-actin-labelled granules to avoid confusion.

P5. L8 Put some description of DIC observation in Materials and Method.

Re: We added a sentence describing this technique at the end of the paragraph together with a new citation: "Nomarski contrast or Differential interference contrast (DIC) is a microscopy technique utilising interferometry principle for improving contrast in transparent objects (Lang, 1968)."

P5. L11 This text contradicts that the cytoplasm has symbiotic algae and is unobservable.

Re: To clarify we re-wrote this sentence so now it says: 'They can be identified in endoplasmic structures within the chambers of non-symbiont-bearing species such as *Quinqueloculina* sp., close to surfaces of internal walls of the test (Fig. 5).'

P5. L12 Had authors mixed and discussed the results of different species? Are there variations among species?

Re: We added names of the species in which we observed ALGs within different cytoplasmic structures to the main text of the manuscript (originally the names of the species were included only in the captions of the figures).

P6. L1 Isn't "SiR-"actin-labelled granules?

Re: As stated in the response to referee's comment to P5. L6, we have change the term to SiR-actin-labelled granules to avoid confusion. We would like to address this question by showing that those granules are stained not only with SiR-actin but also with different probes targeting F-actin such as phalloidin conjugates. We plan such experiments in the next project

P6. L3 Show the variation of dynamics. Can you make a summarized table?

Re: Yes, we included tables summarising information on velocity of the movement of ALGs and a time lapse movie to the supplementary materials.

P6. L6 It is a good approach.

Re: Thank you for that encouraging comment to our statement that "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".

P6. L15 Although it is difficult to measure the dynamics of the granule, the authors observe the movement of the fluorescently labelled granules that were seen along the pseudopod. This makes it possible to observe the movement of granules by limiting into a one-dimensional movement. For example, if time is plotted on the horizontal axis and the coordinates in the pseudopodia on the vertical axis, can it be possible to illustrate temporal changes in granule's position.

Re: Since submission original version of the manuscript we applied another approach for measurement of velocities of ALGs. We employed the TrackMate plug-in in Fiji software to track and calculate their velocities. We present update results with detailed description how we obtained them in the corrected version of manuscript and supplementary materials.

P6. L19 How did you calculate the rate of granule movement? Did you show the measurement method in the materials and methods?

Re: In the corrected version of manuscript we added new section (2.4 Measurement of velocity of the Actin Labelled Granules) explaining measurement of velocities of ALGs.

P6. L22 Indicate the dynamics of other types of a granule.

Re: The dynamics of other types of granules seem to be comparable to ALGs. Nonetheless, specific observations and measurements have not been made. In the future it is worth to compare dynamics of SiR-actin-labelled granules to dynamics of mitochondria.

P7. L5 FLAKOWSKI et al can be found in the reference list. I guess the authors refer to the study here. Discuss the relationship between foraminiferal actin variability and phylogeny.

Re: We added the citation in the section '4.1 Assessment of unspecific fluorescent labelling risk' as suggested by referee, as well as we corrected the typographic error in the surname of the cited author in the section '4.6 Functional implications, evolutionary consequences, and future research prospect'.

P7. L18 "Such effects have not been reported." Did you compare the results with the population of negative controls.

Re: As referee pointed out, this issue needed clarification. To do that we made major changes to the last paragraph of the section '4.1 Assessment of unspecific fluorescent labelling risk'.

P8. L10 Please reconsider the subtitles. It does not match the content.

Re: We changed the subtitle for "4.3 Main hypothesis regarding the function of SiR-actin-labelled granules".

P8. L16 This paragraph plays the role of the just introduction of following chapters not a discussion of results. Reconstruct the chapter structure. e.g. The chapter number 4.4 should be 4.3.1.

Re: We reconstructed structure of this chapter as you recommend and re-numbered sections of this chapter accordingly

P10. L9 4.5.1 is numbered twice.

Re: We changed numbers of the sections in this chapter, hence this error has been corrected.

P10. L13 "representstructurally" insert space.

Re: It has been corrected in the final version. Thank you for finding this error.

P21. L3 What does the cloudy distribution of SiR-actin around endosymbiont? No signal was detected in the same region by calcein-AM.

Re: It is not clear why there is cloudy pattern of red florescence observed around endosymbionts. It may indicate some F-actin structures involved in the movement of endosymbionts within cytoplasm. Other possibility is that it is merely a consequence of technical constraints (not sufficient signal-to-noise ratio) and some optical properties of the test which can diffuse light causing some reflections and other artifacts.

The second part of this question is still difficult to answer at present stage. It is something unexpected, but we observe it regularly for different species of Globothalamea that calcein red-orange AM stains clearly all pseudopodial structures but not the cytoplasm inside the test. At present we can only speculate that actually endo- and ectoplasm may differ in some important manner or they may be separated from each other by some membranous structure. Fluorescent dye in question is membrane-permeable only in the form of acetoxymethyl ester. Upon penetrating into the cell it is transformed in non-permeable form by enzymes that split ester bond, making it impossible to penetrate other areas of the cell enclosed by lipid membranes. That kind of additional internal compartmentalisation of foraminiferal cytoplasm may play a crucial role in physiology. This problem, although very interesting, is beyond the scope of presented study.

References:

Lukinavičius, G., Reymond, L., D'Este, E., Masharina A., Göttfert F., Ta H., Güther A., Fournier M., Rizzo, S., Waldmann, H., Blaukopf C., Sommer, C., Gerlich D. W., Arndt, H.-D., Hell S.W., and Johnsson K.: Fluorogenic probes for live-cell imaging of the cytoskeleton, *Nat. Methods*, 11, 731–733, https://doi.org/10.1038/nmeth.2972, 2014.

Melak, M., Matthias, P., and Robert, G.: Actin visualization at a glance. *J. Cell Sci.*, 130.3, 525-530, https://doi.org/10.1242/jcs.189068, 2017.

~~Dynamics and organization of~~ SiR-actin-labelled granules ~~as a rapid transport mode of actin cytoskeleton components~~ in Foraminifera~~-~~: Patterns, dynamics, and hypotheses

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Abstract. Recent advances in ~~fluorescent~~fluorescence 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~~pattern of SiR-actin staining in Foraminifera is the prevalence of SiR-actin-labelled granules (ALGs) within pseudopodial structures. Fluorescent ~~signal~~signals obtained from granules dominate over dispersed ~~signal~~signals from the actin meshwork. ~~Actin~~SiR-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~~labelled microstructures 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. Several hypotheses are set up to explain either specific or nonspecific actin staining. Two hypotheses regarding their function are proposed, if specific actin labelling is taken into account: (1) ~~They~~Granules 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~~hypotheses 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 SiR-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 for preservation in the fossil record~~, they~~ and are primarily important ~~primarily for~~in Earth ~~Sciences.~~Science disciplines. Application of foraminiferal research includes among others: biostratigraphy, ~~paleoclimatology, paleo~~ palaeoclimatology, palaeo/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~~nuclei, ribosomes, Golgi apparatus are reported to occur only in the endoplasm (Bowser and Travis, 1991). ~~Most~~The most prominent ectoplasmic structures in Foraminifera are pseudopods, which have a 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~~behaviour.

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*~~*A. 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~~.~~ (see Frontalini et al., 2019).

This paper is an attempt to fill the gap in our knowledge on actin organisation and dynamics in Foraminifera. Therefore, the 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~~ identify a relative localisation and dynamics of granules represented by mitochondria and SiR-actin-labelled structures;

(c) identification and detailed description of the actin cytoskeleton organisation in Foraminifera with particular focus on its granularity and dynamics by means of live 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~~SiR-actin labelled granules;

(f) interpretation and discussion of working hypotheses regarding the functionality of ~~ALGs~~actin granularity and its evolutionary consequences. This will take into account the 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~~Experiments performed on ~~various~~3 species of ~~Foraminifera, such as~~ foraminifera *Amphistegina lessonii* d'Orbigny, *Ammonia* sp., ~~*Quiqueloculina*~~*Quinqueloculina* sp~~.~~. They belong to both main classes of multilocular foraminifera, i.e. first two species belong to Globothalamea and the third one to Tubothalamea. We have observed similar staining patterns in other species, such as *Calcarina* sp. and *Peneroplis* sp. Specimens of *A. lessonii* were collected from the coral aquarium ~~in~~of Burgers' Zoo in Arnhem (the Netherlands). This aquarium contains a diverse ~~population~~assemblage of corals and other

organisms from the Indo-Pacific, among them there are around fifty species of benthic foraminifera, including ~~*Amphistegina*~~*A. 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).

We employed two slightly different methods of sample 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 fine paint brush and transferred into a 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~~the 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. and *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 glass bottom ~~glass~~, specimens were stained and observed. ~~In~~At the AWI we conducted the ~~observation~~observations mainly on chamber formation, while at the ING PAN, most investigations were focused on reticulopodia.

2.2 ~~Staining~~ Fluorescent probes and staining procedure

~~In our experiment we~~We focused on staining F-actin with SiR–actin but also used Mitotracker Green to stain mitochondria and calcein Red- Orange AM for staining cytoplasm. ~~At the AWI~~For experiments focusing on actin organization during chamber formation (Figs. 3-4; Figs. S1-S2 and Movie S2 in Supplement) 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~~For experiments regarding raticulopodia at ING PAN (Figs. 1-2, 5-6; Figs. S3-S5 and movie S1 in Supplement) the ~~final~~ concentration of ~~SiRactin~~SiR-actin was 0.5 µM and of 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~~After 15-20 minutes the signal was sufficient to perform observations. Calcein Red-Orange AM is a cell-permeable dye that stains the cytoplasm of living cells and is often used to indicate the viability of cells (Frontalini et al., 2019). Calcein AM is hydrolyzed in the cytosol and fluoresce in the presence of calcium ions. It differs in a chemical structure and fluorescent from the calcein AM used for staining cytoplasm be Ohno et al. (2017). In our experiments the main purpose of using Calcein Red-Orange AM was to indicate the limits of the cytoplasm and to highlight 3D structure of pseudopodia (e.g., globopodium). Live cells structures are stained with this dye even, if they are surrounded by calcium-free artificial sea water, as calcium ions are always present within living cells Table S1 (Supplement) summarizes information on fluorescent probes used in presented research.

Absorption and emission parameters of the probes are overlapping with the autofluorescence of chlorophyll from endosymbionts, thus, distinguishing between SiR-actin signal and autofluorescence emitted from the endoplasm of *A. lessonii* or other species hosting endosymbionts is difficult. To minimise the problems caused by autofluorescence, all specimens were starved for 24 hours prior the staining and observations.

2.3 Fluorescence and transmitted light 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~~fluorescence 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.~~in triplicate in some images. Hardware settings used for obtaining images are summarised in Tables S2 (exposure time, binning mode, objectives and Apotome mode in experiments conducted on Zeiss Axio Observer Z.1.), S3 (hardware settings in experiments conducted on Leica SP5 inverted confocal microscope), and S4 (filter sets used for different fluorescent dyes for experiments performed on Zeiss Axio Observer Z.1.). We optimised these settings using a trial and error method. To provide additional information over all structures of observed individuals, we captured bright field images for experiments with the Leica SP5. and Nomarski contrast imaging for the experiments with the Zeiss Axio Observer Z.1. Nomarski contrast or Differential interference contrast (DIC) is a microscopy technique utilising interferometry principle for improving contrast in transparent objects (Lang, 1968).

2.4 Measurement of velocity of the Actin Labelled Granules

For measuring the velocities of granules in pseudopodia we used time lapse records of pseudopodia labelled with SiR-actin using the Zeiss Axio Observer Z.1. Time lapse movies consisting of 50 frames were recorded (0.419 s time interval). SiR-actin-labelled granules (ALGs) can display very rapid movement, hence tracking requires dense time lapses. To minimize time necessary for capturing single frame we used only one fluorescent channel (without ApoTome).

TrackMate plug-in applied to the Fiji software (Tinevez et al. 2017) was used for calculating the velocities of the ALGs. Calculating velocities required two main steps: (1) annotation of spots representing ALGs in each of the frames of the time lapse and (2) creating links between particular spots in subsequent frames in time lapse (tracks of movement of spots). The software allows for choosing several options for both of the main steps. This can be done either manually or automatically (with several different options in the latter case). We used the LoG (Laplacian of Gaussian) detector for automatic annotation, As the approximate size of the ALGs is 1 µm, we used this "blob" size for the LoG detector. The threshold value was set up to 100. Blob is a technical term used in the tracking of any objects in during time lapses measurements. These values were optimized by trial and error to minimize two types of errors: (1) lack of annotation of some objects that can be clearly identified on the images (2) annotation as spots areas with no apparent ALGs. It is not always easy to track which blobs in frame n+1 match with those in frame n. ALGs may temporally get so close to each other that limits their separation in subsequent frames. To recognize this we allowed for merging and splitting the tracks. After testing automatic and manual options for the second step (creating links between spots), we decided to perform it manually, as automatic methods seem to give random results for the ALGs. When spots are annotated and links between spots in the subsequent frames are created velocities of spots are measured automatically.

3 Results

3.1 Identification of SiR-actin-labelled structures by ~~fluorescent~~fluorescence microscopy

Fluorescent SiR-actin labelling has revealed three considerably different patterns of staining in *Ammonia* sp, 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~~Supplement). The term SiR-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 in *A. lessonii* (Fig. 3; Figs. S1-S2 in ~~supplement~~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~~Supplement). They ~~are also present~~can be identified in endoplasmic structures within the chambers~~,~~ of non-

symbiont-bearing species such as *Quinqueloculina* sp., 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~~behaviours". All fluorescence labelled structures observed ~~under fluorescent light co-localize~~can be matched with pseudopodial structures and granular microstructures identified in ~~the~~ Nomarski contrast (DIC) or in ~~the~~ brightfield image. Figures 1-2 ~~presents~~present a lamellipodial structure attached to the glass surface with a weak, dispersed fluorescent signal of SiR-actin staining the F-actin meshwork. Very fine ~~brighter~~bright spots represent ALGs that ~~co-localize~~match with granules observed with DIC optics.

3.2 Testing the selectivity of ~~granules~~ labelling~~: ALGs~~ of granules: SiR-actin-labelled granules vs mitochondria

Direct comparative analysis of ~~fluorescent~~fluorescence vs DIC images ~~indicates~~of *A. lessonii* indicate that SiR-actin-labelled granules do not overlap with all granules observed in DIC (Fig. S3 in ~~supplement~~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 a double labelling experiments. Mitochondria were the 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~~Supplement). Therefore, they indicate that ~~generally~~ mitochondria and SiR-actin labelled granules ~~form~~are two non-overlapping categories.

3.3 Dynamics of SiR-actin-labelled granules

The dynamics of the ALGs ~~should be~~(velocity and overall pattern of movement) are described separately in ~~pseudopodia~~granuloreticulopodia and in a globopodium during chamber formation. The dynamics ~~(velocity and overall pattern of movement)~~ may ~~differ according to location~~vary for different locations 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 the 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 in *A. lessonii* (Figs. S4-S5 in ~~supplement~~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~~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~~of ALGs due to ~~lack of constant frame~~the absence of a stationary reference frame. 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~~To overcome this problem intervals between subsequent frames 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,~~time lapse movies were minimised. Using this strategy we recorded time lapse movies (Movie S4 in Supplement) showing a wide range of velocities of ALGs in reticulopodia of *A. lessonii* up to ~~9~~15.4 µm/s. (Fig. S8 and Tables S5- S6 in Supplement).

Lamellipodia ~~overlying~~covering the test form two-dimensional sheets, resulting in a more complex pattern of displacement of ~~ALG~~ALGs than the one observed in granuloreticulopodia. There are areas dominated by directional protoplasm streaming ~~that contrast to~~and areas showing less organised behaviour. Accordingly, actin granules can be divided into several categories based on ~~pattern of demonstrated~~dominant movement patterns: (1) stationary or almost stationary ALGs that oscillate within a 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~~velocities 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 a risk of capturing ~~some~~ artefacts instead of imaging target structures. In case of ~~fluorescent~~fluorescence microscopy the greatest danger is unspecific labelling or autofluorescence. ~~It~~Comparison stained individual of *A. lessonii* to unstained control shows that SiR-actin fluorescent probe indeed stains endoplasmic structures in foraminifera (Fig. S6 in Supplement). This may be caused ~~by using a too high~~ when the concentration of the probe is too high or ~~too much~~when the excitation ~~intensity of~~intensities (or emission measurement sensitivity~~.~~) are too high. Another problem might be that the probe ~~may~~is not specific enough and 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~~ should be addressed to avoid confusion and over-interpretation of the results. Foraminifera are placed in the actin phylogenetic tree with Bikonta (Flakowski et al. 2005), thus the amino acid sequence of actin in foraminifera is significantly distinct from the actin sequence in Metazoa or fungi (belonging to opisthokonts) that are subject of most intensive research on actin physiology. Fluorescent probes may therefore interact differently with actin in Foraminifera.

Moreover, Foraminifera may contain other organic compounds that mimic of actin nanostructures and therefore interact with fluorescence probes, such as SiR-actin. It should be noted, however, that our results are reproducible.

As the granular pattern of SiR-actin staining is unusual compared to other eukaryotes, it requires an extensive discussion of all possible scenarios (see Fig. S7). We can see three possible scenarios in which ALGs may represent real F-actin-containing structures that are labelled by SiR-actin probe (Fig. S6A-C in Supplement), and three additional possibilities that would reveal the observed patterns as artifacts (Fig. S6D-F in Supplement). The first and most likely scenario (Fig. S6A in Supplement) assumes that foraminifera possess granular structures filled with densely packed actin filaments that are specifically stained with SiR-actin. These structures possibly correspond to Fibrillar Vesicles known from TEM ultrastructure studies (see below in Section 4.5.1). According to the second scenario, labelled actin filaments surround some membranous vesicles (Fig. 1B in Supplement). These vesicles are possibly involved in transport and endocytosis and F-actin probably plays role in those processes. Alternatively, they may correspond to elliptical fussy-coated vesicles described by Koonce et al. (1986) regulating motility of reticulopodia (see below section 4.5.2 Elliptical fuzzy-coated vesicle). The third scenario assumes that actin filaments are located both inside and outside of some membrane-bound vesicles (Fig. S6C). Alternatively, the observed staining pattern may be explained as an artifact, if SiR-actin binds to another, unidentified, organic molecule that is different from, and not associated with F-actin, either inside (Fig. S6D in Supplement) or outside (Fig.S6E in Supplement) of membranous vesicles. Lastly, SiR-actin may induce assemblage of actin filaments in the areas rich in G-actin (Fig. S6F in Supplement) as suggested by Melak et al. (2017).

The first argument ~~for~~supporting the reliability of SiR-actin live staining ~~using SiR-actin~~ is the fact that attachment sites of pseudopodia to the substratum often demonstrate a strong ~~fluorescent signals~~fluorescence signal (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~~cannot be ~~speculated~~excluded, however, that ALGs ~~might serve as part of~~ are associated to a defence strategy ~~that could~~to remove and dispose toxic compounds introduced into the cell. If this ~~is~~were true, we would expect ~~that~~ vesicles containing those probes ~~would~~to be transported outward. As ~~they~~ALGs are often moving bi-directionally (both in- and outwards~~, see~~) (Figs. S4, S5 in ~~supplement~~Supplement), this hypothesis is not very convincing. ~~Actually~~In fact, ALGs' inward movement is observed when a pseudopodial structure is being withdrawn ~~which~~and seems to indicate relocation of labelled actin into the endoplasm. Such observations support the notion that live staining using SiR-actin ~~specific~~is specifically labelling ~~and~~actin and that the inward movement of ALGs is a functional response ~~of ALGs~~ during withdrawal of pseudopodial structures.

Another issue that needs to be considered is the risk of interference of a probe with the physiology of actin itself~~,~~ as it may, for instance, cause an artificial polymerisation of F-actin (Melak et al., 2017). In that case ~~it~~we would ~~disturb~~expect negative interfence of SiR-actin on morphogenesis and biomineralization of new chambers ~~which has not~~. Nevertheless, such staining artefacts have never 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~~In our observations we did not recognise any apparent long-term differences neither in morphology or nor in dynamics of

reticulopodia after staining. Occasionally, we observed a temporary retraction of pseudopods immediately after adding the staining solution to the petri dish. However, after 10-15 minutes incubation with SiR-actin, this effect was not visible any more, and reticulopodia were spread out again, closely resembling the pre-staining structure and dynamics. There are species-specific differences in strength of this reaction, where *A. lessonii* is apparently less sensitive than *Ammonia* sp. or *Quinqueloculina* sp.

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 the 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 the reticulopodia as a dominant pattern of actin organisation in reticulopodia. Along those structures there are visible granular actin structures in ~~images~~the figures of the cited ~~publication, which presence has~~publications that are 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. the actin cytoskeleton has a different organisation depending on the ~~area of~~location in the reticulopodium: in proximal parts of pseudopodia ~~it is seen as~~a thick linear ~~fibres,~~fibre; in more distal ~~region~~regions flattened on the glass it is visible only in a few ~~foci~~locations, resembling the SiR-actin-labelled granules in our study; in the most peripheral areas actin staining is absent (Bowser et al., 1988, fig. 1C, 2C, 3C). ~~Those foci probably~~We suspect that the structure in the distal regions flattened on the glass correspond to ~~actin-labelled granules as shown~~the ALGs described in our paper.

Although Figure 1 demonstrates an SiR-actin-labelled linear structure and Figure 4d presents indistinct SiR-actin-labelled fibres, clear cable-like structures are ~~almost missing~~absent in our study in comparison to previous publications ~~that~~which 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~~ equally. The effectiveness of staining F-actin using different probes was ~~comported~~compared by Lemieux et al. (2014). ~~Authors of this paper~~They reported that different probes did not stain all of subsets of F-actin equally. ~~Effectiveness of~~Apparently, the staining effectiveness of F actin depends ~~of~~on the location of actin filaments within the cell. Even though this analysis does not include SiR-actin, ~~this problem~~the same issue may ~~also~~ apply to this probe. ~~Moreover,~~The interaction between probes and F-actin may also lead to stabilisation or enhanced polymerisation of F-actin due to its structural similarities to Jasplakinolide (Melak et al., 2017). ~~On the other hand~~In addition, cell fixation procedures may stabilise dynamic structures or create some artefacts.

Previous studies ~~concerning~~on the dynamics of granules in Foraminifera were conducted mostly on *Allogromia* and *Astramina*~~, maximal~~. The maximum speed of granules within reticulopodia was reported to be approximately 25 µm/s but most of them ~~has speed~~have velocities below 10 µm/s (Travis and Bowser, 1991). Velocities of ALGs ~~falls~~fall within this range. ~~Average~~The average speed of granules in foraminiferal pseudopodia reported by Kitazato (1988) is 13 µm/s, what is roughly comparable to our measurement of ~~9~~15.5 µm/s.

4.3 Main hypothesis regarding the ~~physiology~~functionality of ~~actin granules~~

Actin-Labelled Granules

Actin labelled 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~~hypotheses 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~~whose functions are 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~~3.1 Comparison of actin structures in other organisms

Structures similar to SiR-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 SiR-actin-labelled granules in Foraminifera. ~~Maximal~~The maximum 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 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~~provide the pressure ~~requisite~~required for expansion of new protoplasm (Mogilner and Oster, 2003).

4.~~5~~3.2 Comparison of SiR-actin-labelled granules to organelles identified in TEM images of foraminifera

Transmission electron microscopy (TEM) ~~represents~~is a principal method ~~of investigation of~~to investigate 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 the chemical ~~compositions~~composition of certain areas of the cell but in much lower resolution. Thus, ~~combing those~~combining the two approaches is essential to unravel the ultrastructure and chemical make-up and thus provide clues about the 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 the 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~~1 µm in diameter) and vesicular, globular shape (LeKieffre et al., 2018a; Goldstein and Richardson, 2018) correspond to ALGs (Figs. 2-3, 5-6; Figs. S3, S4-S5 in ~~supplement~~Supplement). Fibrillar vesicles appear to be separated from the cytosol by a lipid membrane (Figs. ~~7a, 8~~12a, 13). Membranes enveloping the fibrillar vesicles may not cover the entire vesicle. It may form characteristic open vase-shaped ~~structures~~structures (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~~nano-tomography of lamellipodium in keratocyte of zebrafish (Mueller et al., 2017), as well as in many other eukaryotic organisms (Fig. 7e). ~~Similarity~~The 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~~filaments (Fig. ~~7~~12). Similar but less organised structures of cross-linked fibres form an actin meshwork in ~~pseudopod~~the pseudopods of *Allogromia* (Bowser et al., 1988; Koury et al., 1985).

It is ~~also~~ not clear how FVs are formed~~,~~. LeKieffre et al. (2018a~~), however,~~) proposed that they are produced ~~according~~similar to ~~a~~the 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~~the

cisternae of Golgi apparatus may resolve this issue. ~~Moreover the last~~This assumption agrees with ~~finding~~findings 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, which contained proteins involved in construction of a protective envelope during chamber formation in *Orbulina universa*. However, it is not clear, whether these structures ~~represent~~~~structurally~~represent structurally and functionally analogous organelles to FVs. Spero (1988, - see figs. 4e, f, 5d) documented ~~under TEM~~ vesicles using TEM 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~~contrasts 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~~the rhizopodia of *Orbulina universa*~~, there~~ may ~~be found~~contain 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~~Finally, Elliptical fuzzy-coated vesicles

~~Elliptical fuzzy-coated vesicles~~ are additional ultrastructural cellular components that may correspond to ALGs. These vesicles are structures unique to Foraminifera. They include elongated structures that ~~aretypically~~are typically approximately 300 nm in length identified in TEM images of reticulopodia (Koury et al., 1985; 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.

4.~~6~~4 Functional implications, evolutionary consequences, and future research prospect

~~Actin~~SiR-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~~It seems very likely that they correspond to fibrillar vesicles or fuzzy coated vesicles observed in much higher resolution ~~in~~using 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~~several 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~~containing them have the ability to rapidly expand the volume of protoplasm and actin networks/patches, which 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~~Our hypothesis 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~~), and in consequence protein synthesis is restricted to the endoplasm. ~~Foraminifera~~Therefore, foraminifera must have mechanisms to efficiently transport proteins needed for the formation of extensive pseudopodial networks. This issue applies primarily to the transportation of the 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~~microtubules 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.

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~~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~~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~~Flakowski et al., 2005). There is some evidence that this duplication is shared by the group ~~Acatharea~~Acantharea 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~~suggests 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~~behaviour (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~~presents results of live fluorescent labelling of actin in Foraminifera with a focus on ectoplasmic (pseudopodial) structures during various behavioural and physiological activities. ~~Fluorescent~~Fluorescence 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~~S4-S5 and Movies S2-S3 in ~~supplement~~Supplement).

The granular appearance is the principal characteristic of actin cytoskeleton in all studied foraminiferal taxa. ~~Actin~~SiR-actin-labelled granules (ALGs) have been described as small (~~c~~ca. 1 µm in diameter), oval and dynamic objects that are numerous in pseudopodia, but present in endoplasm as well. Besides ALGs, the 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~~as a test for the selectivity of granules labelling. As presented images

(Fig. 3; ~~Fig S7~~Figs. S2-S3 in ~~supplement~~Supplement) indicate, there is very little co-localization between those two types of organelles, however, ALGs and mitochondria probably constitute the majority of granules present in pseudopodia.

~~Detailed~~A detailed interpretation of ~~obtained~~the images ~~has been~~is given, including the risk that ALGs may be a result of unspecific labelling. Presented arguments allow to exclude this possibility. Furthermore, the 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~~the question regarding the 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 ~~34~~ movies ~~and 5~~, 8 additional figures showing different actin structures in Foraminifera and ~~their dynamics~~6 tables.

Author contributions

Author contributions: J.G. designed research, performed research, analysed data, cultured foraminifera, wrote the paper and prepared graphics; J.T. proposed and supervised research; U.B. and J.B~~., contributed new reagents/analytic tools~~, provided access and infrastructure at AWI; 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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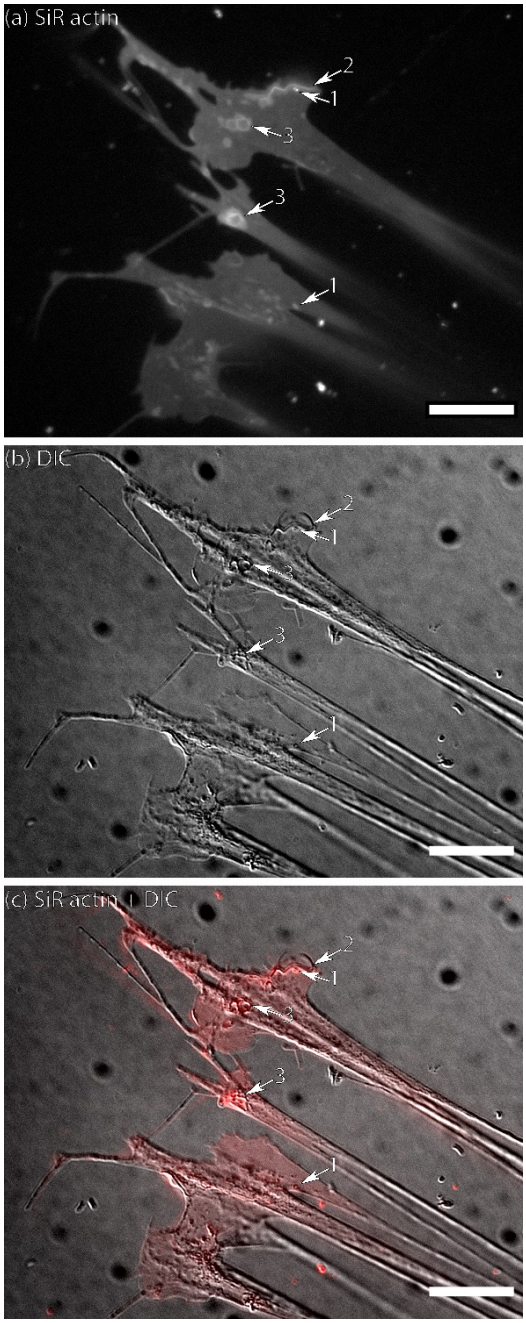


Figure 1: ~~**Frame from time lapse images showing flattened**~~**Flattened** **lamellipodia of living *Ammonia* sp. attached to glass:** (a) fluorescence of SiR-actin-labelled structures, (b) DIC image of the same area, (c) merged image of fluorescence and DIC channels (since the reticulopodia were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

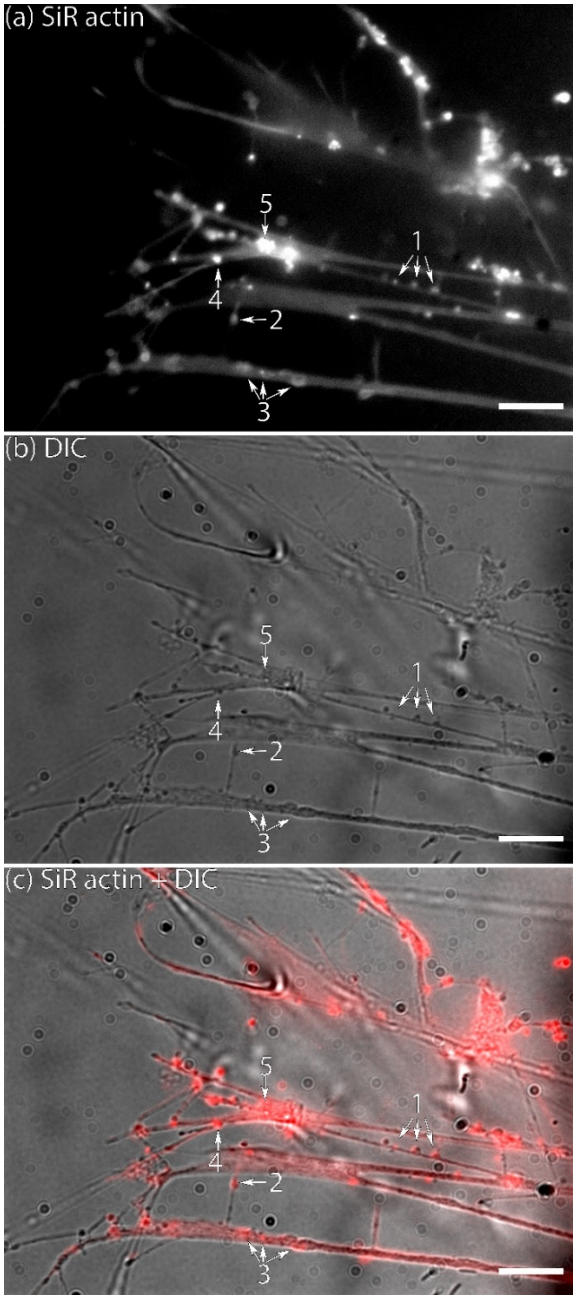


Figure 2: ~~**Frame from time lapse imaging showing pseudopodia**~~**Pseudopodia of living *Ammonia* sp. attached to glass:** (a) conventional fluorescence of SiR-actin-labelled structures, (b) DIC image of the same area, (c) merged image of fluorescence and DIC channels (since the reticulopodia were 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 SiR-actin-labelled granules (ALGs) transported along one thread of pseudopodia; 2 – actin in the tip of thin fillopodium; 3 – larger SiR-actin-labelled areas showing smudgy fluorescence weaker than in most ALGs; 4 – single ~~**4 –**~~ ALG in bifurcation of retiulopodia; 5 – group of very bright densely packed ALGs in the thick reticulopodium. ~~**Images**~~Conventional fluorescence images was obtained with *Zeiss Axio Observer Z.1*. Scale bar 10 μm.

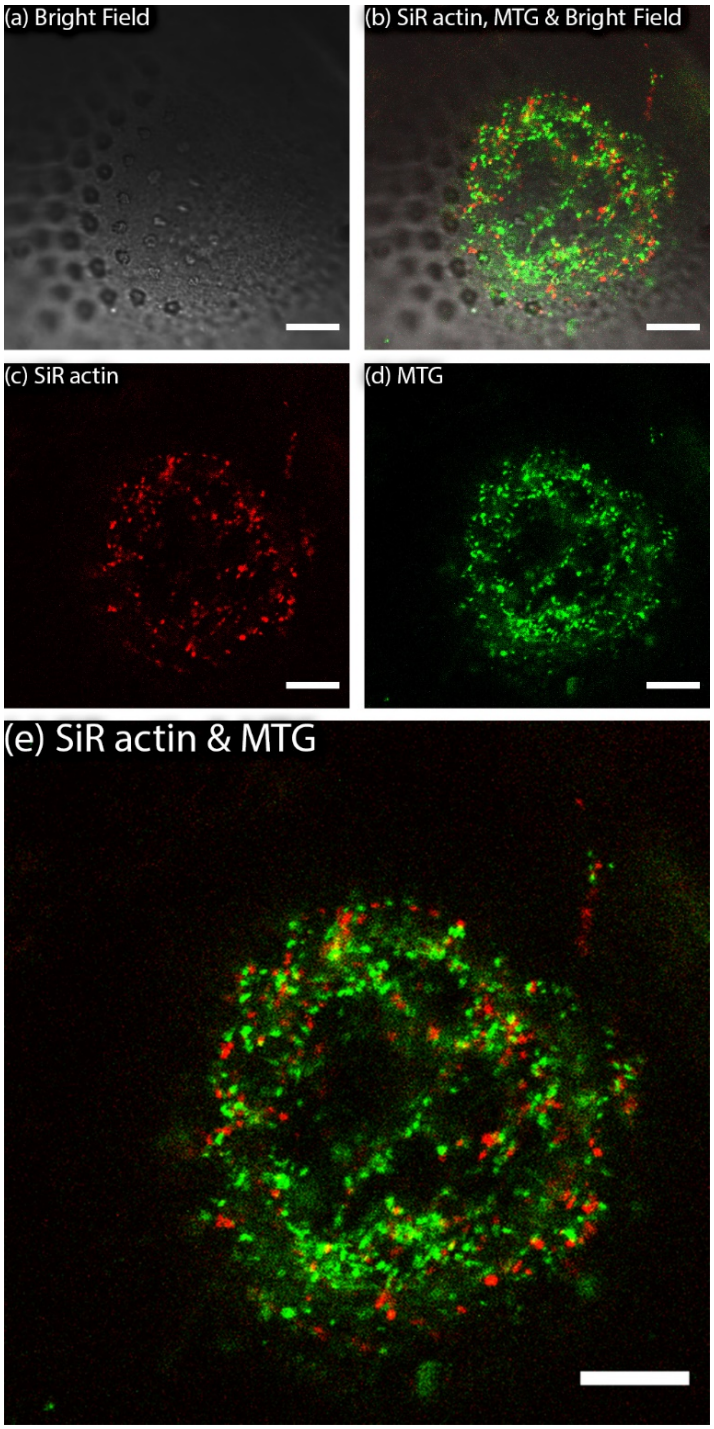


Figure 3: ~~**Single frame from time lapse showing**~~ SiR-**actin-labelled granules (ALGs) and mitochondria in cross-section of a newly forming chamber in *Amphistegina lessonii*** during biomineralization (pores are already visible in transmitted light). ALGs and mitochondria do not show co-localization. Images were obtained with Leica SP5 inverted confocal microscope. For the entire time lapse see ~~**Movie S2.**~~Supplementary Material 2. Scale bar 10 µm.

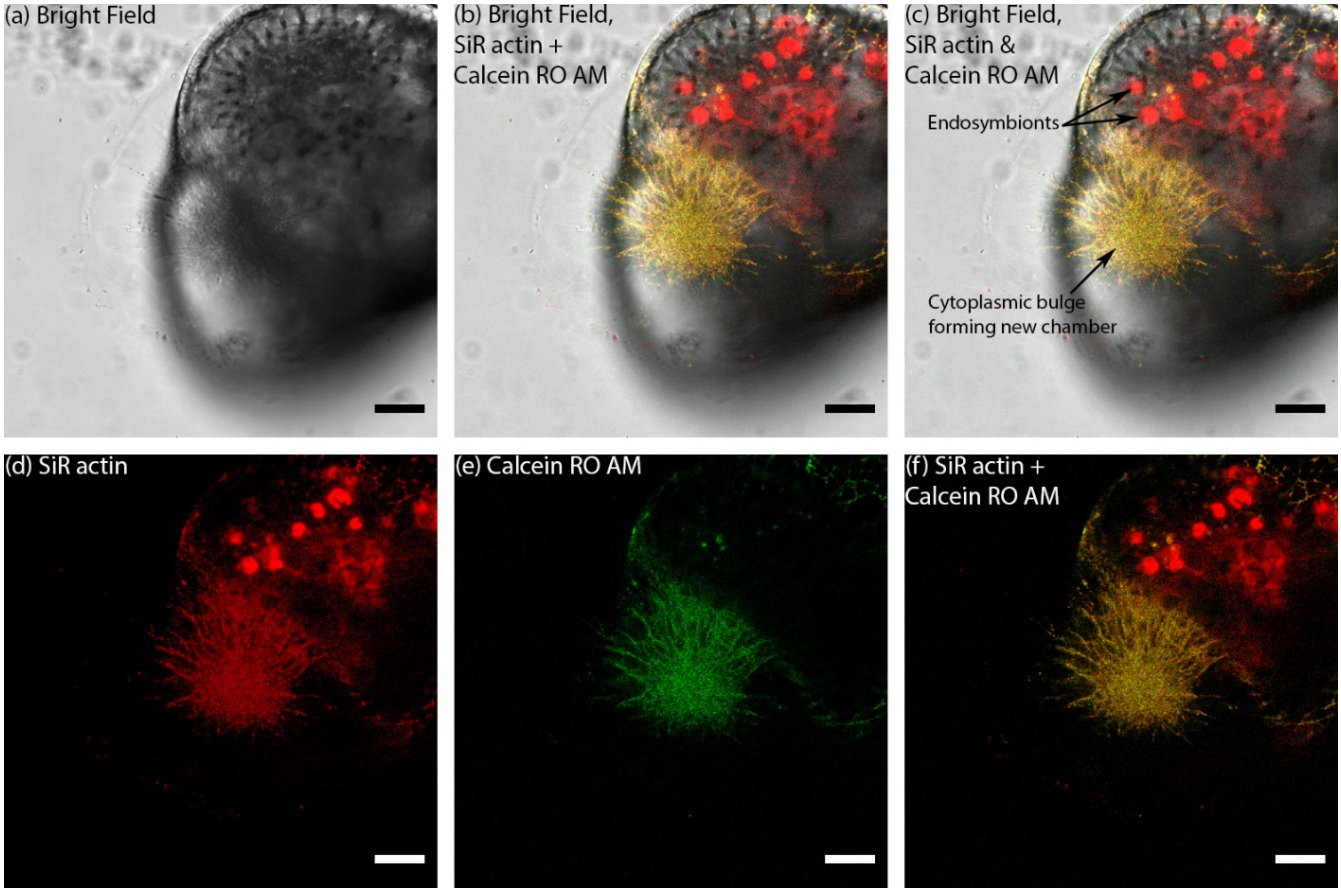


Figure 4: Organisation of actin within in finger-like structure preceding globopodium during chamber formation compared with localisation of cytoplasm stained with calcein red orange AM. Images was obtained with *Leica SP5* inverted confocal microscope. Scale bar 20 μm.

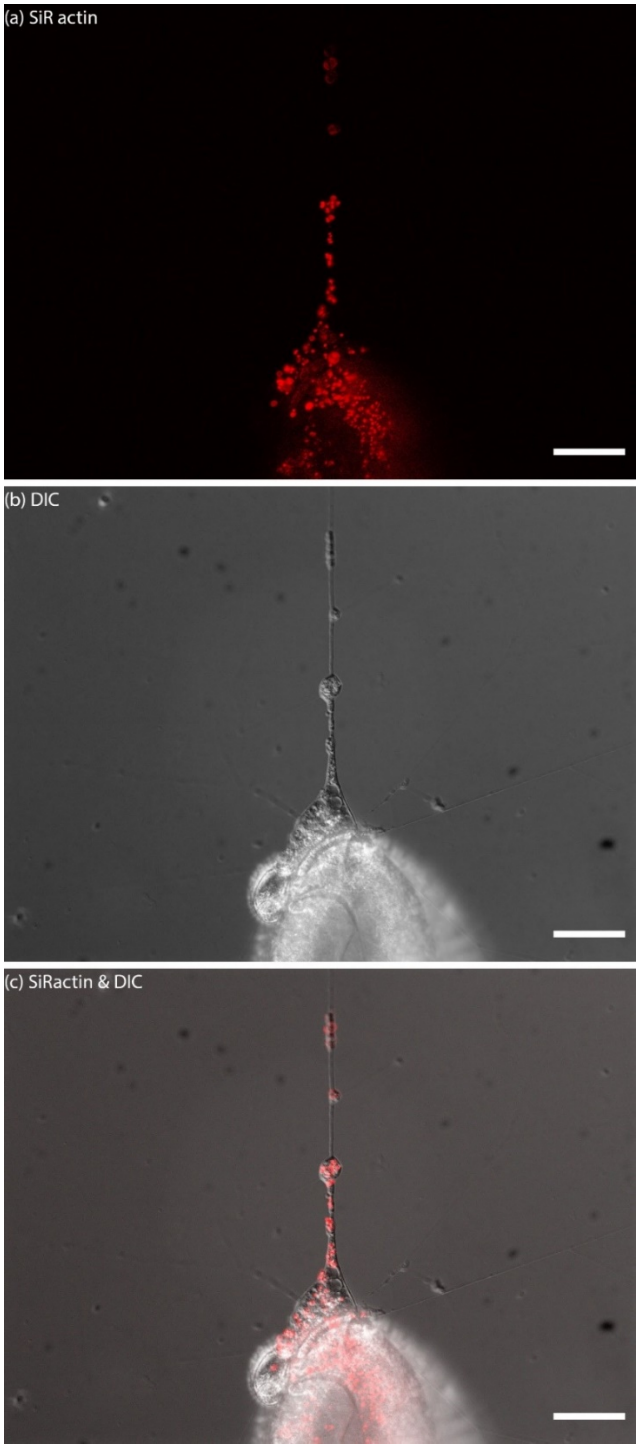
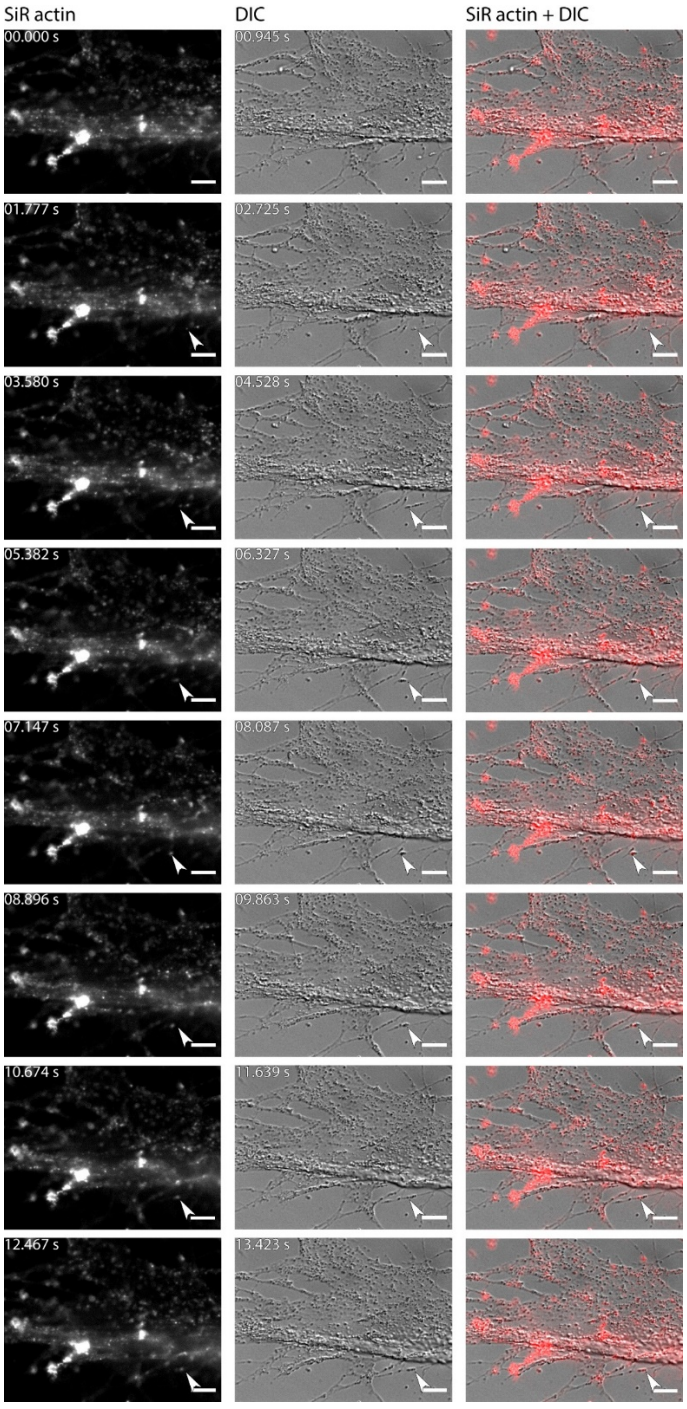


Figure 5: ~~Actin~~SiR-actin-labelled granules (~~ALG~~ALGs) in pseudopodia and endoplasm of *Quinqueloculina* sp. Top image presents actin stained with SiR-actin, middle image presents images obtained with DIC optics (inverted LUT), bottom ~~image~~column presents merged fluorescent and DIC channels.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


SiR actin
DIC
SiR actin + DIC
00.000 s
00.945 s
01.777 s
02.725 s
03.580 s
04.528 s
05.382 s
06.327 s
07.147 s
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08.896 s
09.863 s
10.674 s
11.639 s
12.467 s
13.423 s

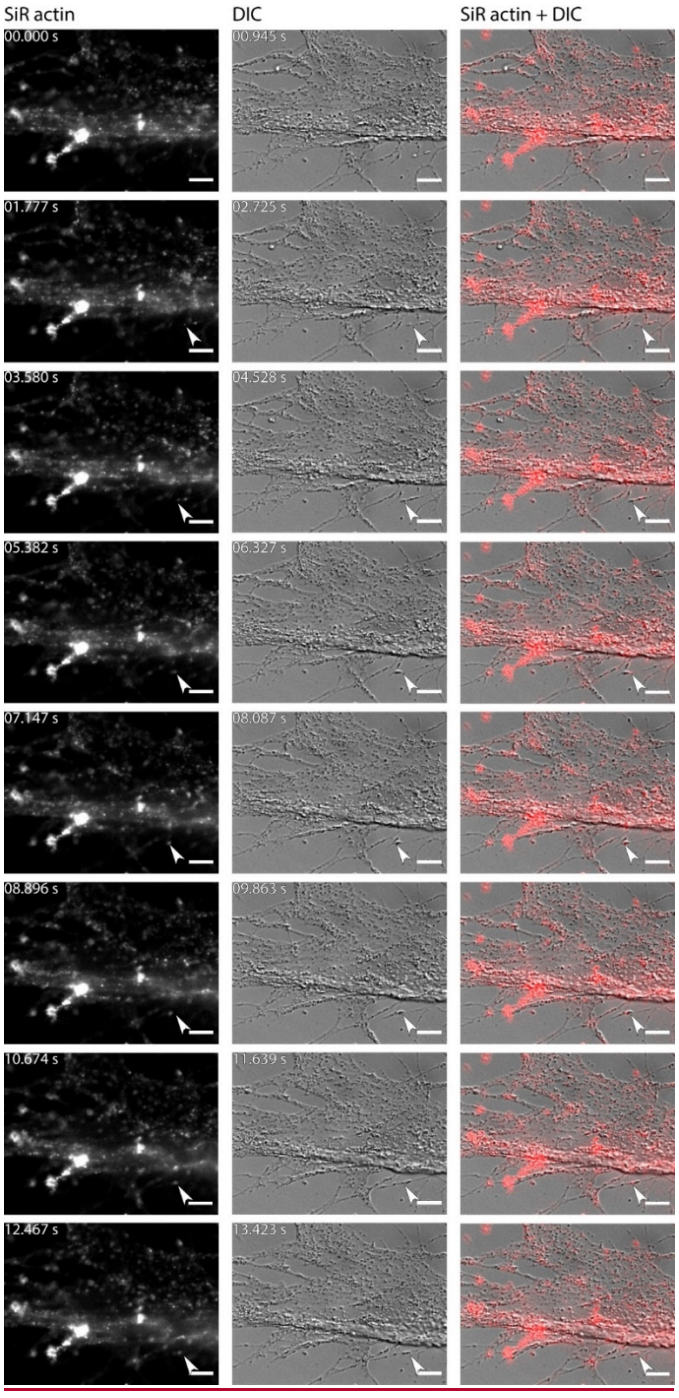

Figure 6: Dynamics of SiR-actin-labelled granules (~~ALG~~ALGs) in reticulopodia of *Amphistegina lessonii*. Eight frames of time lapse. Right column: actin stained with SiR actin; middle column: DIC; right column: overlay of fluorescent and DIC channels. Arrows indicate granule in the tip of one very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

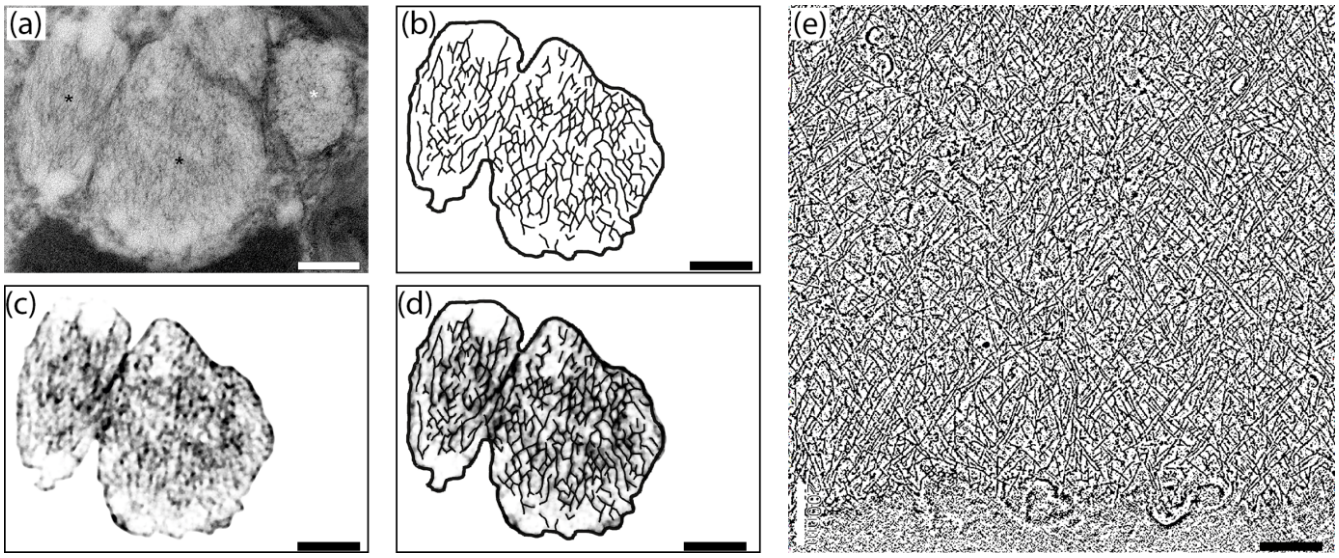

Figure 7: Comparison of internal nanostructure of Fibrillar Vesicles (a–d) to actin meshwork (e), Scale bar 200 nm. (a) TEM image of FV, reprinted from *Mar. Micropaleontol*, 138, LeKieffre et al., An overview of cellular ultrastructure in benthic foraminifera: New observations of rotalid species in 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).

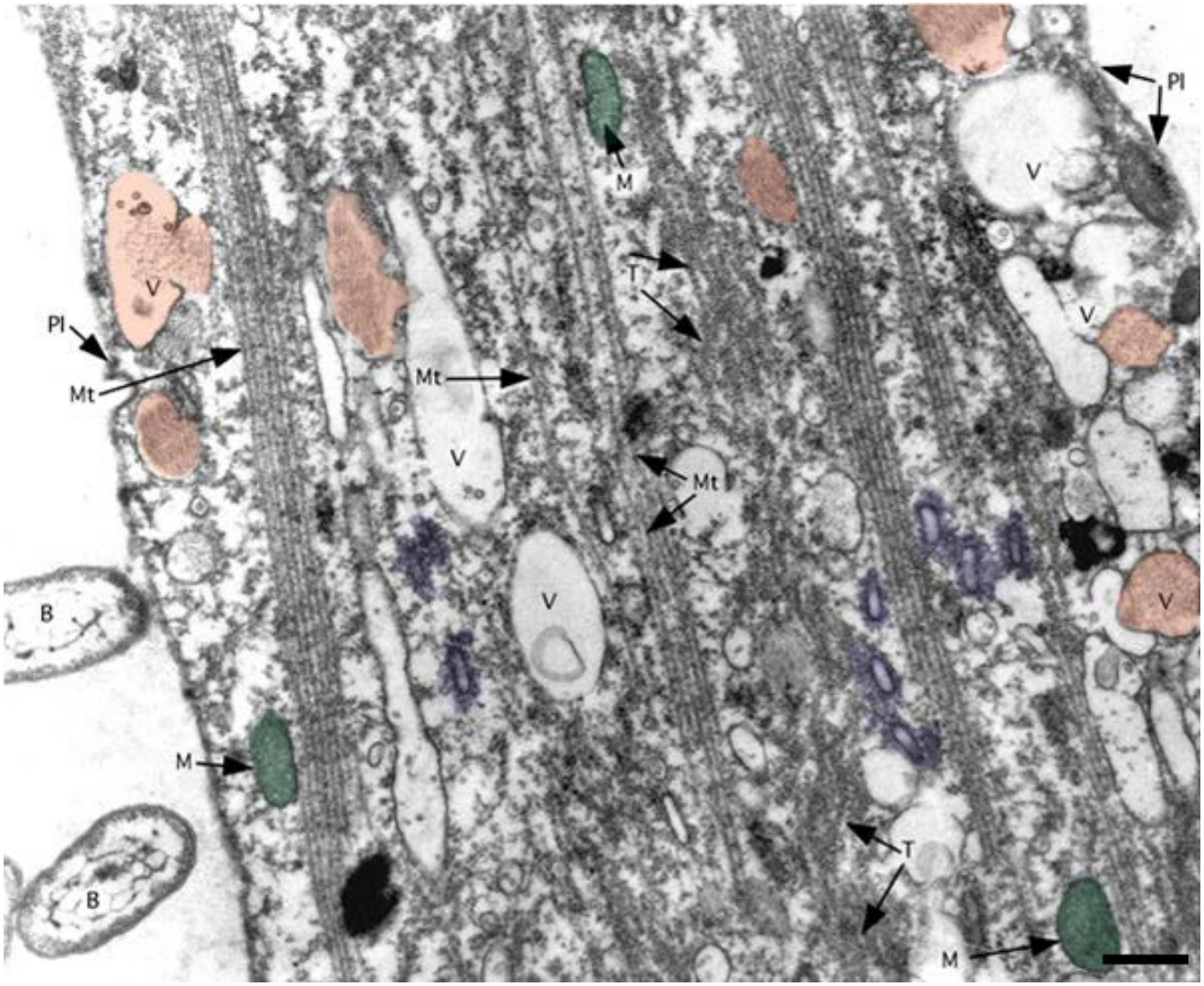

Figure 8. TEM image of ectoplasm of *Assilina ammonoides* (Gronovius) (modified from Hottinger, 2006, fig. 67 based on Creative Commons Attribution 2.5 License~~).~~) Areas marked in ~~**pink**~~red indicates vesicles we interpret as fibrillar vesicels. Areas marked in violet indicates what we interpret as ~~**elliptical fuzzy**~~ 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.