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)

Jan Goleń et al.

Dear Dr. Toyofuku,

we would like to thank you for all 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

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.

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 Dr. Toyofuku for this comment. We agree that other possible scenarios explaining observed staining pattern must be explicitly described in the discussion and interpretation in the final version of manuscript. To avoid risk of over-interpretation, we will follow your comments and propose to re-write the discussion section in the final version of the manuscript. We will 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. 1 – this figure was previously submitted as a supplement to Dr. Bowser's comment). To address both referee's concerns, we would like to propose adding to the section "4.1 Assessment of unspecific fluorescent labelling risk" this following paragraph:

"As the granular pattern of SiR-actin staining is unusual compared to other Eukaryotes, it requires discussion of possible scenarios explaining this pattern (see Fig. 1 [in this response; proposed as a new figure in our paper or supplement]). Three possible scenarios in which ALGs are real F-actin-containing structures that are labelled by SiR-actin probe (Fig. 1A-C), and 3 other possibilities explain observed pattern as artifacts (Fig. 1D-F). First scenario (Fig. 1A), that seems the most likely, assumes that foraminifera possess granular structures willed 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). These vesicles are possibly involved in transport and endocytosis F-actin probably plays role in those processes. Alternatively, they may correspond to elliptical fussy-coated vesicles described by Koonce et al. (1986) regulate motility of reticulopodia (see below section 4.5.2 Elliptical fuzzy-coated vesicle). Next scenario assumes that actin filaments are both inside and outside of some membrane-bound vesicles (Fig. 1C). Furthermore, the observed staining pattern may be explained as an artifact, if SiR-actin binds to some other unidentified organic molecules that are not associated with F-actin, either inside (Fig. 1D) or outside (Fig.1E) of membranous vesicles. Finely SiR-actin may induce assemblage of actin filaments in the areas rich in G-actin (Fig. 1F in supplement) that follows comments by Melak et al. (2017)."

We would like to place this new paragraph in P7.L5.

We noticed already in P7.L15-16 of 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." We agree that we should elaborate it more in the discussion and interpretation section.

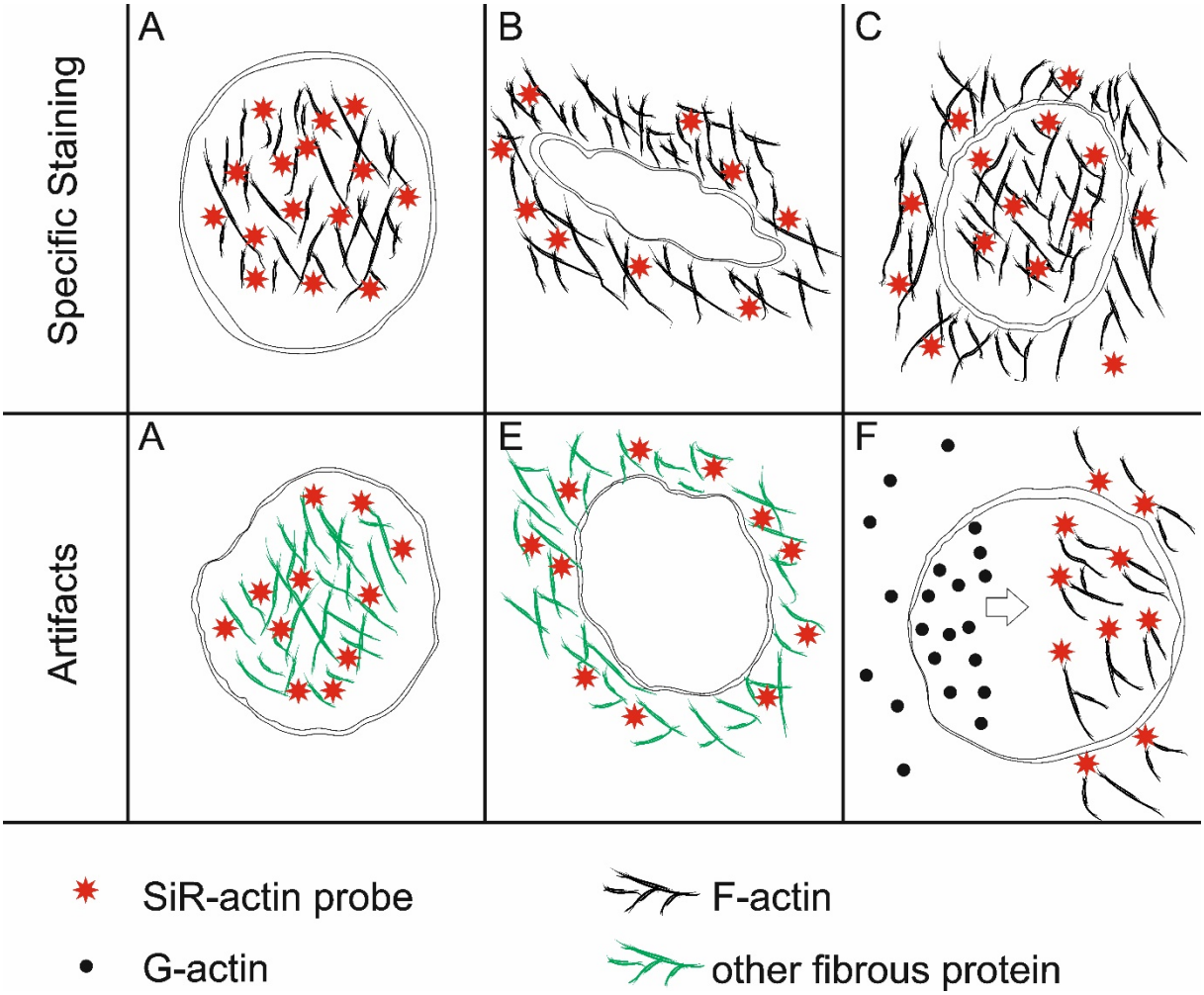

Fig. 1. Graph showing 6 possible scenarios of staining foraminiferal cells with SiR-actin. A-C shows hypothetical scenarios of specific staining: A illustrates a scenario in which SiR-actin stains actin filaments in the interior of vesicles (e.g. Fibrillar Vesicles); B SiR-actin labels actin filaments surrounding vesicles possibly related to movement of these vesicles (e.g. facilitating endocytosis or corresponding to elliptical fussy-coated vesicles). C assumes that actin filaments (stained with SiR-actin) are both inside and outside vesicles. D-E illustrate potential staining artifacts. In those scenarios SiR-actin does not label physiologically active actin filaments in granules in foraminifera (ipso facto observed Actin Labelled Granules consist artifacts), incl. unspecific labelling of some proteins inside (D) or outside (E) of vesicular structures, or (F) SiR-actin induction of F-actin polymerisation. (Published in the supplement to response to referee's comment by Dr. Samuel Bowser on the 4th of July 2018).

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 will leave 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 will re-write the beginning of this paragraph, so it would more clearly specify species used for this study:

"We present here results of experiments performed on 3 species of foraminifera *Amphistegina lessonii, Ammonia* sp., *Quinqueloculina* sp.. They belong to both main classes of multilocular foraminifera (first two belong to Globothalamea and the third one to Tubothalamea). We have observed similar staining pattern 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: Staining with SiR-actin and Mitotracker Green or Calcein red-range AM does not require prolonged incubation. After 15-20 minutes the signal is sufficient enough to perform observations. To minimise the problems caused by autofluorescence we starved all specimens 24 hours prior the observations.

Calcein Red-Orange, AM is a cell-permeable dye that stains cytoplasm of living cells and is often used to indicate viability of the cells. As the regular calcein it is fluorescent in presence of Calcium ions, but unlike usual calcein is cell permeable. It differs in a chemical structure from the regular calcein, and its fluorescent spectrum is shifted towards longer wavelengths.

In our experiments main purpose of using CellTrace™ Calcein Red-Orange was to indicate 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.

P4. L16 Categorize by purpose, not the institute.

Re: We categorised experiments in that way because in different locations we used different equipments and setups that had variable impacts on our observations. We will follow referee's comment and stress differences in hardware used and its constrains than the location itself in the final version of the manuscript. This is true that our experiments in two different labs could be partly categorized "by purpose". Most experiments and confocal observations run at the AWI were focused on chamber formation, in contrast to experiments focused on granuloreticulopodia in the lab in Kraków.

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 will add this reference and stress that fluorescent dye used for staining living cytoplasm was Calcein red-orange AM in contrast to a regular calcein that exhibits green florescence. It should also be mentioned that both fluorescent probes stain same intracellular structures.

P4. L17 remove ")"

Re: Thank you for noticing. It will be corrected in the final version of manuscript, following editorial recommendations.

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

Re: We will summarise necessary information in a new table added to the final version of the supplementary materials (see Table 1 below):

Fluorescent dye	Maximum of absorption	Maximum of emission
SiR-actin	652 nm	674 nm
MitoTracker Green	490 nm	516 nm
Calcein red-orange AM	577 nm	590 nm

Table 1. List of fluorescent dyes used in the presented study with their fluorescent properties.

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 will rewrite 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: Exposure time for experiments performed on Zeiss Axio Observer Z.1. was optimised using trial and error method and it varied between experiments depending on magnification and binning mode. This information will be included in captions to all illustrations in the final version of manuscript. We will add a table summarising this in the final version of supplementary material (see Table. 2 above). We will also add a table summarising information on hardware setting used during confocal experiments (see Table 3 above). We performed negative controls for SiR-actin staining (Fig. 1). We will present results of these experiments in supplementary materials.

In presented time-lapses we set up minimal interval between subsequent frames. Frequency of time lapses in experiments conducted on Zeiss Axio Observer Z.1 was limited by exposure time and time needed for changes the filters and illumination mode used for particular channels. Similarly in confocal experiments performed on Leica SP5 inverted confocal microscope the frequency was limited by scanning time.

Figure or movie number	Fluorescent dye	Exposure time	Binning mode:	Objective	Apotome setting
Fig. 1	SiR-actin	1 s.	1x1	EC Plan-Neofluar 40x/1.30 Oil M27	off
Fig. 2	SiR-actin	1 s.	1x1	EC Plan-Neofluar 100x/1.30 Oil M27	off
Fig. 5	SiR-actin	112.22 ms	1x1	Plan-Apochormat 20x/0.8 M27	off
Fig. 6	SiR-actin	50 ms	3x3	EC Plan-Neofluar 100x/1.30 Oil M27	off
Fig. S3	SiR-actin	282.13 ms	3x3	EC Plan-Neofluar 100x/1.30 Oil M27	ON: 3 frames per image
Fig. S3	MTG	15.38 ms	3x3	EC Plan-Neofluar 100x/1.30 Oil M27	ON: 3 frames per image
Fig. S4., S5	SiR-actin	185.73 mm	3x3	EC Plan-Neofluar 100x/1.30 Oil M27	ON: 3 frames per image
Movie S1	SiR-actin	1 s.	1x1	EC Plan-Neofluar 40x/1.30 Oil M27	off
Movie S3	SiR-actin	20 ms	5x5	EC Plan-Neofluar 100x/1.30 Oil M27	off

Table 2. Summary of hardware settings (exposure time and binning mode) in experiments conducted on Zeiss Axio Observer Z.1.

Figure or movie number	Emission bandwidth (MTG)	Emission bandwidth (Calcein red-orange AM)	Emission bandwidth (SiR-actin)	Excitation Beam Splitter
Fig. 3	500nm – 533nm	N/A	650nm - 690nm for SiR-actin	FW TD 488/561/633
Fig. 4	N/A	596nm - 616nm	656nm - 696nm	FW TD 488/561/633
Fig. S1	500nm - 533nm for MTG	N/A	650nm - 690nm for SiR-actin	FW TD 488/561/633
Fig. S2	N/A	N/A	650nm - 690nm for SiR-actin	FW TD 488/561/633
Movie S2	500nm – 533nm	N/A	650nm - 690nm for SiR-actin	FW TD 488/561/633

Table 3. Summary of hardware settings in experiments conducted on Leica SP5 inverted confocal microscope.

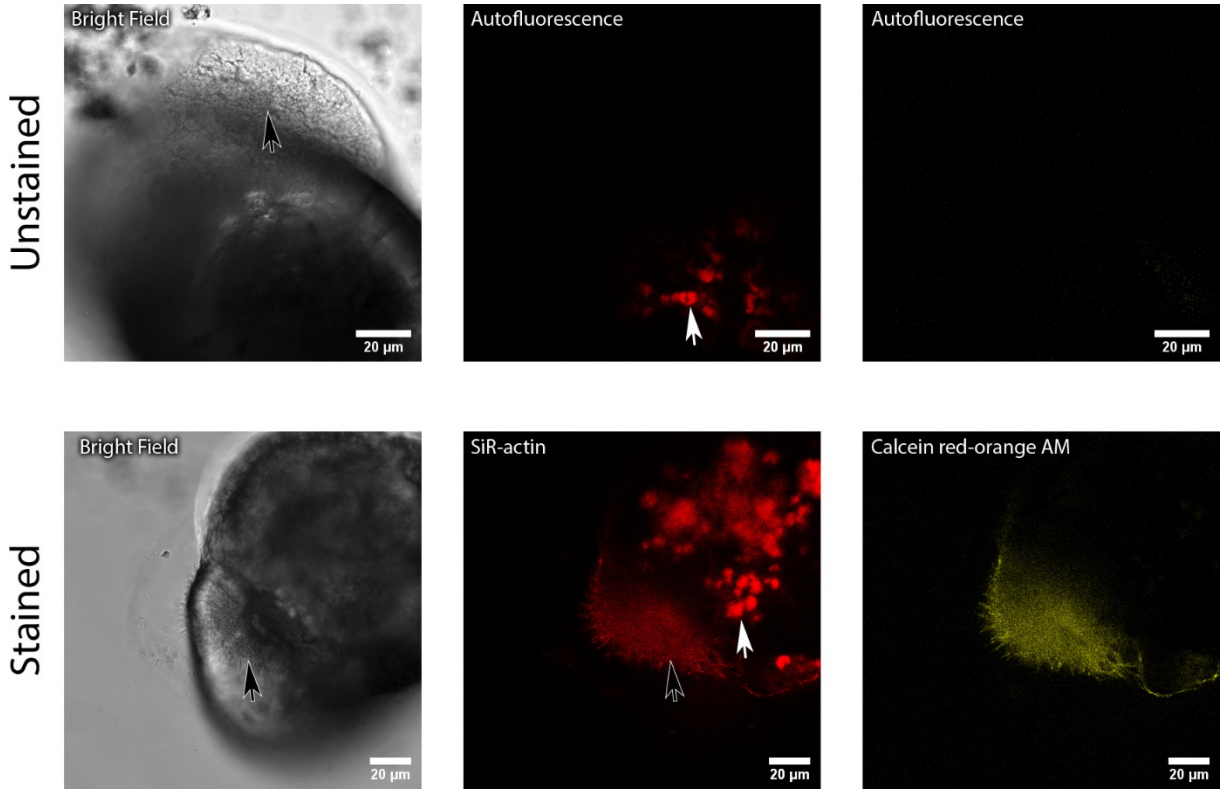

Fig. 2. Globopodia of *Amphistegina lessonii* observed using bright-field and confocal imaging. Images in upper row represent unstained individual (control), images in lower row represent specimen stained with SiR-actin and calcein red-orange AM. The white arrows indicate autofluorescence of endosymbionts, and black arrows indicate globopodium of newly formed chamber.

P4. L22 Indicate the setting of fluorescent cubes.

Re: We would like to add this information to the final version of manuscript. The settings are summarized in Table 4 (see below):

Fluorescent dye	excitation filter	beamsplitter	emission filter
SiR-actin	BP 640/30	FT 660	BP 690/50
MitoTracker Green	BP 500/20	FT 515	BP 535/30

Table. 4. Properties of filter sets used for different fluorescent dyes for experiments performed on Zeiss Axio Observer Z.1.

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

Re: We will follow this instruction. At this stage, confocal images seem to be described clearly enough. We will add 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: Our intention is to introduce a term that would be the most neutral and open for different interpretations. We chose this term because it was based on our observations (i.e. strong signal of probe targeting F-actin), as well as followed reliability of SiR-actin probe tested on other eukaryotic cells (for overview see Lukinavičius et al., 2014; Melak et al., 2017). We agree with the reviewer that at this stage

this term should not imply any specific interpretation of function and ultrastructure of granules in question. Therefore, we would like to define ALGs as fluorescent granules labelled with SiR-actin probe. This definition should precisely describe the term ALGs and avoid misleading interpretations. Nevertheless, further experiments would either prove or disprove whether ALGs represent true actin-labelled granules or just artifacts of unspecific labelling. All these possible scenarios (see Fig. 1 above) are presented and discussed in our response to referee's comment by Dr. Samuel Bowser.

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

Re: We will add a sentence at the end of the paragraph: "Nomarski contrast or Differential interference contrast (DIC) is a microscopy technique utilising interferometry principle for improving contrast in transparent objects."

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

Re: Fig. 5. We refer in this fragment to the image that shows ALGs in endoplasm of *Quinqueloculina sp.* – the species that does not bear the symbionts. We will make it more clear in the final version of manuscript that overlapping of SiR-actin signal and chlorophyll autofluorescence regards only some species of foraminifera. However, we are aware that strong autofluorescence of food particles might occur in all foraminiferal species.

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

Re: We will add name of the species in which we observed ALGs within different cytoplasmic structures (at present this information is only in the caption of the figures, we agree that it is needed to clarify the text). At least in reticulopodia, there seems to be some variations between species, for example *Amphistegina lessonii* appears to have more ALGs than *Ammonia sp.* We did not performed detailed experiments to quantify differences in density of ALGs in different taxa. We would like to dedicate a separate study to analyse this important issue.

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

Re: This is similar question to referee's comment to P5. L6. 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. We agree with our referee that at this stage ALGs are in fact fluorescent granules labelled with SiR-actin probe. This definition would precisely describe the term ALGs and avoid misleading interpretations.

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

Re: Yes, we will include a table in the final version summarising information velocity of the movement of ALGs. We may include detailed table showing data on motility of ALGs, as well as time lapse movies on which our calculations are based.

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: Thank you for this valuable comment. We tried several different approaches for calculating velocity of granules. Presented in the manuscript value of the velocity (9 µm/s) was calculated using manual tracking tool in Fiji software (based on ImageJ).We will add a missing description of these methods and a reference to the authors manual tracking plug-in in the final version of manuscript.

We have tested other methods of quantification of the motility of ALGs including TrackMate plug-in in Fiji software, but we encounter some difficulties using this approach. In first step this plug-in automatically finds spots (in our case ALGs) in each of the frame of the time lapse, and in second step decides, which granules on the frame n+1 correspond to granules on frame n. This software seems to cope very well with the first step in case of images of ALGs, but the second step when done automatically is very prone to errors that can cause strong bias in results. A manual approach is in the contrary very time-consuming, thus, we calculated velocities only of small portion of observed granules, hence, our results should be treated as rather conservative estimates and actual range of the speed of ALGs may be higher.

Since then we learnt that TrackMate plug-in in Fiji software allows for manual tracking of automatically annotated spots. This approach seems promising, and following referee's comment, we would like to present some results obtained with this method in the final version of manuscript. Using this approach and some new time lapses we already recorded higher velocities of ALGs than mentioned in the manuscript (at least up to 13 µm/s).

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

Re: We did not described it materials and methods, what is shortcoming of proposed manuscript. Thank you for pointing it out. We will add necessary information in the final version of manuscript and table with date used for calculation to the final version of supplement.

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.

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: Indeed we have referred to Flakowski et al in P12. L10. However, we made a typographic error (we rote Falkowski et al. instead of Flakowski et al.). We will correct it in the final version of manuscript. In line P12. L8- L17 we discuss evolution of actin-encoding genes in foraminifera and its hypothetical

impact on foraminiferal physiology (especially intracellular transport). In line P12. L10-11 we cited *Flakowski et al* (2005) who presented molecular data indicating duplication of actin genes in many species of foraminifera. As a consequence of this duplication, most foraminifera have at least two paralogs of actin-encoding genes. Hypothetically, this paralogs extend 'molecular tool kit' available to foraminiferal cells, making their physiology more flexible or more precisely controlled.

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

Re: We refer here to possibility of disruptive effect of SiR-actin on reticulopodial motility and morphology. Our statement (lack of such an effect) is based on qualitative observation of stained and unstained individuals. In those observations we did not recognise any apparent long-term differences in morphology or dynamics reticulopodia after stining. Occasionally we observed temporary retraction of pseudopods immediately after adding the staining solution to the petri dish. After 10-15 minutes incubation with SiR-actin this effect was not visible any more, and reticulopodia were spread again, closely resembling their structure and dynamics observed prior staining. There are some differences between species in strength of this reaction (*Amphistegina lessoni* being apparently less sensitive then *Ammonia sp.* or *Quinqueloculina sp.*).

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

Re: We will change 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: Thank you for your suggestion. We will reconstruct structure of this chapter as you recommend.

P10. L9 4.5.1 is numbered twice.

Re: "4.5.1 Fibrillar system of planktic foraminifera" should be changed to "4.5.2 Fibrillar system of planktic foraminifera".

P10. L13 "representstructurally" insert space.

Re: It will be corrected. 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 (similar but less prominent foggy pattern can be seen in the unstained control, see Fig. 1. in this response).

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.