Detailed response to the interactive comment by Takashi Toyofuku.

On behalf of all authors, I would like to thank Dr Takashi Toyofuku for his valuable comments. I would like to present our detailed response to each of the referee's comments. Original referee's comments are in black, and our responses are in blue.

General comment

This research investigated a point that was unclear in the previously published research. Although the content is good, it is questionable whether the purpose and discussion match the common interests of the audience of this journal. In addition, I think many points can be discussed with previous studies, but they are lacking. If these points are improved, I can agree there is a great possibility that the contents of this paper can be published in Biogeosciences.

Thank you for the helpful comment. We will rewrite Introduction to show the significance of our study to Earth Sciences. We will begin with general description of foraminifera and significance, followed by general information regarding pseudopodia and their function. We will add definitions of terms and citation to the relevant literature, wherever it is necessary. As further suggested, we will add references to the literature on morphogenesis of foraminiferal tests, including research using *in silico* and *in vivo* methodologies.

Here we would like to explain important and linked issues: the taxonomy, number of specimens and SEM documentation. During preliminary stage of our experiments we used wide range of tubo- and globothalamean foraminifera (see Table R1 and Fig. R1). Tubothalamea were represented by several specimens of miliolids. By observation under the stereomicroscope, we identified two distinct types among the individuals: first one was elongated and the other is globose to ovate in overall shape of the test. We consulted the original paper that presents the diversity of foraminifera in the Burgers' Zoo marine aquaria (Ernst et al., 2011) and established that our elongate type corresponded to the individual 5 in fig. 4 in this paper. The authors identified this individual as belonging to genus *Quinqueloculina*, without specification of the species. The morphology of most of the globose and ovate individuals in our sample resembled the individual 10 in fig. 4 (identified as *Quinqueloculina bicarinata*) in Ernst et al. (2011). One individual from our individuals (F8 in Table R1) is comparable to individual 11 in fig. 4 (identified as *Miliolinella labiosa*).

Following the referees' suggestion, we conducted additional SEM imaging of specimens stored after observations. State of the preservation of the specimens was not good and a few of them were lost during the transfer to the SEM stubs. Moreover, as mentioned in the manuscript some individuals were embedded in Araldite after fluorescent dye staining (as mentioned in the manuscript). This procedure prevents from imaging them under the SEM. Three individuals we were able to document under SEM include: F1, F3 and F8. However, the specimen F3 was significantly damaged and last two chambers were destroyed. Further consultation with relevant literature allows for conclusion that the elongated individuals likely belong to *Quinqueloculina vandiemeniensis* (Loeblich and Tappan, 1994). Globothalamea were represented by a single specimen of *Heterostegina depressa* and 3 specimens of *Amphistegina lessonii*.

We decided to include into the main manuscript only those well preserved and labelled individuals with intact granular structures observed within reticulopodia. We avoided individuals presenting the beading response after fixation and/or lacking well preserved

granules in pseudopodia (see Table R1). We also excluded the individuals associated with foreign objects, displaying strong fluorescence in each channel (see individuals F3, F8, F11 and F13 – Table R1). Moreover, colocalisation of the fluorescence signal is moderate or strong in all specimens that show well-preserved overall structures of pseudopodia. Even in the absence of the granules the fluorescent signal from SIR actin largely overlaps with the signal form Phalloidin Atto 488 in the actin meshwork. Only within the individuals that show beading response after fixation the colocalisation was significantly weaker. So far, we cannot find compelling explanation for this phenomenon.

We would like to emphasize that the colocalisation between signals from two probes spans across entire granuloreticulopodial network and is not limited to small restricted areas. In fact, all of the areas of the network may be viewed as a separate test of the colocalisation hypothesis.

We agree that proper taxonomic attribution is in principal an important issue that facilitates further independent replication of such experiments. However, limited taxonomic identification of the specimens does not interfere with the presented results. We tested the hypothesis pertaining to all foraminifera that present SiR-actin-labelled granules in their pseudopodial structures, Therefore, testing this hypothesis is not species specific. In light of the additional images presented in the Fig. 1 in this response, we can conclude that our results could be extended to other foraminiferal taxa.

Nevertheless, we have done our best to specify our taxonomic identifications based on available literature. Therefore, the elongated individuals are assigned to *Quinqueloculina vandiemeniensis* Loeblich & Tappan, 1994 (see Fig. R2). This miliolid species presented best labelling results (see Table R1, specimens F1, F2; compare other individuals in Fig. R1). Additional taxa included *Miliolinella labiosa* (d'Orbigny, 1839), *Heterostegina depressa* d'Orbigny (1826), and *Amphistegina lessonii* d'Orbigny (1826).

Specimen No.	Taxonomic identification	Beading response after fixation	Preservation of granules after fixation	Colocalisation between SiR-actin and Phalloidin Atto 488	Presented in the manuscript	Additional information
F1	*Quinqueloculina* sp., cf. *Q. vandiemeniensis*	no	good	strong	Figs 1 and 3 in the manuscript	SEM image
F2	*Quinqueloculina* sp., cf. *Q. vandiemeniensis*	no	moderate	strong	Fig. 2 in the manuscript	Embedded in Araldite (epoxy).
F3	*Quinqueloculina* sp., likely *Quinqueloculina vandiemeniensis*	some	moderate	moderate		Some foreign objects stained with SiR-actin present, SEM image of crushed indvidual
F4	*Quinqueloculina* sp., likely *Quinqueloculina vandiemeniensis*	no	weak	strong		
F5	*Quinqueloculina* sp., likely *Quinqueloculina bicarinata*	yes	moderate	moderate		
F6	*Quinqueloculina* sp., cf. *Q. vandiemeniensis*	no	weak to moderate	moderate to strong		Embedded in Araldite (epoxy).
F7	*Quinqueloculina* sp., likely *Quinqueloculina bicarinata*	yes	weak	weak to moderate		
F8	*Miliolinella labiosa*	no	weak to moderate	moderate		Some foreign objects stained with SiR-actin present, SEM image
F9	*Quinqueloculina* sp., likely *Quinqueloculina bicarinata*	some	weak	week		
F10	*Heterostegina depressa*	no	weak	strong		
F11	*Amphistegina lessonii*	some	moderate	moderate		Some foreign objects stained with SiR-actin present
F12	*Amphistegina lessonii*	no	weak	moderate		
F13	*Amphistegina lessonii*	some	moderate	moderate		Some foreign objects stained with SiR-actin present and in the Phallodin Atto 488

Table R1 *Information regarding the individuals used in the preliminary stage of the study. The level of colocalisation was evaluated by analysing the overlay of the fluorescent images in SiR-actin and Phalllodidin Atto 488 channels (see Fig. R1). Areas that appear yellow in the overlay image indicate higher levels of colocalisation. We excluded form analyses any fluorescent objects outside the pseudopodia.*

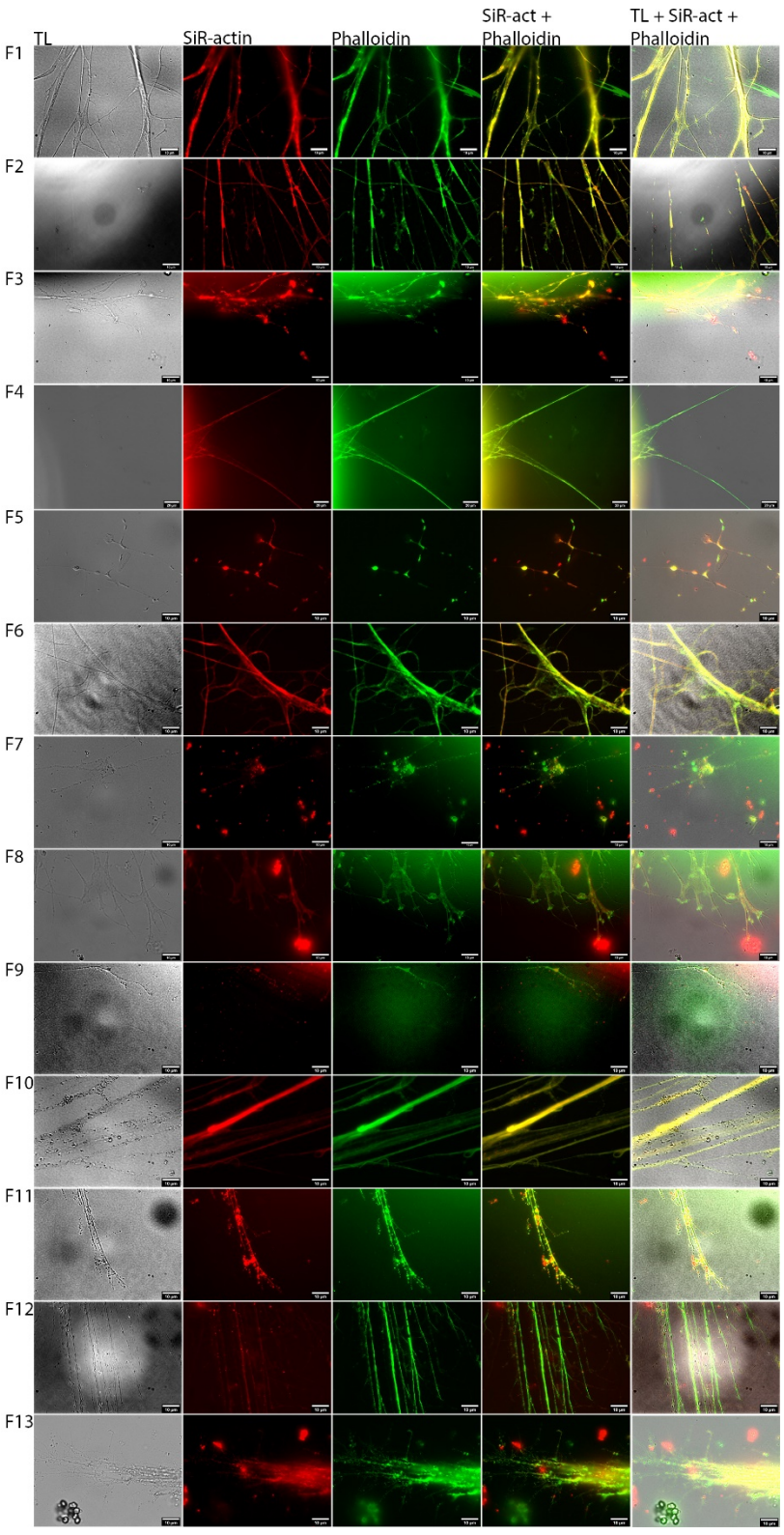

Figure R1 (proposed to be added to Supplement). Compilation of images showing pseudopodia of 13 individuals of foraminifera stained with SiR-actin and Phallodidin Atto 488. Each row represents another individual (individuals F1-F9 represent Miliolida and F10-F13 represent Globothalamea: F10 is *Heterostegina* sp. and F11-F13 are *Amphistegina lessonii*) l. First column (TL) shows the transmitted light channel, second column (SiR-actin) shows the SiR-actin fluorescent channel, the third column shows Phalloidin Atto 488 fluorescent channel, fourth column (SiR-act + Phalloidin) shows overlay of both fluorescent channels, fifth column (TL + SiR-act + Phalloidin) shows overlay of all tree channels.

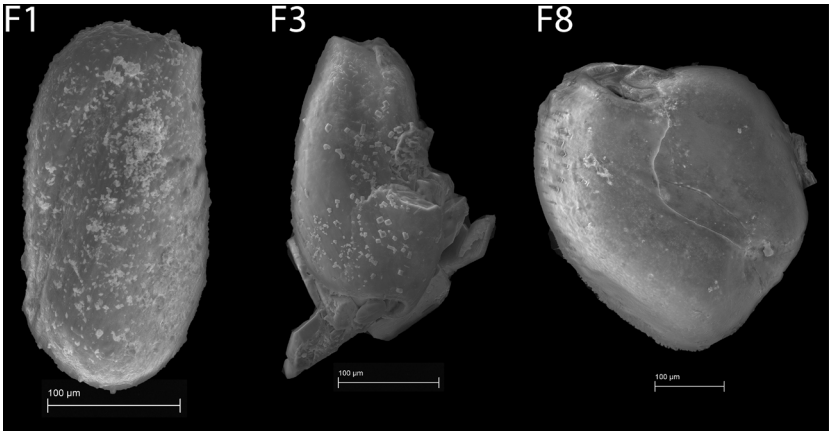

Figure R1 SEM images of 3 individuals used in the study. Numbers correspond to the numbers in Table 1 and Figure 1.

L 21 Introduction

"…To account for this possibility, the term SiR-actin labelled granules has been coined to describe them (GolenÌ et al. 2020). The presented study primarily addresses the question, whether they are experimental artefacts or they represent physiological and functional forms of F-actin in foraminifera."

This targeting is too specific and does not contribute to the general readership.

Too much value is placed on the biological perspective for this study. It will be classified as a more biological and protozoan work. The discussion should return to the proposition that "Foraminifera pseudopodia are very important for understanding the evolution, morphogenesis, physiology, and ecology of these organisms." Otherwise, the position of this research in Biogeoscience and Earthscience will be unclear. It is essential to discuss this research and insights into biomineralization and shell morphology. Considering that cytoskeletal variation governs pseudopod extension and that the three-dimensional structure of the pseudopod unfolding from the aperture governs shell morphology (Tyszka et al, 2005; Tyszka, 2006), it should not be too difficult to connect and discuss the results of this study with these perspectives.

We appreciate these comments and the alternative prospect. In fact, we try our best to fill the gap between Earth sciences and biology in Foraminifera. Why we have chosen ***Biogeosciences*** as a unique journal linking both BIO and GEO perspectives. Our knowledge on intracellular adaptations of foraminifera limits their application as paleoceanographic proxies. Many unsolved questions are attributed to specific “vital effects” that impact our understanding of size, morphology, and highly variable biomineralization patterns. We do believe that unusual cytoskeletal dynamics is directly responsible for impressive adaptability and evolutionary success of Foraminifera.

Anyway, this is our task to convince readers that all these aspects are linked with each other. Therefore, we will rewrite Introduction to show the significance of our study to Earth Sciences and to broaden the scope of the references. We plan to begin with general description of foraminifera and significance, followed by general information regarding pseudopodia and their function. We will add the definition of terms and citation to the relevant literature, wherever it is necessary. As further suggested by the referee, we will add essential references to the literature on morphogenesis of foraminiferal tests, including

research using *in silico* and *in vivo* methodologies. We will also stress importance of the study from the evolutionary perspective of foraminifera and the phylum itself.

Method

L90.

If possible, can the origin of the samples be shown? I would imagine, however, that foraminifera would have been introduced mixed in with corals and other macro-organisms from various origins. Since authors can not know where they originated, it seems like a good idea to identify the species or provide SEM photos. I do not think there is any need to hesitate on account of the deformity. From my own research experience, I am aware that the shell morphology of Miliolid is easily affected in captive environments.

It is true that, since the specimens used in the study came from the marine aquaria in the zoo, we cannot specify their exact origin. However, we mentioned that this assemblage came from the Indo-Pacific area (see Ernst et al. 2011). As suggested by the referee, we performed additional SEM imaging. This issue is explained above in our response to referee's general comment.

L.128

Add "Digital Single Lens Reflex camera" around Cannon DS 126231

Thank you for this suggestion. We will include this information in the final version of the manuscript.

L. 133

3.3 Control for autofluorescence →3.1 Control for autofluorescence

It will be corrected in the verified version of the manuscript.

L. 140

"The staining of reticulopodium with both of fluorescent probes was successful "

Describe and discuss the reasons and conditions for what authors would call a "successful." Just quoting Figure 1-3 is not enough explanation, and the reader will not know if it is successful or not.

The entire section 3.2 will be rewritten in the final version of the manuscript and this unfortunate statement will be avoided. By "successful" we meant that both probes stained some structures within pseudopodia, so that the significant fluorescent signal can be detected. The staining procedure would be "unsuccessful", if in one or both channels, we would not be able to detect any signal above the natural autofluorescence of observed structures.

L. 334

Figure 1→Figure 2

Thank you for noting this error. It will be corrected.

L. 145

"Stanley 1971"

This study focuses on the localization identity, and it is a fundamental issue of this study, then needs a solid evaluation, rather than discussing the possibility ("possibly" in L144).

If this can be technically corrected, and it can be proved that there is no problem, then it is better to state in the method "analyzed by correcting for differences in depth of focus depending on wavelength" and describe the correction methodology in the supplement.

This is an important suggestion. We will move this information to the methodology section either in the main text or manuscript. We will try to find additional arguments for the hypothesis that the effect we observe (i.e. in-focus image of the same object may be in different focal planes for two different channels) is caused by dispersion as we suggested. However, this effect is consistent and, regardless its cause, it must be taken into account, when analysing the images. So far, we cannot find another explanation for it.

This study focuses on the localization identity, and it is a fundamental issue of this study.

L. 159

3.3 MT

Abbreviations that appear for the first time should be accompanied by an explanation.

Thank you for pointing this out. We will add necessary explanations.

L161

"The fact that three independent methods indicate presence of the F-actin …"

What are the three methods, SiR-Actin, Phalloidin Atto 488, and birefringence? I believe that the authors have shown that they existed in the same place. However, it is a leap to say that this is the basis for showing that F-actin is present in the reticulopodium. Authors need to explain it sequentially to complete the logic for example.

The SiR-Actin, Phalloidin Atto 488, and birefringence are three methods we are referring to in this sentence. Our argument is that, the detection of the signal of the two fluorescent probes in question and the birefringence appearing in the same areas is best explained in the light of our hypothesis, i.e. that F-actin is present in this area, including SiR-actin-labelled granules.

Competing hypothesis (SiR-actin does not bind actin specifically in foraminifera or causes the inducing of F-actin assembly) cannot explain these observations.

The signal of SiR-Acrin was detected orthotopically with that of Phalloidin Atto 488. Phalloidin is known to bind specifically to F-actin and is used as a major indicator substrate for F-actin. (Cooper, 1987 DOI: 10.1083/jcb.105.4.1473). It is also known that F-actin bundles exhibit birefringence. (Hodge AJ. J Biophys Biochem Cytol. 1955 Jul 25;1(4):361-80. DOI: 10.1083/JCB.1.4.361; Hodge AJ. J Biophys Biochem Cytol. 1956 Jul 25;2(4 Suppl):131-42. DOI: 10.1083/jcb.2.4.131. These results strongly suggest that the signal of SiR-Actin indicates F-actin.

The above is an example, but this is the kind of discussion that needs to be addressed. This is the primary purpose of this paper. Further, TEM images of granular materials are also demanded in future studies. After that, we can start to discuss whether granular materials have much F-actin or not.

We will add suggested references to the revised version of manuscript. We are grateful for this helpful idea.

L. 195 "suggest that they are key evolutionary adaptation that most likely predated emergence of foraminiferal tests in the early Palaeozoic."

The results of Pawloski et al. (2013: already referred to in this study) should be cited and discussed. It is also essential to compare the results with Habura et al. (2005: https://doi.org/10.1093/molbev/msi190), who attempted to explain the quick movements of pseudopodia from the aspect of Tubulin.

The citation (Pawlowski et al. 2013) will be add in the final version of the paper. We will discuss results presented by Habura et al. (2005) as well. Thank you for pointing out the lack of this reference. Role of tubulin in the formation and movement of pseudopodia is an important is and is better established that role of actin. The fact that tubulin is involved in this process does not exclude the possibility that actin plays a critical role in it as well.

L. 196 "They probably facilitate efficient formation of tests and fast reorganization of pseudopodial structures in Foraminifera. "

Provide evidence for why authors think so, and discuss the connection to shell formation.

There are strong arguments for the connection of actin to the shell formation demonstrated by Tyszka et al. (2019). We will add this reference here. The role in the remodelling of pseudopodia is still under discussion. Such a hypothesis has been proposed by Goleń et al. (2020). We will refer to this paper.

L. 198 cannot be determined without more detailed ultrastructural studies

I agree with this statement, but there are many examples of previous studies that have observed the movement, function, and microstructure of pseudopods during shell formation. Based on the present findings, a discussion of the contents of these previous studies must be made. In particular, I believe that comparisons with and interpretations of the authors' previous studies can be made with certainty.

We would like to thank the referee for this comment. We will broaden our discussion and include suggested issues in it.

Literature:

Ernst, S., Janse, M., Renema, W., Kouwenhoven, T., Goudeau, M. L., & Reichart, G. J. (2011). Benthic foraminifera in a large Indo-Pacific coral reef aquarium. Journal of Foraminiferal Research, 41(2), 101-113.

Goleń, J., Tyszka, J., Bickmeyer, U., & Bijma, J. (2020). SiR-actin-labelled granules in foraminifera: patterns, dynamics, and hypotheses. *Biogeosciences*, *17*(4), 995-1011.

Loeblich, A. R.; Tappan, H. (1994). Foraminifera of the Sahul Shelf and Timor Sea. *Cushman Foundation for Foraminiferal Research Special Publication.* 31: 1-661

Tyszka, J., Bickmeyer, U., Raitzsch, M., Bijma, J., Kaczmarek, K., Mewes, A., ... & Janse, M. (2019). Form and function of F-actin during biomineralization revealed from live experiments on foraminifera. *Proceedings of the National Academy of Sciences*, *116*(10), 4111-4116.