Detailed response to the interactive comment by Jutta Wollenburg.

On behalf of all authors I we would like to thank Dr Jutta Wollenburg for her constructive 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.

The manuscript by Golén et al. is the logical consequence of their previous paper (Golén et al., 2020) where they observed a very distinct F-actin labelling in foraminiferal granule. In this new paper they applied co-labelling with SiR-actin and phalloidin on pseudopodial structure to rule out SiR-actin staining artefacts as a possible cause for the intense F-actin labellin described be Golén et al (2020). The paper shows impressive and stunning results and I have no doubts in the methods. However, the paper is unprecise in how many species and how many specimens were finally successfully investigated with this double staining method. If I am not wrong, only one Quinqueloculina specimen and only one area of its pseudopodial network is shown in the figures investigated. These things need to be clearified. I assume it was a tremendous effort and most attempts failed, anyhow, these are things that are important to show. I suggest to create a table with all species and the number of specimens involved, showing then which steps were successful, which failed, and how many specimens of which species could/was finally investigated for its fluorescence. Having eventually just one positive outcome is no problem but the reader want's to know.

We appreciate constructive comments. We will clarify this issue in the corrected version of the manuscript. We would like to add additional documentation of all individuals imaged, including their taxonomic identification. In the submitted version of the manuscript we included figures presenting images of two different specimens, for one of them we showed images of two different areas of the pseudopodia (Fig. 1 and Fig. 3 are form the same individual and Fig. 2 is form the different one). During the preliminary stage of our experiments we used a wide range of tubo- and globothalamean foraminifera (see Table R1 and Fig. R1). Tubothalamea were represented by 9 specimens of miliolids. By observation under the stereo microscope, we identified two distinct types of morphology among the individuals: first one was elongated and the other was 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*).

Weconducted 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.*

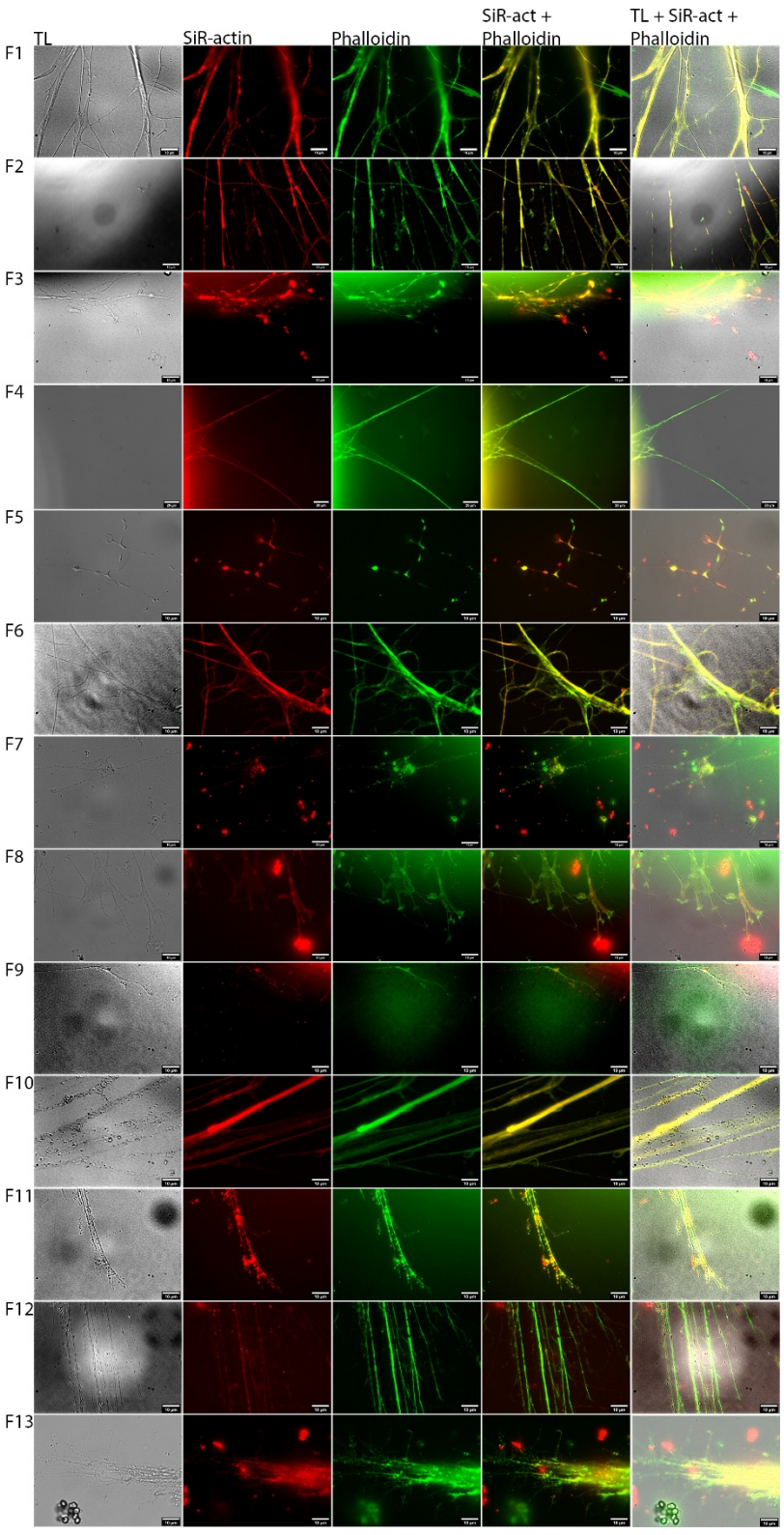

Figure R1 (proposed to be added to Supplement). Compilation of images showing pseudopodia of 13 individuals of foraminifera stained with SiR-actin and Phallodidin Atto 488. Each row represents another individual (individuals F1-F9 represent Miliolida and F10-F13 represent Globothalamea: F10 is *Heterostegina* 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.

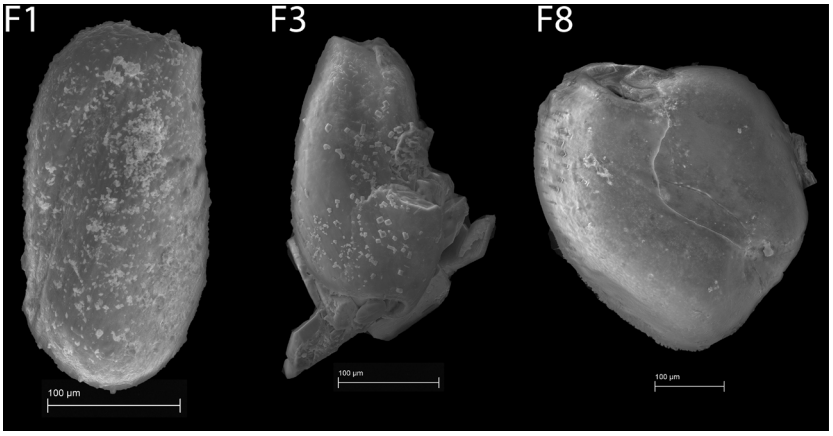

Figure R1 SEM images of 3 individuals used in the study. Numbers correspond to the numbers in Table 1 and Figure 1.

I sometimes had problems to understand the meaning of sentences, thus, I think the revised version should be read by a native speaker before submission.

Thank you for pointing this problem. We will pay close attention to the precision of formulation of sentences and as suggested we will consult the native speaker before submission of the revised version.

The Pdf file lacks line numbers which complicates the review

We will make sure that new version of the manuscript includes the line numbers.

Some further comments

Introduction

The first three sentences lack any references and give incomplete statements. Please streamline these aspects, provide information on the differences in pseudopodia, what is known about the content of granule etc. , and add references on these topics. I would also recommend that the first sentence is written in a way to provide the none-foram reader with some basic informations on what pseudopodia are and for which purposes they are used. Provide some background on granule, historical and information on the kind of granule that exist according to literature. Either here or when you use them, you have to explain terms like 'trunk reticulopodia' as, I guess the majority of readers may not be familiar with such terms.

We are grateful for this valuable comment. We will rewrite the beginning of Introduction taking into account both of the referees' suggestions. We will begin with general description of foraminifera and their significance, then we will present general information regarding pseudopodia and their function. We plan to add definitions of terms and citation to the relevant literature, wherever it is necessary.

2 Material and methods

2.1 Specimens

It is irrelevant how many species are in this tank, it just matters how many were used/investigated for this study.

We entirely agree with this statement and will clarify this issue in the final version of manuscript. For more details, see our response above.

It would be interesting to know which Quinqueloculina species was used and which additional species, not just the genus, were investigated.

We agree that the taxonomy is very important and in the final version of the manuscript we will be as precise in this matter as possible. For more details, see our response to general comment above.

The statement 'This taxon presented the most stable and predictable reticulopodial activity **, thus,** was **most** suitable for replicated experiments.' I would suggest this is a result. I would also modify it in the 'bold' way. I am not sure what you wanted to say with the last sentence of this paragraph.

We would argue that it is not exactly a result. We meant here rather that the stable and predictable reticulopodial activity was a necessary condition, that a given species of foraminifera fulfilled our requirements for designed study. Another similar and necessary condition was that the pseudopodial network must have been preserved in their intact form after fixation. This section will be re-written in the final version of the manuscript to clarify this issue.

2.3 Fixation

You describe that you optimised the fixation method by the trial and error method. It would be good to provide all respective receipts and the results following their application. The reader may want to apply the method in her/his own research and don't have face the same failures. Please also provide the specifications for the glutaraldehyde and what else is used (e.g. Cacodylbuffer??, millepore filtered water??,…). Describe how the fixative was washed out etc.

Thank you for this comment. Apart from glutaraldehyde, for fixation of the pseudopodia of miliolids we did not used any other chemical reagents. As described in the manuscript, we dissolved the 200 µl of glutaraldehyde for the 800 µl of artificial sea water (ASW). We will add the specification of the ASW in the final version of Supplement. For relatively small miliolids such a method of fixation of pseudopodia was sufficient to granular appearance of pseudopodia, when fixation time was short (2 minutes). Only occasionally, we observed the occurrence of beading response during and after fixation. It is more likely to happen when the concentration of glutaraldehyde is lower. For two species of Globothalamea that we used in the preliminary stage the simple solution of glutaraldehyde is not sufficient to preserve the pseudopodia undisturbed. Following the paper of Bowser and Travis (2000) we used low Ca^{2+} ASW.

Later in the text you write 'It is likely that standard fixation methods make ALGs very difficult to preserve during fixation.' It would be good for the discussion to show the fixation receipts in previous and this manuscript/-s, perhaps in the supplements.

Thank you for this suggestion. Such a comparison of the fixation methods will be added to the supplement. It will show not only the differences in the recipe of the fixation solution but also in the fixation time.

2.4 Staining

About the same issue as before. Please provide details. I guess you mixed a stock solution, then stored it at xy temperature, then you applied the staining by adding xy µl of the stock solution to xy ml in your petri dish… How did you wash out the dye? How did you dry the samples? Etc.

We will provide all the details in the final version of manuscript. In case of both of the probes, the stock solution was prepared according to manufacturers' instructions and stored in -20ºC. We did not add the stock directly to the petri dish, but first dissolved in ASW and then we added such dissolved probes to the petri dishes so that the final concentration of probes in the petri dishes was: 1-2 µM of SiR-actin, 0.1-0.2 µM of Phalloidin Atto 488.

2.5 Imaging

'If necessary, fluorescence images were processed using FIJI software to remove the background noise.' Please explain what this software does.

FIJI is a very versatile software enabling many different image processing methods. We used it for general correction of brightness and contrast as well as to remove the background noise. We did it by using the function subtract that subtracts chosen value from the intensity value of each pixel (this function can be accessed in menu Process > Math). To determine the value to be subtracted we measured the pixel intensity in several areas of the background of the image and within the pseudopodia (subtracted value should be significantly lower than the level of the fluorescence in the pseudopodia but higher than the average level of background noise).

'Additionally, imaging of the pseudopodium of an unstained individual with the same light source intensity 130 and exposure time was done to control autofluorescence.' Was this species and its pseudopods fixed in the same way?

Yes, the species and fixation was the same as in the stained individuals.

1. Results

Please rewrite the subchapter 3.3 (should be 3.1) Control for autofluorescence

'Profile of the intensity of fluorescence along the line that crosses the pseudopodium shows low level of the fluorescence intensity in the unstained (control) individual.' Please rewrite starting with 'In the unstained control foraminifera (provide species) the fluorescence intensity profile ..´. I have no idea what you wanted to say. If possible provide a figure or refer to a respective figure for the control specimen (just one???). ´ Also in this individual the variability of the intensity is low, there is no significant difference between the pseudopodium and the background´ Observed with which channels?

´The individual labelled with SiR-actin and Phalloidin Atto 488 displays much higher intensity levels and variation of the intensity with the intensity peaks in the same location. Please be precise, which channels were used for the control, which channels for which dye in which specimen of which species. Refer to the respective figures.

`The relative height of the peak is larger for the SiR actin channel (Fig. S3 in Supplementary Materials).´…is larger for the SiR … than for…

Thank you for these comments. We will rewrite all this section to clarify it. We refer to Fig. S3 in Supplementary Materials. We compered one control specimen to one stained individual.

We compared the intensity of fluorescent signal between the stained and unstained (control) individuals of *Quinqueloculina* sp. observed with set 50 (for SiR-actin) and with set 46 (for Phalloidin Atto 488). The light intensity and exposure time for both individuals were the same. The two individuals show significant differences in the intensity of fluorescent signal (see Fig. S3 in Supplementary Materials). In the unstained (control) specimen, the fluorescence intensity profile along the line that crosses the pseudopodium shows low intensity of signal in both fluorescent channels. Additionally, the variability of the intensity in this individual is low, there is no significant difference between the pseudopodium and the background. On the contrary, the individual labelled with SiR-actin and Phalloidin Atto 488 displays much higher intensity levels and variation of the intensity with the intensity peaks in the same location. This pattern means that both fluorescent probes binds the same type of objects within the pseudopodia.

3.2 Colocalisation of signal from SiR-actin and Phalloidin Atto 488 in fluorescently stained pseudopodia

I really have big problems in understanding some of the sentences in this section. It would be good if a native speaker reads and eventually corrects some parts of the revised manuscript. ‘Moreover, z-position in which given object appeared the sharpest in the Phalloidin Atto was shifted away from the objective for about 620-930 nm in relation to the z-position in which the same object was in focus in the SiR-actin channel .’ I have no idea what is written here, please rewrite. ‘The light of different wavelength is focused at different positions as refractive index of a medium depends on the wave length (Stanley 1971).’ Please rewrite. ‘Both probes stained the most intensely the granular objects (see Fig. 1), however, the whole reticulopodium also displayed weaker fluorescence.’ Are you talking about autofluorescence or is this fluorescence related to the stains?

‘The relative intensity of signal in this area compared to the intensity of fluorescence throughout the whole pseudopodium appeared to be higher for Phalloidin Atto 488 than for SiR-actin.’ Do you mean ‘In this area fluorescence intensity for Phalloidin Atto 488 is much higher than for SiR-actin, whereas, in the remaining pseudopodium…?’

We deeply appreciate all the comments to this section of the manuscript. We admit that in its current form this section is confusing and needs substantial rewriting. Some of the information presented here will be transferred to, section ‘2.5 Imaging’.
In the section ‘2.5 Imaging’ we will include following information:

"For each location within pseudopodium, z-stacks have been taken to capture the 3D structure of pseudopodium. Z-stack is a set (stack) of 2D images of the same area with different distance between the objective and the sample, allowing for imaging of different focal planes (position along z-axis or z-positions). Distance between two subsequent z-position was set up to be constant and equalled to 320 nm. This technique allows for gathering data across the whole 3D structure. The capturing of z-stack images instead of only one image for each part of the pseudopodium was crucial for testing the colocalisation between signals in two fluorescent channels. The excitation light in two fluorescent channels significantly differs in the wave length. The wavelength of the excitation light in two fluorescent channels (Phalloidin Atto 488 and SiR-actin) is significantly different. In this case, the image of a given object may be in-focus in one of the channels and out-of-focus in the other. This is likely caused by the fact that the relative index depends not only on the medium but also on the wave length of the light (Stanley 1971), the pathway of the rays of light of different wavelengths may be different as well as the point of focus. To test the hypothesis assuming the high level of the colocalisation of signal between two channels, we had to correct for this effect, i.e. identify which focal plane of the SiR-actin fluorescent channel is the closest to which plan in the Phalloidin Atto 488 channel. To do that we opened SiR-actin and Phalloidin Atto 488 channels in FIJI software separately and comported individual focal planes of both channels to each other."

After transferring this information, section '3.2 Colocalisation of signal from SiR-actin and Phalloidin Atto 488 in fluorescently stained pseudopodia' will include the description of the observed staining and its interpretation.

Some exemplified typos

Introduction

.. **which** elaborate hierarchical

..critical **in** understanding the evolution

.. see **(**Goldstein, 1999).

.. fluorescen**ce** microscopy

..fixed specimens **of** various species

the classical method of imaging

.. the classical method **for** imaging

… In **an** attempt to **verify if** the possibility that these filaments are the form of **are actin** they tried to **label** them with myosin sub-fragment 1 (myosin S1). As **these** filaments in question did not bind myosin S1, **they concluded** that the nature of these structures differ from the actin.

.. Koonce et al. (1986b) demonstrated by fluorescent imaging, using the rhodamine-phalloidin, **the** presence of microfilaments spread throughout all pseudopods.

Results starts with number 3.3.

Fig. legends, number 2 is missing but there is two times fig. 1.

Thank you for pointing out the typos. We will correct all of them and double check the final version of the manuscript before proposed re-submission.

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.