

Biogeosciences Discuss., referee comment RC1
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Comment on bg-2021-76

Jutta Wollenburg (Referee)

Referee comment on "Fluorescent double labelling of F-actin in Foraminifera: evaluation of granular pattern F-actin organisation in reticulopodia" by Jan Goleń et al., Biogeosciences Discuss., <https://doi.org/10.5194/bg-2021-76-RC1>, 2021

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 labelling described by 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 clarified. 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 wants to know.

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.

The Pdf file lacks line numbers which complicates the review

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.

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.

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

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.

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.

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.

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 μ l 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.

2.5 Imaging

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

'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?

▪ 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...

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...?'

Some exemplified typos

Introduction

.. **which** elaborate hierarchical

..critical **in** understanding the evolution

.. see (**G**oldstein, 1999).

.. fluorescence**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.