

## ***Interactive comment on “Dynamics and organization of actin-labelled granules as a rapid transport mode of actin cytoskeleton components in Foraminifera” by Jan Goleń et al.***

**Jan Goleń et al.**

ndgolen@cyf-kr.edu.pl

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Thank you very much for the 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. Our study is 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

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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 an unexpected finding appears 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.

Referee's comment: The results presented are interesting, but the author's conclusions can only be considered hypothetical at this point.

Response: We agree that our conclusions can only be considered hypothetical at this point. This is partly expressed in the original text, including conclusions. At this point, we can only present staining patterns and propose hypotheses to be tested by future experiments. Our interpretation regarding the function of the observed Actin Labelled Granules is indeed largely hypothetical at this stage.

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.

Response: 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-

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consuming type of experiments that needs to be carried out. Our intention is to run such correlative studies presenting directly what the staining patterns correspond to ultrastructurally. This 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 experiments would need another extensive and well-illustrated publication.

Referee’s 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.

Response: We highly acknowledge all recommendations. We are currently planning additional experiments to address most of these points. At this stage we present results of replicated experiments conducted over last three years. Our intention is to identify

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the problem, then to propose and discuss all working hypotheses. 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. We did not observe any 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 "Actin-labelled granules in Foraminifera: Setting hypotheses based on SiR-actin live experiments". 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

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for designing future experiments.

Here we propose alternative explanations for the observed Actin Labeled Granules (see Fig. 1 in supplement to this response; to be proposed as a new figure in our paper). We describe three possible scenarios in which SiR-actin specifically labels F-actin within structures that have a granular appearance (Fig. 1A-C in supplement), and present possible artifacts caused by staining foraminiferal reticulopodia with SiR-actin (Fig. 1D-F in supplement). 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 from TEM ultra-structure studies (Fig. 1A in supplement). 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. 1B in supplement). 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. 1C in supplement). 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. 1D in supplement) or outside (Fig. 1E in) membranous vacuoles. The last scenario assumes that SiR-actin induces assemblage of actin filaments in specific regions of cytoplasm rich in G-actin (Fig. 1F in supplement) that follows comments by Melak et al. (2017). We will stress multiple scenarios in the final version of the manuscript and discuss them more extensively.

As mentioned above, we will follow up on the suggestion to monitor the movement of granules prior to fixation and perform correlative light-electron microscopy to validate if

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ALGs (or a subset of them) are indeed identical with Fibrillar Vesicles.

We are conducting additional control experiments, including phalloidin and SiR-actin parallel staining and monitoring of Actin Labelled Granules in living specimen treated with inhibitors of F-actin polymerization. We expect a high degree of overlap between the signal from 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 reticulopodia 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).

Referee's 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.)

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Response: 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 described in foraminifera by the reviewer and collaborators. 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 seems to be restricted to endoplasm. Reticulopodia, and especially their distal parts may 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  $1 \mu\text{m/s}$  [..]. In contrast, neurite outgrowth from neurons cultured at  $37^\circ\text{C}$  (albeit only a superficially similar process) occurs at approximately  $10 \mu\text{m/h}$ . Similar growth rates of neurites are found in several other studies as well, e. g. for *Xenopus* a growth rate of  $54.6 \pm 1.22 \mu\text{m h}^{-1}$  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

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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 as 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. 1F in supplement). 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 definitely be an interesting physiological property of Foraminifera (Fig. 1F in supplement). 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.

Referee's comment: 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

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it will receive much more attention.

Response: This true that this paper seems more suitable for a cell biology or protistology journal. It might receive much 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 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 mammalian cells. Just giving a single example, Nature Cell Biology has never referred to Foraminifera in any published paper. 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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Please also note the supplement to this comment:

<https://www.biogeosciences-discuss.net/bg-2019-182/bg-2019-182-AC1-supplement.pdf>

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