Dear Professor Kitazato,

We are pleased to submit a further revised version of our Research Article manuscript "**Weaving of biomineralization framework in rotaliid foraminifera: Implications for paleoenvironmental reconstructions**" (bg-2018-295), which received two reviews for *Biogeosciences*.

We appreciated the constructive criticisms and comments from the two reviewers, and we thank you for providing this opportunity for us to improve this manuscript and submit a revised version. Furthermore, in response to the suggestion from Professor Kitazato, we added more detailed discussions along the reviewers' comments, in order to answer and discuss openly the topics raised.

A point-by-point response to comments is included below.

We hope the present version is acceptable for publication in *Biogeosciences*.

Best regards,

Yukiko Nagai

RESPONSE TO REVIEWER 1 (Inge van Dijk)

The study by Nagai and co autors ('Weaving of biomineralization framework in rotaliid foraminifera: Implications for paleoenvironmental reconstructions', bg-2018-295) shows new insights into the pseudopodial structured during foraminiferal chamber formation, leading to better understanding of processes involved in controlling the chemical signature of the precipitated carbonate shell. By timing calcium carbonate precipitation with the structure of organic layers gives crucial information about the closeness/openness of the site of calcification, and therefore the role of passive transport (seawater exchange), which is still heavily debated. In general, the manuscript is well-structured and well-illustrated

and I just have some minor comments.

> We are grateful for these encouraging words and your constructive review of our manuscript.

Minor comments:

- Change numbering of figures: Reference to figure 7 (page 8/ line16) before Figures 5, 6.

> We preferred to keep the SEM plates of the microstructures together. We have thus added the following to the beginning of section 3.2 to justify the numbering (we also moved Fig. 3, the schematics, to the end as the new Fig. 7 according to a comment from Reviewer 2).
>
> <u>'Figures 3-6 show microstructures of different stages of chamber formation seen by the SEM.</u>

- 4.2 It has been suggested pores are used for gas exchange/respiration (e.g. Berthold, 1976; Leutenegger and Hansen, 1979), and their size might change with e.g. seawater oxygen level (Kuhnt et al., 2013). Would this fit with your observations? Or are the pores closed by pore plates and have no possibility to exchange?

> As O_2 and CO_2 used in respiration are nonpolar molecules, they are able to pass through the cell membrane. As such, the existence of pore plates made from cytoplasm seen in the present study should not influence respiration. Whether their size change with environment differences is a subject for future studies. Added the following in Discussion:
>
> <u>'Pores have been suggested to be used for respiration (e.g., Berthold, 1976; Leutenegger & Hansen, 1979). As O_2 and CO_2 used in respiration are nonpolar molecules, they are able to pass through the cell membrane. As such, the existence of pore plates made from cytoplasm seen in the present study should not influence respiration.'</u>

- 4.3 Are there observed vesicles associated to seawater vacuoles? Was it possible to perform SEM-EDS on vesicles observed during chamber formation to potentially observe (amorphous phase of) calcium carbonate? Did you observe a difference in the intensity/size of vesicles during different phase of calcification and/or chamber size?

In the present study, we were unable to observe amorphous phase of calcium carbonate within the 'vesicles'. In fact, as another reviewer pointed out, we do not have sufficient evidence to prove that these spherical structures indeed correspond to vesicles (and they are outside the cell!). Therefore, we have changed the terminology from 'vesicle' to 'spherical structure' throughout the revised MS. Although we did find these spherical structures with high Ca signals in our SEM-EDS analyses (Figure 6B), we could not know for sure whether these signals are indeed from within the structures or from tests underneath by limitation of the current measurement technology. Added the following in Discussion:

'The number of spherical structures increased as the chamber formation progressed. There is a variation in the size of spherical structure from ca. 50 nm - 1 μm. There are relatively more small ones at the Initial stage, and relatively more large ones at the Late stage, but all sizes of the spherical structures are found across all stages.'

- 4.4 Implications for element distribution: When looking at element distribution across the chamber wall, it has been shown for several elements (e.g. S, Na, Mg) there is a higher concentration band near the POS. The presence of gaps in the organic layers at the initial phase of calcification compared to its absence during llater phases does explain the difference observed in element distribution (i.e. band and no-band).

Yes, we agree.

However, when taking Mg as an example, these Mg/Ca bands close to the POS are still much lower than expected from inorganic precipitation experiments. Based on your observations, is this because the system is not fully open, or simply because inorganic partitioning is different from foraminiferal partitioning, due to presence of other ions (inhibitors) or organic layers (adsorption)? Would this suggest that comparing foraminiferal element partitioning to inorganic precipitation experiments is not useful, since the systems are so different (organics, open/closed system etc.)?

From the present results it is not possible to say how much seawater actually passes through the gaps in organic layers during chamber formation. From the beginning, the Mg content of the fluid is already decreased even the system is not fully closed, because the observed gaps are not sufficiently large to exchange seawater between the site of calcification and the exterior. But this is

just speculative at this point.

We consider comparing foraminiferal elemental partitioning to inorganic precipitation still meaningful, as the partitioning from fluid to crystal follows the same chemical laws in both organic and inorganic systems. The key difference is that the elemental contents of the fluid in the site of calcification is strongly controlled in biomineralisation processes (and very different from inorganic processes). The elemental analysis of fluid at the site of calcification, however, is still currently unmeasurable due to technical limitations. Nevertheless, because magnesium ion is an inhibitor of calcification, it can be speculated that during biomineralization magnesium ions are actively discriminated and removed from the fluid at the site of calcification. Therefore, as pointed out by the reviewers, it is presumed that calcite with low Mg / Ca precipitates around the POS.

We have added the following to Discussion:

"The elemental analysis of fluid at the site of calcification, however, is still currently unmeasurable due to technical limitations. Nevertheless, because magnesium ion is an inhibitor of calcification, it can be speculated that during biomineralization magnesium ions are actively discriminated and removed from the fluid at the site of calcification (Zeebe and Sanyal, 2002). Therefore, it is presumed that calcite with low Mg/Ca precipitates even around the POS. It is reported in many species that the foraminiferal Mg/Ca is high around the POS, but it is still much lower than Mg/Ca estimated from inorganic precipitation experiments (de Nooijer et al., 2014). The elemental partitioning in foraminiferal tests must be strongly controlled through the elemental composition of the fluid in the SOC, which is a key subject for future studies."

Textual suggestions (page number/line number):
2/14 ..from seawater, which implies active ion exchange.

Changed as suggested.

2/24 ..(Haynes, 1981), and each species..

Changed as suggested.

2/25 ..modern days, during which they have..

Changed as suggested.

2/29 Moreover, the tests are..

Changed as suggested.

3/5 Even though the test morphology and chemical composition depend to a certain extent on the environment (), the calcification process..

Changed as suggested.

5/5 For specimens fixed at different time slides during the chamber formation process..

Changed as suggested.

5/15 ..the chamber formation process of A. beccarii with DIC for 59 times in total..

Changed as suggested.

6/19 ..the pseudopodial activity significantly differed. A fan-shaped..

Changed as suggested.

7/13 formed chamber, leaving an empty space in the new chamber..

Changed as suggested.

9/16 ..corresponding to the IOL, the POS, and the OOL respectively from inner to outer side..

Changed as suggested.

10/1 ..has been speculated in previous studies,..

Changed as suggested.

10/6 – in other words the organic layer is part of cytoplasm.

Changed as suggested.

RESPONSE TO REVIEWER 2

A paper by Nagai et al. presents novel and detailed observations on pseudopodial activities and structures during chamber formation in calcareous forams. They also verified that organic layers are a part of pseudopodia/cytoplasm. In addition,

the authors also present the important finding for pore formations on calcareous walls, which have not yet been understood in detail. I think highly of their works which made progress to understand the biomineralization processes in foraminifers. I am sure that their findings would provide many ideas and hints with paleoceanographers and biogeochemists using foraminifers as tools.

> Many thanks for your positive comments and we appreciated your very thorough review of our manuscript.

I think the manuscript is generally well-structured and well-written. However, I feel the Results section and Figures are still not easy to understand for most readers. The authors should take more careful about descriptions of observations, figures and their explanations. I also prefer more explanations in the figure caption to understand without reading the relevant text. I also suggest adding more close-up photographs and movie in particular for Figs. 1&2 as supplementary materials.

> We can agree with these comments in general, and we have increased textual explanations and details on figure captions according to the comments (which are detailed below). We also added higher magnification photos and a video as supplemental materials. As reviewer 2 pointed out, the distribution of pseudopodia during chamber formation is much better visible with a video We add supplementary movie and mentioned about the movie in the text of result section.

I assess some unsolved questions on foram biomineralization can be solved by this paper. However, some new questions are arising after reading this paper. For examples,
1) Are three organic layers produced by separate/independent pseudopodia? I think the initial stage is very important to understand this process. I suggest that the authors should present SEM photographs prior to starting the initial stage as well.

> We consider that all three organic sheets are formed with branched pseudopodia extending from the aperture. We expect these pseudopodia themselves are ultimately expanded from single root, but separate branches are forming each organic sheet. Our recent study (Nagai et al., 2018) showed that all sheets actually converge at the pore plate. Further, pore plates and pore funnels smoothly peeled off each other, which suggests that each sheet is

formed from independent pseudopodia. We think the plates and funnels are gently adhered to each other. We must agree that the very initial stage of these sheet construction must be important, as can be inferred from the details of organic sheet arrangement clarified in the present study.

Regarding early stages before the Initial stage, it was already shown in our recent study (Nagai et al., 2018) that the three sheets (OOL, POS, IOL) are separate in the very early stage (although ultimately from a single root), and thus we refer to SEM figures contained within that study.

Added the following to Discussion to make this clear:

'According to a recent study (Nagai et al., 2018), the three sheets (OOL, POS and IOL) appears initially to be independent even at the very early stage of chamber formation during the total thickness of the whole organic sheet being less than 1 μm. We expect these organic sheets themselves are ultimately expanded from single root, but separate branches of pseudopodia are forming each organic sheet. '

2) Thickness between OOL and POS remain the same or increasing throughout this process? In L256-257, the authors noted chamber thickening.

The distance between OOL and POS increased along the growth of the calcareous wall. Added: 'the chamber wall of which thickens (overall distance between OOL and POS increased over time).

3) Why is the space between IOL and POS narrower than that between OOL and POS?

This is caused by the difference of growth rate of calcareous naterual between the inner side and the outer side. Assuming that the materials (Ca2+, Carbon, among others) are transported from the seawater, it can be presumed that the inner side will become thinner because the chamber wall is formed and material transportation is more restricted in the inner side. Added the following to Discussion:

'The reason why the space between IOL and POS is narrower than between OOL and POS (meaning the inner calcareous layer is thinner than the outer) is presumably caused by the difference of growth rate of calcareous material

between the inner side and the outer side. Assuming that the materials for chamber formation are transported from the seawater, it can be presumed that the inner side will become thinner because the chamber wall is formed and material transportation is more restricted in the inner side.'

4) How can pore plates on POS and pore funnels on OOL align at the same locations if these are separately formed?

At the beginning of construction, all three sheets (OOL, POS and IOL) are converged at the pore site (as was shown by Nagai et al. 2018), in which case these are not truly separately formed and explains the alignment of pore plates and funnels (i.e., we interpret that the sites of pore plates and funnels formation is aligned in very early stage when the sheets are still converged at the pore site). Added the following to Discussion:

'Pore plates and pore funnels smoothly peeled off from one another (Fig. 3B and 7A), suggesting that pore plates and pore funnels belong to independent organic sheets formed from separated pseudopodia. Pore plates and funnels were gently adhered to each other before calcification started. It is thought that pore plates and pore funnels are formed simultaneously face to face, during the organic sheet formation. Pore plates and pore funnels likely function as anchors that hold together all three sheets to result ultimately in a smooth calcareous wall.'

5) Are pore plates on POS biconvex on both side?

Pore plates seems to be dented at the IOL side (see Fig. 3C in Nagai et al. 2018). Added the following in Results:

'Pore plates seems to be dented at the IOL side, from a previous study (see Fig. 3C in Nagai et al. 2018).'

6) Vesicles are usually included in foraminiferal cytoplasm/pseudopodia. Why are these vesicles independently found on the surface of the OOL and POS? Were vesicles originally contained inside the OOL/POS?

Indeed, we agree that vesicles are usually found inside the cell, as the reviewer mentioned. There is no sufficient evidence in the present study to prove that these are vesicles, and thus we changed 'vesicles' to 'spherical structures' across the entire manuscript.

7) Concerning semi-closeness/closeness at site of calcification during early/late stages, maybe your finding is related to passive/active ion transport to change Mg/Ca, but it is still speculative unless the authors verify changes in passive and active ion transport at different stages. I wonder the authors are missing the importance of the space between IOL and POS, which maybe more closed and has not yet fully understood even in this paper. Is there any possibility that differences in Mg/Ca corresponding to an IOL-POS space and a POS-OOL space? To solve this question, I suggest showing Mg/Ca distribution map with OOL/POS lines in Fig. 7. I also wonder if elemental compositions in vesicle have any clues to solve this question.

We approve this point by the reviewer, and agree that the actual amount of seawater exchanged is a key subject to explore in the future. Regarding the differences in Mg/Ca between IOL-POS vs POS-OOL space, measuring the elemental distribution in such a narrow gap is very difficult with existing techniques. This is because chemical compositions are not well preserved during the conventional process of sample prep for electron microscopy. We carried out EDS analyses with the same interest in mind, but have not achieved sufficient results for a publication because Mg signals of the early deposited calcite was below detection level. However, we agree that this is a very important point to mention, and included the following in Discussion:

"The elemental composition of the inner calcified layer formed between the IOL and the POS is probably more closed and strongly affected by cellular processes than the outer calcified layer between the OOL and the POS. Therefore, the magnesium contents of the inner layer may differ from the outer layer with pure calcite may be precipitated at the inner side."

Instead of that, we discussed about elemental (Mg/Ca) heterogeneity within a single wall along the chamber formation process with closeness of the site of calcification, as Reviewer#1 pointed out:

" The elemental analysis of fluid at the site of calcification, however, is still currently unmeasurable due to technical limitations. Nevertheless, because magnesium ion is an inhibitor of calcification, it can be speculated that during biomineralization magnesium ions are actively discriminated and removed from the fluid at the site of calcification (Zeebe and Sanyal, 2002). Therefore,

it is presumed that calcite with low Mg/Ca precipitates even around the POS. It is reported in many species that the foraminiferal Mg/Ca is high around the POS, but it is still much lower than Mg/Ca estimated from inorganic precipitation experiments (de Nooijer et al., 2014). The elemental partitioning in foraminiferal tests must be strongly controlled through the elemental composition of the fluid in the SOC, which is a key subject for future studies."

I anticipate that some questions can be answered in the revised manuscript, but for others I look forward to future works by the authors.

Many thanks. We have attempted to do so, as detailed above.

Specific and technical comments

L1: Weaving? implications for paleoenvironmental reconstructions? I suggest the title rewrite as "Functional role of pseudopodia on biomineralization in rotaliid foraminifers: Implications for paleoceanographic proxies."

We can agree in part, in that saying 'reconstructions' is going too far. So we changed the second part of the title as suggested. It now reads: '*Weaving of biomineralization framework in rotaliid foraminifera: Implications for paleoceanographic proxies*". We hope this is ok.

L22-24: I suggest rewriting as "Elemental and/or isotopic signatures of calcareous tests of Foraminifera are commonly used to reconstruct paleoenvironmental conditions."

Changed as suggested.

L25: differ greatly between taxa/species/individuals/inter-chambers/ intrachambers/layers?

Added '…, as well as between taxa, species, individuals, etc.'

L26: proportional contributions from : : : > relative contributions between : : :

Corrected.

L27: still investigated > still under investigation/unknown/not clear

Changed to '… still under investigation'.

L30: Better to specify what you found for the first time
> To make it clear we moved 'for the first time' to the end, it now reads: 'We document triple organic layers sandwiching carbonate precipitation sites for the first time'.

L33: POS should be explained when first mentioned in the abstract
> Agreed and changed to 'primary organic sheet'.

L40: I do not think so unless the authors verify changes in passive and active ion transport at early and later stages, respectively.
> We agree with you and therefore changed the expression to make it more mild: 'provides insight towards resolving' instead of 'resolved'.

L41: The "vital effect" has broad meanings. Better to specify. You may mean differences in elemental and/or isotopic ratios along chamber walls.
> Specified as follows: 'The 'vital effect', specifically elemental and isotopic ratios along chamber walls,…'

L42-44: Better to conclude how your findings are helpful to interpret and calibrate paleoceanographic proxies and biogeochemical cycles.
> To address this we reorganised the last few sentences of the introduction, as follows, which we hope helps:
> 'Our study provides insight towards resolving a key 'missing piece' in understanding foraminiferal calcification though culture experiments and in-depth observations of living animals. Our findings contribute to interpreting and understanding biogeochemical proxies by showing that the 'vital effect', specifically elemental and isotopic ratios along chamber walls, is directly linked to spatio-temporal organization of the 'biomineralization sandwich' controlled by the three major organic layers.

L47, Keywords: should have more important words.
> *Biogeosciences* does not actually require keywords, so now they have been removed.

L52-66, the first paragraph: this paragraph is jumbling about rotallids and forams

in general, most of which are not directly related to the topic of this manuscript. I guess most BG readers know about forams. So better to start from the introduction of biomineralization of forams.

We can agree with this and have deleted the first paragraph.

L71-73: Better to set this sentence as a topic sentence

Done.

L75: by experiment > by culturing experiments

Corrected as suggested.

L84-87: I think in situ observations and culturing experiments of foraminifera have a long history and many researches, as described in the next paragraph. I suggest deleting these sentences.

Deleted as suggested.

L91-95: too long noun, better to rewrite as "Superfine structure observation by : : : have been reported in order to : : :"

Modified as suggested.

L100-108: The authors should more justify to use the general term "pseudopodium/a" because foraminiferal pseudopodia are usually named as granuloreticulopodia. I would only agree with the authors if foraminifers do not produce any dynamic net-like structures with no any granules visible during chamber formation.

Your suggestion is true, but we prefer to use pseudopodia as it is a more general term. We modified the sentence as follows to make this clear:

'Foraminiferal pseudopodia are usually named granuloreticulopodia (see Travis and Bowser, 1991) to define a granular reticulated pseudopodium responsible for feeding, digestion and locomotion; in the present paper we will simply use pseudopodia as it is a more general term.'

L116-117: The "POS" used to be called as POM (Primary Organic Membrane) in Hemleben et al. (1986).

Changed to '…the one in the middle was initially named the 'Primary organic membrane' (POM) (Hemleben et al., 1986) but later changed to 'Primary

organic sheet' (POS) (Erez, 2003)'

L116-118: OOL and IOL are not first mentioned

True. Changed to simply OOL and IOL instead of spelling out the whole name.

L124: The new term "organic scaffolding" are not easy to imagine and not mentioned as an important term throughout the manuscript. I suggest the author redefine the term "anlage" to confine organic layers.

Since *Anlage* has been defined differently by different people, we disagree and would like to refresh with a new term to avoid confusion in the future.

L126-127: Use POS, OOL and IOL consistently throughout the text except for first mentioned.

Changed to simply OOL and IOL instead of spelling out the whole name.

L130: natural state?

Changed to 'well-preserved morphology'.

L130-131: electron microscopy>SEM/TEM?

Changed as suggested.

L132, (SOC): Move to L129 that is first mentioned

Changed as suggested.

L150-151: paleoenvironments>palaeoceanographic proxies

Changed as suggested

; predicting responses to ongoing climate change > how?

Deleted this part of the sentence.

L153: Better to rewrite as "within a hyaline calcareous wall using the benthic foraminifera"

Changed as suggested.

L157, SEM: Define when first mentioned.

This was defined already in the introduction.

L167: De Nooijer et al., 2009? Check all years of references in the text. I found some typos in other refs as well.

　　　Corrected and checked all years of references.

L208: The first paragraph of the Results section is just an outline and unnecessary. Delete or partly move to the method section.

　　　Deleted.

L223: the last existing calcified chamber > the last chamber

　　　Modified as suggested.

L224: characteristic > morphology

　　　Modified as suggested.

L226: delete "from then"

　　　Deleted.

L231, an aggregation of cytoplasm: Indicate where and which part in the figure,

　　　Added reference to Figure 1B.

L233: retracts until where?

　　　Added 'until the surface of the newly forming chamber'.

L238-239: fine and short pseudopodia? I cannot see it. Need more close-up photos.

　　　We added an enlarged part to Figure 1C, we hope this helps.

L240-241: A brighter band of particles? I cannot see it.

　　　Changed to simply saying 'bright band'.

L242: beyond? inside?

　　　Changed to 'inside' as suggested.

L243: smooth? Fig. 1C looks smoother than 1D

　　　Deleted this part of the sentence.

L251, Calcium carbonate: How do you know it?

Added to 'material, inferred to be calcium carbonate,' at beginning of the paragraph.

L252: I think the overall outline and size are fixed at earlier stages (the middle stage).

We removed 'size' but the outline morphology is actually changing since the middle stage so we left it.

L253: Hard to see pseudopodial movements. Do you have a movie?

We have added a movie as Supplementary Video.

L253: Open triangles in Fig. 2A?

Deleted 'open triangles' (this referred to an earlier version of the figure, apologies).

L256-257: How do you know the chamber wall getting thicker?

This is clearly visible in the supplementary video, so we added reference to that video:

'(from Figure 2B–C; also see Supplementary Video)'

L262: The usual type of pseudopodia movement means reticulopodia?

Added 'usual type of pseudopodia movement (typical of reticulopodia)'.

L266-267: Move to the method section

We do not think this can be moved to Methods because without results from the observation of the stages, we would not have been able to divide the stages as such. So we have left this section here.

L266-277: This paragraph with Fig. 3 should move to the Discussion section because Fig. 3 are mostly schematic models and your interpretations based on observations.

Moved.

L274: gray in Figures 2-4?

Corrected.

L274: um?

Corrected.

L292, vesicle: How do you identify it? Vesicles are usually included in foraminiferal cytoplasm/pseudopodia. Why are these vesicles independently found on the surface of OOL and POS?

This is a good point in that we cannot clearly identify these spherical structures as vesicles with certainly, at this point. Therefore we have changed the name to 'spherical structures' throughout the manuscript and deleted the assumption that these represent vesicles.

L295, pseudopodia: how do you identify it?

We can only identify pseudopodia by morphology, that the elongated structures are inferred to be pseudopodia. Added the following to make this clear: 'elongated structures, inferred to be pseudopodia,'

L311, needle-like structure: Show in the figure.

Added panels to Figure 5E to show this.

L316-317: Show in the figure

Added arrowheads in Figure 5E to show the gaps.

L328-329: Which are algal cysts in Fig. 5A?

These overlay the OOL, so we added 'Algal cysts including *Dunaliella* individuals can be seen overlaying the OOL'.

L335: period>stage

Corrected.

L353: OOL had toward the inner side?

This is a part of a line we thought we have removed, apologies. Deleted now.

L376-377: Indicate which photos clearly show this.

Added '(Figure 4C-E, Supplementary Video)'

L387: Cader>Cadre, Ni>Ní based on references

Corrected.

Figs. 1&2: Add color legend in the figure;

Added.

indicate initial, middle, late stages in the figure;

Indicated

hard to see any bubbles and pseudopodia inside chambers;

This is more visible in the Supplementary Video which we have added now, these represent structures within the chambers.

in Fig. 1C, the frame of new chamber are magenta?;

No, in 1C there is no calcified parts yet, and magenta indicates calcified wall. So this is only present in 1D. We made this more easily visible in 1D now.

I prefer more explanations in the caption to understand without reading the relevant text;

We added more comprehensive explanations to Figs 1 & 2 in the revised captions, as follows. It should be understandable without having to read the relevant text now.

'Figure 1: Time series observation of chamber formation by optical microscopy, as seen in the individual observed on December 7th, 2017 (see Table 1). The initial stage of chamber formation, where the organic framework is built, depicted by A-B. A: Beginning of chamber formation, defined as 0 minute from the start, indicated by a dense radiating spray of pseudopodial network. B: 9 minutes, when an aggregation of cytoplasm becomes visible around the aperture of the last existing chamber. As this cytoplasm expands, the pseudopodial network starts to retract to the surface of the new chamber to complete the framework. The middle stage, where the organic framework is prepared for calcium carbonate precipitation which begins at near the end of this stage, takes place between 15 minutes to 60 minutes, as depicted by C-D. C: 27 minutes, cytoplasm concentrates and outline of newly forming chamber wall now clearly visible, pseudopodia still just visible on the surface. D: 41 minutes, pseudopodial retracts inside the forming chamber wall. Left: optical microscopy image. Right: the same image with schematic overlay; colour legend: deep purple = pseudopodia; light purple = cytoplasm; magenta = calcium carbonate in the newly forming chamber; yellow = previously formed

chambers.

Figure 2: Time series observation of chamber formation by optical microscopy (continued), as seen in the individual observed on December 7th, 2017 (see Table 1). The late stage of chamber formation, where calcium carbonate is extensively precipitated and chamber wall is thickened, taking place from around 60 minutes after the start of chamber formation (total time varies considerably among individuals). A: 65 minutes, pseudopodia expands again to form a dense network but in thicker strands than seen in previous stages. B: 100 minutes, a network of pseudopodia is seen in the new chamber, the chamber wall of which thickens. C: 124 minutes, chamber wall thickening continues. D: 180 minutes, chamber wall thickening is nearly and the pseudopodial network begin to disappear, indicating that the end of the chamber formation process is near (actual completion was at 248 minutes for this individual). Left: optical microscopy image. Right: the same image with schematic overlay; colour legend: deep purple = pseudopodia; light purple = cytoplasm; magenta = calcium carbonate in the newly forming chamber; yellow = previously formed chambers.'

open triangle?;

Deleted, this was from an earlier version which should have been removed before submission.

Did you identify calcareous wall by polarized microscope?:

We inferred the brighter parts to be calcium carbonate based on images from differential interference contrast (DIC) microscopy.

any more magnified images?

We added images in original resolution as supplementary material.

You should have used a fluorescent dye to observe cytoplasm more clearly.

That is a possibility for future research, but we consider that the activity of pseudopodia is already clearly seen in the supplementary video taken with DIC.

Fig. 3: move after Figs. 4-7;

Moved.

indicate initial, middle, late stages in the figure;

Indicated.

for A, indicate which part of close-up in B;

B is not a close-up of A but instead is a cross-section.

colors of outer (blue) and inner sides (purple) are confusing with vesicle and pseudopodia.;

Background colours are deleted.

the shape of carbonate crystals looks like needles. is it OK?;

Yes. We have added a reference to Figure 4E, newly added to show needle-like crystals.

What are the purple colored polygonal shape on the POS?

These are holes, we removed the colours.

Figs. 4-6: Indicate differences between dotted lines and thick lines.

Added 'Thick lines indicate membranous pseudopodia and dotted lines indicate framework pseudopodia.'

Fig. 7: Indicate OOL and IOL lines;

Added this on Panel C.

Add Mg signal and Mg/Ca data

The Mg signals were too weak, therefore we left it out.

Weaving of biomineralization framework in rotaliid foraminifera: Implications for ~~paleoenvironmental reconstructions~~paleoceanographic proxies

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Abstract. Elemental and/or isotopic signatures of calcareous tests of Foraminifera are commonly used to reconstruct paleoenvironmental conditions~~Foraminifera are commonly used to reconstruct paleoenvironmental conditions based on the taxonomical composition, as well as elemental and/or isotopic signatures of their calcareous tests~~. A major problem, often referred to as the 'vital effect', is that such geochemical signatures stored in inorganic calcium carbonates differ greatly under the same environmental conditions, as well as between taxa, species, individuals, etc. This effect was previously explained by relative~~proportional~~ contributions between~~from~~ passive vs active ion transport patterns, but their details are still ~~investigated~~under investigation. In this study, the functional role of pseudopodial structures during chamber formation is elucidated by detailed observation of *Ammonia beccarii* (Linnaeus) using a time-lapse optical imaging system and high-resolution electron microscopy. ~~For the first time, w~~We document triple organic layers sandwiching carbonate precipitation sites for the first time. The three major organic layers (outer organic layer, primary organic sheet, and inner organic layer) are formed by an initial framework of pseudopodia overlaid with further layer-like pseudopodia. The P~~r~~imary ~~O~~organic ~~S~~sheet seems to facilitate early calcium carbonate nucleation, then entrapped by double precipitation sites. We further show that calcification starts when outer/inner organic layers still reveal tiny gaps (holes within the framework) that may serve as pathways for passive ion exchange (e.g., Mg^{2+}) between seawater and the confined precipitation space. Nevertheless, the majority of wall thickening occurs when the precipitation site is completely isolated from seawater, which implies ~~that implicates of~~active ion exchange. This may explain the differences in Mg/Ca ratios in early and later stages of calcification observed in previous studies. Our study ~~resolves~~ provides insight towards resolving a key 'missing piece' in understanding foraminiferal calcification though culture experiments and in-depth observations of living animals. Our findings contribute to interpreting and understanding biogeochemical proxies by showing that ~~T~~the 'vital effect', specifically elemental and isotopic ratios along chamber walls, is directly linked to spatio-temporal organization of the 'biomineralization sandwich' controlled by the three major organic layers. ~~This study exemplifies the importance of culture experiments and in-depth observations of living organisms in order to interpret and calibrate biogeochemical proxies.~~

1 Introduction

~~Rotaliids are calcareous perforate foraminifera representing a group of marine protists classified within the Globothalamea class of the phylum Foraminifera (Pawlowski et al., 2013). They consist of well-established group of benthic and planktonic proxies. Rotaliid foraminiferal tests (shells) grow by additions of small compartments called 'chambers' sequentially with the growth of the cytoplasm (Haynes, 1981), and each species has a characteristic test morphology. Foraminifera as a phylum originated in the Precambrian and survived to modern days, during which they have experienced numerous diversifications and extinctions following global environmental changes. About 4,000 species have been described from the modern environment (e.g. Murray, 2007; Pawlowski et al., 2014). Furthermore, approximately 50,000 to 100,000 species~~

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The calcification process of the foraminiferal test is the phase of growth in which the elemental and isotopic compositions of the test is determined, and is also the key generating their morphological diversity. In recent years, the foraminiferal test has become widely applied as a palaeoenvironmental proxy, and its geochemical / isotopic composition has become one of the major tools in palaeoenvironmental reconstructions. Even though t~~T~~he test morphology and chemical composition depend to

10 a certain extent ~~depend~~ on the environment (Schiebel *et al*., 2017; D~~d~~e Nooijer *et al*., 2014)~~.~~. ~~tThe calcification process of the foraminiferal test is the phase of growth in which the elemental and isotopic compositions of the test is determined, and is also the key generating their morphological diversity.~~ To this end, elucidating the detailed mechanisms of foraminiferal calcification has been treated with great interest in the field of geosciences. For example, it has been proven by culturing experiments that the seawater temperature and the Mg/Ca ratio of foraminifera show a strong linear correlation (Nürnberg *et*

15 *al*., 1996; Toyofuku *et al*., 2000). Meanwhile, it is also known that the incorporation ratio Mg/Ca is variable and species specific (summary in Toyofuku *et al*., 2011). The chemical distribution, however, vary among even individuals of the same species and exhibit zonation, corresponding to the test wall structure (Kunioka *et al*., 2006; Van Dijk *et al*., 2017). These variations in chemical composition, both inter- and intraspecific, are inclusively termed 'vital effect' (Urey, 1951). In order to reconstruct accurate palaeoenvironments, it is important to utilize reliable proxies, such as the chemistry and isotopic

20 composition of foraminifera tests. Therefore, the biological processes of chamber formation is of great importance and interest. ~~Fortunately, since foraminifera still survive till modern days, it is possible to carry out *in situ* observations and design culture experiments for learning their biology and further improving palaeoenvironmental analysis. Despite this, the biomineralization process foraminifera is much less studied compared to that of bivalves and coccolithophores.~~

Observation of the foraminiferal chamber formation has been reported from as early as 1854 using the genus *Peneroplis*

25 (Schultze, 1854), and many species have been documented thereafter (e.g., Myers, 1935, 1940, 1943; Jepps, 1942; Sliter, 1970; Berthold, 1976; Spindler and Rottger, 1973). Superfine structure observation by scanning and transmission electron microscopy (SEM and TEM) have been reported in order to carry out more detailed documentation of the cellular process of calcite precipitation during chamber formation in the benthic species *Rosalina floridana* (Angell, 1967) and the planktonic species *Globorotalia truncatulinoides* (Hemleben *et al*., 1986), *Orbulina universa* (Spero, 1988) ~~have been reported~~. The

30 common features summarized from these detailed observations on benthic and planktonic species, points to the fact that cytoplasm and the many types of organic sheet-like structures (i.e., organic layers like Outer Organic layer (OOL) and Inner Organic Layer (IOL)) play fundamental roles in calcification, as opposed to simple chemical reactions between calcium and carbonate ions.

Pseudopodium is one of the key features of foraminiferal biology. Pseudopodia form a part of the cytoplasm consisting of cytoskeleton structures, such as microtubules and actin filaments, as well as other organelles like mitochondria, ~~vesicles~~spherical structures, and vacuoles (Marszalek, 1969, reviewed in Travis and Bowser, 1991). Pseudopodium represents a multi-functional cellular structure serving various purposes such as locomotion, feeding, digestion, and chamber

5 formation. Foraminiferal pseudopodia are usually named ~~G~~granuloreticulopodia~~um~~ (see Travis and Bowser, 1991) ~~is included in this general term~~ to define a granular reticulated pseudopodium responsible for feeding, digestion and locomotion; in the present paper we will simply use pseudopodia as it is a more general term. The appearance of pseudopodia changes during chamber formation and a fan-like array of pseudopodia develops (Bé *et al*., 1979). Then, an organic structure that forms the framework for chamber formation, called *Anlage*, is formed (Angell, 1967). In benthic

10 foraminifers, an algal cyst composed of foreign detritus and other materials is constructed around this *Anlage* (Angell, 1967). *Anlage* is largely constructed by foamy and spherical microstructures (<1 μm) (Angel, 1967; Hemleben *et al*., 1986), and is bulging in shape which led some authors to call it a 'bulge' in early studies using planktonic foraminifers (e.g., Bé *et al*., 1979). This bulging *Anlage* is the three-dimensional structure that becomes the precursor of the chamber. There are three organic layers in the *Anlage*, one on the outer surface has been termed the ~~'Outer organic layer'~~OOL (~~OOL;~~ Spero, 1988),

15 the one in the middle ~~was~~is initially named the 'Primary organic membrane' (POM) (Hemleben *et al*., 1986) but later changed to 'Primary organic sheet' (POS) (~~Hemleben *et al*., 1986;~~ Erez, 2003), and the innermost one is called the ~~'Inner organic layer'~~IOL (~~IOL;~~ Spero, 1988). Precipitation of calcium carbonate microcrystals takes place on both sides of the POS, sandwiched between the Outer and Inner organic layers. In addition to these three organic layers, the term *Anlage* is now loosely accepted to include the numerous pseudopodial cytoplasm that are present around them during calcification. Since

20 different authors have different views and definitions as to what *Anlage* means (e.g., Angell, 1979; Bé *et al*., 1979; Hemleben *et al*., 1986), hereafter we refrain from using the term *Anlage* and instead use 'organic scaffolding' to refer to the organic framework which the chamber wall is built upon.

In order to investigate the fundamental functions of the POS, the ~~Outer organic layer~~OOL and the ~~Inner organic layer~~IOL during chamber formation, Nagai *et al* (2018) conducted focused ion-beam (FIB) processing on a foraminifera specimen

25 during calcification, which allows the thin-sectioning of the site of calcification (SOC) without decalcification to observe cytoplasm and well-preserved morphology~~the natural state~~ of the calcifying test (calcium carbonate crystals) together using ~~electron microscopy~~SEM/TEM. Their observations clearly show that the organic scaffolding has numerous voids and empty spaces within the membranous structure of the ~~site of calcification~~ SOC~~(SOC)~~. The presence of calcification liquid and exo/endocytosis are inferred, and the growth of calcium carbonate could be shown using time series samples. However, they

30 have not documented the processes which leads to the construction of the POS and other organic structures during chamber formation.

Undoubtedly, the organic scaffolding built prior to chamber formation is an important factor shaping the characteristic morphology of foraminifera, serving as a template for calcification. When the foraminiferal test is dissolved, the organic structure is revealed and it has the same overall morphology as the calcareous part (Banner and Williams, 1973). Despite it

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has been suggested that pseudopodial activity plays a key role in this process, little is known about the mechanism. Spindler and Röttiger (1973) first stated that it is pseudopodia that secrete the organic layer using optical microscopy, working with *Heterostegina depressa*. However, due to the low resolution of optical microscopy, they were unable to see the details of the process and this had no evidence other than largely speculation.

5 Although foraminifera are widely used for palaeoenvironment modelling, a total understanding of the foraminiferal calcification process is still lacking, impacting the accuracy of predictions made from foraminifera-based data. An accurate overview and model of the chamber formation by pseudopodia and the calcification process in calcareous foraminifera is therefore urgently needed to better our understanding of ~~palaeoenvironments~~ paleoceanographic proxies ~~as well as predicting responses to ongoing climate change~~. To fill this knowledge gap, this study aims to elucidate the role of pseudopodial

10 activities on the formation process of foraminiferal chamber and its organic structures within the within a hyaline calcareous wall using the benthic foraminifera ~~calcareous wall~~ using the benthic hyaline foraminifera *Ammonia beccarii*, which has been used in a few relevant previous studies (e.g., Toyofuku *et al.*, 2017), as a model system. We combine differential interference contrast (DIC) microscopy and scanning electron microscopy, capturing DIC images through a time-lapse to document the pseudopodial activities during chamber growth and carried out SEM observations. Ffor specimens fixed at~~in~~ different time

15 slides during the chamber formation process to visualize organic structures at the sub-micron order.

2 Materials and Methods

2.1 Sample Collection and Laboratory Culture

Living foraminifera were collected from brackish water salt marsh sediments of Hiragata Bay, Natsushima-cho Yokosuka, Japan (35°19'21''N, 139°38'5''E) in the spring of 2015. Surface (top 5 mm) sediments were collected and transported to the

20 laboratory to serve as a stock from which individuals of the benthic calcareous foraminifera *Ammonia beccarii* (*sensu* De Nooijer *et al.*, 2009~~8~~) were isolated. Living specimens were recognised by their bright yellow colour and visible pseudopodial activity. They were cleaned from excess sediment and debris under a stereo microscope (SteREO Discovery V12, Zeiss Co. Ltd.) and transferred to filtered (0.2 μm) natural seawater (salinity ca. 35) and placed in a Petri dish. The Petri dishes were maintained at 20°C and twice a week, a small amount of live microalgae (*Dunaliella tertiolecta*, NIES-

25 2258) were added. Within a few days of feeding, some individuals started chamber formation and were selected for observation.

2.2 Optical Observation Settings of Chamber Formation

Chambers in the process of formation were observed using an inverted differential interference contrast (DIC) microscope (Axio Observer Z1, Zeiss, Germany). Time-lapse images were captured automatically by the digital microscope software

30 Axiovision (Version 4.6). Time intervals between shots varied from 10 seconds to 10 minutes, but typically the interval was

1 minute. Magnifications of the available objective lenses were x10, x20 x40, and x63. A heat cut filter was applied to reduce damage on the living individuals inflicted by the image capture process.

2.3 Microstructure Observation and EDS Analysis

All specimens were fixed simultaneously using a fixing solution (3% paraformaldehyde, 0.3% glutaraldehyde, 2% NaCl in PBS buffer, pH 7.8) and subsequently stored in 2.5% glutaraldehyde at 4°C to avoid any morphological changes in the cell material through dehydration. They were then washed in 0.2 μm filtered seawater, post-fixed with 2% osmium tetraoxide filtered seawater solution for 2 hours at 4°C. Following that the specimens were rinsed with distilled water and conductive staining was performed by incubating in 0.2% aqueous tannic acid (pH 6.8) for 30 minutes (Willingham and Rutherford, 1984). After another wash with distilled water, specimens were further treated with 1% aqueous osmium tetraoxide for 1 hour. Finally, they were dehydrated in a graded ethanol series and critical point dried (JCPD5; JEOL Ltd., Tokyo, Japan). SEM observations were carried out on a JSM6700F field emission scanning electron microscope (FE-SEM) in Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka, Japan. Elemental composition of all specimens was analysed using a JED 2300 (JEOL) dispersive spectrometer (EDS) equipped on the same JSM6700F FE-SEM at JAMSTEC. Selected specimens processed for SEM observation were embedded in epoxy resin for the purpose of measuring elemental composition of the newly forming chamber wall. The epoxy resin fully filled the chamber cavities and were polished to expose the chamber wall being formed, and the exposed surface was coated with a ca. 3-nm-thick osmium foil. After rinsing with distilled water, this polished block was sectioned using an automicrotome to generate relief-free sections of foraminiferal tests revealing fresh calcite surfaces of chamber walls.

3 Results

We succeeded in observing the chamber formation in *Ammonia beccarii* using DIC and SEM techniques. Time-lapse imaging with DIC observation was able to capture the process of chamber formation and the change in morphology over time at a micrometer order resolution, importantly also capturing the movement of cytoplasm and pseudopodia in detail. SEM observation revealed the fine submicron order processes leading to the construction of organic structures as well as the precipitation of calcium carbonate.

3.1 Time Series Observation with Optical Microscopy

We were able to observe the chamber formation process of *A. beccarii* with DIC for 59 times in total with DIC. Depending on the size of the chamber, it took about 5–8 hours to complete the whole process (Table 1). Prior to the start of chamber formation, exceptional activities were exhibited by the expanded pseudopodia. Usually for the purpose of feeding and moving, pseudopodia randomly branches at irregular intervals to arbitrary direction with variable lengths. During the chamber formation process, however, the pseudopodial activity significantly differed., Ain that a fan-shaped complex

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pseudopodial network was constructed (Figure 1A), expanding from the aperture of the last ~~existing calcified~~ chamber. This pseudopodial network is arranged in a dense, radiating spray resembling that of a dandelion flowerhead. This unique ~~characteristic~~ morphology allowed us to recognise individuals in the beginning of chamber formation and start our time-lapse observation ~~from there~~ (as 0 min). For an average individual, the events of chamber formation can be sequentially

5 divided into three steps, outlined as follows in a typical time sequence (see supplementary movie)~~.~~

The initial stage of chamber formation was from 0 to ~~about~~ approximately 15 minutes, although this varied considerably among individuals and size of the newly forming chamber observed, as with all stages (Table 1). ~~This~~ The initial stage is the stage where the organic framework for chamber formation is built. Following the pseudopodial network construction which takes place from 0 minute, an aggregation of cytoplasm (Figure 1B) quickly became visible around the aperture of the last

10 existing calcified chamber (~~15 minutes;~~ Figure 1B). As the cytoplasm expands, the pseudopodial network retracts until the surface of the newly forming chamber. We consider the completion of the organic framework to be the end of the initial stage.

The middle stage of chamber formation took place ~~at~~ approximately at around 15–60 minutes (Figure 1C-D). During this stage, the foraminifer prepares the organic scaffolding for calcium carbonate precipitation which begins during this stage. By

15 about 30 minutes, the cytoplasmic aggregation concentrates in the same shape of a newly forming chamber like a hemi-sphere (Figure 1C). At this point, fine and short pseudopodia have retracted to a certain extent but still seen on the surface of the structure. A bright~~er~~ band ~~of particles~~, probably representing calcium carbonate starting to become formed, can be seen on the surface of this. This proceeds to become the chamber wall. ~~At around 60 minutes,~~ Finally, the pseudopodia retracts ~~beyond~~ inside the forming chamber wall ~~and the wall surface becomes smooth~~ (Figure 1D).

20 The late stage of chamber formation is defined as the stage where material, inferred to be calcium carbonate, is precipitated extensively to thicken the chamber wall in the newly forming chamber, and ~~takes~~ took place~~s~~ between around 60–400 minutes (total time varied among individuals; Table 1). We define the start of the late stage as when the pseudopodia beings to expand again to cover the organic scaffolding, and also the whole organic scaffolding is covered by a layer of calcium carbonate (with pores becoming visible under light microscopy). At the start of this stage ~~From around 60–100 minutes~~, the

25 pseudopodia expand again to form a dense network, this time in thicker strands (Figure 2A). The length of all the pseudopodia appear remarkably regular. Calcium carbonate continues to be precipitated in the forming wall. At this point, the overall outline ~~and size~~ of the newly forming chamber is basically fixed. Pseudopodial movement can be seen inside the forming chamber (~~open triangles in~~ Figure 2A). Cytoplasm aggregate that filled the newly forming chamber retreats to the previously formed chamber~~,~~ leaving~~So~~ an empty space ~~is made~~ in the new chamber. ~~At 150 minutes,~~ After that, a network

30 of pseudopodia is present in the forming chamber, the chamber wall of which thickens (overall distance between OOL and POS increased over time) and the pores become increasingly and clearly visible (from Figure 2B–C; also see Supplementary Video). Chamber thickening continues to occur from this point onwards, generally at around 1~~5~~20–400 minutes (Figure 2C). During this process, the density of the pseudopodial network on the chamber wall surface is increased and wraps the chamber wall like a mesh. As the chamber wall thickening completes (approximately 400 minutes but actually quite variable,

the specimen shown in Figures 1-2 had completed this by 248 minutes)at around 400 min, the mesh-like pseudopodial network on the surface disappears (Figure 2D). We consider this to indicate the termination of chamber formation process. After this, the individual starts to show the usual type of pseudopodia movement (typical of reticulopodia).

3.2 Ultramicro Observations on the Forming Chamber Wall

5   The process of chamber formation is classified into three stages, as outlined above. Specimens exemplary of each stage were observed with a scanning electron microscope. A schematic diagram is presented in Figure 3, which outlines the general observations. The basis of organic layer formation is the interweaving of a pseudopodial framework (Figure 3A), the interspaces of which is then filled in with a further layer of pseudopodial material, resulting in a complete organic layer. The pseudopodia are observed to form a dense framework (purple dotted lines in Figures 2A, 3 and 4), which is then overlaid by

10 a layer of membranous pseudopodia which fills the interspaces (Figure 4E). In the OOL, numerous spaces of 100 nm – 1 μum can be seen (gray in Figures 3 and 4, 62–4), which represents the interspaces between the framework which is yet to be filled. In some instances, the membranous pseudopodia were observed during the process of filling the interspaces, sometimes from more than one direction (e.g., Figure 4E), by a gradual, webbed expansion. Figures 3-6 show microstructures of different stages of chamber formation seen by the SEM.

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Initial Stage

In the initial stage of chamber formation, the test was entirely covered with pseudopodia and organic layer-like structures (Figure 43A), some parts of these covering structures were peeled off during the sample preparation process. Focusing on the chamber being formed, it was possible to observe the OOL and the POS (Figure 34B), with the POS being visible from gaps

20 in the OOL. The interspace between the two layers was narrow (Figures 34B-C). Even at high magnification, the outer surface of the OOL itself is relatively smooth layer-like structure (OOL in Figure 34B-C). In some cases, the pseudopodia can be seen directly expanding from the OOL (Figure 34C). The primary organic sheet (POS) can also be observed (Figure 43B) and is relatively robust, covered by numerous protrusions. These are convex, frustoconical structures about 1 μm in width (Figure 43A-B), and represent pore plates which corresponds to pores. These are simultaneously formed when the

25 POS was constructed (green coloured in Figure 43C-D). Projections (<1 μm) were observed on the cytoplasmic surface of OOL (light green in Figure 43B). Spherical structuresVesicles can be seen on the OOL (blue coloured in Figure 43C, some appeared crushed probably due to the critical point drying process), and similar structures could also be found on the POS (blue coloured in Figure 43D and E). The size of vesicles these spherical structures varied from 50 nm to 500 nm, and these likely represent vesicles. On the OOL, some elongated structures, inferred to be pseudopodia, appeared to have a form like

30 that of a sausage chain (Figure 34B), the diameter and interval of contractions were variable. The bulging part contained only cavities and this form might be associated with peristalsis. It is known pseudopodia transport mitochondria and vesicles (Travis and Bowser, 1991; Cedhagen and Frimanson, 2002), and it is possible that this peristaltic structure has important roles in such transportation.

No crystals were found between the POS and the OOL, indicating that no calcium carbonate has been deposited at this stage, supported by the fact that the SEM-EDS analyses showed an absence of calcium signals (Figure ~~7~~6A).

Middle Stage

At the middle stage, the interspaces among the framework structure constructed by the pseudopodia has been filled to a much larger extent than in the initial stage, with much fewer gaps (about 5 nm – 200 nm; grey coloured in Figure ~~4~~5B and C) that could be seen. Nevertheless, calcium carbonate precipitation has already started between organic layers in some parts of the forming chamber (Figure ~~5~~4D). Upon closer observation, these were revealed to consist of needle-like structures that covered the surface of the POS, close to the previously formed chamber. These needle-like structures were confirmed to be crystals of calcium carbonate precipitating vertically between the OOL and the POS by EDS observation (Figure ~~6~~7B). Therefore, the precipitation does not start at the same time across the entire chamber, but instead begins locally right after the completion of organic layer construction. At this point, there are still small gaps between independent crystals. It can also be noted that the framework structure formed by pseudopodia appears to have a certain directionality in growth.

Numerous, rather regularly spaced pores (about 1 μm) can be clearly observed on the crystalline layer (Figure ~~4~~5A). In the part where OOL was curled up to reveal the inner side (see Figure 3B), convex structures corresponding to pore lining were seen (Figure ~~5D~~4D). This has been termed 'pore funnel' by Hottinger (2006), which we adopt here. Interestingly, pores cannot be seen at this stage from the outer side on the OOL with SEM observation (Figure ~~5A~~4A-B), and the OOL appears entirely smooth in the parts where the framework has been filled completely. We interpret this as due to a layer of cytoplasmic material also fills the pore lining (i.e., the 'well') during chamber formation, which regresses after the completion of chamber formation (and therefore becomes visible under SEM). As discussed previously, however, pores can still be seen during chamber formation using light microscopy due to the semi-transparent nature of the organic layers as well as the thin calcium carbonate layer. Algal cysts including *Dunaliella* individuals can be seen overlaying the OOL (Figure ~~5A~~4A). The OOL is a continuous structure that envelopes the entire test, and it extends to the newly forming chamber from the aperture of the previously formed chamber. In some parts where calcium carbonate precipitation has not yet taken place, the outer surface of the POS can be seen (Figure ~~5A~~4A and ~~5C~~4C) and like in the initial stage, many frustoconical structures about 1 μm in width are seen (Figure ~~5C~~4C). Spherical structures ~~Vesicles~~ (blue coloured in Figure ~~5B~~4B-C), about 50 nm – 500 nm in size, could be seen on both the OOL and the POS as in the initial stage~~period~~.

Late Stage

In this final stage (Figure ~~6~~5), the construction of organic layers has been fully completed, and a layer of calcium carbonate began precipitation across the entire forming chamber. The OOL is therefore seen as uniformly smooth and without gaps from the outer side (Figure ~~6B~~5B). Cross-section through the forming chamber wall at the late stage clearly shows three completed layers (corresponding to the IOL, the POS, and the OOL respectively from the inner ~~to~~side outwards in that order) and two layers of precipitating calcium carbonate sandwiched between the IOL and the POS as well as between the POS and

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the OOL (Figure ~~6C~~5C). EDS analyses obtaining signals of Ca and C, O simultaneously (Figure ~~7C~~6C) clearly indicated high Ca signal distribution being detected these two layers, showing that these layers are calcium carbonate in nature.

The precipitation of calcium carbonate crystals, continuing from the middle stage, leads to carbonate crystals to become increasingly densely packed, with gaps between crystals completely disappearing by the end of the late stage (which marks the end of chamber formation). In the figured specimen observed in Figure ~~5~~4, the thickness of the calcium carbonate layer is about 1 μm between the OOL and the POS, and about 0.3 μm between the POS and the IOL. ~~OOL had toward the inner side (Figure 65D).~~

As in the middle stage the exterior of the OOL appears smooth (i.e., pores cannot be seen yet) (Figure ~~5D~~4D). The IOL, however, when seen from the cytoplasm side, is seen to be covered by regular depressions that corresponds to the convex side of the pore plate on the POS (which may be named the 'inner pore') (Figure ~~6D~~5D). The IOL can therefore be considered to have the same shape as the POS. Spherical structures~~Vesicle-like structures~~ could also be observed in the late stage on the surface of the OOL but the size of these structures was more variable than in the earlier stages, ranging from 50 nm to 1 μm (Figure ~~5C~~4C-D). Furthermore, similar structures could also be observed on the IOL (not shown).

4 Discussion

4.1 The Weaving of Organic Layers During Chamber Formation

This study is the first to observe the detailed making of organic layer during chamber formation, and revealed that the layers are actually woven by pseudopodial activity. A schematic diagram is presented in Figure 7, which outlines the general observations. The basis of organic layer formation is the interweaving of a pseudopodial framework (Figure 7A), the interspaces of which is then filled in with a further layer of pseudopodial material, resulting in a complete organic layer. The pseudopodia are observed to form a dense framework (purple dotted lines in Figures 2A, 3 and 7), which is then overlaid by a layer of membranous pseudopodia which fills the interspaces (Figure 3E). In the OOL, numerous spaces of 100 nm – 1 μm can be seen (gray in Figures 3, 5, and 7), which represents the interspaces between the framework which is yet to be filled. In some instances, the membranous pseudopodia were observed during the process of filling the interspaces, sometimes from more than one direction (e.g., Figure 3E), by a gradual, webbed expansion. ~~Initially~~In short, a framework is constructed by a pseudopodial network, which is then overlaid and the interspaces filled in by a layer of membranous pseudopodia. According to a recent study (Nagai et al., 2018), the three sheets (OOL, POS and IOL) appears initially to be independent even at the very early stage of chamber formation during the total thickness of the whole organic sheet being less than 1 μm. We expect these organic sheets themselves are ultimately expanded from single root, but separate branches of pseudopodia are forming each organic sheet. ~~even the very early stage of chamber formation during the total thickness of the whole organic sheet was less than 1 μm. We expect these organic sheets themselves are ultimately expanded from single root, but separate branches of pseudopodia are forming each organic sheet. but all sheets actually converge at the pore plate,~~

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~~indicating they probably ultimately expand from a single root. This, however, warrants confirmation by higher resolution investigation of the very start of the initial stage in future studies.~~

The importance of organic layers in the early stages of chamber formation has been speculated in ~~the~~ previous studies~~y~~, but little was known about its origin. It was previously thought that the organic layer was secreted from the pseudopodia (e.g., Angell, 1967; Röttiger, 1974; Hemleben *et al.*, 1986), and Spindler and Röttiger (1973) reported that the organic layer seems to be connected with pseudopodia. These studies were largely limited in that their magnification (only light microscopy was available then) was not sufficient resolution to observe the detailed process. The process documented herein provides evidence for an entirely novel model in that the pseudopodia itself weaves the organic layers (Figure 3C-E, Supplementary Video)– in other words the organic layer is ~~the~~ part of cytoplasm.

The reason why the space between IOL and POS is narrower than between OOL and POS (meaning the inner calcareous layer is thinner than the outer) is presumably caused by the difference of growth rate of calcareous material between the inner side and the outer side. Assuming that the materials for chamber formation are transported from the seawater, it can be presumed that the inner side will become thinner because the chamber wall is formed and material transportation is more restricted in the inner side.

4.2 Pore Formation

Fine-scale observations from the present study allowed us to reconstruct the actual steps in pore formation. As shown already in previous studies (Bé *et al.*, 1979; Spero, 1988), the structure known as 'pore' in foraminifera is actually a composite structure formed by two opposing wells converging at the POS, one opening towards the outer side located on the OOL and one opening towards the cytoplasm side located on the POS (and same on the IOL). The POS/IOL well has been called the pore plate in previous studies (e.g., Haynes, 1981). These pore plates can also be seen on the organic layer template when fossil foraminiferal tests are dissolved (Bannar *et al.*, 1973; Banner and Williams, 1973; Hottinger and Dreher, 1974; Cad~~e~~re *et al.*, 2003; Ní~~Ní~~ Fhlaithearta *et al.*, 2013). Pore plates seems to be dented at the IOL side, from a previous study (see Fig. 3C in Nagai et al. 2018). Therefore, the pores are not actually pass-through structures formed at once but are instead formed in unison by separate processes on the OOL and the IOL. Pores have been suggested to be used for respiration (e.g., Berthold, 1976; Leutenegger & Hansen, 1979). As O_2 and CO_2 used in respiration are nonpolar molecules, they are able to pass through the cell membrane. As such, the existence of pore plates made from cytoplasm seen in the present study should not influence respiration. Our observations show that in the initial stage of chamber formation, the pore plate (visible as frustoconical structures of about 1 μm) is already present when the POS is woven, at the growth front. Pore funnels, about 0.5 μm in size, that pair up with the pore plate in the same location (but open to the opposite direction) are formed on the OOL. This structure and the pore plate collectively form the pore, and there is no space between the two for calcium

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carbonate to precipitate, and therefore the pore is not calcified.~~-~~ Pore plates and pore funnels smoothly peeled off from one another (Fig. 3B and 7A), suggesting that pore plates and pore funnels belong to independent organic sheets formed from separated pseudopodia. Pore plates and funnels were gently adhered to each other before calcification started. It is thought that pore plates and pore funnels are formed simultaneously face to face, during the organic sheet formation. Pore plates and

5 pore funnels likely function as anchors that hold together all three sheets to result ultimately in a smooth calcareous wall.~~Pore plates and pore funnels are smoothly peeled off each other (Fig. 3B and 7A). It is suggesting that pore plates and pore funnels are belonging to independent organic sheets formed from separated pseudopodia. Pore plates and funnels are gently stuck each other before calcification started. It is thought that pore plate and pore funnel are formed face to face during the organic sheet forming and it would be functioning as anchoring the all three sheet at numerous points of the newly~~

10 ~~forming chamber to arrange the calcareous wall smooth.~~ All hyaline foraminifera that have been observed in detail possess pores. Since pores are not pass-through and formed as the framework for organic layer (i.e., OOL, POS, and IOL) formation is woven and that the layers are somewhat flexible before calcification, one possible speculative function for pores is to serve as a connective structure between OOL and IOL. In this scenario, the pores 'staple' the organic layers of the forming chamber together, so that the sites of calcification maintain a consistent thickness and form throughout the chamber while

15 calcification occurs.

4.3 ~~Vesicles~~Spherical structures

The existence of ~~vesicles~~ spherical structures on the surface of organic layers have been reported in previous studies (Angell, 1967; Spero, 1988), but their function and significance have not been mentioned. A recent study (Nagai *et al.*, 2018) that utilized Focused Ion Beam (FIB) technology to process SEM samples in order to visualize calcium carbonate and organic

20 layers on the same semi-thin section. They were able to observe the presence of ~~vesicles~~ spherical structures in the site of calcification, and that they might be responsible for exo- and endocytosis. The ~~vesicles~~ spherical structures increase the surface area and probably serve to improve the material exchange efficiency, by increasing the contact surface area with seawater. In the present study, we could observe numerous ~~vesicles~~ spherical structures on all three organic layers, including the OOL, the POS, and the IOL. This indicates that the ~~vesicles~~ spherical structures probably play important roles in material

25 exchange during calcification for both the outer and inner calcified layers, and as the ~~vesicles~~ spherical structures are inferred to result from the activity of the organic layers this further strengthens the active role of these layers in calcification (i.e., they are not mere templates). The number of spherical structures increase~~s~~d as the chamber formation progresse~~s~~d. There is a variation ~~of~~in the size of ~~the~~ spherical structure ~~of~~from ca. 50 nm - 1 μm. There are ~~many~~relatively more small~~er~~ ones at the Initial stage, and relatively ~~many ones~~more large~~are larger~~ ones at the Late stage, but all sizes of the spherical structures ~~can~~

30 ~~be~~are found ~~-~~across all ~~t any~~stages.

4.4 Prospects for Calcification Model

Until now, the exact process of calcium carbonate precipitation, in terms of how precipitation was related to the degree of isolation of the site of calcification, remained largely unclear (Erez, 2003; De Nooijer *et al.*, 2014). In the present study, the sequence of events during calcification was made clear by time-series observations, and importantly both the formation of organic layer and calcium carbonate precipitation were observed together. It is significant that during the Middle Stage, although the overall shape of the forming chamber has already been formed by framework-like pseudopodia, the precipitation of calcium carbonate was seen to initially start before the framework pseudopodia have been fully covered and filled by membranous pseudopodia. The organic layers (especially well-observed in the OOL and the POS) still contained numerous gap <1 μm in size, which we interpret to maintain the exchangeability of seawater and elements contained within, for the initial part of calcium carbonate precipitation. The site of calcification is therefore interpreted to be still open in the Middle Stage. In the Late Stage, however, the organic layers have been completely filled by membranous pseudopodia and no such gaps remain. At this stage, therefore, the site of calcification is closed from the surrounding seawater. Hence, we interpret that during the Late Stage the elements require for calcification must be selectively taken up by biological means such as exo-endocytosis or ion pumps through the OOL. Although we could not observe the IOL in detail (due to its position below the POS) during this process, the IOL most likely receives the required elements through pseudopodial transport during the Late Stage, although whether this originate directly from the forming chamber or the previous chambers cannot be ascertained yet. Previous evidences (e.g., Toyofuku *et al.*, 2008; De Nooijer *et al.*, 2009) appear to suggest that calcium and carbonate are transferred from the cellular material inside the previously formed chamber. The elemental composition of the inner calcified layer formed between the IOL and the POS is probably more closed and strongly affected by cellular processes than the outer calcified layer between the OOL and the POS. Therefore, the magnesium contents of the inner layer may differ from the outer layer with pure calcite may be precipitated at the inner side. The POS has been widely considered to be the only template for calcification (Hemleben *et al.*, 1986), but recent research has revealed that calcium carbonate precipitation also occurs on the other organic layers (Nagai *et al.*, 2018). It was also shown that the POS gradually becomes obsolete as the chamber matures towards completion of thickening. Therefore, the true role played by the POS during calcification should be reconsidered. A likely function of the POS is that by doubling the surface area on which precipitation occurs, the existence of the POS doubles the rate of chamber formation. Considering that the mobility of foraminifera is highly limited during chamber formation, increasing the efficiency of chamber formation is probably beneficial and adaptive to the foraminifera.

It is well known that the chemical and isotopic compositions of calcareous foraminifera tests differ significantly from those precipitated inorganically, and the compositions also differ among different species. This effect is collectively known as the 'vital effect' (Urey *et al.*, 1951), and has been a great hindrance to the use of foraminifera tests as geochemical proxies, for example to reconstruct palaeoclimates. In attempt to explain the vital effect, Nehrke *et al.* (2013) proposed a transmembrane

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transfer / passive transfer (TMT/PT) model by observing Mg/Ca ratio during calcification, assuming that low ratio indicates active transport (i.e., transmembrane transfer *sensu* Nehrke *et al.*, 2013) and high ratio indicates passive transport; as Mg is discriminate against in Ca channels in active transport. Their observations indicated that that passive transport predominates at the early period of calcification, with active transport becoming dominant at later periods. This is consistent with the results outlined above from our observations during the present study, but we were able to reveal the reasons behind the differences in Mg/Ca ratios in early and later periods of calcification, which is that during the Middle Stage the site of calcification has not yet been fully enclosed. This is a key piece of finding as to what actually causes the vital effect, in that the construction process of the organic layers can significantly influence when the site of calcification becomes isolated, leading to differences in chemical and isotopic compositions of the test by the proportion of contributions from passive vs active transport. The elemental analysis of fluid at the site of calcification, however, is still currently unmeasurable due to technical limitations. Nevertheless, because magnesium ion is an inhibitor of calcification, it can be speculated that during biomineralization magnesium ions are actively discriminated and removed from the fluid at the site of calcification (Zeebe and Sanyal, 2002). Therefore, it is presumed that calcite with low Mg/Ca precipitates even around the POS. It is reported in many species that the foraminiferal Mg/Ca is high around the POS, but it is still much lower than Mg/Ca estimated from inorganic precipitation experiments (D~~d~~e Nooijer et al., 2014). The elemental partitioning in foraminiferal tests must be strongly controlled through the elemental composition of the fluid in the SOC, which is a key subject for future studies.~~Unfortunately the elemental analysis of fluid of site of calcification is still unable to measure due to technical limitation. However, because magnesium ion is an inhibitor of calcification, it is speculated that biology actively discriminate and remove magnesium ion from fluid (Zeebe and Sanyal, 2002). Therefore, it is presumed that calcite with low Mg/Ca precipitates even around POS. It is reported in many species that the foraminiferal Mg/Ca is high around POS, but it is still enough lower than Mg/Ca estimated from inorganic precipitation experiments (de Nooijer et al., 2014).~~

5 Conclusion

Calcareous foraminifera are a highly important group in palaeoclimate reconstruction and as indication fossils, by using their chemical and isotopic composition as a geochemical proxy. A major problem was that such compositions differed greatly from inorganic calcium carbonate under the same environment. The key finding of the present study is that one main contributor to this 'vital effect' is in fact the proportion of contributions from passive vs active transport in material transfer during calcification, which is directly linked to how the three major organic layers (i.e., the OOL, the POS, and the IOL) are constructed. For the first time, this study revealed that the organic layers are in fact woven by a framework-like pseudopodia network that are then overlaid by an overlaying layer of membranous pseudopodia, closing the gaps in the framework and thus forming a complete organic layer. We show that calcification has already started when the site of calcification is still able to passively exchange elements (e.g., Mg) with seawater; but the majority of wall thickening occurs when it is completely isolated and the only means of element exchange is through active transport. This agrees with and explains the

differences in Mg/Ca ratios in early and later periods of calcification observed in previous studies (e.g., Nehrke *et al.*, 2013). As such, we resolved a key 'missing piece' in understanding foraminiferal calcification that has mystified us for more than a decade. This study exemplifies the importance of extensive rearing and in-depth observations of a living species in order to correctly use biominerals as a geochemical proxy.

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Author Contributions

Scientific conception and experimental design: YN and TT. Data acquisition and analysis: YN, TT and KU. Data processing: YN. Data interpretation: YN, TT, CC, JT. Manuscript writing and editing: YN, TT, CC, KU, RW and JT. YN and TT contributed equally to this work.

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Competing interests

The authors declare that they have no conflict of interest.

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Figure 1: Time series observation of chamber formation by optical microscopy, as seen in the individual observed on December 7th, 2017 (see Table 1). The initial stage of chamber formation, where the organic framework is built, depicted by A-B. A: Beginning of chamber formation, defined as 0 minute from the start, indicated by a dense radiating spray of pseudopodial network. B: 159 minutes, when an aggregation of cytoplasm becomes visible around the aperture of the last existing chamber. As this cytoplasm expands, the pseudopodial network starts to retract to the surface of the new chamber to complete the framework. The middle stage, where the organic framework is prepared for calcium carbonate precipitation which begins at near the end of this stage, takes place between 15 minutes to 60 minutes, as depicted by C-D. C: 3027 minutes, cytoplasm concentrates and outline of newly forming chamber wall now clearly visible, pseudopodia still just visible on the surface. D: 6041 minutes, pseudopodial retracts inside the forming chamber wall. Open triangles indicate pseudopodia inside the newly forming chamber. Left: optical microscopy image. Right: the same image with schematic overlay; colour legend: deep purple = pseudopodia; light purple = cytoplasm; magenta = calcium carbonate in the newly forming chamber; yellow = previously formed chambers.

Figure 2: Time series observation of chamber formation by optical microscopy (continued), as seen in the individual observed on December 7th, 2017 (see Table 1). The late stage of chamber formation, where calcium carbonate is extensively precipitated and chamber wall is thickened, taking place from around 60 minutes after the start of chamber formation (total time varies considerably among individuals). A: 10065 minutes, pseudopodia expands again to form a dense network but in thicker strands than seen in previous stages. B: 10050 minutes, a network of pseudopodia is seen in the new chamber, the chamber wall of which

thickens. C: ~~201~~204 minutes, chamber wall thickening continues. D: ~~400~~180 minutes, chamber wall thickening is nearly and the pseudopodial network begin to disappear, indicating that the end of the chamber formation process is near (actual completion was at 248 minutes for this individual). ~~Open triangles indicate pseudopodia inside the newly forming chamber.~~ Left: optical microscopy image. Right: the same image with schematic overlay; colour legend: deep purple = pseudopodia; light purple = cytoplasm; magenta = calcium carbonate in the newly forming chamber; yellow = previously formed chambers.

Figure ~~4~~3: Microstructures during the initial stage of the chamber formation shown by SEM images on the left, supplemented by schematic explanation on the right. A: Overview of a specimen showing the OOL covering both the newly forming and older chambers. B–C: Magnified images showing the OOL and the POS. D–E: Magnified image of the POS construction front showing the weaving action of pseudopodia. E: The same POS construction front showing the membranous pseudopodia extending so as to close a large hole (white arrow). Colour legend: brown = OOL; orange = POS; purple = pseudopodia/cytoplasm; light green = pore funnel on the OOL; green = pore plate; blue = ~~vesicles~~spherical structures; gray =gap. Thick lines indicate membranous pseudopodia and dotted lines indicate framework pseudopodia.

Figure ~~5~~4: Microstructures during the middle stage of the chamber formation shown by SEM images on the left, supplemented by schematic explanation on the right. A: Overview of the ventral side of a specimen, showing the cytoplasm covering the newly forming chamber. B: Magnified image showing the OOL on the suture, between the new chamber and the previous chamber. C: A higher magnification image of the POS showing spherical structures on the POS. D: Image showing the matching relationship between convex structures on the cytoplasmic surface of the OOL and the pore. E: Needle-like crystals of calcium carbonate between IOL and OOL, with white arrowheads indicating clear gaps between the crystals. Colour legend: brown = OOL; orange = POS; purple = pseudopodia/cytoplasm; light green = pore funnel on the OOL; green = pore plate; magenta = calcium carbonate; blue = ~~vesicles~~spherical structures; gray =gap. Thick lines indicate membranous pseudopodia and dotted lines indicate framework pseudopodia.

Figure ~~6~~5: Microstructures during the late stage of the chamber formation shown by SEM images on the left, supplemented by schematic explanation on the right. A: Overview of the dorsal side of a specimen, with the newly forming chamber on the bottom. B: Magnified image of the OOL seen from the outside. C: Image showing a cross-section through the forming chamber wall. D: A magnification of the IOL seen from the inner side, showing pores and lots of spherical structures. Colour legend: brown = OOL/IOL; orange = POS; purple = pseudopodia/cytoplasm; light green = pore funnel on the OOL; green = pore plate; magenta = calcium carbonate; blue = ~~vesicles~~spherical structures.

Figure ~~6~~7: Elemental maps of cross-sections through the forming chamber wall at different stages, shown by SEM-EDS analyses. A: Initial stage. B: Middle stage. C: Late stage. White lines indicate the position of the POS. The false color maps indicate the intensity of calcium signals, corresponding to the legend shown on the bottom. Thick lines indicate membranous pseudopodia and dotted lines indicate framework pseudopodia.

Figure ~~3~~7: Schematic illustrations of chamber formation. A: Construction of organic layers by pseudopodial weaving and subsequent gap-filling. B: The entire chamber formation process from the initial stage on the left side to the late stage on the right side. Colour legend: brown = OOL /IOL; orange =POS, purple = pseudopodia/cytoplasm; light green = pore funnel on the OOL; green = pore plate; magenta = calcium carbonate (see Figure 5E); blue = ~~vesicles~~sperical structures; gray =gap.

Supplementary Video: Time-lapse observation of the entire chamber formation process an *Ammonia beccarii* individual, taken on December 7th, 2017. This is the same individual used for Figures 1-2.

Supplementary Figures: Original high-resolution photographs used in Figures 1-2.

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