Dear Dr. Chan,

Your manuscript JoVE55164 "Characterization of calcification events using live optical and electron microscopy techniques in the marine tubeworm" has been peer-reviewed and the following comments need to be addressed. Please keep our formatting requirements in mind while revising the manuscript to address peer review comments. Please maintain the overall manuscript changes, e.g., if formatting changes were made, commercial language was removed, etc.

Please track the changes to identify all of the manuscript edits. After revising the submission, please also upload a separate document that addresses each of the editorial and peer review comments individually with the revised manuscript. For each comment, please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed or out of the scope of this work for publication in JoVE.

Your revision is due by Nov 23, 2016.

Please note that due to the high volume of JoVE submissions, failure to meet this deadline will result in publication delays. To submit a revision, go to the JoVE Submission Site and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Sincerely,

Patrick Lansing,

Peer Review Manager

JoVE

617-401-7717

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Editorial comments:

The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55164\_R0\_072816.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please abbreviate all journal titles.

3. Please define all abbreviations before use.

Reply: Yes we have taken actions to respond to points 1-3

4. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution’s human research ethics committee.

Reply: Our work involves a marine invertebrate which lacks a vertebrate, hence, is exempted from research ethics concerns.

5. Please specify all microscope parameters: what zoom, aperture, etc.

Details on relevant microscope parameters were added to the protocol .

6. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

The figure legends were revised to show only the data presented.

Changes to be made by the Author(s) regarding the video:

Video file issues

 • The video file name should contain the article ID number (55164). The wrong ID number was on the file for this submission, which caused some confusion.

The video file has been renamed accordingly

Graphics/screen capture issues

 • 5:22-6:10 - When the video is scaled to our webplayer's size, the text in the screen capture is impossible to read. If seeing details in these sections is important for the viewer, areas of interest should be zoomed in on, so that text is legible.

Editing issues

 • 6:56-7:00 - The use of fast motion seems out of place here. I would recommend using the footage at standard speed and letting there be more of a pause between steps.

Audio issues

 • 10:38 - It sounds like there is an audio glitch here that should be fixed.

 • 15:28-15:32 - There is a bit of dialogue that was caught and is looping during this time.

Text/formatting issues

 • In addition to the production credits at the end of the video, there should be a title card listing the authors and their affiliations.

 All of the above suggestions have been accepted in the new video.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is an excellent contribution.

Major Concerns:

I don't have any major concerns.

Minor Concerns:

I don't have any minor concerns either.

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

This is a very elegant manuscript showing the use of several microscopy methods to observe calcification of an important fouling serpulid polychaete.

Major Concerns:

None

Minor Concerns:

None

Additional Comments to Authors:

N/A

Reviewer #3:

Manuscript Summary:

The authors provide a guide to using a range of advanced microscopy techniques to study the early stages of biomineralisation, which are applied to the study of a marine tubeworm, but should be fairly generally applicable across a range of sample types.

Major Concerns:

N/A

Minor Concerns:

1) Title: change "the marine tubeworm" to "a marine tubeworm".

  The title slide has been changed

2) The species name (Hydroides elegans) of the tube worm being used should be given early in the manuscript, ideally in the abstract, or even in the title. Currently it is first encountered in the figure legends.  
Yes, we have added the species name to both the short and long abstract and last paragraph of the introduction.

3) Spell out acronyms in full when they are first used. For example, in the Long Abstract expand "SEM-EDS" and "SEM-EBSD, just as has been already done for 'TEM' and 'FIB'.

The full names for SEM-EDS and SEM-EBSD are energy-dispersive X-ray spectroscopy (EDS) and electron backscatter diffraction (EBSD), they are now spelt out in the long abstract.

4) Long Abstract: change "In this protocol, Focused Ion Beam (FIB) is used to fabricate typical dimensions of sample suitable for TEM analysis" to, "In this protocol, Focused Ion Beam (FIB) is used to isolate samples with dimensions suitable for TEM analysis".  
This is a nice sentence to illustrate the application of FIB, the change is made accordingly.

5) Introduction, lines77-79: change the ending of the sentence to something like, "… hence, identifying the life stage that has an increased pH reveals the time when calcification is likely to be occurring".

Thank you for the excellent suggestion, the changes are made.

6) Check the formatting of the units. In my copy of the manuscript pdf some of the "micro" symbols appeared to be absent.

There may be file differences, in the file we do not see such a problem.

7) The source of the tubeworms should be given as well as their culture or maintenance conditions. The method by which the authors obtain larvae should also be given. All of this information should be the first protocol provided, before describing how these larvae can be stained and observed.  
These larvae were cultured and maintained from a dedicated marine facility in Hong Kong at Dr. Rajan's lab. Previously described methods and their citations have been added to the first line of the protocol to for interested reader to look up specific details regarding larval culture. (line 101)

 8) What is the recipe (or supplier details) for the artificial seawater?  
The artificial seawater was Rei-sea Marine by Iwaki Co. Ltd. This information was provided in the excel spread sheet.

9) Line 187: How long are the larvae fixed with 4%paraformaldehyde for?  
They were kept in the fixative for about 1 week to enable transportation from laboratory in University of Hong Kong to Clemson University.   
  
This information about fixation is added to line 190.

10) Line 197: spell-out "HMDS".  
The full name Hexamethyldisilazane has been added.

11) Line 248: change "fills up" to "filling up".  
Changes were made.

12) Line 253: the wording of the first part of this line does not seem to make sense. Is a word missing?  
"To obtain point-quantification data, change the process time to 6 and adjust the electron probe to acquire a dead time of ~30%. Run alignment steps and optimize the image as necessary."

The line has been corrected, see line 258 for updated version

13) Line 317: is "0.04 in" giving a distance in inches? This should be changed to a decimal measurement if so, in order to be consistent with elsewhere in the manuscript.  
I agree, the use of inch is inconsistent. However, it has been the unit as displayed in the video (display was showing 0.04). We decide to write in 1mm, but keep 0.04 inches in a bracket.

14) Line 345 uses "1kx" but line 366 uses "1000x". I presume that this represents the same magnification(?). Be consistent in what is used. "1000x" is more conventional. See also line 377. And "5kx" on line 421. And "10kx" on line 440.  
Thank you for spotting the inconsistency, we have discarded the use of k, and added 000 to the values.

15) In Fig.1 indicate on the picture exactly where the collar region is, to help readers not familiar with tubeworm anatomy.

Yes, the following anatomical labels are added to the picture. c, collar; b, branchial lobes; ant, anterior; post, posterior

16) Figure legends: Fig.2, please clarify what precisely is being observed in Fig.2C. The yellow Ca signals are distributed over a dispersed area of the worm and are not tightly associated with the collar region. Does this represent dispersed pockets of calcification happening in the mucus tube scaffold that tends to be deposited first? Or are these signals really localised within tissues of the animal?  
Unfortunately, this dataset is limited to spatial description only as not enough temporal information is available.  
This is an interesting question. Since most of the tube materials in the later stage of calcification (Fig. 2b) are around the collar region, we investigated the collar region with FIB-TEM to demonstrate the involvement of these tissues in Ca deposition. They appeared to be within the tissue of the animal because the specimen has gone through washing and serial dehydration steps. Ca deposition over the tail region is actually consistent to a study in 1973, tail and the collar regions were two types of tissues that produce CaCO3 with different mechanisms. This is an interesting topic for further research, but is too much beyond the scope of the current demonstration of methods.   
 **A brief description of this observation was added to th.**

17) Fig. 3 legend: the panels are now labelled with Roman numerals, in contrast to to use of letters in Fig.2. Be consistent. Presumably throughout the Fig.3 legend the authors mean things like "3i" and "3ii" rather than "2i" and "2ii"?

The figure labelling systems are now standardized, and the errors in the legend of Fig.3 have been fixed.

Additional Comments to Authors:

N/A