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Dr. Bing Wu

Review Editor

Journal of Visualized Experiments

Ph.: (617) 674-1888

Email: bing.wu@jove.com

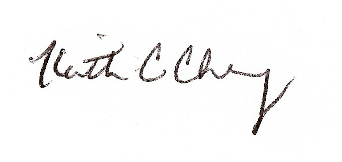
Dear Editor,

Attached is the requested revision of our original submission, “Rigid Embedding of Fixed and Stained, Whole, Millimeter-Scale Specimens for Section-free Imaging by Micro-CT” by Alex Lin et al. (ms. JoVE58293).

We thank the reviewers for their thoughtful reviews and constructive suggestions that have resulted in an improved manuscript. We address comments made by the editor and for each reviewer in a point-by-point manner.

We trust that the updated manuscript is now of sufficient quality and rigor to be suitable for publication in JoVE. Feel free to contact us as needed. Thank you for your consideration.

Sincerely,



Keith C. Cheng

Distinguished Professor of Pathology and Director, Division of Experimental Pathology

Department of Biochemistry & Molecular Biology

Curator, Zebrafish Atlas of Microanatomy (now renamed Bio-Atlas)

Founding Director, Penn State Zebrafish Functional Genomics Core

**Editorial comments**

**Editor comment 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.

Response 1: Done.

**Editor comment 2.** Please revise title, summary, and abstract to avoid previously published text.

Response 2: Done.

**Editor comment 3.** Figures: Please remove the titles and Figure Legends from the uploaded figures and include all the Figure Legends together at the end of the Representative Results in the manuscript text.

Response 3: Titles and Figure Legends have been removed from uploaded figures and inserted at the end of the Representative Results section of the manuscript.

**Editor comment 4.** Please provide an email address for each author.

Response 4: Done.

**Editor comment 5.** Abstract: Please include a statement about the purpose of the method.

Response 5: The text has been revised to clearly state the purpose of the method.

**Editor comment 6.** Introduction: Please rephrase to include a clear statement of the overall goal of this method.

Response 6: We have revised the text to explicitly state the overall goal of the method.

**Editor comment 7.** Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Response 7: An ethics statement is now included.

**Editor comment 8.** 3.1: Is 1:1 the mass ratio or volume ratio? Please specify.

Response 8: We apologize for this error. It is a 1:1 volume ratio, as is now specified in the text.

**Editor comment 9.** 4.3.1: Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response 9: The protocol text has been revised to remove personal pronouns.

**Editor comment 10.** There is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response 10: The essential steps of the protocol for the video have now been highlighted in yellow, totaling less than 2.75 pages.

**Editor comment 11.** Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Response 11: The filmable content has been highlighted and edited accordingly.

**Editor comment 12.** Line 272: Please change “ml” to “mL”.

Response 12: Correction has been made to the text.

**Reviewer #1 Comments**

This manuscript entitled "Rigid embedding of fixed and stained, whole, millimeter-scale specimens for section-free imaging by Micro-CT" presents a clever way to embed biological specimens for 3D imaging. The developed embedding apparatus is tricky and the adapter is well designed. An optimized sample preparation (i.e. ensuring sample immobility and good contrast) represents a crucial step to obtain high-quality 3D image. This protocol works well for relatively large and robust specimen. The use of this apparatus appears more delicate for very fragile and small sample as the suction is used to transfer the specimen into the tube and as the inner diameter of the polyimide tubing is only slighter larger than the width of the specimen.

**Reviewer 1 Comment 1.** My first major concern is about the spatial resolution. Images shown as examples can be used to study observable traits and their variations, to perform morphology and volumetric analyses of tissues, as mentioned by the authors. But I wonder if it is possible to go until cellular resolution with such spatial resolution. What the authors precisely mean with the term "cell"?

R1, Response 1: Cell resolution is defined as the ability to resolve individual cells in the reconstructed image files. Cell nuclei are the most prominent organelle in a cell and typically have diameters in the range of ~2-10 microns; therefore, individual cells can be distinguished by identifying cell nuclei. We reference several studies which show that micro-CT can be used to resolve specimens at sub-micron voxel resolution. We have revised the text.

We would like to emphasize that the focus of our method paper is not imaging, *per se*, rather on sample preparation - specifically sample embedding. We present micro-CT scans of *Danio*, *Daphnia*, and *Drosophila* rendered in such a way to show the staining of the specimens with some contours of the acrylic. Also, we show scans of the same zebrafish imaged twice over the period of two years to show that rigidly embedded samples allow sample preservation and the ability to re-image over time without dramatic reduction to signal-to-noise.

**Reviewer 1 Comment 2.** Lines 365-368 the authors write, "re-imaged at sub-micron resolution so that cell counting is possible". It would be nice to demonstrate that cell counts for individual tissues can be generated, e.g. from zebra fish image with a sub-micron resolution (voxel size of 0,7 µm).

R1, Response 2: We thank the reviewer for the suggestion. We agree that this is an important aspect for automated image analytics and phenotyping. We are actively pursuing computational approaches for cell counting which will be presented in another manuscript.

**Reviewer 1 Comment 3.** Lines 377-379, the authors explain, "cellular resolution imaging can be performed with micro-CT" and they wish to expand the analyses to human samples. But the discussion is based on unpublished results "previous work of Thomas et al. examining mutant and diseased fish histology". Moreover, high-resolution CT scan (at sub-micron or nano resolution) can be generally performed only for very small sample. I wonder if cell resolution can be obtained for e.g. mouse embryo.

R1, Response 3: We have properly referenced the discussion. The reviewer is correct in stating that high-resolution CT is generally performed for small samples. We are not claiming that cell resolution can be obtained for a whole mouse embryo. Our inclusion of the mouse embryo is to demonstrate that our embedding protocol can be adjusted to accommodate larger samples. That being said, cell resolution can be obtained for a smaller region of interest of the mouse embryo. One feature of our embedding method is that if/when technology becomes available to scan larger fields-of-view at higher resolution, the same sample can be readily scanned.

**Reviewer 1 Comment 4.** Line 384: given reference (n°29) is for unstained tissue scanned by nano-CT. I also wonder if this sample preparation is adapted to image unstained tissue. Did the authors perform test on unstained sample? In the literature, you can find recent studies on unstained tissue (e.g. Walton et al., 2014, Scientific Reports; Chaurand et al., 2018, Scientific Reports; …).

R1, Response 4: We thank the reviewer for bringing these studies to our attention, but the type of imaging done in the referenced articles was not tested in our embedded samples; we therefore cannot comment on the relevance of our embedding method for unstained tissue scanning. However, the success of embedding is not affected by staining. We have embedded unstained samples with our method to image bony structures of a juvenile zebrafish, but without the use of the type of phase contrast described in the cited studies. In histology, staining is done to visualize cellular features because only faint outlines can be visualized from sections in unstained histology sections. Similarly, stain is required to achieve histology-like results for microCT.

**Reviewer 1 Comment 5.** Finally, in the introduction the presentation of methods for sample immobilization (lines 45-46 and 96-105) should be completed with drying procedures. Drying protocols following a fixation step (e.g. HDMS, critical point drying CPD) should be mentioned as they provide good quality images (see for example Zysk et al., 2012, Biomedical Optics Express or Chaurand et al., 2018, Scientific Reports). These procedures could be compared to the procedure developed by the authors (rigid embedding of fixed and stained sample).

R1, Response 5: Sample drying is practiced in electron microscopy because liquid-removal is necessary for samples to be placed in vacuum. Ethanol-based dehydration is needed in our sample preparation for the purpose of replacement by resin. In the context of handling soft tissue for histology, we have not encountered the use of CPD. The embedding procedure described would not expected to be improved by the addition of CPD.   
  
**Reviewer 1 Comment 6.** Keywords/title: for better visibility, I recommend using in the title, keywords or summary at least one time the non-abbreviated name of the imaging technique, i.e. X-ray micro-computed tomography (for micro-CT)

R1, Response 6: We have now included the non-abbreviated name of micro-CT in the keywords.  
  
**Reviewer 1 Comment 7.** Summary, line 31: "we are developing a non-destructive, isotropic, 3D imaging modality … " not exactly. Authors have developed a way for sample preparation, not the 3D imaging technique.

R1, Response 7: It is true that we do not describe that work in this paper. We were referring to ongoing work for the purpose of context. We have therefore revised the text to reflect, more accurately, our emphasis on sample preparation procedures.  
  
**Reviewer 1 Comment 8.** Line 53: "long-term preservation". This point was validated for a 2-years period, is it long enough? Also, line 282: authors write: "can be stored indefinitely", it should be nuanced.

R1, Response 8: We agree. We have therefore revised the text to add the caveat that the full time limit of sample preservation remains to be determined.  
  
**Reviewer 1 Comment 9.** Line 91: Authors can also cite Pauwels et al., 2013, Journal of Microscopy

R1, Response 9: Done.  
  
**Reviewer 1 Comment 10.** Line 94: "Ding et al." is an unpublished reference

R1, Response 10: We have removed the reference and revised the text accordingly.  
  
**Reviewer 1 Comment 11.** Lines 115-116: "excess resin must be removed to reduce imaging artifacts in micro-CT" please explain why and how resin induces imaging artifacts;

R1, Response 11: Removal of excess resin is beneficial for keeping the center of rotation close to the center of the specimen for micro-CT imaging. Bulky resin also introduces extraneous edges that may interfere with any phase-based enhancements in micro-CT imaging at the synchrotron. We have revised the text accordingly.

**Reviewer 1 Comment 12.** Protocol: some precisions are missing, Line 156: precise % vol. ? type of agitation, shaking table ?

R1, Response 12: We apologize for the lack of clarity. The protocol has been revised to include more details. The device used for agitation is now specified in the text.

**Reviewer 1 Comment 13.** Steps 4.1 and 4.2 are not well described in details. Fresh resin of 4.2 should replace resin of step 4.1 ?

R1, Response 13: We have clarified these points in the revised text.

**Reviewer 1 Comment 14.** Line 243: what do you mean by "clay" ? could you precise.

R1, Response 14: We have modified “clay” to “oil-based soft modeling clay”, whose supplier and catalog number are provided in the Table of Materials.

**Reviewer 1 Comment 15.** Step 5: authors should add some details on sample mounting for micro-CT. Is Kapton tubing removed before analyze? Moreover, a minimum of scan parameters should be detailed (only an unpublished reference is given).

R1, Response 15: We thank the reviewer for the suggestion. We have revised the text to include this information.  
  
**Reviewer 1 Comment 16.** Lines 279-280: it would be nice to also see the 3D images of mouse embryo and drosophila.

R1, Response 16: We thank the reviewer for the suggestion. Unfortunately, the embedded mouse embryo has not been imaged (see Response, Reviewer 1 Comment 3). Our inclusion of the mouse embryo is to demonstrate that our embedding protocol can be adjusted to accommodate larger samples. That being said, per the Reviewer’s suggestion, we have now included the 3-D image of the *Drosophila* specimen. We trust that this addition will add to demonstrating the versatility of our method to accommodate a variety of samples.

**Reviewer 1 Comment 17.**Line 379: "dissecting microscope" what does it mean ?

R1, Response 17: We apologize for the lack of clarity. The dissecting microscope used was a low power stereo microscope and is now properly referenced.

**Reviewer 1 Comment 18.** Reference 29 is uncompleted, year of publication?

R1, Response 18: The year of publication for this reference is now included.

**Reviewer 1 Comment 19.** Figure 2: scale bar is missing

R1, Response 19: We have added scale bars to Figure 2.

**Reviewer 1 Comment 20.** Figure 3: scale bar would be more clear than a dime picture

R1, Response 20: Dime pictures are removed and scale bars are now added to Figure 3.

**Reviewer 1 Comment 21.** Figure 4B: why the voxel size of daphnia is larger than the voxel of the zebrafish larva? However the samples have nearly the same size.

R1, Response 21: We agree that a clarification is called for. The voxel sizes are different because they were imaged with different micro-CT machines with different field-of-view and nominal resolution. We have revised the figure legend to clarify this point.

**Reviewer 1 Comment 22.** Figure 5: "comparable image quality": should be justified, how the authors estimate more quantitatively the image quality? How the authors can compare the quality of two images obtained with different scanning parameters?

R1, Response 22: We agree that a more precise and quantitative evaluation would be valuable. We have therefore updated Figure 5 to include an image analysis comparison (Relative Intensity Profile and Regional Signal-to-noise) across select regions of the registered scans of the same specimen. The basic scanning parameters were the same between both acquisitions (e.g., beam energy, sample to scintillator distance). The main point of this figure, that specimens can be re-imaged without detectable diminution of image quality, is unchanged.

**Reviewer #2 Comments**  
  
**Reviewer 2 Comment 1.** Overall, I found this manuscript well written and I believe that this protocol could be of interest and useful to other groups. The steps appear to be methodical and easy to follow but specifically I think that the use of the adaptor and the addition of the CAD image file for the adaptor is what makes this a particularly attractive and appealing method. However, removing excess resin from a block to scan for MicroCT is not a particularly difficult task so I feel that there should be some indication as to the ease of successfully using this device. What is the average success rate of not damaging your sample or not having air bubbles that can jeopardize the quality of the scan? If the adaptor proves to be able to achieve regularly reproducible successfully embedded samples then this could be a method routinely employed by a number of groups who are interested in MicroCT and other 3-dimensional high resolution imaging techniques.

R2, Response 1: Removal of excess resin is not difficult for blocks. However, we found early on that straight knife edges associated with the squaring-off of blocks cause edge artifacts in micro-tomography. Those artifacts are eliminated by making the samples cylindrical, motivating the presently described methods. In our hands, the average success rate of not damaging the sample or having air bubbles is > 95%. Air trapped during the embedding process can also be kept toward the edge of the tubing and away from the sample by tilting the tubing during the polymerization process as described in the note of step 4.9 of the protocol. Also, as discussed in *critical steps, modifications, and troubleshooting*, random formation of air is also associated with some resins, which is the reason we switched to LR White for its minimal shrinkage.

**Reviewer 2 Comment 2.** lines 45-46: The use of resin in MicroCT for sample immobilization and correlative work is already being employed by a number of groups, e.g. Handschuh, S. et al., Front. Zool 2013 and Bushong, E.A. et al., Microscopy & Microanalysis 2015, so it is inaccurate to describe the only methods of sample immobilisation relied on in previously published methods as paraffin wax, agarose or liquids. This gives the impression that resin has not previously been used, which is not the case so I feel that this should be addressed.

R2, Response 2: We appreciate the citations in which plastic embedding was used for the purpose of subsequent electron microscopy. We have revised the text to include those references.

**Reviewer 2 Comment 3.** lines 112-114: The manuscript states that resins such as EPON 812 are commonly used in electron microscopy. This is correct but LR White is also used in electron microscopy. I am a little confused as to why the manuscript specifically mentions an epoxy resin used in electron microscopy in the introduction whilst in the method proposed in this manuscript an acrylic resin is used which is also widely used in electron microscopy?

R2, Response 3: We have revised the text to more accurately represent our course of experiments that lead to the switch from an epoxy resin to an acrylic resin (LR White).

**Reviewer 2 Comment 4.** line 120: How much difference does scanning with the polyamide tubing still in place rather than removing the tubing make to the scan? Is it not beneficial to remove the tubing prior to scanning? If there is any difference does removing the tubing risk damaging the sample and that is why it is left in place?

R2, Response 4: We have chosen polyimide for its high X-ray transmittance; leaving the tubing in place has minimal effect on scanning. Removal of the tubing described in the protocol requires scoring the tubing along the side with a razor blade, which risks damaging the resin surface. Scratched, uneven resin surface can result in increased artifacts. On the other hand, polyimide tubing is dark, which is less satisfactory for photography of the specimen. We therefore remove the tubing when photography of the sample was needed. We have revised the text for clarification.

**Reviewer 2 Comment 5.** line 134: Should this be 'Neutral' rather than Normal Buffered Formalin?

R2, Response 5: The error has been corrected.

**Reviewer 2 Comment 6.** line 146: There is much debate regarding the benefits of reagents being chilled or used at room temperature. Unless I have misunderstood it, the work by J.E. Copper et al., Comparative Biochemistry & Physiology Part C: Toxicology & Pharmacy 2018 suggests that room temperature is better at preserving structure so the statement that chilled reagents minimize degradation needs to be validated.

R2, Response 6: The recent manuscript by J.E. Copper *et al*. suggests that room temperature is better at preserving structure. We agree that there is debate regarding chilled or room temperature reagents. Importantly, our choice of chilled reagents (i.e., NBF and Tricaine-S) was also to ensure a more rapid and humane euthanasia. Notably, our pre-chilled reagents were left to equilibrate to room temperature and the overnight fixation was performed at room temperature as stated in 1.6. We have revised the text for clarification.

**Reviewer 2 Comment 7.** line 326: Is there a reference to support the statement JB4 shrinks ~6-8%? If not, where have these figures been obtained from?

R2, Response 7: We were informed of shrinkage of the JB4 Plus resin through communications with the supplier. Furthermore, JB4 Plus is a glycol methacrylate-based resin and volumetric shrinkage of methacrylates have been documented (Pate, M.P. *et al*. *Biomaterials* 1987, Fróes-Salgado *et al*. Journal of Applied Polymer Sciences 2012). We are however unaware of published data on polymerization shrinkage of the JB4 Plus resin. We have revised the text accordingly.

**Reviewer 2 Comment 8.** Figure 2: From line 317 I understood that air can become trapped during the embedding process but this figure suggests that this is only the case with JB4 Plus. Can the authors please clarify and make this clearer in the figure if that is the case? If this issue does only arise when using JB4 then the manuscript itself needs to be clearer on this.

R2, Response 8: We agree with this point. Air can become trapped during the embedding process with the use of other resins. We have revised the figure legends of Figure 2 for clarification.

**Reviewer 2 Comment 9.** Figure 3: For anyone who is not from the US, the size of a dime is not obvious. Assuming that the paper is intended for an audience also outside of the US it would be far more appropriate and informative to use a ruler or scale bar to illustrate dimensions.

R2, Response 9: Dimes have been removed and scale bars have been added to Figure 3 to better illustrate dimensions.