Journal of Visualized Experiments

Rigid Embedding of Fixed and Stained, Whole, Millimeter-Scale Specimens for Section-free Imaging by Micro-CT

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58293R2
Full Title:	Rigid Embedding of Fixed and Stained, Whole, Millimeter-Scale Specimens for Section-free Imaging by Micro-CT
Keywords:	X-ray micro-computed tomography, micro-CT acrylic resin, embedding, model organisms, zebrafish, millimeter-scale, three-dimensional imaging, tissue architecture, complete morphological phenotyping, phenomics
Corresponding Author:	Alex Yu-Shun Lin, M.S. Penn State College of Medicine Hershey, PA UNITED STATES
Corresponding Author's Institution:	Penn State College of Medicine
Corresponding Author E-Mail:	aylin197@gmail.com
Order of Authors:	Alex Yu-Shun Lin, M.S.
	Yifu Ding
	Daniel J Vanselow
	Spencer R Katz
	Maksim A Yakovlev
	Darin P Clark
	David Mandrell
	Jean E Copper
	Damian B van Rossum
	Keith C Cheng
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	500 University Drive Rm7804, Hershey, PA, 17033

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Cover Letter

PENNSTATE

1855

Jake Gittlen Cancer Research Foundation

Keith C. Cheng, M.D., Ph.D.
Distinguished Professor
Department of Pathology
Department of Biochemistry
and Molecular Biology

Penn State College of Medicine
The Milton S. Hershey Medical Center
Jake Gittlen Cancer Research Foundation, H059
500 University Drive
Hershey, PA 17033-2390

Voice: (717) 531-5635
Fax: (717) 531-5634
E-mail: kcheng76@gmail.com

April 12, 2018

Dear Dr. Singh:

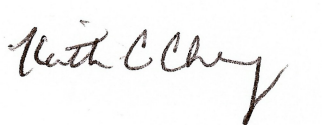
We are pleased to submit our original research article entitled “Rigid embedding of fixed and stained, whole millimeter-scale specimens for section-free imaging by micro-CT” for consideration for publication in *The Journal of Visualized Experiments*. The potential for 3-dimensional imaging using micro-CT for morphometric studies in the life sciences is huge. The current practice of sample preparation, however, is subject to potential movement during acquisition, poor alignment between imaging sessions, and is ill-suited for long-term storage. This manuscript details a straightforward protocol to generate rigidly embedded samples that are refractory to damage, ideal for permanent preservation, and suitable for re-interrogation.

In this manuscript, we present an embedding methodology that combines the use of an acrylic resin and X-ray transparent polyimide tubing to immobilize specimens and minimize optical artifacts. We show that this technique accommodates a wide variety of biological samples that can be re-imaged over years and achieve comparable image quality. The capability for permanent preservation and re-imaging leads to multiple benefits including: (1) creation of long-term repositories of samples that are difficult to generate or prepare such as those from genetic or chemical phenome projects; (2) resistance to damage; (3) ease of sample transport; (4) image re-acquisition; (5) serial imaging across multiple imaging modalities; and (6) the generation of stable standards for calibration and technology development. We believe that this manuscript is appropriate for publication by JoVE because ease of sample preparation such as described will be necessary for the propagation of the application of micro-CT imaging for biological samples, particularly for scientists with limited access to this technology.

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agree with its submission to JoVE. We have no conflicts of interest to disclose.

Thank you for your consideration!

Sincerely,



Keith Cheng, MD, PhD
Distinguished Professor and Chief, Division of Experimental Pathology
Professor of Pathology, Biochemistry & Molecular Biology, and Pharmacology
Co-Director of the Penn State IBIOS Bioinformatics and Genomics graduate program
Director, Penn State Zebrafish Functional Genomics Core

Manuscript Click here to download Manuscript 58293_R2.docx

TITLE:

Rigid Embedding of Fixed and Stained, Whole, Millimeter-Scale Specimens for Section-free Imaging by Micro-Computed Tomography

AUTHORS AND AFFILIATIONS:

Alex Y. Lin[1,2], Yifu Ding[1,2,3], Daniel J. Vanselow[1,2], Spencer R. Katz[1,2,3], Maksim A. Yakovlev[1,2], Darin P. Clark[4], David Mandrell[5], Jean E. Copper[1,2], Damian B. van Rossum[1,2], Keith C. Cheng[1,2]

[1]The Jake Gittlen Laboratories for Cancer Research, Penn State College of Medicine, Hershey, PA, USA.

[2]Division of Experimental Pathology, Department of Pathology, Penn State College of Medicine, Hershey, PA, USA.

[3]Medical Scientist Training Program, Penn State College of Medicine, Hershey, PA, USA.

[4]Center for In Vivo Microscopy, Duke University Medical Center Durham, NC, USA.

[5]KTM Research, Tualatin, OR, USA

Corresponding Author:

Keith C. Cheng

Email: kcc2@psu.edu

Tel: (717) 531-5635

KEYWORDS:

X-ray micro-computed tomography, micro-CT acrylic resin, embedding, model organisms, zebrafish, millimeter-scale, three-dimensional imaging, tissue architecture, complete morphological phenotyping, phenomics

SUMMARY:

We developed protocols and designed a custom apparatus to enable embedding of millimeter-scale specimens. We present sample preparation procedures with an emphasis on embedding in acrylic resin and polyimide tubing to achieve rigid immobilization and long-term storage of specimens for the interrogation of tissue architecture and cell morphology by micro-CT.

ABSTRACT:

For over a hundred years, the histological study of tissues has been the gold standard for medical diagnosis because histology allows all cell types in every tissue to be identified and characterized. Our laboratory is actively working to make technological advances in X-ray micro-computed tomography (micro-CT) that will bring the diagnostic power of histology to the study of full tissue volumes at cellular resolution (*i.e.*, an X-ray Histo-tomography modality). Toward this end, we have made targeted improvements to the sample preparation pipeline. One key optimization, and the focus of the present work, is a straightforward method for rigid embedding of fixed and stained millimeter-scale samples. Many of the published methods for sample immobilization and correlative micro-CT imaging rely on placing the samples in paraffin wax, agarose, or liquids such as alcohol. Our approach extends this work with custom procedures and the design of a 3-dimensional printable apparatus to embed the samples in an acrylic resin directly into polyimide tubing, which is relatively transparent to X-rays. Herein, sample preparation procedures are

described for the samples from 0.5 to 10 mm in diameter, which would be suitable for whole zebrafish larvae and juveniles, or other animals and tissue samples of similar dimensions. As proof of concept, we have embedded the specimens from *Danio*, *Drosophila*, *Daphnia*, and a mouse embryo; representative images from 3-dimensional scans for three of these samples are shown. Importantly, our methodology leads to multiple benefits including rigid immobilization, long-term preservation of laboriously-created resources, and the ability to re-interrogate samples.

INTRODUCTION:

Phenotypes are observable traits of an organism that represent the consequences of unique interactions between its genetic background and the environment. These characteristics can include behavioral, biochemical, morphological, developmental, and/or physiological properties. Importantly, differences in traits between wild-type organisms and genetic mutants can provide key insight into the mechanisms and functions of affected genes. With respect to morphology, histopathology is the gold standard for assessing phenotypes at the cellular level but suffers from mechanical artifacts and does not allow accurate quantitative volumetric analysis[1]. Our laboratory is motivated to overcome the barriers to the application of the diagnostic power of histology to full tissue volumes at submicron resolution.

A survey of available technologies suggests that imaging by X-ray micro-computed tomography (micro-CT) may provide ideal capabilities needed for millimeter-scale whole-animal 3-dimensional (3D) histology. Micro-CT enables non-destructive, isotropic, 3D visualization and the ability to conduct quantitative analysis of tissue architecture[1,2]. In recent years, 3D imaging of unstained and metal-stained zebrafish by micro-CT has gained increasing traction and has been utilized for volumetric analysis of several tissues, including muscle, teeth, bone, and adipose tissues[3-10]. Other model organisms and tissue specimens are also amenable to micro-CT imaging. For instance, a pipeline has been introduced for quantifying dense mesoscale neuroanatomy of mouse brains imaged via synchrotron micro-CT[11]. Similarly, an eosin-based staining protocol has been shown to be suitable for soft tissue micro-CT imaging of whole mouse organs[12]. Morphometric analysis of unstained human L3 vertebrae and the visualization of silver-stained human lungs using micro-CT have demonstrated the utility of this technology for human samples[13,14].

In order to actualize the great promise of micro-CT for complete morphological phenotyping of intact small animals and tissue specimens, a number of hurdles need to be overcome in relation to throughput, resolution, field-of-view, comprehensive cell staining, and long-term preservation. While each of these aspects is critically important, the focus of the present manuscript is the optimization of embedding procedures, with additional notes on sample fixation and staining. Heavy metal staining is useful because the inherent contrast between different soft tissues in micro-CT images is low. The potency of various metal stains, such as osmium tetroxide, iodine, phosphotungstic acid (PTA), and gallocyanin-chromalum, to enhance contrast for micro-CT imaging has been studied[15,16,17]. Uranyl acetate has also been used as a contrasting agent for micro-CT imaging of bone and cartilage[18,19]. PTA as used in our protocol leads to consistent staining of nearly all tissues and cells in whole zebrafish specimens, yielding

images that are potentially compatible with histology-like studies through full volumes of tissue.

During micro-CT image acquisition, a 2-dimensional (2D) projection is taken as the sample is rotated by a fraction of a degree and repeated until the sample has completed a 180° or 360° rotation, generating a series of thousands of 2D projections used for the reconstruction of the 3D volume[20]. In this process, any perturbation of the specimen will cause the corresponding 2D projections to be out of alignment, resulting in poorly reconstructed 3D volumes. Sample immobilization is a way to correct for this issue and some current strategies involve submerging the sample in alcohol or embedding in agarose in polypropylene tubes or micropipette tips[3,5,15,16,21,22]. Liquid immersion methods are not ideal because sample movement during image acquisition can occur between imaging sets, resulting in shifted reconstructions of 3D volumes[23]. Further, it is well known that the physical stability of liquid-stored tissue samples is poor in time scales of months to years, as a result of chemical instability and surface abrasion associated with movement of contact points between sample and the container wall (personal observations with fixed animal and human tissue samples).

To reduce the potential for sample movement during imaging, samples can be embedded in agarose[24-26], but this practice is associated with the risk of the stain diffusing into agarose thus reducing image contrast[26]. Furthermore, liquid or agarose preparations are prone to physical damage and degrade over time, making them unsuitable for long-term sample storage. We reasoned that a potentially successful and straightforward sample immobilization method could be adapted from that used for electron microscopy specimens. Polymerized resins such as EPON 812 (discontinued and replaced by EMbed 812) are commonly used to provide the hardness required to generate ultrathin sections for electron microscopy[27,28]. Indeed, several comparative studies between X-ray imaging and electron microscopy have demonstrated that the samples embedded in resin can be imaged by X-ray microscopy[29,30]. However, some of the standard practices of electron microscopy do not translate directly to micro-CT imaging. For example, micro-CT imaging of samples with squared resin edges of typical resin blocks and samples cut from those blocks is associated with edge diffraction artifacts that can interfere with imaging. Smoothing resin edges, while possible, is laborious and time-consuming.

While a variety of plastic embedding resins are employed in electron microscopy, the high background associated with harder resins such as Embed 812 led us to test others. We chose LR White due to its low viscosity, low shrinkage, low tendency to form bubbles during polymerization, and lower background. To create edge-free samples, and to minimize the quantity of resin surrounding the sample, we developed the protocols to draw specimens in liquid resin into polyimide tubing prior to the polymerization. Polyimide was chosen for its high thermal stability and high X-ray transmittance so that the removal of the tubing is not necessary for imaging. Finally, we designed a custom micropipette adapter for our sample embedding technique in order to reliably hold the tubing and prevent the contamination of micropipettes. The adapter can be 3D printed from a CAD image file. Taken together, the goal of the sample preparation methods presented here is to make embedding of small samples more straightforward, achieve enhanced staining contrast, rigid immobilization, and long-term storage of intact millimeter-scale specimens.

PROTOCOL:

All procedures on live animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the Pennsylvania State University.

1. Day 1: Fixation

1.1 To improve the fixation and reduce the volume of gut contents, starve the juvenile fish for at least 24 h for a 10 mm specimen, or longer for larger specimens (*e.g.*, 72 h for a 14 mm specimen).

1.2 Pre-chill 10% Neutral Buffered Formalin (NBF) and 2x Tricaine-S (MS-222, 400 mg/L) on ice to 4 °C.

1.3 Double the fish water volume with chilled 2x MS-222 for rapid and humane euthanasia.

1.4 Wait for 30 s (larvae) to 60 s (juveniles) after the fish stops moving.

1.5 Immerse the fish in chilled 10% NBF for 10 min.

1.6 Fix the fish in chilled 10% NBF solution overnight in flat-bottomed containers at room temperature.

Note: Flat-bottomed containers are required to minimize the bending of the specimen. The volume of fixative and solutions of all subsequent steps should be at least 20 times the sample volume unless otherwise specified. For 1-5 larval zebrafish, the recommended solution volume is at least 1 mL. Insufficient fixative results in poor fixation, and a surplus of reagent volume does not negatively impact the outcome of the described procedure. For complete fixation of larger fish, slit the belly starting slightly anterior to the anal pore. Use minimal penetration of scissors or knife and apply gentle force away from the fish while cutting to minimize damage to internal organs. Fixation protocols have been described by others[31-33].

2. Day 2: Dehydration & Staining

2.1. Rinse fixed specimens 3 times in 1x phosphate buffered saline (PBS) pH 7.4 for 10 min.

2.2. Submerge the samples in 35% ethyl alcohol (EtOH) for 20 min at room temperature with gentle agitation.

Note: For all gentle agitation steps, use a tabletop shaker and set for a mixing effect while carefully avoiding bumping and rubbing of the sample against the container surfaces.

2.3. Submerge the samples in 50% EtOH for 20 min at room temperature with gentle agitation.

2.4. Dissolve phosphotungstic acid (PTA) powder in water to prepare a 1% w/v stock solution.

2.5. Dilute the 1% PTA stock solution 3:7 in 100% EtOH to obtain a 0.3% PTA staining solution.

2.6. Stain the samples overnight in the 0.3% PTA solution at room temperature with gentle agitation.

3. Day 3: Infiltration

3.1 Prepare 1:1 v/v mixture of 100% EtOH and LR White acrylic resin and set aside.

3.2 Rinse 3 times in 70% EtOH for 10 min.

3.3 Submerge the samples in 90% EtOH for 30 min at room temperature with gentle agitation.

3.4 Submerge the samples in 95% EtOH for 30 min at room temperature with gentle agitation.

3.5 Submerge the samples twice in 100% EtOH for 30 min at room temperature with gentle agitation.

3.6 Submerge the samples in the 1:1 EtOH and LR White acrylic resin mixture overnight at room temperature with gentle agitation.

4. Day 4: Embedding

4.1. Submerge the samples in 100% LR White resin for 2 h at room temperature with gentle agitation.

4.2. Replace the resin in step 4.1 with fresh 100% LR White resin and incubate for 1 h at room temperature with gentle agitation.

4.3. Assemble the embedding apparatus (**Figure 1**).

4.4. Cut polyimide tubing of appropriate diameter and length.

Note: The tubing with an inner diameter that is at least 0.1 mm larger than the diameter of the specimen is recommended. For typical specimens, a standard length of 30 mm of tubing is used for each sample. When desired, elongated specimens can be accommodated by using longer tubing.

4.4.1. Attach a P1000 micropipette to the wide end of the embedding adapter and insert the polyimide tubing to the narrow end (**Figure 1C**). If the embedding adapter is not available, go to Step 4.3.3. Otherwise, continue to Step 4.4.

4.4.2. Clip off the end of a micropipette tip straight across such that the polyimide tubing fits snugly. Cut at 44 mm from the wide end of a 200 µL yellow micropipette tip for the 0.0403" tubing and 59 mm from bottom of 1 mL blue tip for the 0.105" tubing. Trim as needed.

4.4.3. Attach the cut micropipette tip to a micropipette.

4.5. Transfer the samples to a small weigh boat or V-shaped solution basin and fully submerge the samples in 100% LR White resin.

4.6. With the embedding apparatus from Step 4.3, aim the tubing at the wide end of the fixed sample (or towards the head in the case of animal specimens) and pipette the specimen slowly into the tubing. Position the sample in the middle of the tubing and ensure the tubing above and below the specimen is filled with resin.

4.6.1. Pipette the sample into the tubing such that the sample is moving in the natural, forward direction.

Note: Slow pipetting avoids air bubbles and specimen damage caused by abrasion against the tubing edge. Backward movement into the tubing can easily damage extremities (limbs, wings, fins, *etc*.). It is recommended to allow some resin overflow in the embedding apparatus so that, later, air does not enter the tubing during removal.

4.7. Immediately seal the open end with oil-based soft modeling clay.

4.7.1. Flatten the clay into a 1 mm-thick sheet.

4.7.2. Stabilize the tubing between index and middle fingers.

4.7.3. Slowly press the clay against the end of the tubing with thumb.

4.7.4. Remove excess clay.

4.8. Remove the embedding apparatus from the micropipette.

4.9. Pull out the tubing by gentle rotation and seal the other end with the clay.

4.9.1. Hold a finger against the sealed end to prevent the ejection of the clay.

4.9.2. Slowly push the unsealed end of the tubing into the clay.

4.10. Place the tubing horizontally and polymerize the resin for 24 h at 65 °C.

Note: Horizontal placement avoids the sample movement during polymerization. If a small amount of air was trapped in Step 4.8, the end with the air bubble may be elevated slightly to

prevent air bubble movement toward the specimen. Importantly, too much elevation during polymerization may cause the specimen to fall towards the low end due to gravity. A properly embedded sample is shown compared to one with an air bubble, which should be avoided because the refraction from air/resin interfaces can degrade image quality (**Figure 2**).

5. Day 5: Collection

5.1 Collect the samples for image acquisition at the end of Day 5.

Note: The removal of polyimide tubing is possible but not necessary for imaging. Synchrotron-based micro-CT imaging for the zebrafish sample was performed at Beamline 2-BM at Advanced Photon Source (APS) in Argonne National Laboratory (Lemont, IL, USA). For PTA-stained zebrafish, an optimal X-ray energy range of 5-30 keV was determined, and an X-ray energy of 13.8 keV was used for imaging. 1501 projections were obtained at 13.8 keV over 180 degrees (1 projection every 0.12 degrees) with a 2048-by-2048 pixel camera. Additionally, two flat-field (gain) images (one at the beginning and one at the end of the acquisition) and one dark-field image were also acquired. Flat-field and dark-field corrections, ring artifact reduction, and image reconstruction were done using the open source TomoPy toolkit. PTA-stained *Daphnia* and *Drosophila* samples were imaged on an X-ray microscope[12,34]. Specific micro-CT settings are dependent on the object and the quality of the stain. For example, the *Drosophila* specimen was scanned at 40 kVp, 74 mA, and 15 s at a voxel resolution of $3.10 \times 3.10 \times 3.10$ μm^3.

REPRESENTATIVE RESULTS:

The protocol described above details the sample preparation procedure for whole zebrafish larvae and juveniles for micro-CT imaging. Notably, this method is readily adapted to other samples types (*e.g.*, *Drosophila*, *Daphnia*, *Arabidopsis*, mouse organs). Incubation times in various steps are appropriate for zebrafish larvae and samples of similar size; larger samples may require longer incubation. To assist with the sample embedding steps, we designed an adapter that attaches to a 1 mL micropipette, and can be customized for various sizes of polyimide tubing (**Figure 1**). This custom adapter was 3D printed from a CAD file that is associated with this manuscript and available for download from http://publications.chenglab.com/LinEtAlJOVE/. Since all readers may not have access to a 3D printer, assembly of a homemade embedding apparatus using micropipette tips is described in the protocol (Step 4.3). A successfully embedded zebrafish larva is shown compared to a specimen with an air bubble (**Figure 2**), which will likely degrade the image quality. To demonstrate the versatility of the embedding procedure, we show the specimens from various model organisms (*i.e.*, *Danio*, *Drosophila*, *Daphnia*, mouse embryo) that have gone through the sample preparation pipeline (**Figure 3**). Reconstructed 3D volumes of whole *Danio*, *Daphnia*, and *Drosophila* specimens imaged by micro-CT are shown (**Figure 4**). Importantly, as demonstrated by the *Danio* sample, these specimens can be stored for an extended period of time and reused for multiple imaging sessions (**Figure 5**).

FIGURE LEGENDS:

Figure 1. Embedding apparatus. (A) An adapter was designed to facilitate the embedding using common laboratory tools. (B) A cross-sectional illustration of the adapter. (C) The embedding

apparatus after the insertion of the polyimide tubing at adapter point (a) and attaching the micropipette at adapter point (c). Adapter segment (b) is an overflow chamber designed to accommodate excess resin and protect the micropipette from contamination.

Figure 2. Successfully embedded specimens are devoid of air bubbles. (A) An embedded 3 day post-fertilization (dpf) larval zebrafish without air bubble. (B) An embedded 3 dpf zebrafish with an air bubble. Air trapped during the embedding process can move toward the specimen if the sample is not placed horizontally during the polymerization. Scale bars = 1 mm.

Figure 3. A wide variety of specimens can be embedded with our protocol. (A) 7 dpf zebrafish larva. (B) Adult *Drosophila*. (C) Adult *Daphnia*. (D) Mouse embryo. Larger samples such as the mouse embryo are accommodated by a few modifications to the protocol. Briefly, the polyimide tubing was filled to 1/3 of its length with resin and polymerized prior to embedding. Fixed and stained sample was placed on top of the pre-polymerized resin. The tubing was then filled with un-polymerized resin followed by a second polymerization. The polyimide tubing was removed prior to photography to allow better visualization of the sample in this figure. The removal of the tubing is not necessary for successful image acquisition by micro-CT. Scale bars: specimen images, 5 mm; enlarged insets, 1 mm.

Figure 4. 3-dimensional renderings of embedded specimens. (A) Reconstructed 3 dpf zebrafish larva at $0.743 \times 0.743 \times 0.743$ μm^3 voxel resolution. (B) Reconstructed adult *Daphnia* ($3 \times 3 \times 3$ μm^3 voxel resolution). (C) Reconstructed adult *Drosophila* ($3.1 \times 3.1 \times 3.1$ μm^3 voxel resolution). Digital cross-sections can be generated at any angle as shown on the left column. Surface renderings are shown on the right, rendered using commercial software. Embedding resin can be seen in all scans outside of the sample (noted by *), but does not interfere with the sample itself. The zebrafish larva was imaged at Argonne National Laboratory at the Advanced Photon Source that is synchrotron-based. The *Daphnia* or *Drosophila* specimens were imaged with a commercial X-ray microscope.

Figure 5. Samples embedded with our protocol can be re-imaged with micro-CT. The same 5 dpf zebrafish larva was imaged in (A) 2011 and (B) 2013, presented as average intensity projections of the sagittal view. Image comparison between scans after the storage shows the preservation of anatomical features. (C) A close-up of muscle from both scans is presented, with segmented lines indicating corresponding regions used for (D) intensity profile generation. Intensity profiles normalized to their corresponding averages along both lines show spatially matching local peaks in both scans. (E) Average intensity values for selected regions in A and B belonging to background (Bg, purple), neuronal nuclei (N, green), white matter (W, blue), and the muscle (M, orange) were divided by an average for background (white) and used to generate signal-to-noise ratios (SNR), which corresponds well between scans. Scale bars = 100 µm.

DISCUSSION:

In this manuscript, we present a detailed protocol for rigid immobilization of fixed and stained millimeter-scale (0.5 to 2.5 mm diameter) specimens for micro-CT imaging. Given the rapid advance of micro-CT technology with regard to resolution and speed, a method of permanent

sample preservation with re-imaging capabilities is highly desirable. Traditionally, dense embedding resin are commonly used in electron microscopy to provide structural support to cut ultrathin sections and minimize the damage to the specimen. Our method adapted its use for rigid immobilization during non-destructive imaging. To minimize the imaging artifacts from the presence of excess resin, we have implemented the use of X-ray transparent polyimide tubing to create round samples without edges, and to restrict the volume of resin around the sample.

Optimal outcome of the embedding procedure is contingent upon careful execution of several critical steps and attention to sample integrity throughout the procedure. Indeed, damaging the specimen will result in a compromised final image regardless of the success of execution of imaging. Since chilling contributes to a diminution of pain responses, and unfixed tissue degrades more rapidly at higher temperatures, we use pre-chilled reagents. Large specimens may need to be cut to allow the entry of fixative into the inside of the specimen so that internal organs such as liver, pancreas, and gut are completely fixed. Unfixed tissues deteriorate and lose structural integrity, destroying biological structure. Another key step is to maintain the specimen in its natural alignment. Therefore, we advocate for the use of flat-bottomed containers, which we use throughout the procedure and are particularly important during fixation. Fixation in polypropylene tubes or conical tubes that usually have a V-shaped bottom should be avoided because they can cause elongated samples to bend, which distorts the natural morphology of the specimen. In addition, to minimize the use of resin, the inner diameter of the polyimide tubing is only slightly larger than the width of the specimen. Bending of the specimen can also result in damage to the specimen as it enters the tubing. It is also recommended to transfer the specimen into the tubing in a natural forward manner to avoid damaging extremities. Proper dehydration is another critical step. This is accomplished with small increments of increasing EtOH concentrations to slowly replace water with EtOH, which is more miscible with the resin. Large increases in EtOH concentration are associated with tissue shrinkage and can be ameliorated by additional increments of EtOH incubation to make the transition more gradual. Finally, to minimize the movement of the specimen or air pockets, if there are any, the samples are placed horizontally during the resin polymerization (end of Day 4). Air pockets or sealant next to the specimen causes optical artifacts with X-ray imaging associated with edge diffraction, reducing image quality.

With respect to possible modifications to the protocol, other embedding resins and metal stains can be used in place of LR White acrylic and PTA, respectively. Embed812, an epoxy resin commonly used in electron microscopy, is highly viscous, more difficult to transfer, may cause the distortion of the specimens, and is associated with higher background than acrylic or glycol methacrylate resins. While JB4 Plus, a glycol methacrylate, is less viscous than EMbed812 and has lower background, random formation of air pockets frequently appear in the vicinity of the specimen. As mentioned, air pockets degrade the image quality and are particularly problematic for precious samples. It is worth noting that Technovit 7100, another glycol methacrylate, interferes with PTA staining, resulting in poor contrast in the final image. Comparatively, LR White acrylic has water-like viscosity, low background, and does not interfere with PTA staining. In our hands, the success rate of embedding in LR White without sample damage or air bubbles is greater than 95%. For these reasons, we advocate its use as the embedding resin for tissue micro-

CT. With respect to stains, the outcome of various metal stains such as osmium tetroxide, iodine, and PTA in micro-CT imaging has been compared and discussed elsewhere[15,16,17] and is outside the scope of this manuscript.

The sample size is a major limitation to our current protocol. Since we employ suction to transfer specimens into the tubing, the ability to see the specimen is critical for orientation and ensuring that it is indeed in the tubing. As a result, sub-millimeter samples such as *Tardigrade* (*i.e.*, water bears) are extremely difficult to embed because they require the use a microscope to be visualized. Once suction is applied, microscopic specimens are easily lost from the focal plane and become challenging to rediscover, making it difficult to determine if they passed too far through the attached end of the tubing. Larger samples, such as mouse embryos, (3 – 10 mm) can be embedded with slight modifications to the embedding protocol (**Figure 4D**). In particular, the polyimide tubing was filled to 1/3 with liquid resin, which was polymerized prior to embedding. The fixed and stained sample was placed on top of the pre-polymerized resin. The tubing was then fully filled with un-polymerized resin and followed by a second polymerization.

The significance of our embedding protocol is manifold. Rigidly immobilized whole animal or tissue specimens are desirable for reasons including: (1) creating long-term repositories of samples that are difficult to generate or prepare; (2) re-acquisition of data; (3) enabling serial imaging using multiple imaging modalities; and (4) providing standards for calibration and technology development. Samples prepared with our embedding procedure are encased in solid resin that is resilient against damage and can therefore be easily stored perhaps indefinitely. Long-term storage is particularly useful for rare or laboriously generated specimens such as those associated with phenome projects. Further, in the event that the digital data are lost, the data can be generated from the original sample. The capability of re-imaging over a long period of time potentially allows the same sample to be interrogated with more advanced imaging modalities in the future. Since the samples are physically stable between imaging instances, images are acquired from the same specimen in the same orientation, facilitating registration between earlier and later data sets. For example, a low-resolution image may only allow segmentation of tissues in a zebrafish larva. The same larva can later be re-imaged at higher-resolution so that more detailed computational analyses at cellular resolution. This capability for direct comparison between registered scans suggests resin-embedded samples as a potential standard for technology development of micro-CT. The effects of any alteration to the imaging method can be assessed by imaging of the same sample so that all variables in the sample preparation step are controlled. Finally, a resin-embedded standard sample can be used for instrument calibration to test for consistency of imaging.

Our previous work examining mutant and diseased fish histology showed that cellular resolution allows detection of subtle abnormalities in tissue structure that are overlooked in gross examination using the dissecting microscope[35], or a low-power stereo microscope. We wish to expand our high-resolution analyses to human samples. Zebrafish larvae, which comprise the primary subject of our development work, are similar to human needle biopsies in their fragility, cellular and intercellular tissue heterogeneity, size (1-3 mm diameter), and elongated shape. Based on our extensive experience with zebrafish, and other work showing that micro-CT

successfully stained and scanned human tissue, albeit at lower resolution[36], we expect our approaches to bring added value to imaging and analysis of needle biopsies. We have estimated that the assembly of a kit sufficient for one preparation of up to 20 samples of the same condition to be potentially less than 30 USD. The samples prepared by our embedding method are resistant to physical damage and therefore easy to transport. The low cost and ease of transport suggest the possibility of collection of samples from across the world, particularly areas in which cellular resolution imaging with micro-CT is not readily accessible. Our vision is for high resolution digital files of needle biopsies (ranging from 0.1 to several terabytes) to enable the creation of a digital atlas of human tissues, and at the same time allow the researchers to refine and enhance current analysis pipelines for localization and quantitative characterization of cellular and tissue architecture within the full 3D context of the tissues scanned.

ACKNOWLEDGMENTS:

The authors would like to thank Roland Myers for kindly providing the original TEM protocols. In addition, we would like to thank Dr. John Colbourne for providing the *Daphnia* specimen, Dr. Santhosh Girirajan for providing the *Drosophila* specimen, and Dr. Fadia Kamal for providing the mouse embryo. The investigators acknowledge funding support from the NIH (1R24OD18559-01-A2, PI: KCC), the Jake Gittlen Laboratories for Cancer Research, and from pilot award funding from the PSU Huck Institutes of the Life Sciences and the Institute for CyberScience.

DISCLOSURES:

All of the authors declare that there are no conflicts of interests.

REFERENCES:

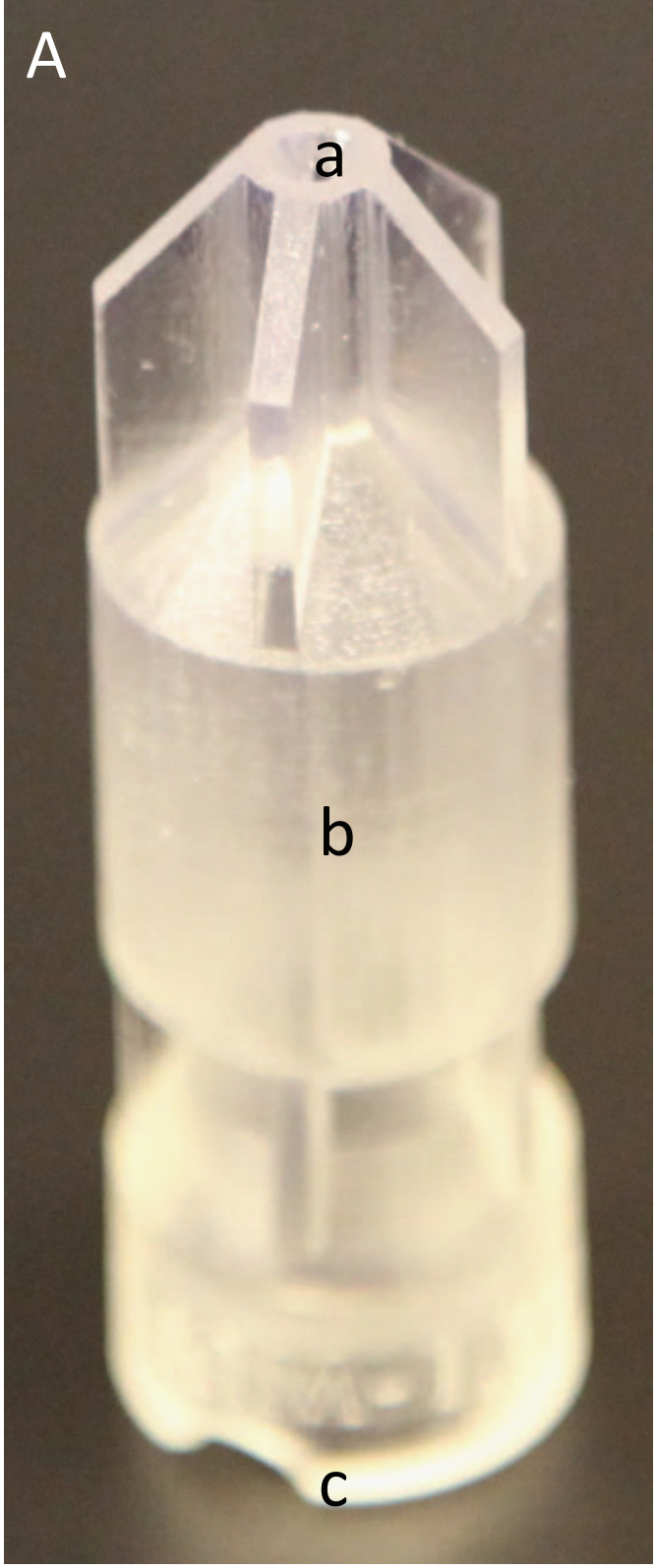
1. Cheng, K.C., Katz, S.R., Lin, A.Y., Xin, X., Ding, Y. Whole-organism cellular pathology: a systems approach to phenomics. *Advances in Genetics*. **95**, 89-115 (2016)
2. Cheng, K.C., Xin, X., Clark, D., La Riviere, P. Whole-animal imaging, gene function, and the zebrafish phenome project. *Current Opinion in Genetics & Development*. **21** (5), 620-629 (2011)
3. Vågberg, W., Larsson, D.H., Li, M., Arner, A., Hertz, H.M. X-ray phase-contrast tomography for high-spatial-resolution zebrafish muscle imaging. *Scientific Reports*. **5**, 16625 (2015)
4. Bruneel, B. *et al*. Imaging the zebrafish dentition: from traditional approaches to emerging technologies. *Zebrafish*. **12** (1), 1-10 (2015)
5. Charles, J.F. *et al*. Utility of quantitative micro-computed tomographic analysis in zebrafish to define gene function during skeletogenesis. *Bone*. **101**, 162-171 (2017)
6. Silvent, J. *et al*. Zebrafish skeleton development: High resolution micro-CT and FIB-SEM block surface serial imaging for phenotype identification. *PLoS One*. **12** (12), e0177731 (2017)
7. Hur, M. *et al*. MicroCT-based phenomics in the zebrafish skeleton reveals virtues of deep phenotyping in a distributed organ system. *eLife*. **6**, e26014 (2017)
8. Seo, E., Lim, J.H., Seo, S.J., Lee. S.J. Whole-body imaging of a hypercholesterolemic female zebrafish by using synchrotron X-ray micro-CT. *Zebrafish*. **12** (1), 11-20

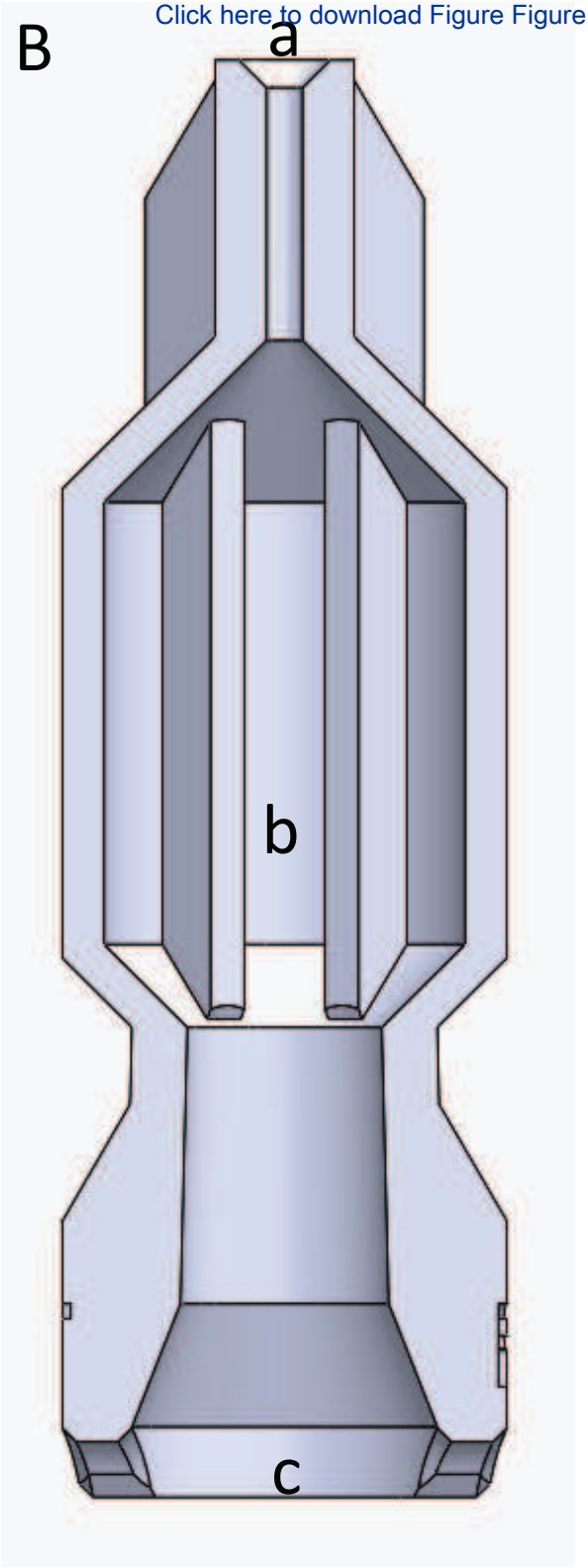
9. Hasamura, T. *et al*. Green tea extract suppresses adiposity and affects the expression of lipid metabolism genes in diet-induced obese zebrafish. *Nutrition and Metabolism*. **9** (1), 73 (2012)
10. Meguro, S., Hasumura, T., Hase, T. Body fat accumulation in zebrafish is induced by a diet rich in fat and reduced by supplementation with green tea extract. *PLoS One*. **10** (3), e0120142 (2015)
11. Dyer, E.L. *et al*. Quantifying mesoscale neuroanatomy using X-ray microtomography. *eNeuro*. **4** (5), doi: 10. 1523/ENEURO.0195-17.2017
12. Busse, M. *et al*. Three-dimensional virtual histology enabled through cytoplasm-specific X-ray stain for microscopic and nanoscopic computed tomography. *Proc Natl Acad Sci U.S.A*. **115** (10), 2293-2298
13. Perilli, E., Parkinson, I.H., Reynolds, K.J. Micro-CT examination of human bone: from biopsies towards the entire organ. *Ann Inst Super Sanita*. **48** (1), 75-82 (2012)
14. Watz, H., Breithecker, A., Rau, W.S., Kriete, A. Micro-CT of the human lung: imaging of alveoli and virtual endoscopy of an alveolar duct in a normal lung and in a lung with centrilobular emphysema – initial observations. *Radiology*. **236** (3), 1053-1058 (2005)
15. Metscher, B.D. MicroCT for developmental biology: A versatile tool for high-contrast 3D imaging at histological resolutions. *Developmental Dynamics*. **238** (3), 632-640 (2009)
16. Metscher, B.D. MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. *BMC Physiology*. **9**, 11 (2009)
17. Pauwels, E. Van Loo, D., Cornillie, P., Brabant, L., Van Hoorebeke, L. An exploratory study of contrast agents for soft tissue visualization by means of high resolution X-ray computed tomography imaging. *Journal of Microscopy*. **250** (1): 21-31 (2013)
18. Kün-Darbois, J.D., Manero, F., Rony, L., Chappard, D. Contrast enhancement with uranyl acetate allows quantitative analysis of the articular cartilage by microCT: Application to mandibular condyles in the BTX rat model of disuse. *Micro*. **97**, 35-40 (2017)
19. Tang, S.Y., Vashishth, D. A non-invasive *in vitro* technique for the three-dimensional quantification of microdamage in trabecular bone. *Bone*. **40** (5), 1259-1264 (2007)
20. Elliott, J.C., Dover, S.D. X-ray microtomography. *Journal of Microscopy*. **126** (Pt 2), 211-213 (1982)
21. Johnson, J.T. *et al*. Virtual histology of transgenic mouse embryos for high-throughput phenotyping. *PLoS Genetics*. **2** (4), e61 (2006)
22. Larsson, D.H., Vågberg, W., Yaroshenko, A., Yildirim A.Ö., Hertz, H.M. High-resolution short-exposure small-animal laboratory x-ray phase-contrast tomography. *Scientific Reports*. **6**, 39074 (2016)
23. Pleissis, A., Broeckhoven, C., Guelpa, A., Roux, S. G. Laboratory x-ray micro-computed tomography: a user guideline for biological samples. *GigaScience*. **5**, 1011 (2017)
24. Wong, M.D., Spring, S., Henkelman, R.M. Structural stabilization of tissue for embryo phenotyping using micro-CT with iodine staining. *PLoS One*. **8** (12), e84321 (2013)
25. Wong, M.D., Maezawa, Y., Lerch, L.P., Henkelman, R.M. Automated pipeline for anatomical phenotyping of mouse embryos using micro-CT. *Development*. **141** (12), 2533-2541 (2014)

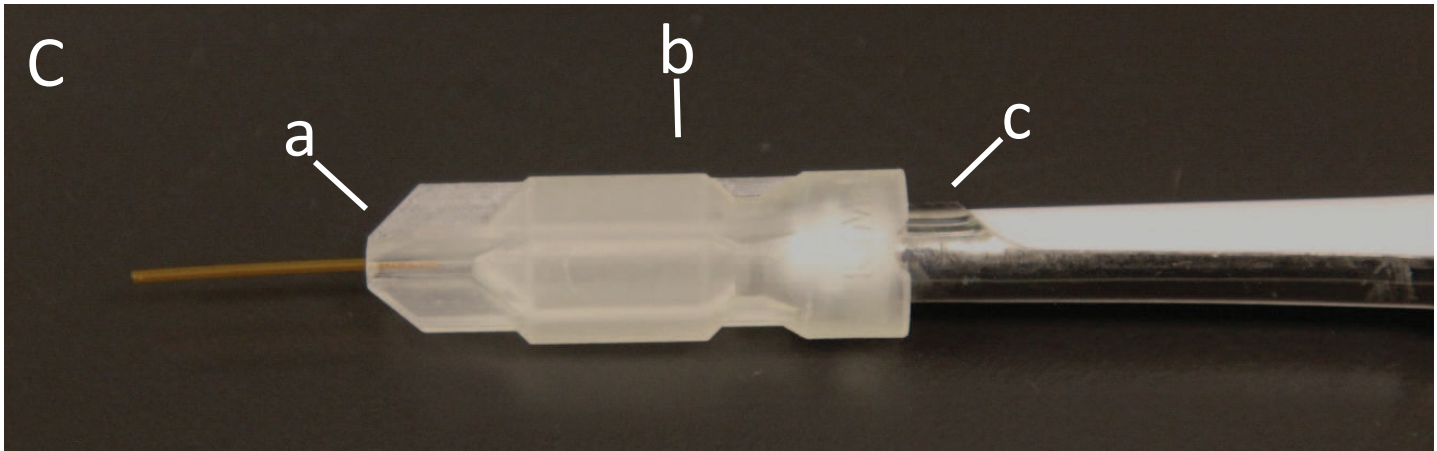
26. Hsu, C.W. *et al*. Three-dimensional microCT imaging of mouse development from early post-implantation to early postnatal stages. *Developmental Biology*. **419** (2), 229-236 (2017)
27. Luft, J.H. Improvements in epoxy resin embedding methods. *The Journal of Biophysical and Biochemical Cytology*. **9**, 409-414 (1961)
28. Hayat, M.A., Giaquinta, R. Rapid fixation and embedding for electron microscopy. *Tissue Cell*. **2** (2), 191-195 (1970)
29. Handschuh S., Baeumler, N., Schwaha, T., Ruthensteiner, B. A correlative approach for combining microCT, light and transmission electron microscopy in a single 3D scenario. *Frontiers in Zoology*. **10**: 44 (2013)
30. Bushong, E. A. *et al*. X-ray microscopy as an approach to increasing accuracy and efficiency of serial block-face imaging for correlated light and electron microscopy of biological specimens. *Microscopy and Microanalysis*. **21** (1): 231-238 (2015)
31. Tsao-Wu, G.S., Weber, C.H., Budgeon, L. R., Cheng, K.C. Agarose-embedded tissue arrays for histologic and genetic analysis. *Biotechniques*. **25** (4): 614-8 (1998)
32. Sabaliauskas, N.A. *et al*. High-throughput zebrafish histology. *Methods*. **39** (3): 246-54 (2006)
33. Copper, J.E. *et al*. Comparative analysis of fixation and embedding techniques for optimized histological preparation of zebrafish. *Comparative Biochemistry and Physiology*. **208**: 38-46 (2018)
34. Merkle, A.P., Gelb, J. The ascent of 3D X-ray microscopy in the laboratory. *Microscopy Today*. **21** (2), 10-15 (2013)
35. Thomas, G.K. A molecular and systems biology analysis of pleiotropic vertebrate phenotypes (Doctoral dissertation). Retrieved from the Penn State Electronic Theses and Dissertations Database. (2009)
36. Walton, L.A. *et al*. Morphological characterisation of unstained and intact tissue micro-architecture by X-ray computed micro- and nano- tomography. *Scientific Reports*. **5**, 10074 (2015)

Figure

Click here to download Figure Figure 1.eps

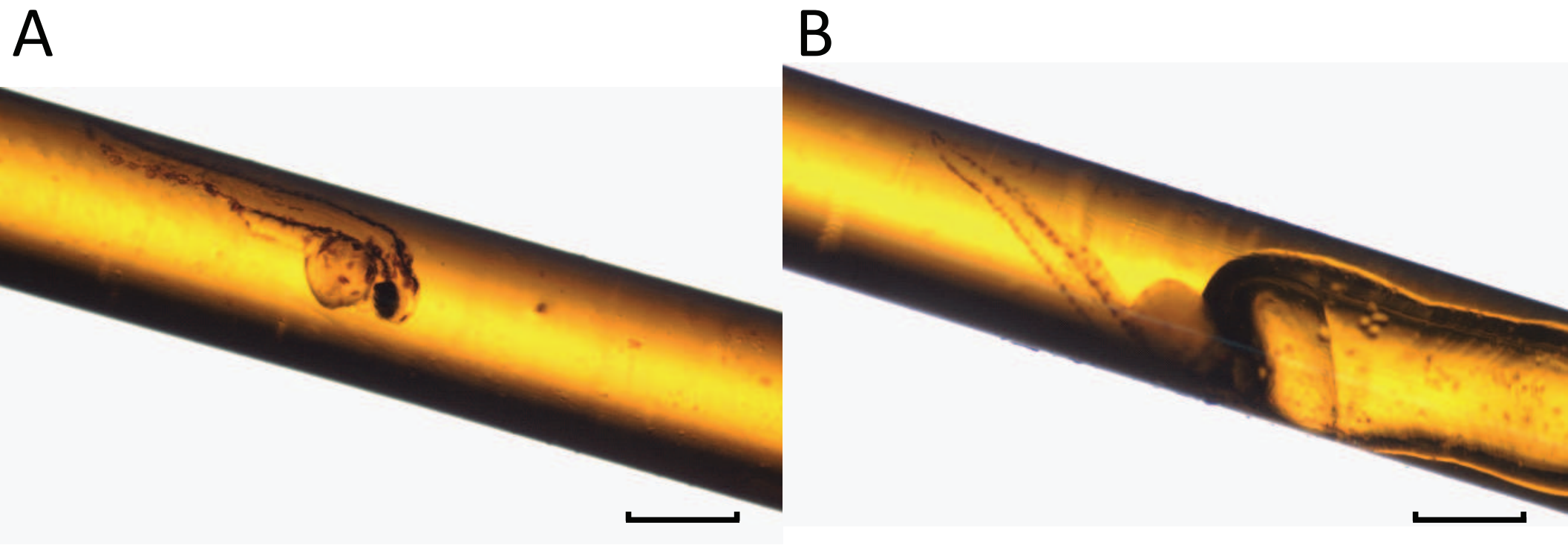






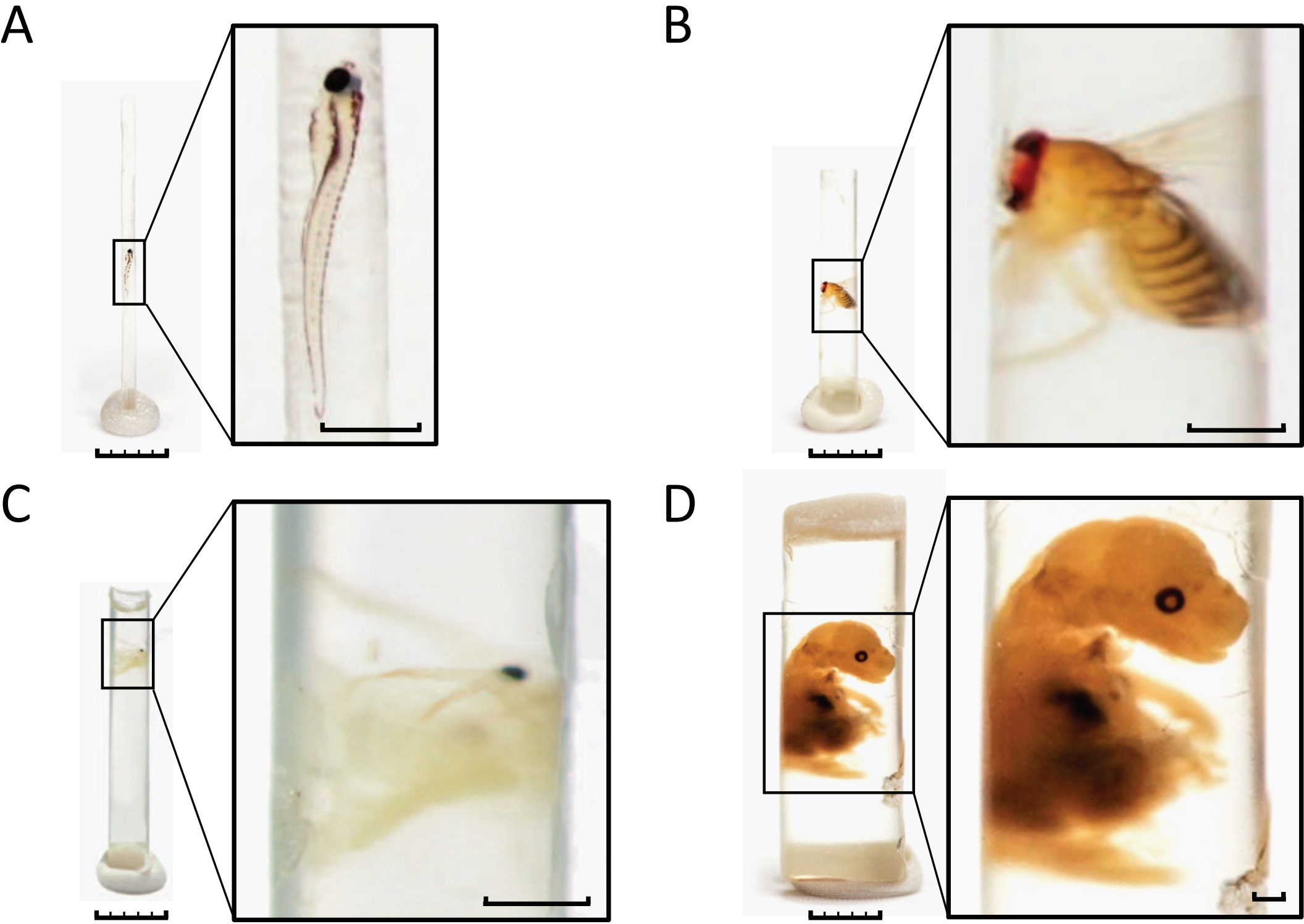
Figure

Click here to download Figure Figure 2.eps

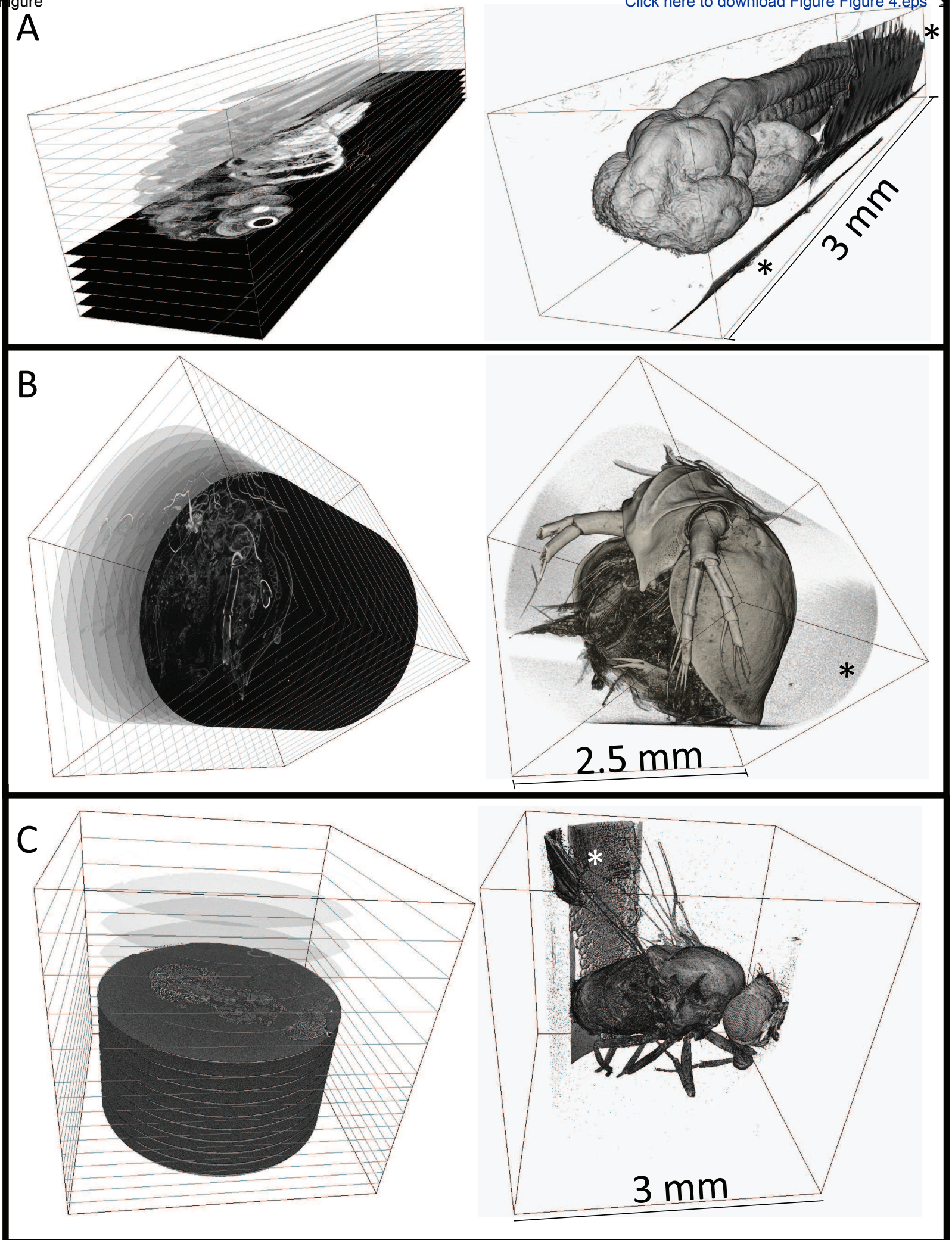


Figure

Click here to download Figure Figure 3.eps

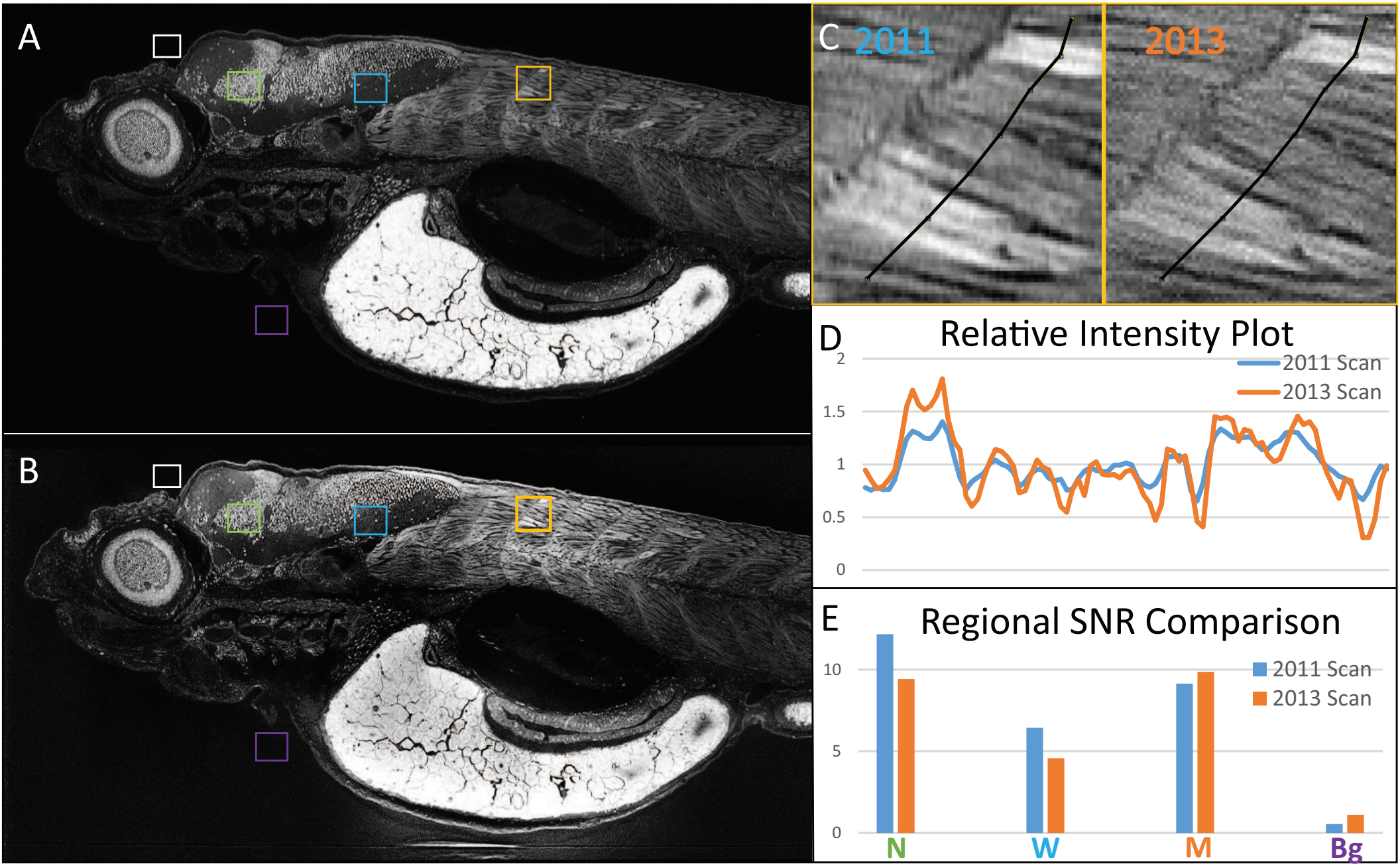


Click here to download Figure Figure 4.eps



Figure

Click here to download Figure Figure 5.eps



Click here to download Table of Materials JoVE - Table of Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10X Neutral buffered formalin	Fisher Scientific	SF100-4	
Phosphotunstic Acid	VWR	MK282402	
Tricaine-S (MS-222)	Western Chemical	MS-222	
LR White	Electron Microscopy Sciences	14380	
Polyimide tubing (ID 0.04")	Nordson Medical	141-0065	The exact polyimide tubings described in the manuscript are no longer available. A close match is provided here. Tubing with custom diameter can be manufactured upon request.
Ethyl Alcohol 200 Proof	Pharmco-Aaper	111000200	
Oil-based soft modeling clay	Sculpey	S302 001	
Micropipette P200	Gilson	F123601	
Micropipette P1000	Gilson	F123502	
Glass vials	VWR	66015-042	
V-shaped basin	VWR	89094-676	
Weigh boats	VWR	10803-148	
200 µL yellow micropipette tip	Fisher Scientific	02-707-500	
1 mL blue micropipette tip	Fisher Scientific	02-681-163	
Tabletop shaker	Thermolyne	M71735	
Camera	Photometrics	CoolSNAP HQ2 CCD	
X-ray microscope	Zeiss	Xradia 520 Versa	

Author License Agreement (ALA)

Click here to download Author License Agreement (ALA) JoVE - Author_License_Agreement 2018.pdf

jove JOURNAL OF VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Rigid embedding of fixed and stained, whole millimeter-scale specimens for section-free imaging by micro-CT

Author(s): Alex Y. Lin, Yifu Ding, Daniel J. Vanselow, Spencer R. Katz, Darin P. Clark, David Mandrell, Jean E. Copper, Damian B. van Rossum, and Keith C. Cheng

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☐ Standard Access ☒ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Alex Yu-Shun Lin
Department:	Pathology
Institution:	Penn State College of Medicine
Article Title:	Rigid Embedding of Fixed and Stained, Whole, Millimeter-Scale Specimens for Section-free Imaging by Micro-CT
Signature:	 Date: Apr. 12, 2018

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

PENNSTATE

JAKE GITTLEN LABORATORIES FOR CANCER RESEARCH

Keith C. Cheng, M.D., Ph.D.
Distinguished Professor,
Division of Experimental Pathology
Department of Pathology
Department of Biochemistry
& Molecular Biology

Penn State College of Medicine
The Milton S. Hershey Medical Center
Jake Gittlen Laboratories for Cancer Research H059
500 University Drive
Hershey, PA 17033-2390

Voice: (717) 531-5635
Fax: (717) 531-5634
E-mail:
kcheng@pennstatehealth.psu.edu

June 06, 2018

Dr. Bing Wu
Review Editor
Journal of Visualized Experiments
Ph.: (617) 674-1888
Email: bing.wu@jove.com

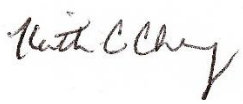
Dear Editor,

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.

Click here to download Rebuttal Letter Reponse to editorial comments 06-024-2018.docx

PENNSTATE

JAKE GITTLEN LABORATORIES FOR CANCER RESEARCH

Keith C. Cheng, M.D., Ph.D.
Distinguished Professor,
Division of Experimental Pathology
Department of Pathology
Department of Biochemistry
& Molecular Biology

Penn State College of Medicine
The Milton S. Hershey Medical Center
Jake Gittlen Laboratories for Cancer Research H059
500 University Drive
Hershey, PA 17033-2390

Voice: (717) 531-5635
Fax: (717) 531-5634
E-mail:
kcheng@pennstatehealth.psu.edu

June 06, 2018

Dr. Bing Wu
Review Editor
Journal of Visualized Experiments
Ph.: (617) 674-1888
Email: bing.wu@jove.com

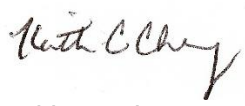
Dear Editor,

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 you for the careful review of our manuscript and the constructive suggestions that have resulted in an improved manuscript. We address these comments 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

Editorial Comment 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response 1: Done.

Editorial Comment 2. JoVE cannot publish manuscripts containing commercial language. This company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

Response 2: All commercial company names have now been removed from the manuscript and appropriately referenced in the Table of Materials.

Editorial Comment 3. Step 5.1: Please write this step in imperative tense.

Response 3: Done.