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# Use of Micro X-ray Computed Tomography with Phosphotungstic Acid Preparation to Visualize Human Fibromuscular Tissue

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| **Corresponding Author:** | Hun-Mu Yang<br>Yonsei University College of Medicine<br>Seoul, KOREA, REPUBLIC OF |
| **Corresponding Author's Institution:** | Yonsei University College of Medicine |
| **Corresponding Author E-Mail:** | YANGHM@yuhs.ac |
| **Order of Authors:** | Jehoon O |
| | Hyun-Jin Kwon |
| | Shin Hyung Kim |
| | Tae-Hyeon Cho |
| | Hun-Mu Yang |
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**TITLE:**

# Use of Micro X-ray Computed Tomography with Phosphotungstic Acid Preparation to Visualize Human Fibromuscular Tissue

**AUTHORS AND AFFILIATIONS:**

Jehoon O[1], Hyun-Jin Kwon[1], Shin Hyung Kim[2], Tae-Hyeon Cho[1], Hun-Mu Yang[1,3]

[1]Department of Anatomy, Yonsei University College of Medicine, Seoul, Republic of Korea

[2]Department of Anesthesiology and Pain Medicine, Anesthesia and Pain Research Institute, Yonsei University College of Medicine, Seoul, Republic of Korea

[3]Surgical Anatomy Education Centre at the Yonsei University College of Medicine, Seoul, Republic of Korea

**Corresponding Author:**

Hun-Mu Yang

yanghm@yuhs.ac

**E-mail Addresses of Co-authors:**

Jehoon O (jhoon81@yuhs.ac)

Hyun-Jin Kwon (hjkwon128@yuhs.ac)

Shin Hyung Kim (tessar@yuhs.ac)

Tae-Hyeon Cho (choth@yuhs.ac)

**KEYWORDS:**

micro X-ray computed tomography, phosphotungstic acid, human tissue, fibromuscular tissue, 3D anatomy, contrast agent

**SUMMARY:**

Micro X-ray computed tomography is effective in obtaining three-dimensional information from undamaged human specimens but has limited success in observing soft tissues. The use of phosphotungstic acid contrast agent can resolve this issue. We implemented this contrast agent to examine human delicate fibromuscular tissues (the orbicularis retaining ligament).

**ABSTRACT:**

Manual dissection and histological observation are common methods used to investigate human tissues. However, manual dissection can damage delicate structures, while processing and histological observation provide limited information through cross-sectional imaging. Micro X-ray computed tomography (microCT) is an effective tool for obtaining three-dimensional information without damaging specimens. However, it shows limited efficiency in differentiating soft tissue parts. Use of contrast-enhancing agents, like phosphotungstic acid (PTA), can solve this problem by improving soft tissue contrast. We implemented microCT with PTA to investigate the human orbicularis retaining ligament (ORL), which is a delicate structure in the orbit area. In this method, harvested specimens are fixed in formalin, dehydrated in serial ethanol solutions, and stained with a PTA solution. After staining, microCT scanning, 3D reconstruction, and analysis are performed. Skin, ligaments, and muscles can be clearly visualized using this method. The

specimen size and duration of staining are essential features of the method. The suitable specimen thickness was about 5–7 mm, above which the process was slowed, and the optimum duration was 5–7 days, below which an empty hole in the central area occasionally occurred. To maintain the location and direction of small pieces during cutting, sewing on the same region of each part is recommended. Furthermore, preliminary analyses of the anatomical structure are needed to correctly identify each piece. Parafilm can be used to prevent drying, but care should be taken to prevent specimen distortion. Our multidirectional observation showed that the ORL is composed of a multilayered meshwork of continuous plates, rather than thread-like fibers, as reported previously. These results suggest that microCT scanning with PTA is useful for examining specific compartments within complex structures of human tissue. It may be helpful in the analyses of cancer tissues, nerve tissues, and various organs, like the heart and liver.

**INTRODUCTION:**

Manual dissection and histological observation are typically used to examine human tissues, such as muscles and connective tissues. However, manual dissection can easily damage delicate structures, and histological observation provides limited information about flat cross-sectional surfaces[1,2]. Therefore, improved methods are needed to examine tissues more precisely and efficiently.

Conventional computed tomography (CT) is generally used in clinical practice, but it lacks the ability to distinguish small structures[2,3]. Micro X-ray CT (microCT) is an effective tool for obtaining three-dimensional (3D) information of small structures from specimens, without destroying them. However, microCT has limited applications because only dense tissues can be visualized clearly; it cannot be used to differentiate soft tissues. To overcome this limitation, staining agents can be used. Contrast-enhancing agents, like phosphotungstic acid (PTA), phosphomolybdic acid, and Lugol's iodine, improve the soft tissue contrast rate during scanning[4,5]. Several studies comparing these agents suggest that PTA demonstrates good performance and is easy to handle[6-8].

The orbicularis retaining ligament (ORL) is a delicate structure around the orbit, which can be easily damaged during conventional observation[9]. We examined and successfully retrieved 3D information on this structure using microCT with PTA as a contrast agent. This method can be applied to studies on other human tissues, such as the heart and liver, with appropriate modifications[10-12]

**PROTOCOL:**

All cadavers utilized in this study were legally donated to the Surgical Anatomy Education Centre at Yonsei University College of Medicine.

**1. Obtaining samples**

1.1. Draw an incision line on the cadaver with a colored pencil to indicate the cutting area for sample harvesting. Check that the incision line drawn extends medially to a medial canthus,

laterally to a lateral canthus, superiorly to a superior border of the lower eyelid, and inferiorly to 1 cm below the line from the orbital rim.

NOTE: Consider the sample size based on the maximum scanning size of the micro-CT equipment (our equipment could acquire an image with a maximum object dimension of 7 × 7 cm). Here, a sample approximately 1 cm in width, 3 cm in length, and 1.25 g weight was harvested from the ORL region.

1.2. Cut the facial tissues following the incision line with a blade. Make sure the cut is deep such that the tip of the blade touches the bone. The sample must include the skin, subcutaneous tissue, muscle, fat, and periosteum.

1.3. Fix the sample in 10% formalin immediately and preserve it for 5 to 7 days at room temperature (**Figure 1A**).

NOTE: Both embalmed and fresh cadavers can be used for this study. However, the fixation solution for cadavers might differ slightly from the solution used in a biological experiment. Therefore, we suggest fixing the sample with 10% formalin again even after obtaining the sample from embalmed cadavers.

## 2. Preparation for staining

2.1. After fixing, slice the sample into 3 pieces (5–7 mm in thickness). Do not lose the location and direction of each piece during this process.

NOTE: The microCT scanner we use can cover a maximum size of 7 $cm^3$, but the PTA solution cannot penetrate the sample successfully if it is too thick.

2.2. Sew the superolateral side of each piece using a needle and black thread such that the direction of the sample can be checked later.

2.3. Dehydrate the sample in a series of 30%, 50%, and 70% ethanol solutions for 1 day each.

2.4. Place the sample in 70% ethanol until staining.

## 3. PTA preparation

3.1. Begin the PTA staining process 1 week before microCT scanning is scheduled.

3.2. Prepare 70 mL of 70% ethanol solution and add 0.7 g of PTA power to it. Mix well using a shaker at 55-60 rpm.

NOTE: The concentration of the PTA solution should be 1% in ethanol.

3.3. Prepare several 70 mL plastic containers of PTA solution for each sliced piece. Soak the specimens into the containers and place them on a shaker for effective penetration. Leave the samples for 5–7 days (**Figure 1B**).

3.4. When the staining is completed, store the sample in 70% ethanol to prepare for scanning.

NOTE: The stained samples can be maintained for several months, but it is recommended that the samples be scanned as soon as possible to ensure full staining.

## 4. MicroCT scanning

4.1. Wrap the sample with parafilm to prevent drying. Do not wrap the samples too tight, as that may lead to deformation.

4.2. Open the scanner and place the sample on the tray (**Figure 2**).

4.3. Set the scanning parameters as follows: source voltage (kV) = 70, source current (µA) = 114, Al filter = 0.5 mm, image pixel size (µm²) = 20, pixels = 2240 × 2240, exposure (ms) = 500, rotation step (deg) = 0.3.

NOTE: The parameters may be modified according to the samples and/or scanners used.

4.4. Start scanning.

NOTE: Scanning takes 30 to 60 min depending on the intended resolution and the speed of the scanner.

## 5. Reconstruction and optimization of data

5.1. Run the reconstruction software. Select **Open Dataset** on the **Actions** menu to launch the scanned files.

5.2. Select the **Settings** tab on the **Reconstruction** window. Set the parameters as follows: Ring artifacts reduction = 7, Beam-hardening correction (%) = 40.

NOTE: The parameters can be modified according to the sample.

5.3. Begin reconstruction by selecting **Start** on the **Start** tab. The final data will be stored in the designated folder.

5.4. Run the file resizing software. Select **Source data set** to launch the reconstructed files.

5.5. Select **jpg** on the **Destination data set** tab.

5.6. Choose the **Resizing** option **1/2** with a **Quality** option of **No interpolation (fast).**

5.7. Adjust the slide bar to **100 (highest)** in the **Image compression** tab. Start converting.

NOTE: The resizing option is to avoid slowing down the computer speed when 3D rendered; however, it can result in lower resolution when resized extensively. We suggest resizing in half for acceptable resolution with better handling.

**6. 3D reconstruction**

6.1. Run the 3D volume rendering software.

6.2. Select **Actions** > **Load volume data** to launch the dataset.

6.3. Adjust the brightness and contrast level by modifying shape transfer function in histogram in the **Transfer Function Editor** tab.

6.4. Select **Options** > **Lighting**.

6.5. Select **Shadows** and **Surface Lighting** icons. These effects provide a realistic modeling tone.

6.6. Find the best view by moving (**click and drag**), rotating (**right-click and drag**), and zooming in or out of (**scroll**) the model.

6.7. Slide the plane (**shift + click and drag** in the inner direction) to show the sectional images (**Figure 3**).

6.8. Turn on **Light** icon. Adjust the lighting indication bar and find the best lighting for viewing. Then, turn off the icon and close the **Lighting** tab.

6.9. Select **Actions** > **Save image** to store the image.

**REPRESENTATIVE RESULTS:**

The detailed reconstruction of the ORL was achieved by microCT with PTA preparation (**Figure 4**). The ligamentous fibromuscular structure extending obliquely between the dermis and periosteum was distinctly observed (**Figure 4A**). In the coronal view (**Figure 4B**), there were fewer, more intricate fibers posterior to the muscle layer than those seen anterior to the muscle layer. In the horizontal view (**Figure 4C**), an elaborate meshwork with an arborized formation was observed. We observed a shape characterized by continuous plates, rather than thread-like fibers, as reported previously. In the sagittal view (**Figure 4D**), the thicknesses of the ORL fibers decreased inferiorly. Furthermore, the amount and complexity of fibers increased laterally. Overall, this multidirectional observation proved that the ORL is made up of a multilayered meshwork of continuous plates with variation in the number and thickness depending on the

location.

**FIGURE AND TABLE LEGENDS:**

**Figure 1. Samples were harvested and then stained with PTA solution. (A)**. Samples were fixed in 10% formalin after harvesting. **(B)**. Samples were cut into thinner pieces to enhance the penetration and then placed into the PTA solution.

**Figure 2. The microCT scanner.** Arrow indicates the tray where the specimen is placed.

**Figure 3. 3D reconstruction.** Slide the plane into the inner direction to view the sectional images inside.

**Figure 4. 3D images of the ORL.** (**A)**. The overall image of the ORL. **(B)**. Coronal view. **(C)**. Horizontal view. **(D)**. Sagittal view.

**Figure 5. Analyzed structures of the ORL.** Yellow, red, and green indicate the skin, the muscle, and the ligament, respectively. S, Superior; P, posterior.

**Supplemental Figure 1. Comparison between 3D and 2D images. (A)**. Volume rendered 3D image. **(B)**. Cross sectional 2D image. Scale bar = 1 mm.

**Supplemental Figure 2. Wrapping and fixing of the parafilm. (A)**. Wrapping the parafilm over the whole sample to prevent drying out. **(B)**. Parafilm helps fix the sample firmly on the scanner. **(C)**. Parafilm is not visible on the microCT scanning and could be subtracted easily.

**Supplemental Figure 3. Insufficient staining of PTA.** A hollow space at the center shows where the PTA solution has not penetrated sufficiently**.** (**A)**. Volume rendered 3D image. (**B)**. Cross sectional 2D image. Scale bar = 1 mm.

**Supplemental Figure 4. Comparison between fresh and embalmed cadavers.** No differences were found between fresh and embalmed cadavers to apply the protocol. The picture shows another feature could be taken by the same method as well. **(A)**. The ORL obtained from a fresh cadaver. **(B)**. The nasolabial crease obtained from an embalmed cadaver.

**DISCUSSION:**

We implemented microCT with PTA preparation in the examination of human soft tissues. Briefly, specimens are harvested and fixed in formalin for a few days, followed by dehydration in serial ethanol solutions. Placing the sample into the PTA solution directly after formalin fixation can result in some tissue cracking due to rapid dehydration. Therefore, serial dehydration is needed before PTA staining. Next, the samples are stained using PTA solution for about a week. MicroCT scanning, 3D reconstruction, and analysis can then be performed. Our goal was to observe the ORL and adjacent structures using this method. We successfully presented the tissue as a 3D model. Skin, ligaments, and muscles were distinctly visualized (**Figure 5**).

Several points should be considered while processing the samples. The size of a specimen and the duration of staining are principal concerns. After several pilot studies, we found that the proper thickness of a specimen is about 5–7 mm and the proper duration of staining is 5–7 days. In these conditions, the PTA solution penetrates the specimen at a rate of approximately 1 mm/day. If the thickness exceeds 7 mm, the processing time increases. When the duration of staining is insufficient compared to the volume of a specimen, the final image may include an empty hole in the central area of the specimen. This often occurs, especially at the skin level, and removing unnecessary skin can improve staining efficiency. When the duration is too long, the entire specimen will be overstained, making it difficult to identify each compartment. Further study on the optimal duration for staining larger specimens could prove useful.

Usually, the specimen is divided into pieces to enhance penetration; it is important to remember the location and direction of each specimen during this process. To maintain this information, sewing on the same region of each part is recommended. The thread will be seen in the final image, and one should be careful that the thread does not interfere with the main area. For example, sewing on the superolateral region of each piece might be helpful. Additionally, preliminary analyses of an anatomical structure are needed to recognize each tissue part owing to their complexity.

Parafilm and other materials are used to prevent specimens from drying out. However, slight deformities can occur when wrapping specimens. It is important to preserve the original shape to the greatest extent possible. Sometimes a liquid tube is used, instead of parafilm. However, even the slightest trembling of a machine has the potential to affect the tube during scanning and might reduce the clarity of the final image.

There are several limitations to this approach. First, this protocol cannot be done with a living object. Furthermore, the sample size is restricted by the maximum scanning size of the microCT scanner. There could be errors while analyzing the rendered image by the naked eye; therefore, additional histological experiments may be needed to confirm the findings. There might be slight dimensional distortion during preparation; however, we believe that this does not significantly affect the result of the study.

MicroCT scanning with PTA preparation is advantageous for examining specific compartments within a complex structure. This study focused on the development of a method to enhance the contrast rate using PTA preparation, and other features, like scanning and reconstruction processes, were indicated briefly. However, readers should be able to get the same result if they use contemporary microCT scanners and image-analyzing programs after the staining process. This method could prove helpful in the analyses of cancer tissues and structures, nerve contribution in specific areas, and high-resolution anatomical structures of organs, such as the heart and liver[13-15].

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1

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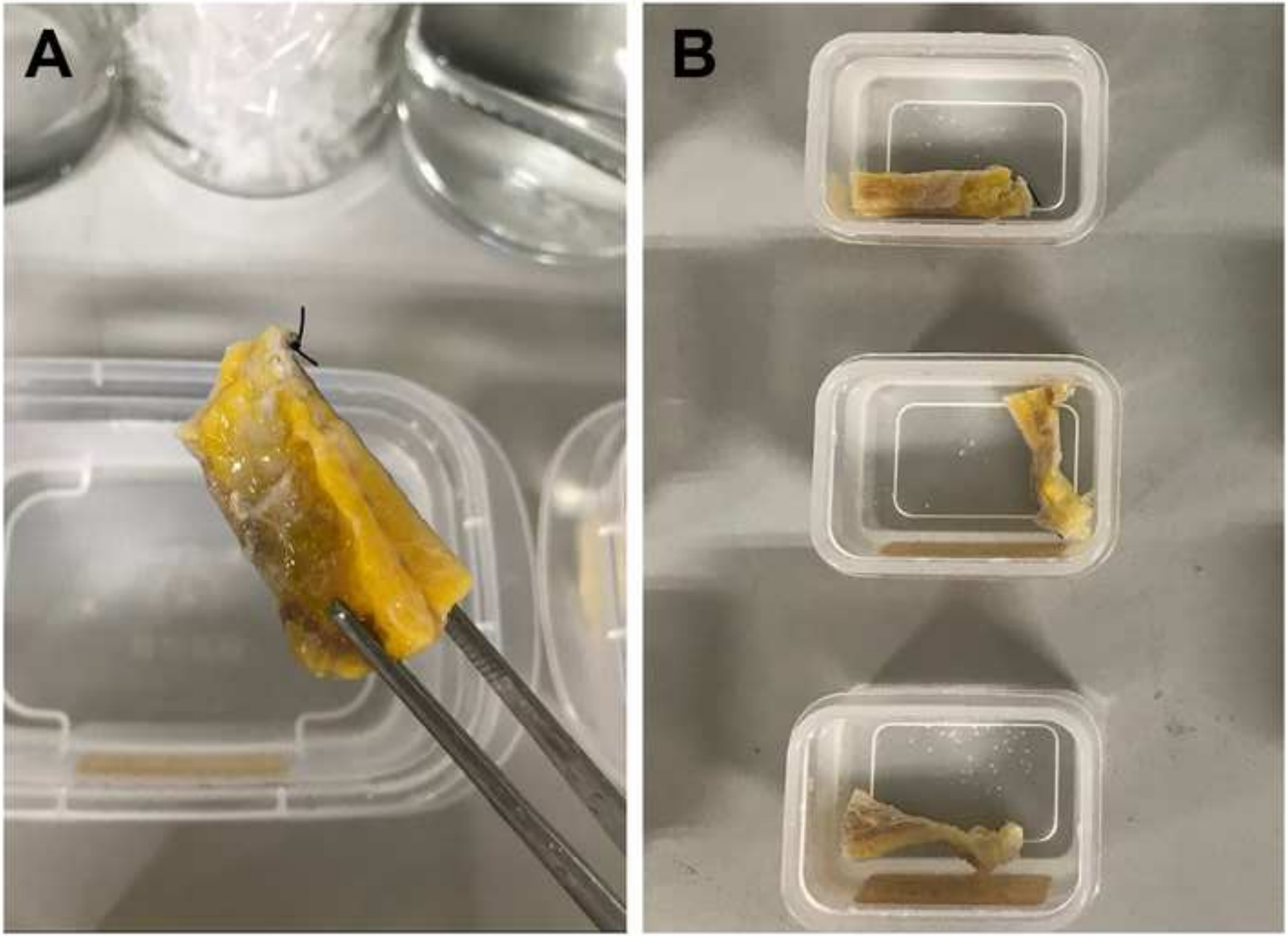

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Figure 3

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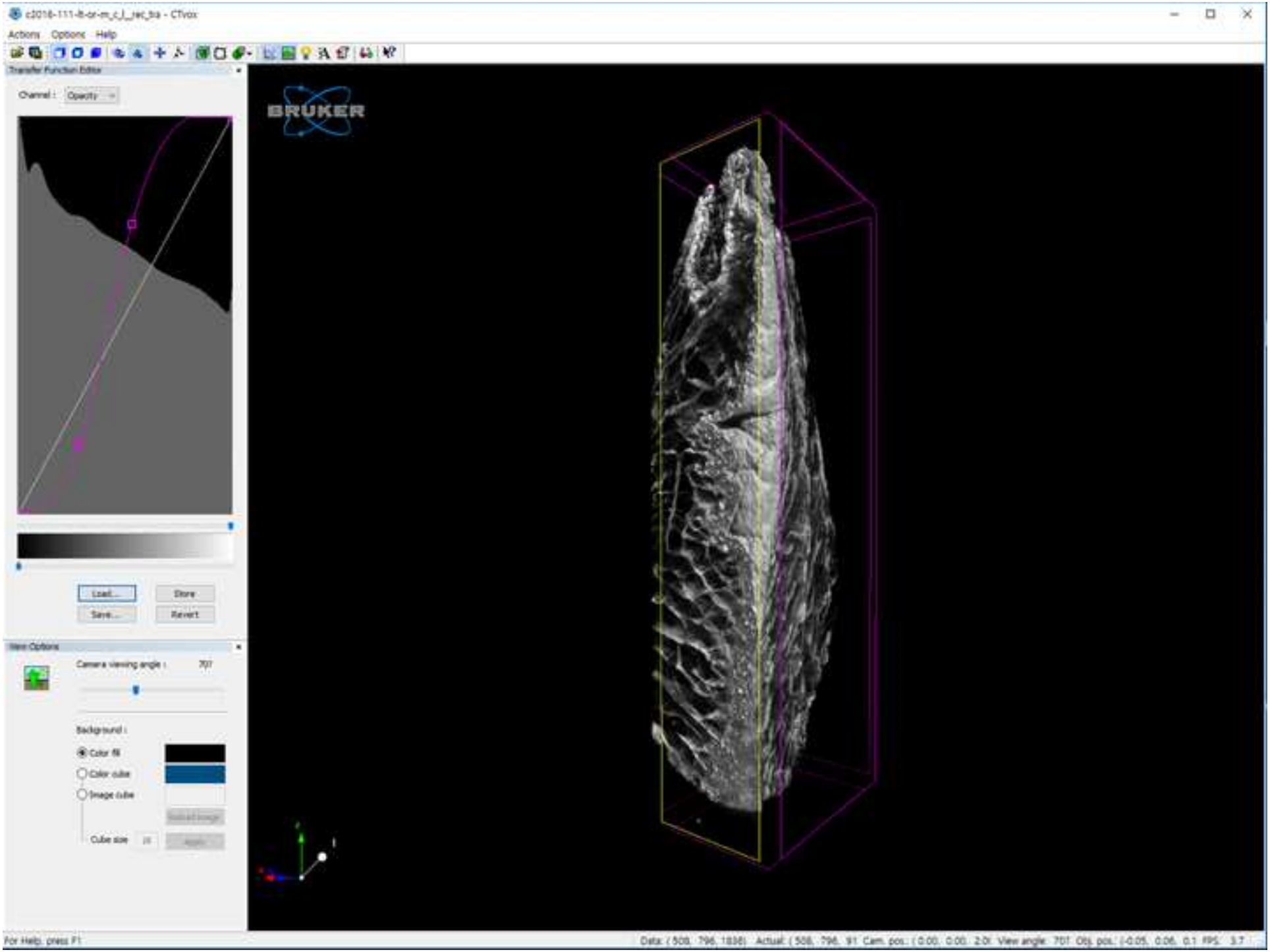
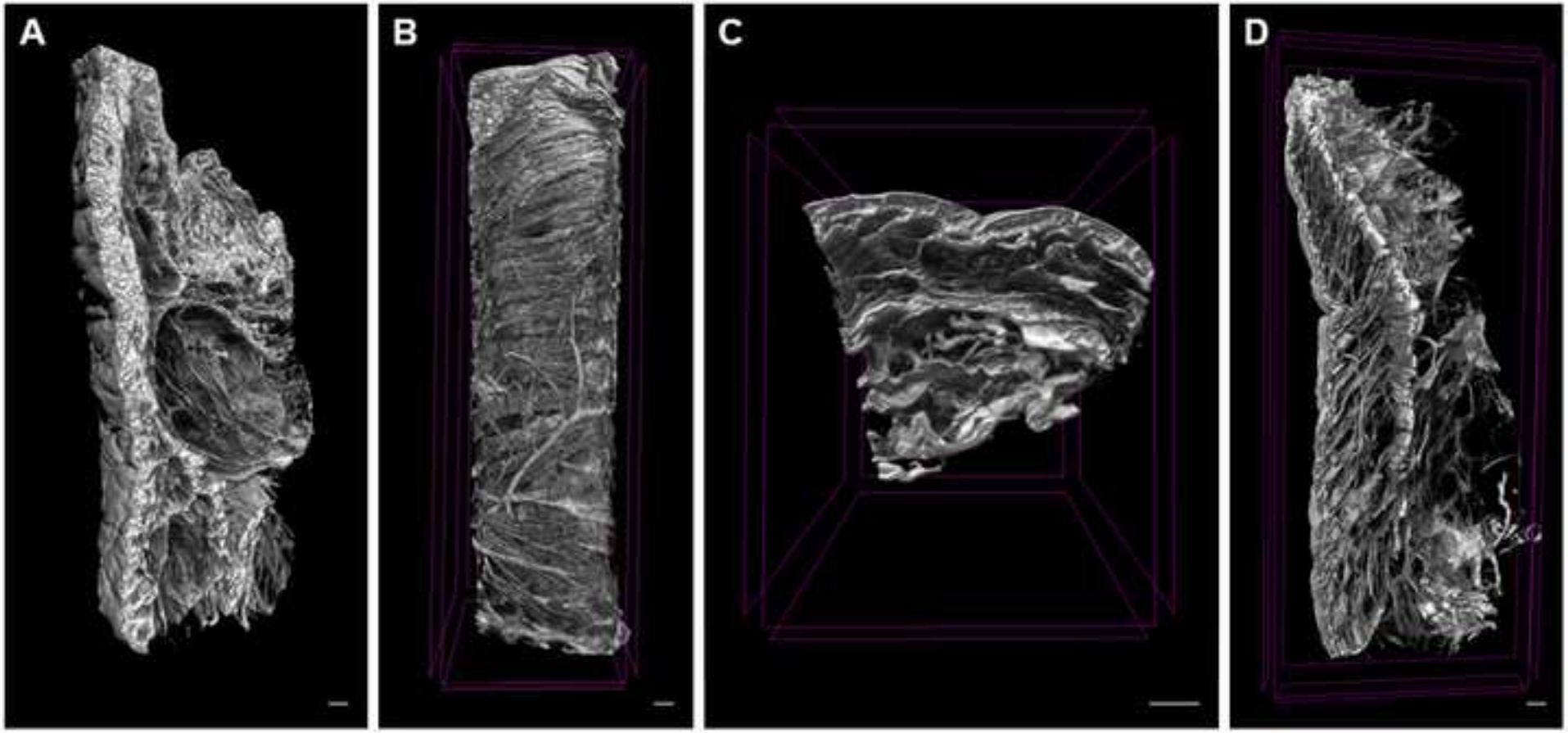


Figure 4





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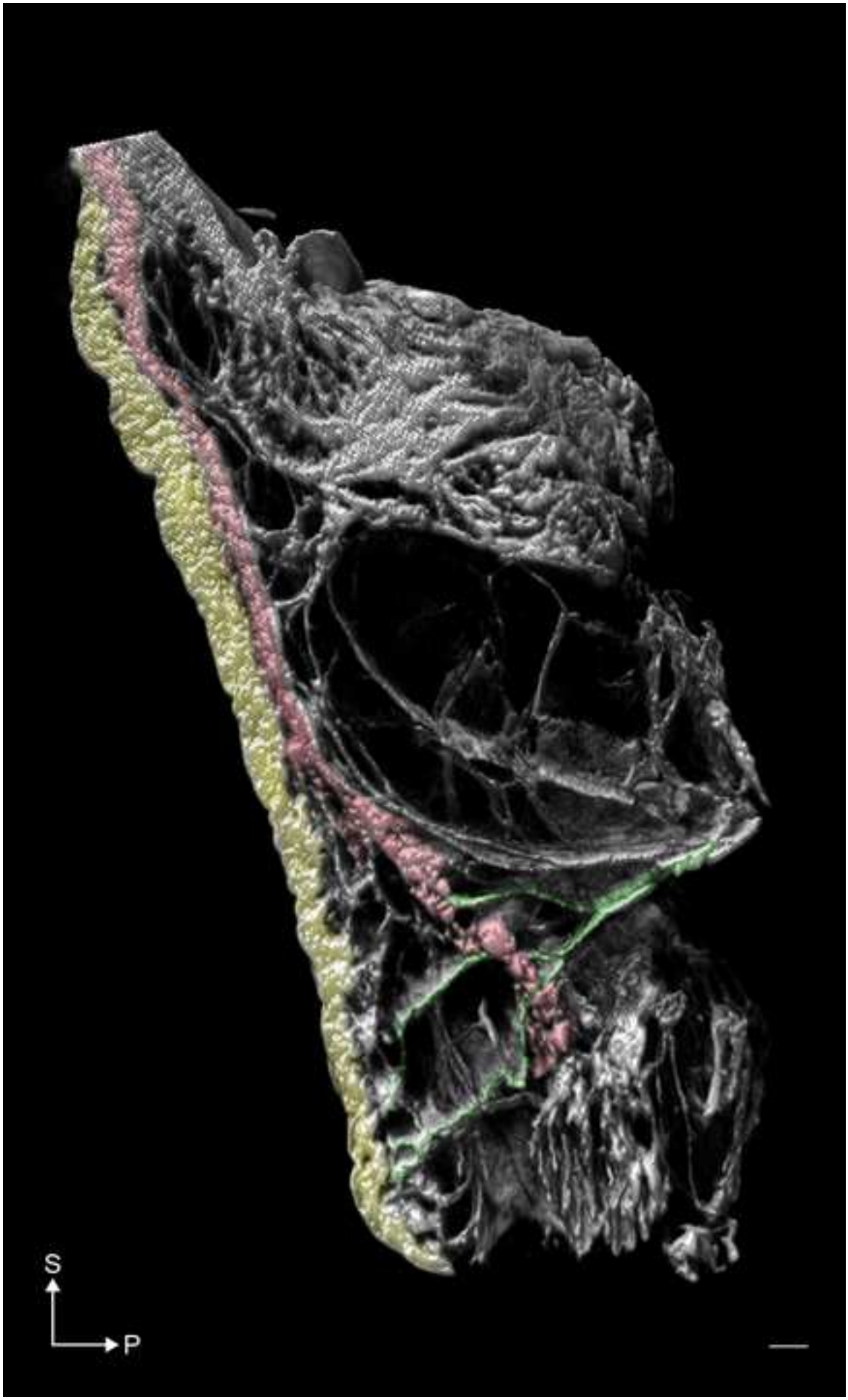

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| Phosphotungstic acid | | | |
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| Nrecon | Bruker | ver 1.7.0.4 | Reconstruction software |
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**CORRESPONDING AUTHOR**

| | |
|---|---|
| Name: | Hun-Mu Yang |
| Department: | Department of Anatomy |
| Institution: | Yonsei University College of Medicine |
| Title: | Assistant professor |
| Signature: | Yang(HM) |
| Date: | 2019. 1. 30. |

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25th April 2019

Editorial Board

*Journal of Visualized Experiments*

Dear Editor:

We thank you for the thorough and constructive reviews.

On our manuscript, we discussed the issues raised by you and revised some sentences.

We think that we have done a good job in addressing the points raised and hope the m anuscript is now ready to be published in *Journal of Visualized Experiments*.

We look forward to your favorable consideration.

Sincerely,

Hun-Mu Yang

Department of Anatomy, Yonsei University College of Medicine, Seoul, Republic of Korea

yanghm@yuhs.ac

Tel.: +82-2-2228-1649

**Author's responses about the Editorial comments:**

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

We proofread the manuscript thoroughly and revised some sentences. We checked the modified parts as red colored text for tracking.

2. Step 1.1: Please ensure that all text is written in the imperative tense.

We modified the text as following your recommendation.

"1.1. Draw an incision line on the cadaver with a colored pencil to indicate the cutting area for sample harvesting. Check the incision line drawn medially to a medial canthus, laterally to a lateral canthus, superiorly to a superior border of the lower eyelid, and inferiorly to 1 cm below the line from the orbital rim.

Note: Consider the sample size based on the maximum scanning size of the micro-CT equipment (our equipment could take an image with a maximum object dimension of 7 × 7 cm). Here, a sample approximately 1 cm in width, 3 cm in length, and 1.25 g weight was harvested from the ORL region."

3. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

We had removed all the commercial language in the first revision and therefore had generalized the relating steps as well. We added the detailed usage of the software (buttons) again, but we wonder if it is okay to add it without the names of the program.

4. Please provide a title for each supplementary figure in Figure Legend.

We added the title of supplemental figures also with short descriptions. Many supplemental figures were added to the manuscript after the review process, but we have not seen many supplemental figures from other JoVE articles. So, we wonder if it is better to change the Supplemental figures to Figures.

Supplemental Figure 2

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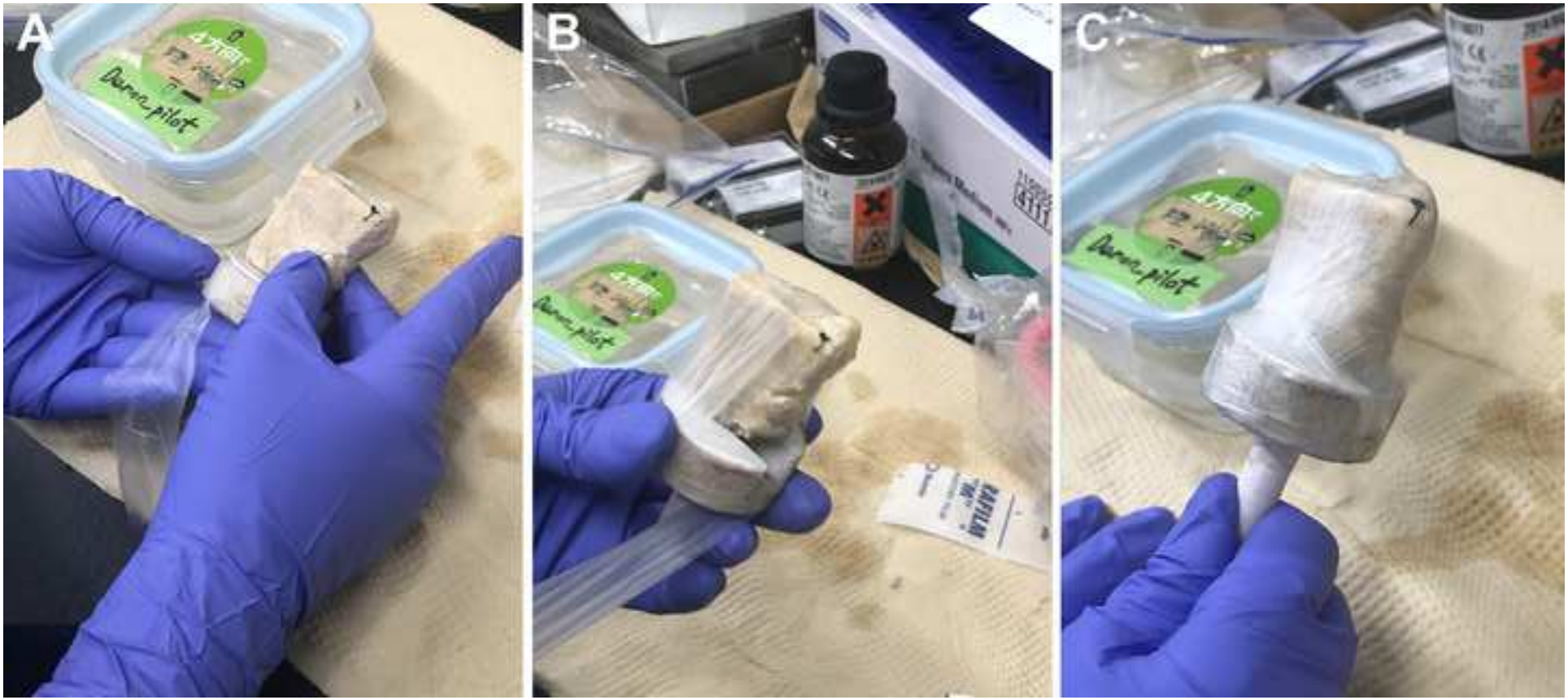

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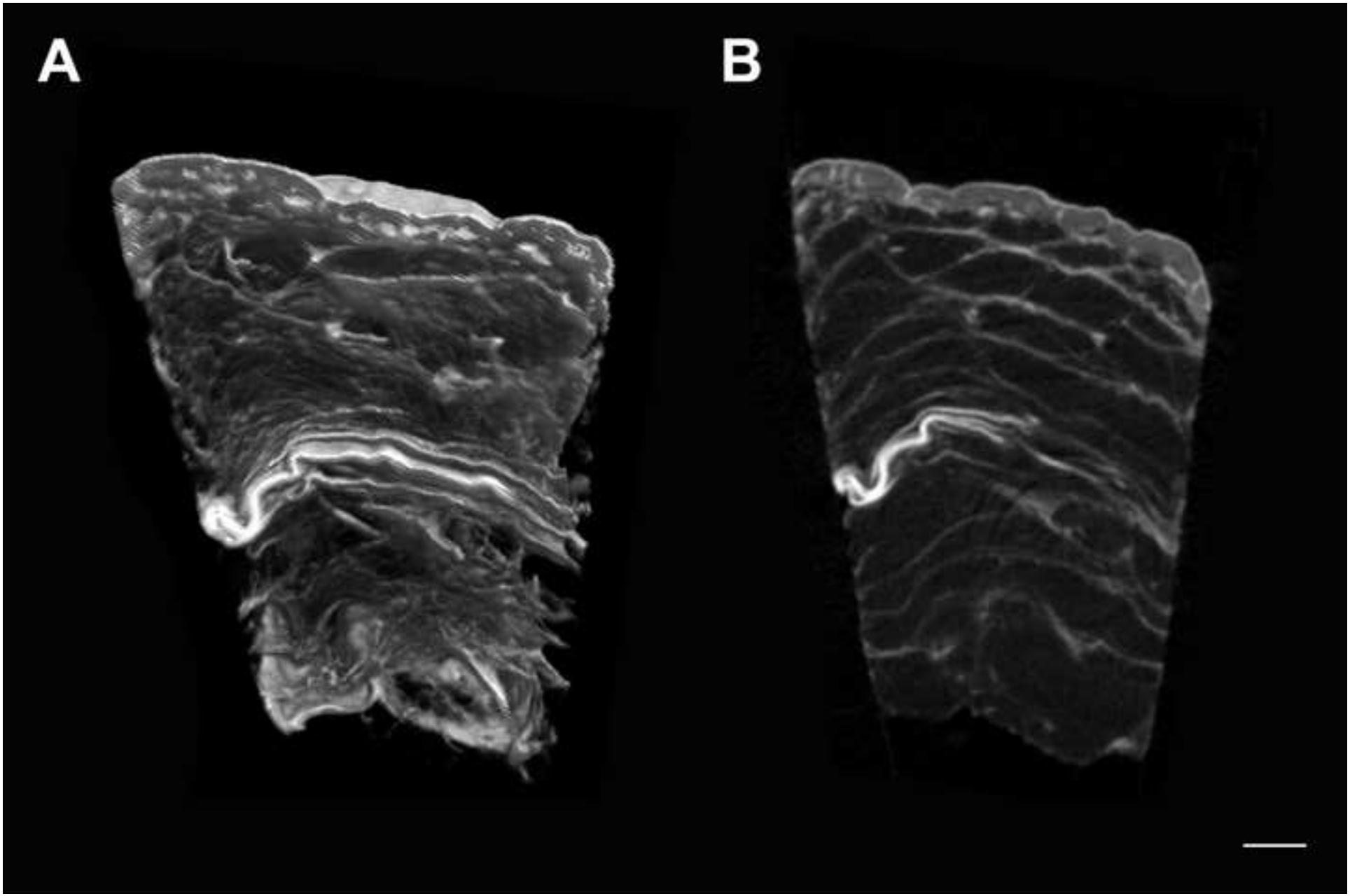

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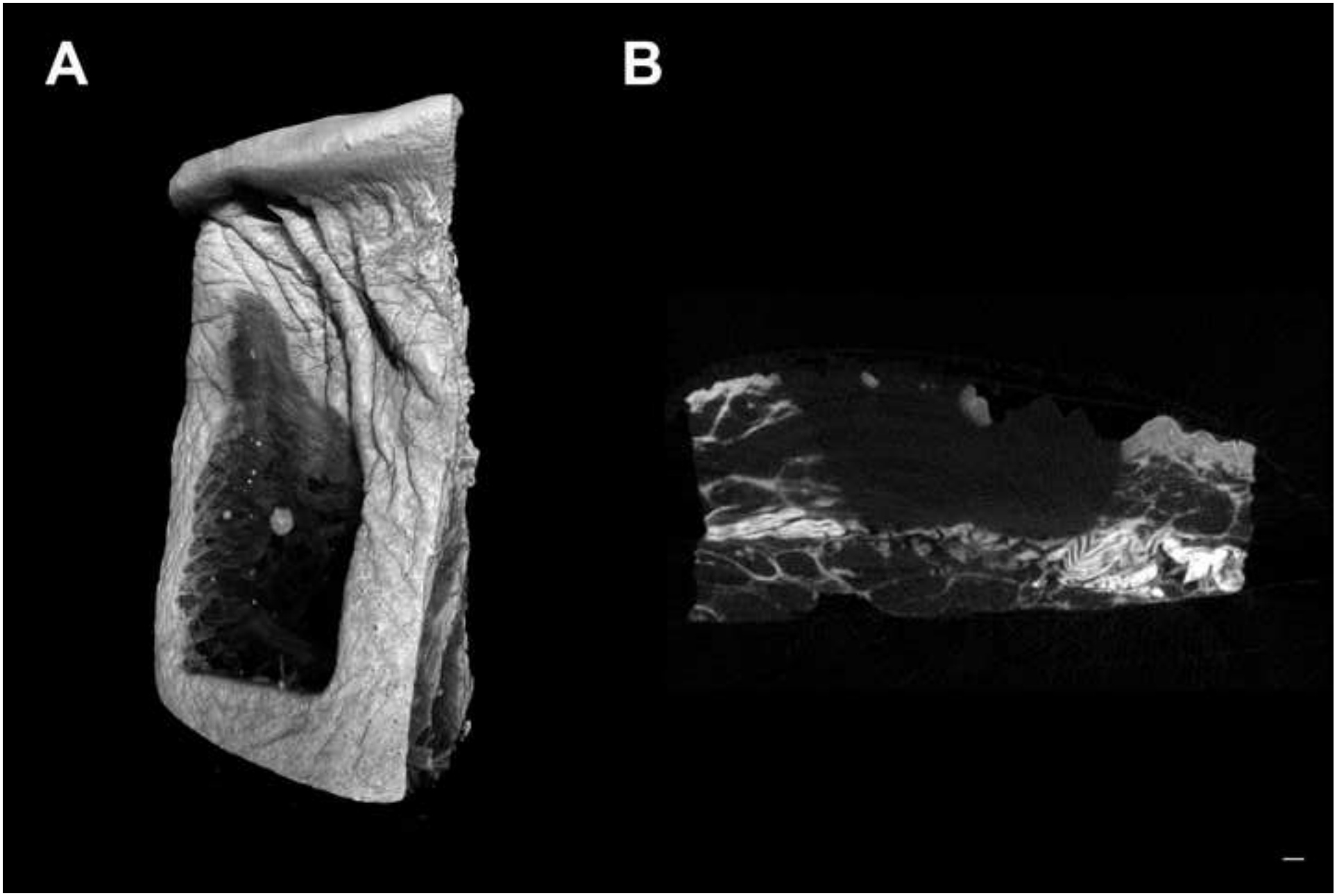

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