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# Microfocus X-ray computed tomography(microCT) imaging of Actinia equina(Cnidaria), Harmothoe sp.(Annelida), and Xenoturbella japonica(Xenacoelomorpha)

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**TITLE:**

# Microfocus X-ray CT (microCT) Imaging of *Actinia equina* (Cnidaria), *Harmothoe* sp. (Annelida), and *Xenoturbella japonica* (Xenacoelomorpha)

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**KEYWORDS:**

microCT, Lugol solution, iodine, *Actinia*, Cnidaria, *Harmothoe*, Annelida, *Xenoturbella*, Xenacoelomorpha, invertebrates

**SUMMARY:**

Here, protocols for performing microfocus X-ray computed tomography (microCT) imaging of three marine invertebrate animals are explained in detail. This study describes steps such as sample fixation, staining, mounting, scanning, image reconstruction, and data analyses. Suggestions on how the protocol can be adjusted for different samples are also provided.

**ABSTRACT:**

Traditionally, biologists have had to rely on destructive methods such as sectioning in order to investigate the internal structures of opaque organisms. Non-destructive microfocus X-ray computed tomography (microCT) imaging has become a powerful and emerging protocol in biology, due to technological advancements in sample staining methods and innovations in microCT hardware, processing computers, and data analysis software. However, this protocol is not commonly used, as it is in the medical and industrial fields. One of the reasons for this limited use is the lack of a simple and comprehensible manual that covers all of the necessary steps: sample collection, fixation, staining, mounting, scanning, and data analyses. Another reason is the vast diversity of metazoans, particularly marine invertebrates. Because of marine invertebrates' diverse sizes, morphologies, and physiologies, it is crucial to adjust experimental conditions and hardware configurations at each step, depending on the sample. Here, microCT

imaging methods are explained in detail using three phylogenetically diverse marine invertebrates: *Actinia equina* (Anthozoa, Cnidaria), *Harmothoe* sp. (Polychaeta, Annelida), and *Xenoturbella japonica* (Xenoturbellida, Xenacoelomorpha). Suggestions on performing microCT imaging on various animals are also provided.

**INTRODUCTION:**

Biological researchers generally have had to make thin sections and perform observations by light or electron microscopy in order to investigate the internal structures of opaque organisms. However, these methods are destructive and problematic when applied to rare or valuable specimens. Furthermore, several steps in the method, such as embedding and sectioning, are time consuming, and it can take several days to observe a sample, depending on the protocol. Moreover, when handling numerous sections, there is always a possibility of damaging or losing some sections. Tissue-clearing techniques are available for some specimens[1-5] but are not yet applicable for many animal species.

To overcome these problems, some biologists have started using microfocus X-ray computed tomography (microCT) imaging[6-15]. In X-ray CT, the specimen is irradiated with X-rays from various angles that are generated from an X-ray source moving around the sample, and the transmitted X-rays are monitored by a detector that also moves around the sample. The X-ray transmission data obtained are analyzed to reconstruct cross-sectional images of the specimen. This method enables the observation of internal structures without destruction of the sample. Because of its safety and ease, it is commonly used in medical and dental applications, and CT systems can be found in hospitals and dental centers worldwide. Moreover, industrial X-ray CT is frequently used for observing non-medical samples for inspection and metrology in the industrial field. In contrast to medical CT, in which the X-ray source and the detectors are mobile, the two parts are fixed in industrial CT, with the sample rotating during scanning. Industrial CT generally produces higher resolution images than medical CT and is referred to as microCT (micrometer-level resolution) or nanoCT (nanometer-level resolution). Recently, research using microCT has rapidly increased in various fields of biology[14-34].

Biological studies using CT initially targeted internal structures that mainly consist of hard tissue, such as bone. Advances in staining techniques using various chemical agents enabled the visualization of soft tissues in various organisms[6-9,14-34]. Of these reagents, iodine-based contrast agents are relatively safe, inexpensive, and can be used for the visualization of soft tissues in various organisms[7,14]. Concerning marine invertebrates, microCT has been widely used on such animals as molluscs[6,25,32,33], annelids[18-20,28], and arthoropods[21,23,29,31]. However, there have been few reports on other animal phyla, such as bryozoans[6], xenacoelomorphs[26], and cnidarians[24,30]. In general, there have been fewer studies using microCT on marine invertebrates than those on vertebrates. One major reason for this limited use on marine invertebrates is the vast diversity observed in these animals. Because of their diverse sizes, morphologies, and physiologies, each species reacts differently to different experimental procedures. Therefore, it is crucial during sample preparation to choose the most appropriate fixation and staining reagent, and to set conditions at each step, adjusted for each species. Similarly, it is also necessary to set the

scanning configurations, such as the mounting method, voltage, current, mechanical magnifying rate, and the space resolution power, appropriately for each sample. To overcome this problem, a simple and comprehensible manual that covers all of the necessary steps, explains how each step can be adjusted depending on the specimen, and shows detailed examples from multiple samples is essential.

In the present study, we describe the microCT imaging protocol step-by-step, from sample fixation to data analysis, using three marine invertebrate species. Specimens of the sea anemone *Actinia equina* (Anthozoa, Cnidaria) were collected near the Misaki Marine Biological Station, University of Tokyo. They had a spherical, soft body that was about 2 cm in diameter (**Figure 1A-C**). *Harmothoe* sp. (Polychaeta, Annelida) samples were also collected near Misaki Marine Biological Station. They were slender worms that were about 1.5 cm in length, with tough chaetae present along the whole body (Fig. 1D). A *Xenoturbella japonica*[35] (Xenoturbellida, Xenacoelomorpha) specimen was collected near Shimoda Marine Research Center, University of Tsukuba. It was a soft-bodied worm that was about 0.8 cm in length (**Figure 1E**). Adjustments made for the conditions and configurations of each sample are explained in detail. Our study provides several suggestions on how to perform microCT imaging on marine invertebrates, and we hope that it will inspire biologists to utilize this protocol for their research.

**PROTOCOL:**

**1. Fixation**

1.1. For *Actinia equina*, relax the animals in 10% $MgCl_2$ seawater for about 15 min at room temperature. Transfer to 70% ethanol and store at room temperature.

1.2. For *Harmothoe* sp., anesthetize the animals by placing them in ice-cold seawater for about 15 min. Transfer to 10% (v/v) formalin solution with seawater and store at room temperature.

1.3. For *Xenoturbella japonica*, relax the animal using 7% $MgCl_2$ in freshwater. Fix in 4% paraformaldehyde (PFA) in filtered seawater overnight. Place in 70% ethanol and store at 4 °C.

CAUTION: PFA is hazardous and must be handled with care.

**2. Staining**

2.1. Transfer the samples to 50% ethanol and store at room temperature for 15 h. Replace the 50% ethanol with 25% ethanol and store at room temperature for 2 h.

NOTE: This is not necessary for the *Harmothoe* sp. sample in 10% (v/v) formalin solution with seawater.

2.2. Replace the solution with distilled water (DW) and store the samples in DW at room temperature for 2 h. Repeat this step three times.

2.3. Prepare 25% Lugol solution by diluting the stock solution (below) to 25% with DW. Stock solution (100% Lugol solution) contains 10 g of KI and 5 g of $I_2$, adjusted to 100 mL with DW.

NOTE: Lugol solution is light-sensitive, so store and handle the solution protected from light. Follow the regulations of each country and institution for iodine handling and waste disposal.

2.4. Decant the DW from the samples and pour in the 25% Lugol solution. Stain for 24 h at room temperature.

**3. Stage Mounting**

3.1. Prepare 0.5% agarose by dissolving 500 mg agarose in 100 mL of DW in a 250 mL conical flask in a microwave (800 W, about 1–3 min). Cool to about 30–40 °C by keeping at room temperature.

CAUTION: Do not overheat or completely seal the flask when heating to prevent the agarose from boiling over.

3.2. Mount large (>2 cm) samples using a 50 mL tube.

3.2.1. Place the stained sample in a 60-mm dish with DW to wash off excessive staining solution from the surface.

3.2.2. Gently pour 5 mL of 0.5% agarose into a 50 mL tube and harden the agarose on ice. Be careful not to make bubbles in the agarose.

3.2.3. Gently add 20 mL of 0.5% agarose to the 50-mL tube and place the specimen within the 0.5% agarose using forceps. Be careful not to make bubbles in the agarose.

3.2.4. Adjust the position and orientation of the sample with forceps and harden the agarose on ice.

3.2.5. Place clay on the microCT mounting stage and set the 50 mL tube on the clay (**Figure 2A**).

3.3. Mount small (<2 cm) samples using a 1000 µL micropipette 'blue' tip.

3.3.1. Draw up 100 µL of 0.5% agarose into a 1000 µL micropipette 'blue' tip and harden the agarose on ice, making a plug in the tip (**Figure 2B-a**).

3.3.2. Decant the stained sample into a 60 mm dish without using forceps.

3.3.3. Gently transfer the sample using ring tweezers into another 60-mm dish with DW to wash off excessive staining solution from the surface.

3.3.4. Add 1000 µL of either DW or 0.5% agarose into the plugged tip (step 3.3.1) using a micropipette.

3.3.5. Gently transfer the sample from the 60-mm dish into the DW or agarose in the plugged tip using ring tweezers.

3.3.6. Gently adjust the position and orientation of the sample with a petiolate needle or precision tweezers so that the sample is stable between the walls of the tip. Be careful not to make bubbles in the agarose. Harden the agarose on ice.

3.3.7. Cut the tip off a new 1000 µL micropipette 'blue' tip (**Figure 2B-b,c**) and insert the tip of the plugged tip into the new tip.

3.3.8. Place clay on the microCT mounting stage and set the tips with the sample inside on the clay (**Figure 2C,D**).

NOTE: The staining solution will start to wash off the sample once it is placed in DW, so proceed to the next scanning step promptly.

**4. MicroCT scanning**

4.1. Turn on the X-ray beam at 80 kV, 100 µA.

4.2. While observing the X-ray transmission image at the center of the screen (**Figure 3A**), move the stage so that the whole sample can be seen by clicking on the **X** and **Z axis** buttons (**Figure 3A**). Set the contrast of the image so that the internal structures can be observed by adjusting the contrast conditions (**Figure 3A**: **Image contrast**).

4.3. Adjust the orientation of the sample by changing the angle of the tube/tip in the clay (**Figure 2B**). Rotate the stage 90° by setting the rotation axis (**Figure 3A**) to 90 and clicking on the relative movement button (**Figure 3A**). Perform the same maneuver four times to complete a full rotation.

NOTE: Manually turn off the X-ray beam each time the sample door is opened, unless the system turns it off automatically.

4.4. Move the stage so that the sample is at the center of the view by clicking on the **Z axis** button (**Figure 3A**) and by manually adjusting the Y axis knob on the mounting stage (**Figure 3B**). Turn the stage by 90° and do the same. Turn the stage 360° and check that the sample is at the center of the view from all directions.

4.5. Move the stage along the x-axis toward the X-ray beam source by clicking on the **X axis** button (**Figure 3A**) to enlarge the sample so that it just fits in the view (**Figure 3C**).

4.6. Turn the stage 360° and check that the sample fits within view from all directions.

4.7. Adjust the scanning conditions as shown in **Table 1**.

4.8. Start scanning; it takes about 10 min.

**5. Image reconstruction**

5.1. Start up the microCT system's accessory software (see the **Table of Materials**) and open the scanned data.

5.2. Adjust differences in the rotation axis of the sample during scanning by clicking on the automatic shift value calculation button (**Figure 4A**: green box).

5.3. Adjust the orientation of the image by rotating the orange arrows (**Figure 4B**). If the orientation was changed, repeat step 5.2.

5.4. Click on the area tab (**Figure 4C**: magenta box) and trim areas where samples are not present (**Figure 4C**: yellow box).

5.5. Click on the reconfiguration tab (**Figure 4D**: magenta box) and set the filters as follows to remove noise. Ring artifact reduce filter: Median filter -3; Noise elimination filter: Average filter-1.

5.6. Perform reconfiguration by clicking on the reconfiguration button (**Figure 4D:** green box).

5.7. Adjust image brightness and contrast by setting the black and white values as black value 0, white value 250 (**Figure 4D**: blue box).

5.8. Save the reconstructed TIFF image dataset as an 8-bit TIFF by clicking on the save button. Rename TIFF files as following: Date_sample_resolution (µm)_number.tiff.

NOTE: The original microCT datasets from this study are available in the Figshare repository, doi:10.6084/m9.figshare.7670837[36].

**6. Data analyses**

6.1. Start up the data analysis software (see the **Table of Materials**) and enable importing of TIFF files by clicking on the **Database** icon (**Figure 5A**: magenta box) and turning off the box shown in **Figure 5B**.

6.2. Click on the **import** icon (**Figure 5C**: magenta box), select the dataset saved in section 5, and click **open**.

6.3. Click on the **copy links** button (**Figure 5D**) to import the data.

6.4. Display the 2D cross-section by clicking on the **2D viewer** icon (**Figure 5C**: blue box).

6.5. Calibrate the dataset by clicking on the **3D viewer** tab (**Figure 5E**: magenta box) and entering the resolution value at scanning (which was 0.018 in this study [**Figure 5F**]).

6.6. Click on the **brightness/contrast** icon (**Figure 5E** green box). Adjust the brightness and contrast by moving the cursor inside the displayed 2D image and changing the window level and window width (**Figure 5G**).

6.7. Check other cross-section images by moving the scrollbar (**Figure 5G:** box).

6.8. Change the orientation of the cross-section by clicking on the orientation icon (**Figure 5E**: blue box) and check images at all orientations (**Figure 5H**).

6.9. Click on the export icon to store cross-section images to save.

**REPRESENTATIVE RESULTS:**

We performed microCT imaging on *A. equina* (Anthozoa, Cnidaria), *Harmothoe* sp. (Polychaeta, Annelida), and *X. japonica* (Xenoturbellida, Xenacoelomorpha) after staining the samples with 25% Lugol solution. The staining successfully enhanced the contrast of the internal structures in all specimens, enabling observations of internal soft tissues (**Figure 6**). Together with past reports[6,7,16,19,22-26,28,30-33], this shows that microCT can be used on various marine invertebrates for visualizing their morphology, including soft internal tissues. Clear images were obtained even with the *X. japonica* specimen, whose epidermis was badly damaged (**Figure 6F,G**), showing that this method is applicable to fragile specimens with external damage.

Scanning only the region of interest, in contrast to a wider area, greatly increased the clarity and resolution of the image (compare **Figure 6F** and **Figure 6G**). However, a single high-resolution dataset of a whole specimen was reconstructed for *Harmothoe* sp. (**Figure 6C**) and *X. japonica* (**Figure 6**F) from multiple scans performed on different (but overlapping) parts of the specimen. The seams between each scan were inconspicuous in the reconstructed images. Our study shows that single high-resolution images can be obtained with cone-beam microCT systems. By scanning a larger area at high resolution, there is a smaller risk of overlooking small structures. Another advantage is that it is easier to locate the relative positions of structures that are situated far apart, such as the anterior and posterior tips of an elongated annelid.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Marine invertebrate animals observed in this study.** (**A**–**C**) *Actinia equina* (Anthozoa, Cnidaria). (**A**) Distal end of a live animal relaxed in 10% $MgCl_2$ seawater. Distal (**B**) and proximal (**C**) ends after fixation in 70% ethanol. (**D**) Live anesthetized *Harmothoe* sp. (Polychaeta, Annelida), dorsal view with anterior to the left. Most of the elytra were already missing at this stage, with only four remaining near the posterior end. (**E**) *Xenoturbella japonica* (Xenoturbellida, Xenacoelomorpha) fixed in 70% ethanol. Right view, with anterior to the top. Because of circumstances at collection, its epidermis was starting to come off. Scale bars = 3 mm.

**Figure 2: Mounting samples on the microfocus X-ray computed tomography system**. (**A**) Mounting samples in a 50 mL tube using clay. The orientation of the sample could be adjusted using the clay. (**B**) Preparation of a 1000 µL micropipette 'blue' tip for mounting small samples. a: Tip with its end plugged with 100 µL of 0.5% agarose (diagonal lines). The samples were placed in this tip. The tip with the sample was inserted into another 1000 µL micropipette 'blue' tip (b, c) for mounting. b was used for *Xenoturbella japonica*, and c was used for *Harmothoe* sp. (**C**) Mounted *X. japonica* sample, overview (left) and close up (right). X-ray source can be seen to the right of the sample. (**D**) Diagrams for mounting samples in a 1000 µL micropipette 'blue' tip. a: *X. japonica* sample in distilled water. b: the sample was in contact with the tip wall (arrows), so that it does not move while scanning. c: *Harmothoe* sp. sample in 0.5% agarose.

**Figure 3: Scanning samples on the microfocus X-ray computed tomography system**. (**A**) Operating screen during scanning of the microfocus X-ray computed tomography system showing an X-ray transmission image of an *Actinia equina* specimen. Adjust the contrast and brightness with the 'Image contrast' at the lower left. (**B**) View of the mounting stage showing the Y axis knob. (**C**) X-ray transmission image of the *A. equina* specimen after the mounting stage was moved closer to the X-ray beam source. Notice it is enlarged when compared to the image at the center of (A).

**Figure 4: Operating screen of the image reconstruction system**. (**A**) Screen for adjusting differences in the rotation axis of the sample during scanning, showing an *Actinia equina* specimen. Magenta box: shift tab; green box: automatic shift value calculation button. (**B**) Screen for adjusting the orientation of the image, with *Harmothoe* sp. shown. (**C**) Screen during the image reconstruction of *A. equina*, trimming the area outside the yellow box where no samples are present. Magenta box: area tab. (**D**) Screen during image reconstruction, showing the reconstructed image of *A. equina*. Magenta box: reconfiguration tab; green box: reconfiguration button; blue box: black and white value adjustment.

**Figure 5: Operating screen of the image analysis system**. (**A**) Preference window. The Database icon (magenta circle) was clicked to open the database file management window. (**B**) Database file management window. In this software, the box shown with an arrow needs to be off to enable importing TIFF files. (**C**) Menu and tool bars of the Database screen. Magenta box = import icon; blue box = 2D viewer icon. (**D**) Dataset import window. Magenta circle = copy links button. (**E**) Menu and tool bars of the 2D viewer screen. Magenta box = 3D viewer tab; green box =

brightness/contrast icon; blue box = orientation icon. (**F**) Calibration setting window. Enter the desired resolution values within the columns in the magenta box. (**G**) Cross-section of an *Actinia equina* specimen displayed in the 2D viewer window for adjusting brightness and contrast. Magenta box: scrollbar for checking other cross-sections. (**H**) Cross-section of *A. equina* displayed in the 2D viewer window with a different orientation to (**G**).

**Figure 6: Scanned and reconstructed images of marine invertebrates.** (**A**) Transverse and (**B**) longitudinal sections of *Actinia equina*. The area inside the dotted yellow box in (B) is enlarged in the inset. Abbreviations: dm, pair of directive mesenteries; m, pair of perfect mesenteries; mf, mesenterial filament; p, pharynx; si, siphonoglyphs; t, tentacle; arrows, oral disc; white arrowheads, pedal disc; black arrowheads, sphincter muscle. Scale bars in A, B = 3 mm. (C–E) *Harmothoe* sp. (**C**) Sagittal section of the anterior part. (**D**, **E**) Transverse section at the dotted lines d and e in (C). Abbreviations: aci = aciculum; acim = acicular muscle; coe = coelom; dlm = dorsal longitudinal muscle; elp = elytrophore; eye = eye; int = intestine; jaw = jaw; mant = median antenna; mo = mouth; mp = muscles of proboscis; palp = palp; pha = pharynx; prob = proboscis; vlm = ventral longitudinal muscle; vnc = ventral nerve cord. Scale bars: C = 1 mm; D, E = 0.3 mm. (**F**, **G**) *Xenoturbella japonica*. (**F**) Sagittal section of the whole sample. (**G**) Sagittal section of the anterior part. bl = basal lamina; int = intestine; ml = muscle layer; mo = mouth; nn = intraepidermal nerve net; white arrow = statocyst; black arrow = frontal pore; white arrowheads = ventral glandular network; black arrowheads = oocytes. Scale bars: F = 1 mm, G = 0.5 mm.

**Table 1: Sample preparation and scanning protocol for each specimen.**

**DISCUSSION:**

Fixatives using formalin, such as the 10% (v/v) formalin solution in seawater used in this study, are known to preserve the morphology of diverse marine invertebrates and are often used for microCT imaging[18,24-26,28,30,33]. However, restrictions on the use of this chemical have become strict in some countries in recent years, and substitutes such as paraformaldehyde or glutaraldehyde may be used. If there are plans to extract DNA after scanning, it is better to avoid using formalin as a fixative, because it is known to fragment DNA. In this case, the use of fixatives that preserve DNA, such as 70% ethanol, is recommended. In this study, the cnidarian *A. equina* was fixed using 70% ethanol, and clear microCT images were obtained from the 70% ethanol-fixed samples (**Figure 6A, B**).

In a previous study that performed microCT scanning of various cnidarian taxa, many samples were dehydrated in 100% ethanol, and some were critical-point-dried prior to scanning[24]. Although soft internal organs such as tentacle clusters, muscles, and gonads were successfully observed in their study, dehydration and drying processes are known to result in major artefacts such as the deformation and contraction of soft tissues[11,21]. In the present study, we were able to observe the internal structures of the cnidarian *A. equina* fixed in 70% ethanol and stained with 25% Lugol solution (**Figure 6A,B**). Our protocol, without any dehydration or drying steps, is preferable, and should be performed whenever possible to reduce the risk of damage to the specimens and artefacts during scanning.

Lugol solution, iodine solution, and phosphotungstic acid (PTA) are staining solutions that are often used on biological samples in microCT imaging[6,7,9,14,16,17,20,26,27,38]. From our experience of using various biological samples, Lugol solution provided the best results for many samples, with dark staining in a relatively short amount of time. Iodine solution yielded only very weak stains, and PTA required a long time for sufficient staining and the stained specimens showed strong contractions. Therefore, all specimens were stained with Lugol solution in this study. However, although Lugol solution is recommended, the appropriate staining solution differs between specimens, and we suggest that trials using other staining solutions be performed if there are enough specimens. Irrespective of the staining solution, samples do contract during staining[37,38], so it is important to keep the staining time short.

A critical step in microCT scanning is to mount the sample so as to prevent it from moving. In this study, this was performed in two steps, first using agarose as the direct mounting medium, and then using clay to mount the tube that contained the sample to the stage. For the first step, various low-density mounting media have been used in previous studies, including ethanol[6,17,20,25,30], agarose[9,29], and floral foam[15,22,31]. Agarose was selected in this study as it is a low-cost chemical that is accessible worldwide. A disadvantage of agarose is that it may be difficult to retrieve the sample from the hardened agarose after scanning but using low-melting-point agarose makes this retrieval step easier. For the second step, jaw clamps or screws are often used[6,9,17]. Clay was selected in this study as it enables fine adjustments in the orientation and angle of the sample. Caution is needed for experiments with long scanning times, as the possibility of the sample moving is higher when using clay rather than jaw clamps or screws.

A previous study conducted microCT scanning on seven polychaete species with body lengths of 2–8 mm, smaller than the *Harmothoe* sp. used in this study[16]. They were able to generate high-resolution images, and showed organs such as vascular systems and individual chaeta clearer than in the present study. The main cause of this difference was not the protocol, but the specifications of the microCT systems used. The system used in the previous study was equipped with an 11-megapixel charge-coupled device camera (4000 x 2672 pixels) with a maximum resolution of <0.8 μm/pixel[16]. The active image matrix size of the system used in this study was 992 x 992 pixels, with a maximum resolution of >5 μm/pixel. Therefore, the spatial resolution of the microCT system used in this study was inferior to the high performance microCT system used in Faulwetter et al.[16]. This difference was particularly noticeable when scanning specimens smaller than 8 mm, in which we experienced a lack of resolution (data not shown). However, because fewer data were obtained during scanning in this study, the scanning time was much shorter than in the previous study[16] (data: 992 x 992 and 4000 x 2672 pixels, respectively; scanning time: 10 to 26 min and 30 min to several hours, respectively). A short scanning time reduces the discoloration of the iodine staining, allowing the use of Lugol solution, which is a good staining solution with a high penetration rate, but easily diffuses in DW[34]. A short scanning time also decreases the possibility of the sample moving during scanning, which enabled the use of a simple mounting method using agarose or DW (**Figure 2**). Longer scanning times also have the disadvantage of possible sample shrinkage blur in images. Several other mechanical and

hardware problems that can occur during long scans have also been reported[39]. Therefore, when using microCT systems, it is important to accurately understand the specification of each system, and to choose the right system in terms of specimen size or research aim. In some cases, a microCT system with low resolution may be sufficient.

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**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES:**

1. Susaki, E. A., Tainaka, K., Perrin, D., Yukinaga, H., Kuno, A., Ueda, H. R. Advanced CUBIC protocols for whole-brain and whole-body clearing and imaging. *Nature Protocols.* **10**, 1709 – 1727, (2015).

2. Susaki, E. A., Ueda, H. R. Whole-body and whole-organ clearing and imaging techniques with single-cell resolution: toward organism-level systems biology in mammals. *Cell Chemical Biology.* **23**, 137 – 157, (2016).

3. Silvestri, L., Costantini, I., Sacconi, L., Pavone, F. S. Clearing of fixed tissue: a review from a microscopist's perspective. *Journal of Biomedical Optics.* **21**, 081205, (2016).

4. Greenbaum, A., et al. Bone CLARITY: clearing, imaging, and computational analysis of osteoprogenitors within intact bone marrow. *Science Translational Medicine.* **9**, eaah6518, (2017).

5. Konno, A., Okazaki, S. Aqueous-based tissue clearing in crustaceans. *Zoological Letters.* **4**, 13, (2018).

6. 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, (2009a).

7. Metscher, B. D. MicroCT for developmental biology: a versatile tool for high-contrast 3D imaging at histological resolutions. *Developmental Dynamics.* **238** (3), 632 – 640, (2009b).

8. Degenhardt, K., Wright, A. C., Horng, D., Padmanabhan, A., Epstein, J. A. Rapid 3D phenotyping of cardiovascular development in mouse embryos by micro-CT with iodine staining. *Circulation Cardiovascular Imaging.* **3** (3), 314 – 322, (2010).

9. Metscher, B. D. X-ray microtomographic imaging of intact vertebrate embryos. *Cold Spring Harbor Protocols* **12**, 1462 – 1471, (2011).

10. Boistel, R., Swoger, J., Kržič, U., Fernandez, V., Gillet, B., Reynaud, E. G. The future of three-dimensional microscopic imaging in marine biology. *Marine Ecology.* **32**, 438 – 452, (2011).

11. Mizutani, R., Suzuki, Y. X-ray microtomography in biology. *Micron.* **43**, 104 – 115, (2012).

12. Merkle, A. P., Gelb, J. The ascent of 3D X-ray microscopy in the laboratory. *Microscopy Today.* **21**, 10 – 15, (2013).

13. Ziegler, A., Menze, B. H. Accelerated acquisition, visualization, and analysis of zooanatomical data. In: Zander, J., Mosterman, P. J., eds. *Computation for humanity. Information technology to advance society.* CRC Press, Boca Raton, USA, 233 – 260, (2013).

14. Gignac, P. M., et al. Diffusible iodine-based contrast-enhanced computed tomography (diceCT): an emerging tool for rapid, high-resolution, 3-D imaging of metazoan soft tissues. *Journal of Anatomy.* **228** (6), 889 – 909, (2016).

15. du Plessis, A., Broeckhoven, C., Guelpa, A., le Roux, S. G. Laboratory x-ray micro-computed tomography: a user guideline for biological samples. *GigaScience*. **6** (6), 1 – 11, (2017).

16. Faulwetter, S., Vasileiadou, A., Kouratoras, M., Dailianis, T., Arvanitidis, C. Micro-computed tomography: Introducing new dimensions in taxonomy. *ZooKeys*. **263**, 1 – 45, (2013).

17. Staedler, Y. M., Masson, D., Schonenberger, J. Plant tissues in 3D via X-ray tomography: simple contrasting methods allow high resolution imaging. *PLoS One.* **8** (9), e75295, (2013).

18. Fernández, R., Kvist, S., Lenihan, J., Giribet, G., Ziegler, A. Sine Systemate Chaos? A Versatile Tool for Earthworm Taxonomy: Non-Destructive Imaging of Freshly Fixed and Museum Specimens Using Micro-Computed Tomography. *PLoS One.* 9 (5), e96617, (2014).

19. Paterson, G. L. J., et al. The pros and cons of using micro-computed tomography in gross and microanatomical assessments of polychaetous annelids. *Memoirs of Museum Victoria*. **71**, 237 – 246, (2014).

20. Faulwetter, S., Dailianis, T., Vasileiadou, K., Kouratoras, M., Arvanitidis, C. Can micro-CT become an essential tool for the 21st century taxonomist? An evaluation using marine polychaetes. *Microscopy and Analysis.* **28**, S9 – S11, (2014).

21. Sombke, A., Lipke, E., Michalik, P., Uhl, G., Harzsch, S. Potential and limitations of X-ray micro-computed tomography in arthropod neuroanatomy: a methodological and comparative survey. *Journal of Comparative Neurology.* **523**, 1281 – 1295, (2015).

22. Landschoff, J., Plessis, A., Griffiths, C. L. A dataset describing brooding in three species of South African brittle stars, comprising seven high-resolution, micro X-ray computed tomography scans. *GigaScience*. **4** (1), 52, (2015).

23. Keiler, J., Richter, S., Wirkner, C. S. The anatomy of the king crab *Hapalogaster mertensii* Brandt, 1850 (Anomura: Paguroidea: Hapalogastridae) – new insights into the evolutionary transformation of hermit crabs into king crabs. *Contributions to Zoology.* **84** (2), 149 – 165, (2015).

24. Holst, S., Michalik, P., Noske, M., Krieger, J., Sötje, I. Potential of X-ray micro-computed tomography for soft-bodied and gelatinous cnidarians with emphasis on scyphozoan and cubozoan statoliths. *Journal of Plankton Research.* **38**, 1225 – 1242, (2016).

25. Moles, J., Wägele, H., Ballesteros, M., Pujals, Á., Uhl, G., Avila, C. The End of the Cold Loneliness: 3D Comparison between *Doto antarctica* and a New Sympatric Species of *Doto* (Heterobranchia: Nudibranchia). *PLoS One.* **11** (7), e0157941, (2016).

26. Nakano H., et al. A new species of *Xenoturbella* from the western Pacific Ocean and the evolution of *Xenoturbella*. *BMC Evolutionary Biology.* **17**, 245, (2017).

27. Tsuda, K., et al. KNOTTED1 Cofactors, BLH12 and BLH14, Regulate Internode Patterning and Vein Anastomosis in Maize. *Plant Cell.* **29** (5), 1105 – 1118, (2017).

28. Parapar, J., Candás, M., Cunha-Veira, X., Moreira, J. Exploring annelid anatomy using micro-computed tomography: A taxonomic approach. *Zoologischer Anzeiger.* **270**, 19 – 42, (2017).

29. Akkari, N., Ganske, A. S., Komerički, A., Metscher, B. New avatars for Myriapods: Complete 3D morphology of type specimens transcends conventional species description (Myriapoda, Chilopoda). *PLoS One*. **13** (7), e0200158, (2018).

30. Gusmao, L. C., Grajales, A., Rodriguez, E. Sea anemones through X-rays: visualization of two species of *Diadumene* (Cnidaria, Actiniaria) using micro-CT. *American Museum Novitates*. **3907** (2018).

31. Landschoff, J., Komai, T., du Plessis, A., Gouws, G., Griffiths, C. L. MicroCT imaging applied to description of a new species of *Pagurus Fabricius*, 1775 (Crustacea: Decapoda: Anomura:

559 Paguridae), with selection of three-dimensional type data. *PLoS One*. **13** (9), p.e0203107, (2018).
560
561 32. Machado, F. M., Passos, F.D., Giribet, G. The use of micro-computed tomography as a
562 minimally invasive tool for anatomical study of bivalves (Mollusca: Bivalvia). *Zoological Journal of*
563 *the Linnean Society*. zly054, (2018).
564
565 33. Sasaki, T. et al. 3D visualization of calcified and non-calcified molluscan tissues using
566 computed tomography. In: Endo, K., Kogure, T., Nagasawa, H., eds. *Biomineralization*. Springer,
567 Singapore, 83 – 93 (2018).
568
569 34. Maeno, A., Tsuda, K. Micro-computed Tomography to Visualize Vascular Networks in
570 Maize Stems. *Bio-protocol*. **8** (1), e2682, (2018)
571
572 35. Nakano, H., et al. Correction to: A new species of *Xenoturbella* from the western Pacific
573 Ocean and the evolution of *Xenoturbella*. *BMC Evolutionary Biology*. **18**, 83, (2018).
574
575 36. Maeno, A., Kohtsuka, H., Takatani, K., Nakano, H. MicroCT files from 'Microfocus X-ray
576 computed tomography (microCT) imaging of *Actinia equina* (Cnidaria), *Harmothoe* sp. (Annelida),
577 and *Xenoturbella japonica* (Xenacoelomorpha)'. *figshare*. (2019). doi:
578 10.6084/m9.figshare.7670837
579
580 37. Vickerton, P., Jarvis, J., Jeffery, N. Concentration-dependent specimen shrinkage in
581 iodine-enhanced microCT. *Journal of Anatomy* **223** (2), 185 – 193, (2013).
582
583 38. Buytaert, J., Goyens, J., De Greef, D., Aerts, P., Dirckx, J. Volume shrinkage of bone, brain
584 and muscle tissue in sample preparation for micro-CT and light sheet fluorescence microscopy
585 (LSFM). *Microscopy and Microanalysis* **20** (4), 1208 – 1217, (2014).
586
587 39. Sasov, A., Liu, X., Salmon, P. L. Compensation of mechanical inaccuracies in micro-CT and
588 nano-CT. *Proceedings of SPIE*. **7078**, 70781C, (2008).

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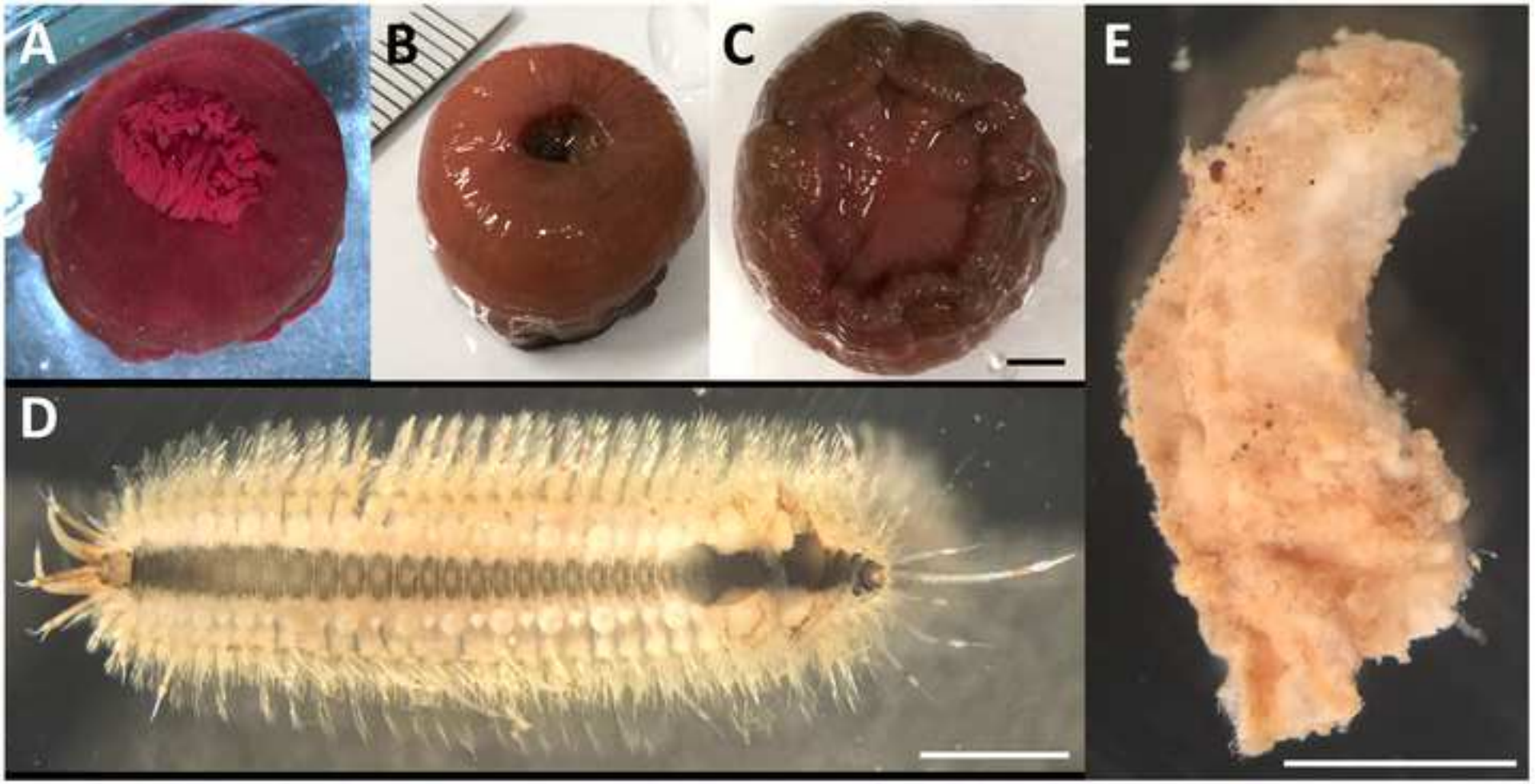

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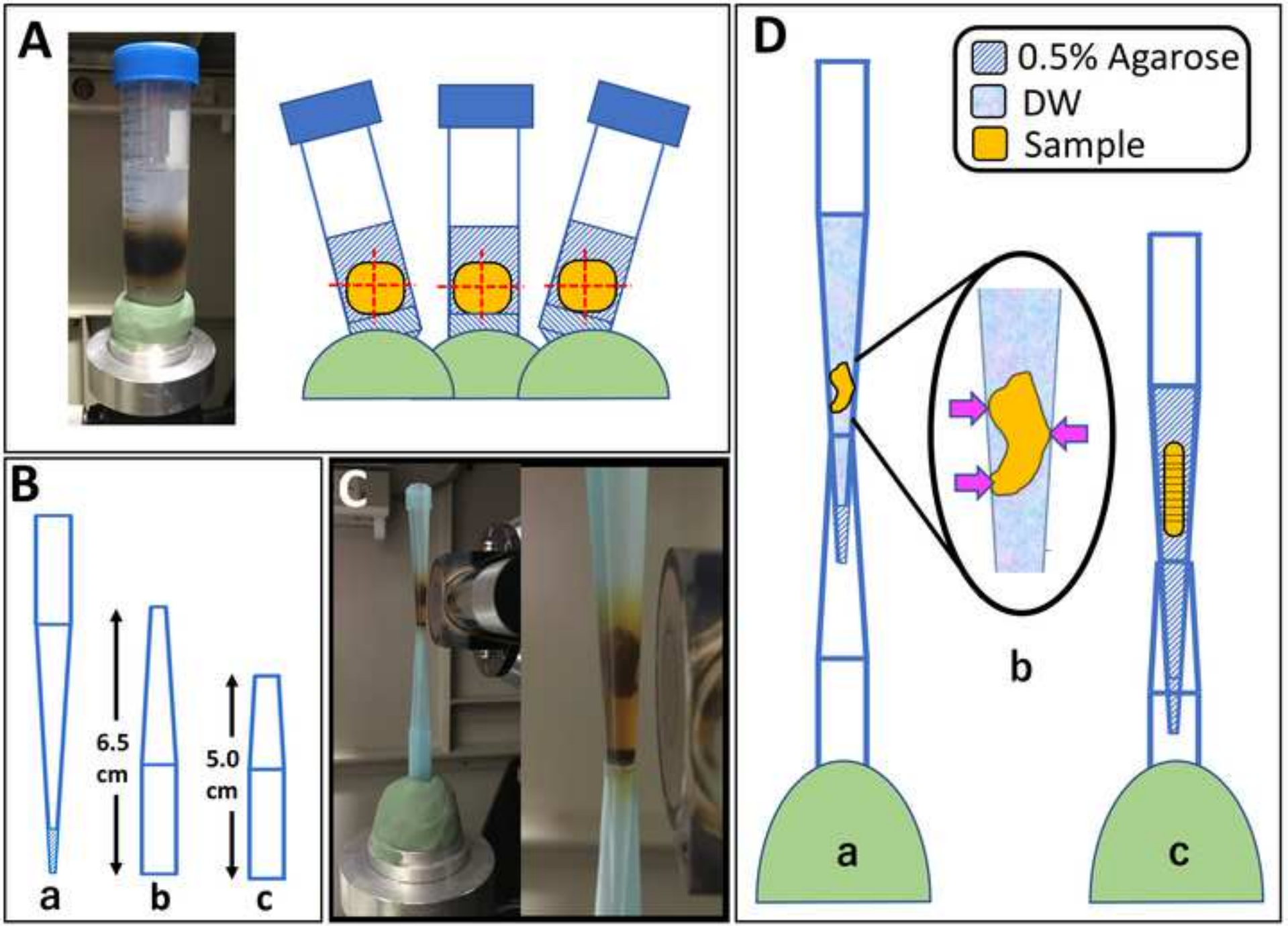

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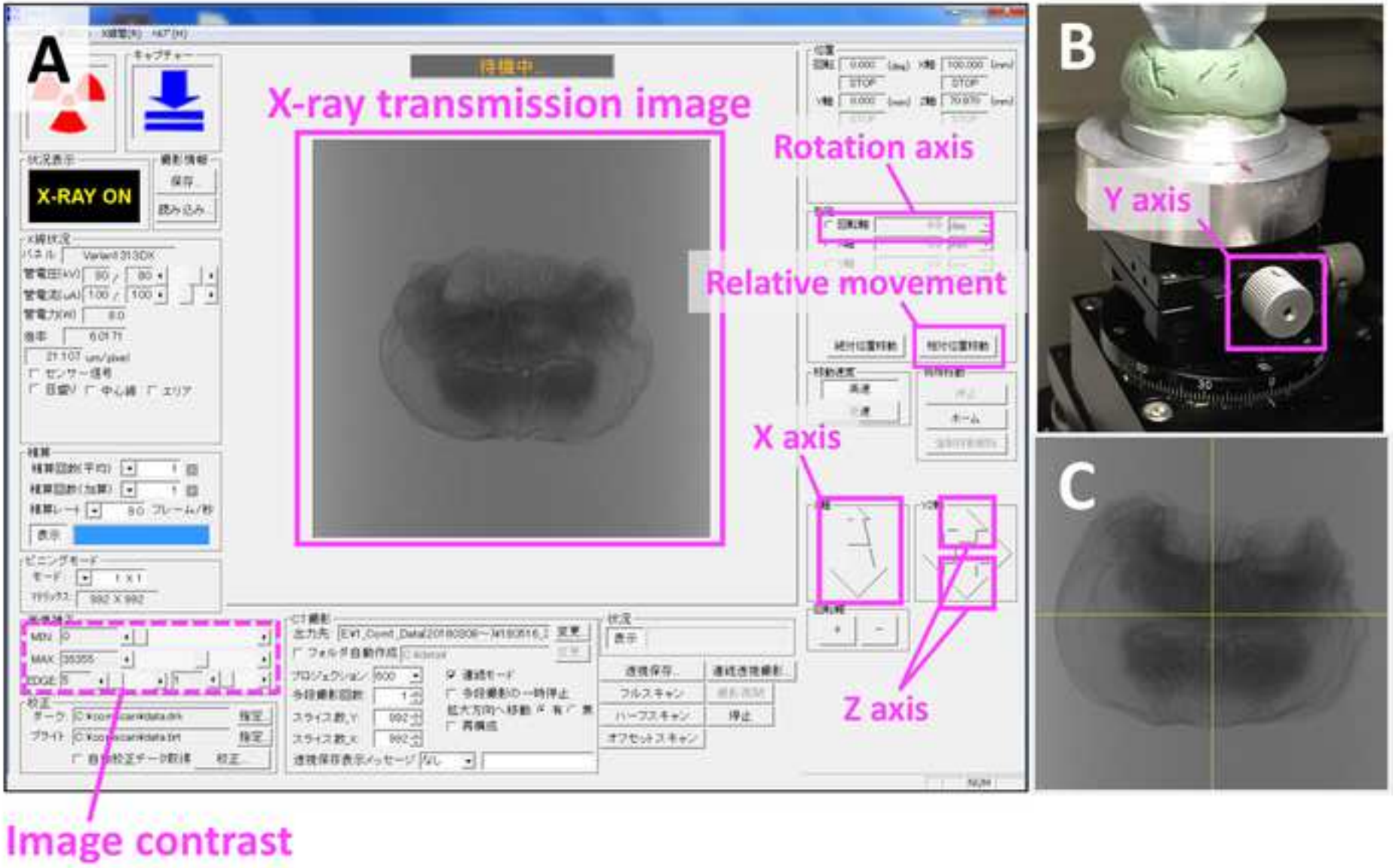

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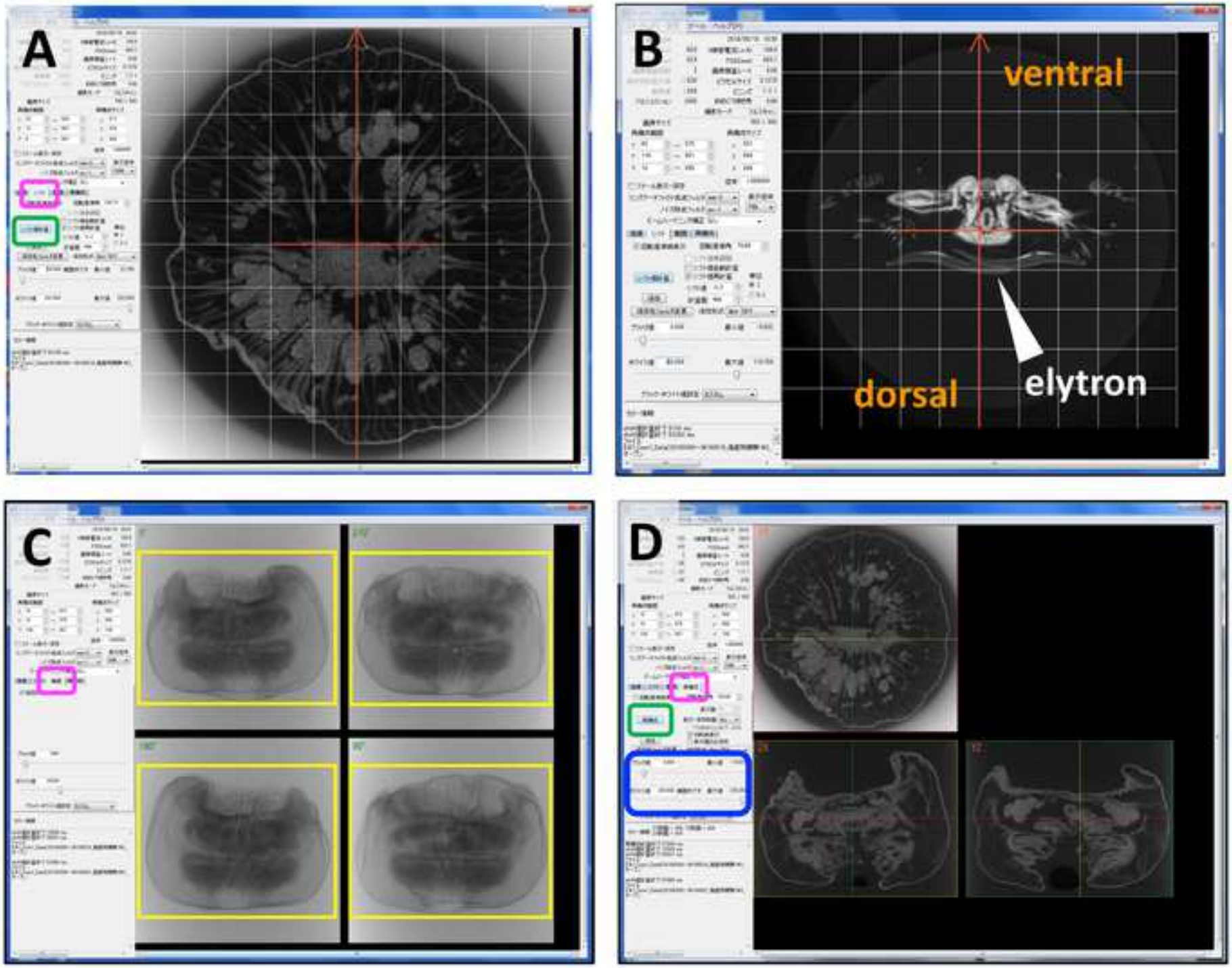

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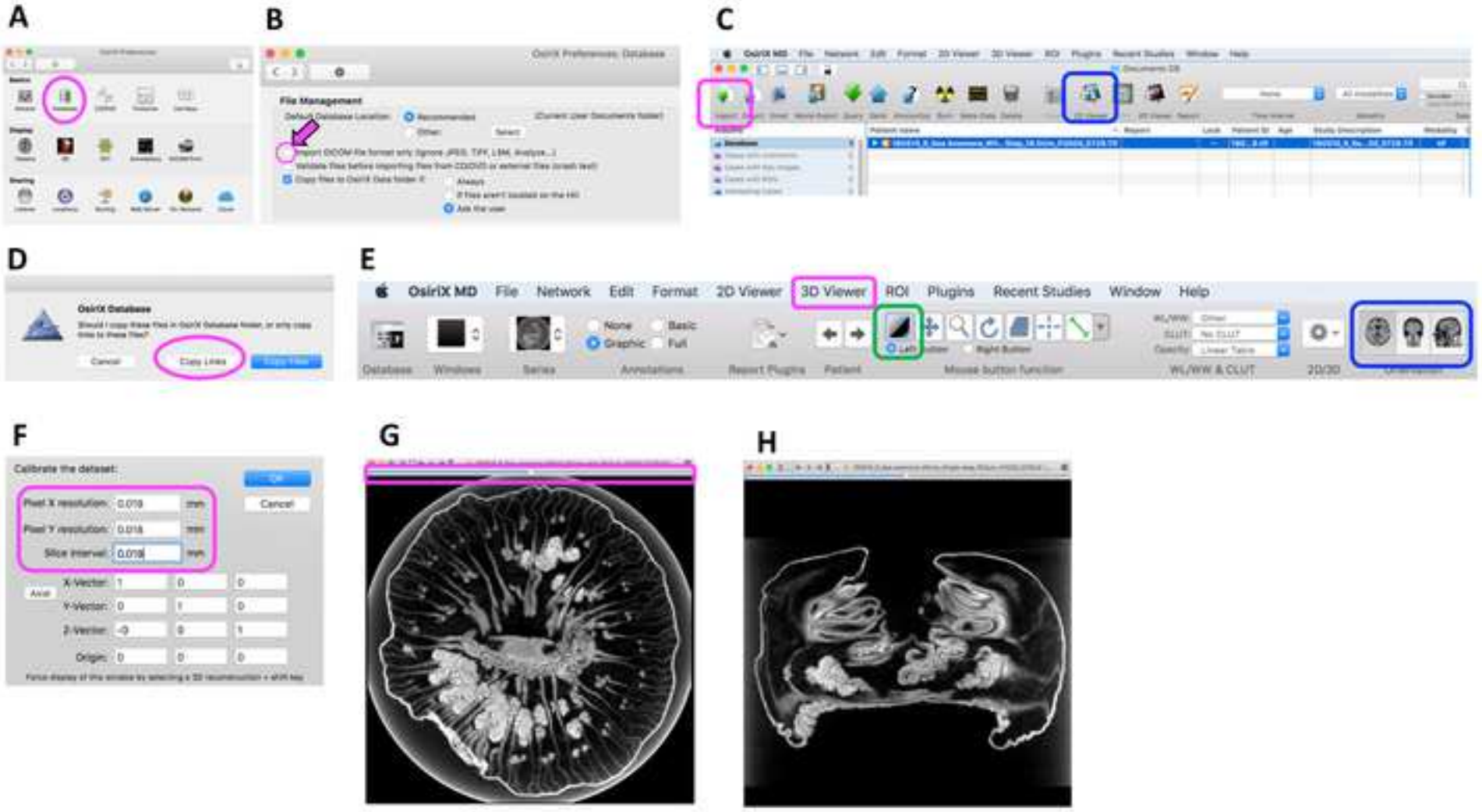

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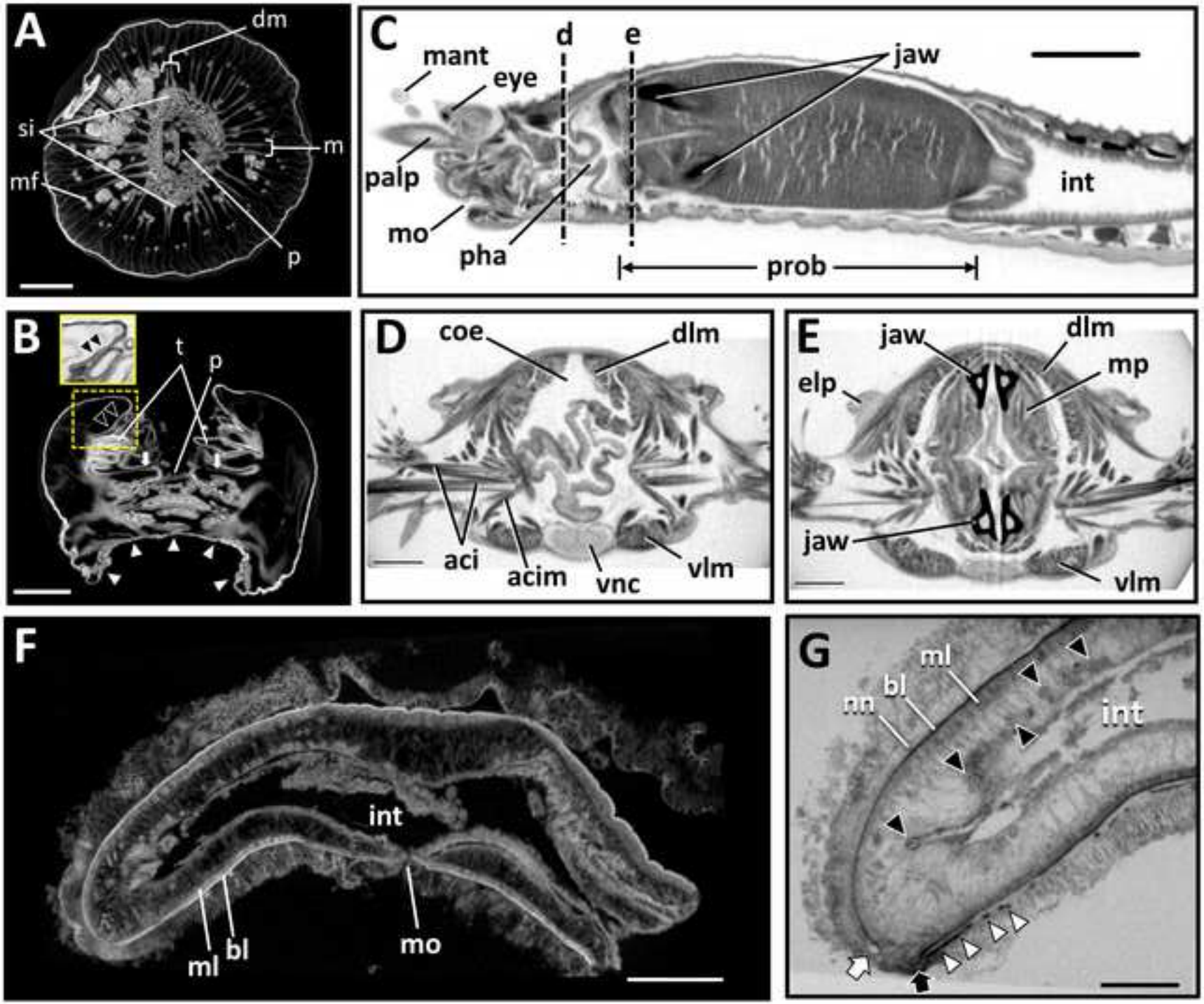

Table 1



**Table 1 : Details on sample preparation and scanning**

| | Deposition Number | Fixation and Storage | | Staining | | MicroCT Scanning | | | | | | | | | Data | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| specimen | Deposited in Shimoda Marine Research Center, University of Tsukuba | Fixative and storage solution | Storage time before staining | solution | Time | Mounting medium | Scanned parts | Voltage, current | Frame average | Frame rate | Rotation steps | Number of projections | Voxel size ( µm ) | Total scanning time (minutes) | Dataset size ( GB ) | Figure |
| *Actinia equina* (Anthozoa, Cnidaria) | SMRC-Actinia1 | 70% ethanol | 61 days | Lugol 1 day | | 0.5% agarose | whole body | 80 kV 100 µA | 2 | 8 fps | 0.18° | 2000 | 18.0 | 10 | 0.68 | 6A, B |
| *Harmothoe* sp. (Polychaeta, Annelida) | SMRC-Harmothoe1 | 10% formalin | | | | | whole body | | | | | | 6.5 | 26 | 2.07 | 6C |
| | | | | | | | anterior end | | | | | | 3.0 | 10 | 0.61 | 6D, E |
| *Xenoturbella japonica* (Xenoturbellida, Xenacoelomorpha) | SMRC-Xenoturbella1 | 4% PFA 70% ethanol | | | | DW | whole body | 60 kV 130 µA | | | | | 4.2 | 18 | 1.45 | 6F |
| | | | | | | | anterior end | | | | | | 2.8 | 10 | 0.80 | 6G |

PFA, paraformaldehyde; DW, distilled water; fps, frames per second

Table of Materials

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| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 250-ml Erlenmeyer flask | Corning | CLS430183 | |
| 5-ml  Sampling tube ST-500 | BIO-BIK | 103010 | |
| 50-ml Polypropylene tube | Greiner Bio One International | 227261 | |
| 60-mm Non-treated Dish | IWAKI | 1010-060 | |
| Agarose | Promega | V3125 | |
| Ecological grade tip (blue) 1000 µl | BMBio | BIO1000RF | |
| Ethanol | Wako Pure Chemical Industries | 057-00451 | |
| Formalin | Wako Pure Chemical Industries | 061-00416 | |
| Iodine | Wako Pure Chemical Industries | 094-05421 | |
| Magnesium chloride hexahydrate | Wako Pure Chemical Industries | 135-00165 | |
| OsiriX DICOM Viewer | Pixmeo SARL | OsiriX MD v10.0 | https://www.osirix-viewer.com |
| Paraformaldehyde | Wako Pure Chemical Industries | 163-25983 | |
| Petiolate needle | AS ONE | 2-013-01 | |
| Pipetman P200 Micropipette | GILSON | F123601 | |
| Pipetman P1000 Micropipette | GILSON | F123602 | |
| Potassium iodide | Wako Pure Chemical Industries | 166-03971 | |
| Precision tweezers 5 | DUMONT | 0302-5-PS | |
| QuickRack MultI fit tip (yellow) 200 ul | Sorenson | 10660 | |
| Razor blades | Feather | FA-10 | |
| Ring tweezers | NAPOX | A-26 | |
| Stereoscopic microscope | Leica | MZ95 | |
| X-ray Micro-CT imaging system | Comscantechno | ScanXmate-E090S105 | |

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>> All commercial language has been deleted and/or referred to in the Table of Materials and Reagents.

4. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

>> The protocol section has been revised accordingly.

5. Lines 137–150: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2–3 actions per step and a maximum of 4 sentences per step. Use sub–steps as necessary. Please move the discussion about the protocol to the Discussion.
>>This part has been rewritten, with the discussion moved elsewhere.

6. 2.4: Please specify what is stored at room temperature.
>> We have revised the sentence (samples at stored at room temperature).

7. 3.1.3.1, 3.2.2.1, 3.2.3.1: Please indicate the specific steps that are repeated here.
>> The specific steps have been indicated.

8. Lines 241–252: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
>> The paragraph has been moved to the Discussions.

9. 4.1.1–4.1.4, 4.2.1–4.2.11: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).
>> Software steps are more explicitly explained with, more specific details added.

10. Line 385: Please describe specific actions being performed in the imperative tense in complete sentences.
>> The specific steps have been indicated.

11. 5.5: Please specify the graded ethanol series used and describe how dehydration is done.
>> The dehydration steps have been specified.

12. Lines 401–404: Such experimental details should be included in a separate step.
>> Based on comments from the reviewers, this step was deleted from the protocol section.

13. Please combine some of the shorter Protocol steps so that individual steps contain 2–3 actions and maximum of 4 sentences per step.
>>The Protocol section have been revised according to this comment.

14. Please include single-line spaces between all paragraphs, headings, steps, etc.
>> The manuscript has been revised accordingly.

15. After you have made all the recommended changes to your protocol (listed above), 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.
>> Parts of the manuscript have been highlighted accordingly.

16. 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.
>> Parts of the manuscript have been highlighted accordingly.

17. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
>> Parts of the manuscript have been highlighted accordingly.

18. Please number the figures in the sequence in which you refer to them in the manuscript text. Currently, Figure 10 is introduced in line 286, before Figures 4–9.
>> This has been corrected.

19. Figures 1 and 7–9, supplemental video: Please include a space between the number and the units of the scale bar.
>> Number and units have been deleted from the figures.

20. Figure 4(C–F): Please include a scale bar for the images.
>> These images have been deleted.

21. Table 1: Please include a space between all numbers and their corresponding units.
>> Space has been inserted between the numbers and their corresponding units.

22. What is the attached video for? If it is to be included in the manuscript, please describe in the figure legend and reference it in the manuscript.
>> The video was deleted.

23. Table of Equipment and Materials: Please use SI abbreviations for all units (L, mL, µL) and include a space between all numerical values and their corresponding units (15 mL, 37 ° C, etc.). Please sort the items in alphabetical order according to the Name of Material/ Equipment.
>> The Table of Equipment and Materials has been revised according to these comments.

24. Discussion: As we are a methods journal, please also discuss critical steps within the protocol and any limitations of the technique.
>> Discussions on the critical steps in the protocol (e.g. fixation, staining and mounting) have been added.

25. References: Please do not abbreviate journal titles.
>> The journal titles are now fully written.

**Reviewers' comments:**
**Reviewer #1:**
Manuscript Summary:
In general the MS is well written and brings new ideas of protocols for the microtomography of some species of marine invertebrates.
Major Concerns:
Video:
The video is interesting and very well elaborated, shows in detail some important structures for the taxonomy of Polychaeta (*Harmothoe* sp). However, I really missed the other species. Why not include *A. equina* and *X. japonica* in the same video? In addition, the knowledge about Polychaeta using Micro-CT has already been widely discussed in many papers (some cited by the authors), while Cnidaria and Xenacoelomorpha not.

>> The video was deleted based on the comments of others.

Figures:
I suggest a standardization of the plates/figures. Note that in Figure 6 and 10, for example, there are no values above the scale bars while in the Figure 7 and 9 they were placed. I think that putting the values on the plates can generate a 'visual pollution', so I prefer to put this information in the legend of the figures.
>> Values above the scale bars have been deleted from these figures.

About the legend of the figures some of them are very large, perhaps it would be interesting to remove all the abbreviations and put them in a separated table. In addition, if this is not done, please insert the word 'Abbreviations' before them.
>> As most of the abbreviations are not used in multiple figures, we think it would be easier for the reader to find the abbreviation explanation in the legends rather than a separate table. Therefore, we have inserted the word 'Abbreviations' in the legends.

Specimens:
These specimens are deposited in a museum? I was not able to find the voucher numbers of these specimens in the text. If not, I strongly suggest that they be, since other researchers might want to access them, right?
>> The voucher numbers of these specimens were added to Table 1.

If possible, to insert in Table 1 the amount of time (days, hours) that the specimens were stored before the image acquisition.
>> The storage time of these specimens were added to Table 1.

Datasets' storage:
Where will be deposited all the micro-CT datasets?. The datasets should be available to all researchers for future reanalyzes of these species. It might be interesting to put all these datasets in the Harvard Dataverse, a free and online repository.
>> The datasets were deposited in the figshare repository, doi: 10.6084/m9.figshare.7670837 and will be made public after the publication of this manuscript.

Missing references:

I missed some important references on the use of Micro-CT for the study of marine invertebrates. I suggest that the references listed below should also be considered by the authors.

Ziegler A, Menze BH. 2013. Accelerated acquisition, visualization, and analysis of zoo-anatomical data. In: Zander J, Mosterman PJ, eds. Computation for humanity. Information technology to advance society. Boca Raton: CRC Press, 233-260.

Faulwetter S, Dailianis T, Vasileiadou K, Kouratoras M, Arvanitidis C. 2014. Can micro-CT become an essential tool for the 21st century taxonomist? An evaluation using marine polychaetes. Microscopy and Analysis 28: S9-S11.

Machado FM, Passos FD, Giribet G. 2018. The use of micro-computed tomography as a minimally invasive tool for anatomical study of bivalves (Mollusca: Bivalvia). Zoological Journal of the Linnean Society, https://doi.org/10.1093/zoolinnean/zly054.

>> We have inserted the above references to appropriate sentences.

Minor Concerns:

Contrast solution:

Why did the authors only use Lugol? Just for curiosity, I wonder why not PTA or Iodine?.

>> We added a paragraph in the discussion explaining the use of Lugol.

Table:

The Table 1 could be a bit more 'clean'. Maybe removing some borders/lines.

>> Some borders/lines were removed from Table 1.

**Reviewer #2:**

Manuscript Summary:

Well done on a nice paper, and especially the figure 7 is very good microCT visualizations. I enjoyed reading the paper and have some comments which will help improve it. The same must be done for the short abstract.

>> The short abstract has been rewritten according to the revised manuscript.

As a suggestion the following references might also be relevant as recent cases of microCT of marine invertebrates:

Landschoff, J., Komai, T., du Plessis, A., Gouws, G. and Griffiths, C.L., 2018. MicroCT imaging applied to description of a new species of Pagurus Fabricius, 1775

(Crustacea: Decapoda: Anomura: Paguridae), with selection of three-dimensional type data. PloS one, 13(9), p.e0203107.
Landschoff, J., Du Plessis, A. and Griffiths, C.L., 2018. A micro X-ray computed tomography dataset of South African hermit crabs (Crustacea: Decapoda: Anomura: Paguroidea) containing scans of two rare specimens and three recently described species. GigaScience, 7(4), p.giy022.
Landschoff, J., Plessis, A. and Griffiths, C.L., 2015. A dataset describing brooding in three species of South African brittle stars, comprising seven high-resolution, micro X-ray computed tomography scans. GigaScience, 4(1), p.52.
>> We have inserted the above references to appropriate sentences.

Major Concerns:
1. Title: removing the word "system" is suggested, as the focus here is on a scanning protocol, not a system description. Not much image analysis is done so consider removing the words "image analysis", a shorter title will also read better
>> "system" and "image analysis" have been deleted from the title.

2. use of agarose. This is new to me and is a clever way of loading samples. I suggest to explain some more or give at least some introduction to it, and suggest alternative ways. Usually in our lab we use florist oasis and other low density mounting materials that can be shaped by hand, for example. Disadvantages of agarose should therefore also be mentioned, eg. if scan times are longer, you might not get the sample out nondestructively?
>> Agarose has been used in previous studies, so only a brief introduction is added. Alternative ways and disadvantages of agarose have been mentioned.

3. Use of clay as mounting material to stage. This is quite new to me also as we use jaw-clamps, fixed using screws. Using clay might have some disadvantages, such as sample movement during scanning if the clay does not hold the tube rigidly, this becomes excessively important as the resolution increases and scan time increases. It might be worth to mention this and to implement this yourself
Use of clay has been described in the discussions.

4. "Fluoroscopic image" must be reworded everywhere in the text and the figure – it is a digital X-ray image, also known as X-ray projection image, or 2D X-ray image. Fluoroscopic refers to fluorescence which is not what we are looking at.

>> Fluoroscopic image was changed to X-ray projection image.

5. Step 3.2.1.3 The adjustment of the sample must take place with X-ray switched off for safety. This is obvious but important to note in a protocol paper
>> Turning off the X-ray has been added as a NOTE.

6. scan times are quite short, which in this case helps to get higher quality images due to some sample shrinkage. It might be worth noting that longer scan times allow higher quality but with the disadvantage of possible sample shrinkage blur in images. Typical microCT scan times are in the region of 1 hr in my experience
>> Discussion on scan time has been expanded.

7. There is no reason to refer to "low cost" CT machine, consider to remove this
>> "low cost" have been removed.

Minor Concerns:
Nothing further

**Reviewer #3:**
Authors present protocols for performing microfocus X-ray computed tomography systems (microCT) imaging on three different marine invertebrates. The subject presented here is within the scope of JoVE. In terms of the scientific contents of the work, I found that the exposition in the Introduction of the background as well as the methodology (protocols) used/proposed and bibliography used are appropriate. In terms of the presentation of the manuscript, the paper is well-written and all the figures is of good quality and necessary for the understanding of the text. The video of the *Harmothoe* polychaete is of high quality and very didactic. The main elements of the internal anatomy are well shown. Totally agree with authors that fail in DNA extraction was not because of mCT scanning, but because of the use of formalin in fixation process.
Minor concern:
The authors discuss on the processes of contraction of the body tissues and, as a consequence, the distortion of the organs both in their form and in their position in the body of the animal. One aspect that perhaps the authors could also add in relation to this, is the possibility of the creation of artifacts due to a long period of

conservation of the animals, as is the case of the museum collections specimens (e.g. type series) and, as a consequence, in a misinterpretation of internal structures. My conclusion is that the work has substance enough to be published in JoVE.
>> The storage time of the specimens were added to Table 1.

**Reviewer #4:**
Manuscript Summary:
The authors claims to present effective new detailed method to obtain good results in micro-CT use by solving the lack of publish paper on this field.
In fact they give exhaustive description of their methods that did not solve the problem of shrinkage (as they admit), and moreover as they use agarose the recognized the ulterior problem to clean up this from the specimens. The way in which the paper is presented did not five a real solution to the problems that authors pretend to. The paper should have been presented as another micro-CT study of the anatomy of invertebrate specimens, describing the methodology that they used and discussing the advantages and disadvantages of what has been already used. So in summary the manuscript needs of a "major" revision rewriting it.
>> We are not trying to solve the problem of shrinkage or agarose cleanup, and do not claim that we have done so. We apologize if our manuscript was unclear, and have revised some sentences that may have been misleading. Furthermore, to meet the aims of the journal, the manuscript was revised to be more focused on methodology.

**Reviewer #5:**
Manuscript Summary:
I have now gone through the manuscript draft "Microfocus X-ray computed tomography system (microCT) imaging and image analysis of marine invertebrates". The study is a welcome contribution to the CT scanning of marine invertebrates. The authors provide a detailed protocol on sample preparation and the scanning of the specimens. The mounting of specimens in agarose gel is interesting. The authors also use three different microCT methods, which will be interesting to read by newcomers to this field of research. However, I have a number of points of critique about the manuscript
Major Concerns:
General comments on the study and its design. The authors claim to have chosen specimens of different size with a range of 0.8-2.0cm. I would argue that all these

specimens are in a similar size class (even if the scanned objects of interest, i.e. the field of view in the final scan does not need to be). Furthermore, the chosen samples, an anemone, a polychaete, and a flat worm are (although phylogenetically distant) not strategically selected from the vast diversity of marine tissues to be scanned. For example, one could expect that the soft-bodied anemone and flat worm samples are similar in scanning properties. The authors do not particularly state why they chose any of these specimens and why these are exactly valuable to the protocol of microCT. Do these animals belong to groups in which microCT scanning could help the most? If so, for which field of research? It therefore seems that the authors did not design this paper as a study to demonstrate the protocol. If so, this does not have to be a problem and is in line with the Journal objectives, but then the title and presentation of the study are misleading. Also, the animal groups chosen are not necessarily exclusively marine. I would suggest to narrow the scope down, pinpointing that this study describes a protocol for the organisms scanned.
>>We have revised the title to refer to the three specific animals used in the study, and have revised the sentences that may have been misleading.

Furthermore, in my view the main limitation and the reason why not more working groups are performing microCT scanning in biology is not the unavailability of scanning facilities or knowledge on how to scan. The problem is rather the time consuming, costly, and very often very difficult segmentation of biological tissues in the reconstructed images. This manuscript claims to present imaging and image analysis, yet falls entirely short on the software analysis part. It is not clear to the reader what the authors did to retrieve the images. The biological observations are nicely presented and illustrated, but the biological findings are rather side-results and not really in line with the scope of the journal. If important enough then the biological findings should be presented in a separate biological paper, not into a paper that focusses on the methods.
>> We have clarified the image retrieval part, and deleted most of the biological observations.

In conclusion, a step-by-step microCT protocol for any groups of marine invertebrates is very welcome. The authors should, however, be encouraged to state the aim of the paper and stay within these goals and within the scope of the journal. In particular, this should include the protocol steps for the most difficult part for the

analysis of biological CT data, which is the segmentation of the different tissues in the visualization software.
>> We have clarified the image retrieval part, and deleted most of the biological observations. Describing the segmentation of the different tissues (which is quite complicated and deserves a different paper itself) is not the central aim of the manuscript, and since it requires steps specific to each visualization software, it has not been added.

Minor Concerns:
I am not native English speaking myself, but in general the manuscript would benefit from editing the grammar and language, which can be quickly resolved by a native speaker or edit and proof-reading service.
>> The grammar and language of the manuscript was reviewed by Editage language editing service provided by Cactus Communications (https://www.editage.jp).

In line 80 the authors make a rather absolute statement that the problem of soft-tissue visualizations in microCT "was solved". I would argue that this problem will never be entirely solved. While it is probably possible to get good image contrast using microCT on many tissues, it will not be entirely possible for all of them, particularly not at the same time. Various staining techniques may also damage the sample, taking away one of the benefits of this otherwise non-destructive technique. This has been discussed in the literature, and in fact mentioned in this paper. In conclusion, I would simply weaken this statement.
>> The statement was weakened.

Line 406. There is no protocol given for the data analysis.
>> Protocols for data analysis has been added.

Line 459. The authors say here that intense signals disappeared after storage. It is unclear how the specimens were examined before? Were they scanned twice, once before and once after staining?
>> The sample was scanned twice, once before and once after 22 days storage at room temperature. We apologize if our manuscript was unclear, and have revised some sentences that may have been misleading.

**Reviewer #6:**

Manuscript Summary:
The article by Maeno et al. provides a welcome addition to the rapidly growing field of contrast-enhanced μCT imaging. Of particular interest is the fact that the authors introduce a number of practical tips based on their own experience that will provide future researchers with further ideas on how to handle their specimens. However, the manuscript suffers at present from a number of issues that require thorough attention and review. I therefore would like to recommend this article for publication provided that a MAJOR REVISION is undertaken. I would be happy to review a revised version of this manuscript.
Major Concerns:
I will here highlight a number of broad, but important topics that each will require a considerable amount of attention, time, and work – but, I believe that the article is bound to profit considerably from these requested changes.
– Language: the entire manuscript needs to be thoroughly checked for spelling and grammatical errors. Furthermore, the text would significantly profit from a review that includes a native/good English speaker with a background in this type of analysis (e.g. Dr Metscher).
>> The grammar and language of the manuscript was reviewed by Editage language editing service provided by Cactus Communications (https://www.editage.jp).

At present, the text mixes several important topics without providing a clear guideline. This issue can presumably only be resolved by a full review of the structure of the entire text (with respect to its logic) or by removing several points that at present are only partially touched by the authors. In fact, the authors could consider stripping the text almost entirely down to its practical core, i.e. the protocols provided in the "Protocol" section. This is clearly the strong point of the present contribution.
>> We have revised the manuscript so that it is more focused on methods.

– Manuscript format: at present, the article is a mix of Comment and Methods paper. With respect to my previous point, I would suggest rethinking the structure of the manuscript. What kind of article would you like to read yourselves and what type of article is presently missing in the literature (see below)?
>> Since JoVE is a protocol-centered journal, we have revised the manuscript so that it is more focused on methods.

– Data transparency: one of the main advantages of the application of a non–invasive imaging technique is that the raw as well as most derived image data are available in digital form and hence are suitable for deposition in online data repositories. This approach is standard in the molecular domain and increases data transparency, but also facilitates knowledge gain through reuse of data (i.e. data mining). Therefore, I would request that the authors upload their entire digital data to such a repository (e.g. Dryad, Morphobank, GigaScience, etc.).
>> The datasets were deposited in the figshare repository, doi: 10.6084/m9.figshare.7670837 and will be made public after the publication of this manuscript.

– Specimens: the chosen species are not at all representative of the structural diversity that marine invertebrate organisms display on the whole. In fact, organisms like *Xenoturbella* and *Actinia* are highly similar with regard to their internal composition in terms of the technique employed. Why not scan a crustacean (chitinous exoskeleton plus soft parts), an echinoderm (calcareous endoskeleton plus soft parts), a cephalopod (external or internal shell plus soft parts), etc. as well? If this is not within the scope of the study, I would suggest to tone down some of the statements made in the Title, Abstract, Introduction, and Discussion (″...of marine invertebrates″).
>> We have revised the manuscript, including the title, to tone down some of the statements.

– *Harmothoe* sp.: please identify this species
>> As the sample was damaged during handling, it is impossible to definitely identify the species, between *H. glomerosa*, *imbricata*, *praeclara*, *sexdentata*, or *spinifera*. As the main aim of the manuscript is methodology, we hope that identification to the genus level is satisfactory.

– *Xenoturbella*: the actual description of the species *X. japonica* was published in a Corrigendum by Nakano et al. (2018). This paper should be cited instead of the 2017 article.
>> We have added Nakano et al. (2018) to the references, but since the microCT images are reported in Nakano et al. (2017) and not (2018), Nakano et al. (2017) is still retained in the references.

– Specimen fixation: fixation of a soft-bodied organism like *Actinia* in 70% ethanol (even after MgCl treatment) is a pretty rough step. Why was this specimen not fixed in formalin buffered in sea water? Please provide some more information and discussion about your choice of protocol.
>> We have added a new paragraph discussing the fixation step and our preference of 70 % EtOH.

– Chosen contrast agent: 25% Lugol's solution is a very high concentration of iodine. This metal has been shown to lead to strong shrinkage artifacts under high concentrations – some of the artifacts observed in your scans might be related to this. Please see the following article and papers cited therein for more information: Hedrick BP, Yohe L, Vander Linden A, Dávalos LM, Sears K, Sadier A, Rossiter SJ, Davies KTJ, Dumont E (2018) Assessing soft-tissue shrinkage estimates in museum specimens imaged with diffusible iodine-based contrast-enhanced computed tomography (diceCT). Microscopy and Microanalysis. In my personal experience, PTA (phosphotungstic acid) has several advantages: no (or very little) shrinkage, no overstaining possible, excellent contrast – but longer staining times. Please provide a discussion of potential contrast agents and why iodine was chosen here.
>> We have added a new paragraph discussing the staining step and our preference of Lugol.

– Figures: the figure design at present is very "nervous" – there are dozens of single images in total and it is very difficult for the reader to follow a consistent track. Please reconsider the entire figure design and try to reduce the number of figure plates as well as individual images.
>> The number of figure plates and individual images were reduced.

– Techniques: some of the techniques used are several years old now and have been shown elsewhere already, e.g. specimen setup (Fig. 2), batch scanning (Fig. 4), scout scanning (Fig. 5), etc. Please take a look at the literature listed below to see where your approach provides novel clues. In my opinion, some of the steps mentioned in the Protocols section should be mentioned in greater detail and should also be figured instead of some of the very conventional steps shown at present in your figures. (Pinpoint is conventionally called ROI scanning = region of interest scanning; multi-step is conventionally called batch scanning).

>> We have rewritten the protocols and revised the figures and deleted detailed explanations on some very conventional steps. Furthermore, we have reworded the method names as suggested.

– Equipment: as noted in the text, the system used by your team is not a scanning system of the latest generation. While this is absolutely OK, you should try to remove all system–specific, crucial information from your text as future users will very likely not have access to your particular system. Examples are in particular the specific terminology used to describe software features as well as figures showing screenshots from your software. You are addressing an international audience where 90% of the readers will use software in English instead of Japanese.
>>Since the editorial comments required that software steps must be explicitly explained ('click', 'select', etc.) and that more specific details are needed (e.g. button clicks for software actions, numerical values for settings, etc.), we believe the figures showing screenshots from our software is necessary. We are aware that the words in the screenshot are Japanese, and have translated them in the figures and manuscript, hoping that it will be useful for readers using a different but similar software. Unfortunately, due to software limitations, the words in screen could not be changed to English.

– Tables: the materials table is incomplete (Comments/Description) and not very informative. Instead of providing catalog numbers that may change rapidly, overall pricing might be more interesting for the user.
>> Since the catalog number is required by the journal, we have left them in the materials table. On the other hand, since prices vary depending on the country (and change rapidly), we do not think it is very informative and have not added them to the table.

Table 1 also needs to be improved and restructured as well as checked for spelling mistakes.
>> Table 1 has been revised and checked for grammar and spelling by Editage language editing service provided by Cactus Communications (https://www.editage.jp).

– Video: I do not understand the purpose of the video file. Will this video be integrated into the final JoVE video? If not, please remove or deposit this video in an online repository (see above).
>> The video file has been deleted.

– The authors need to read and cite this important paper, as it is – to my knowledge – the first and only true "µCT plus staining" protocol: Metscher BD (2011) X-ray microtomographic imaging of intact vertebrate embryos. Cold Spring Harbor Protocols 12: 1462–1471. This article might also help you to restructure and more precisely focus your text.
– Further techniques: if the authors decide to provide a broader text that not only focuses on their specific protocol(s), but on µCT + staining on the whole, then they should integrate further techniques that provide soft part contrast as well, e.g. phase contrast µCT, SRµCT, OPT, LSM, or MRI (Ziegler A, Kunth M, Mueller S, Bock C, Pohmann R, et al. (2011) Application of magnetic resonance imaging in zoology. Zoomorphology 130: 227–254)
– Literature: the authors clearly need to have a much broader overview of the pertinent literature. Many of the points raised in the present contribution have already been worked on or even have been solved. Here are some examples:
>> We thank reviewer #6 for suggestions on further references and we have added some of them to our manuscript. But since the aim of our manuscript is to provide specific protocol(s) of our methods and not a broader review-like text to the readers, not all of the literature has been added.

~~TITLE:~~

**Microfocus X-ray computed tomography ~~system~~ (microCT) imaging of *Actinia equina* (Cnidaria), *Harmothoe* sp. (Annelida), and ~~image analysis of marine invertebrates~~*Xenoturbella japonica* (Xenacoelomorpha)**

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~~**KEYWORDS**~~**Keywords**:
microCT, ~~diceCT,~~ Lugol solution, iodine, ~~volume rendering, image reconstruction,~~ ~~*Actina*~~*Actinia*, Cnidaria, *Harmothoe*, Annelida, *Xenoturbella*, Xenacoelomorpha, invertebrates

**SHORT ABSTRACT**~~**: (10 words minimum, 50 words maximum)**~~

Here, protocols for performing microfocus X-ray computed tomography ~~systems~~ (microCT) imaging ~~on~~of three marine invertebrate animals are explained in detail. This study describes steps such as sample fixation, staining, mounting, scanning, image reconstruction, ~~specimen retrieval,~~ and data analyses. ~~Ideas~~Suggestions on how the protocol can be adjusted ~~to each sample is~~for different samples are also provided.

**LONG ABSTRACT**~~**: (150 words minimum, 300 words maximum)**~~

Traditionally, biologists have had to rely on destructive methods such as sectioning in order to investigate the internal structures of opaque organisms. ~~But recently, non~~Non-destructive microfocus X-ray computed tomography (microCT) imaging has become a powerful and emerging protocol in biology, due to ~~the~~ technological advancements in sample staining methods and innovations in microCT hardware, processing computers, and data ~~analyzing~~analysis software. However, this protocol is ~~still limited to a number of research groups, and~~ not commonly used, as it is in the medical and industrial fields. ~~Even in biological facilities that possess this high-performance system, instances in which experienced users with accurate knowledge of its advantages and disadvantages operate the system to its maximum extent are uncommon.~~ One of the reasons for this limited use ~~in the field~~ is the lack of a simple and comprehensible manual ~~covering~~that covers all of the necessary steps: sample collection, fixation, staining, ~~observations~~mounting, scanning, and data analyses~~, and sample retrieval.~~. Another reason is the vast diversity ~~observed in~~of metazoans, ~~especially in~~particularly marine invertebrates. ~~Due to the~~Because of marine invertebrates' diverse ~~size, morphology~~sizes, morphologies, and ~~physiology~~physiologies, it is crucial to adjust experimental conditions and hardware

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configurations at each step, depending on the sample. Here, three microCT imaging methods are explained in detail using three phylogenetically diverse marine invertebrates: ~~*Actina*~~*Actinia equina* (Anthozoa, Cnidaria), *Harmothoe* sp. (Polychaeta, Annelida), and *Xenoturbella japonica* (Xenoturbellida, Xenacoelomorpha). ~~Ideas~~Suggestions on performing microCT imaging on various animals are also ~~suggested~~provided.

## INTRODUCTION

Biological researchers generally have had to make thin sections and perform observations by light or electron microscopy in order to investigate the internal structures of opaque organisms. ~~But~~However, these methods are destructive~~,~~ and problematic when applied to ~~apply on~~ rare or valuable specimens. Furthermore, several steps in the method, such as embedding and sectioning ~~is~~, are time consuming, ~~taking~~and it can take several days to observe a sample, depending on the protocol. Moreover, ~~due to the~~when handling ~~of~~ numerous sections, ~~the~~there is always a possibility of damaging or losing some sections ~~were always present.~~. Tissue ~~-~~clearing techniques are available for some specimens[1-5]~~,~~ but are not yet applicable ~~to~~for many animal species.

To overcome these problems, some biologists have started using microfocus X-ray computed tomography ~~systems~~ (microCT) imaging[6-13][15]. In ~~general~~ X-ray ~~computed tomography (~~CT~~),~~, the specimen is irradiated with X-rays from various angles that are generated from an X-ray source moving around the sample, and the transmitted X-~~ray is~~rays are monitored by a detector that also moves around the sample. The ~~obtained~~ X-ray transmission data ~~is~~obtained are analyzed to reconstruct cross-sectional images of the specimen. This method enables the observation of internal structures without destruction of the sample. ~~Due to~~Because of its safety and ease, it is commonly used in medical and dental applications, and CT systems can be found in hospitals and ~~dentists~~dental centers worldwide. Moreover, industrial X-ray ~~computed tomography (industrial~~ CT~~)~~ is frequently used for observing non-medical samples for inspection and metrology in the industrial field. In contrast to medical CT, in which the X-ray source and the detectors are mobile, the two parts are fixed in industrial CT, with the sample rotating during scanning. Industrial CT generally produces higher resolution images than medical CT and is referred to as microCT (~~in~~

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the case of micrometer--level resolution) or nanoCT (nanometer--level resolution). Recently, research using microCT ishas rapidly increasingincreased in various fields of biology[12-22]biology[14-34].

Biological studies using CT initially targeted internal structures consistingthat mainly consist of hard tissuestissue, such as bone. Soft tissues were difficult to visualize with CT since X-rays easily penetrate these structures. This problem was solved with advancesAdvances in staining techniques using various chemical agents, and enabled the visualization of soft tissues have been visualized in various animals[6-8,12] and plants[21,23]organisms[6-9,14-34]. Of these reagents, iodine-based contrast agents are relatively safe, inexpensive, and can be applied to the visualization of soft tissues in various organisms. CT using these agents, referred to as diceCTused for diffusible iodine-based contrast-enhanced CT, is today the most popular method for visualizing soft tissue structures in the field of biology[12].the visualization of soft tissues in various organisms[7,14]. Concerning marine invertebrates, microCT has been widely used on such animals as cnidarians[18], arthropods[24], molluscs[6,19], bryozoans[6], and annelids[14,16,25].[32,33], annelids[18-20,28], and arthoropods[21,23,29,31]. However, there have been few reports ofon other animal phyla, such as bryozoans[6], xenacoelomorphs[26], and cnidarians[24,30]. In general, there have been fewer studies using microCT on marine invertebrates are considerably less than those ofon vertebrates, and is limited to a number of research institutions. One major reason for this limited use on marine invertebrates is the vast diversity observed in these animals. Due to theBecause of their diverse size, morphologysizes, morphologies, and physiologyphysiologies, each species reacts differently to variousdifferent experimental procedures, such as the rate of contractions during fixation and the efficiency of staining. Therefore, it is crucial during sample preparation to choose the most appropriate fixation and staining reagent, and to set conditions (e.g. staining time and temperature) at each step, adjusted tofor each species. Similarly, it is also necessary to set the scanning configurations, such as the mounting method, voltage, current, mechanical magnifying rate, and the space resolution power, suitable for each sample to maximize the potentials of each microCT systems. These variable conditions and configurations enable the visualization of various biological samples, but on the other hand, this flexibility in the protocol may seem complicated to inexperienced users and could become a major barrier for researchers who wish to start using microCT. Even in facilities with this high-performance system, it is rarely operated to its maximum extent, and instances in which experienced users are accurately grasping its advantages and disadvantages are

~~scarce. Hence, to overcome this problem, a simple and comprehensible manual is essential,~~appropriately for each sample. To overcome this problem, a simple and comprehensible manual that covers all of the necessary steps, explains how each step can be adjusted depending on the specimen, and shows detailed examples from multiple samples is essential.

~~We have previously reported a new species of *Xenoturbella* from the western Pacific[20], and microCT imaging played a crucial role in its description, especially in the discovery of a new organ, the frontal pore. We have performed microCT imaging on several other marine invertebrate species since.~~

In the present study, we describe the microCT imaging protocol step-~~-~~by-~~-~~step, from sample ~~collection,~~ fixation~~, preparation, scanning,~~ to data analyses, ~~to sample retrieval. Three samples with different size, morphology, physiology, and conditions are used in this study to show the versatility of the method. The~~ using three marine invertebrate species. Specimens of the sea anemone ~~specimen, *Actina*~~*Actinia equina* (Anthozoa, Cnidaria~~),~~) were collected near the Misaki Marine Biological Station, University of Tokyo. They had a spherical, soft body that was about 2 cm in diameter~~.~~ (Fig. 1A-C). *Harmothoe* sp. (Polychaeta, Annelida) ~~sample was a~~samples were also collected near Misaki Marine Biological Station. They were slender ~~worm~~worms that were about 1.5 cm in length, with tough chaetae present along the whole body~~.~~ (Fig. 1D). A *Xenoturbella* ~~*japonica*~~*japonica*[35] (Xenoturbellida, Xenacoelomorpha) specimen was collected near Shimoda Marine Research Center, University of Tsukuba. It was a soft-~~-~~bodied worm that was about 0.8 cm in length~~. Due to the circumstances at collection, its condition was rather poor, with the epidermis starting to come off. Compared to the first two species in which numerous samples can be caught in a single collection, this is a rare species in which only a limited number of specimens available. Therefore, the main objectives for observing *X. japonica* was to investigate the newly reported frontal pore and to retrieve the sample after scanning. For the other two specimens, one objective was to check the effects of staining and storage.~~ (Fig. 1E). Adjustments made ~~on~~for the conditions and configurations ~~for~~of each sample are explained in detail. Our ~~present~~ study provides several ~~tips and ideas~~suggestions on ~~performing~~how to perform microCT imaging on ~~various~~ marine invertebrates, and we hope that it will inspire biologists to utilize this protocol for their research.

**PROTOCOL**

~~Details are summarized in Table~~

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## 1. Fixation

1.

1. ~~**Sample collection and fixation**~~

~~Specimens of *Actina*~~[*Actinia equina* ~~(Fig. 1A-C) were collected near Misaki Marine Biological Station, The University of Tokyo. Animals were relaxed~~ ]

Relax the animals in 10% $MgCl_2$ ~~sea water~~seawater for about 15 minutes at room temperature~~, fixed in~~ . Transfer to 70% ethanol~~,~~ and ~~stored in the fixative~~store at room temperature.

~~Specimens of~~ 1.2. [*Harmothoe* sp~~. (Fig. 1D) were collected near Misaki Marine Biological Station, The University of Tokyo. The animals were anesthetized~~.]

Anesthetize the animals by placing them in ice-cold ~~sea water~~seawater for about 15 minutes. ~~They were fixed in~~Transfer to 10% (v/v) formalin solution with seawater and ~~stored in the fixative~~store at room temperature.

~~A specimen of~~ 1.3. [*Xenoturbella japonica* ~~(Fig. 1E) was collected near Shimoda Marine Research Center, University of Tsukuba. It was relaxed~~]

Relax the animal using 7% $MgCl_2$ in ~~fresh water and fixed~~freshwater. Fix in 4% paraformaldehyde in filtered seawater overnight~~, washed, and kept~~ . Place in 70% ethanol and store at 4 °C.

## 2. ~~2.~~ Staining

2.1. ~~For~~ Transfer the samples ~~in 70% ethanol (*A. equina* and *X. japonica*), transfer the samples into~~to 50% ethanol and store at room temperature for 15 hours.

~~2.2.~~ Replace the 50% ethanol with 25% ethanol and store at room temperature for 2 hours. Note: Not necessary for the *Harmothoe* sp. sample in 10% (v/v) formalin solution

~~2.3. For the *A. equina* and *X. japonica* samples, replace the 25% ethanol~~ with seawater.

2.2.~~distilled water (DW). For~~ Replace the ~~*Harmothoe* sp. sample, replace the fixative~~ solution with distilled water (DW~~).~~) and store the samples in DW

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2.4. Store at room temperature in DW for 2 hours. Repeat this step three times.

2.3. Prepare 25% Lugol solution by diluting the stock solution (below) to 25% with DW. Stock solution (100% Lugol solution) contains KI 10 g and $I_2$ 5 g, adjusted to 100 ml with DW. Note: Lugol solution is light-sensitive, so store and handle the solution away from light. Follow the regulations of each country and institution for iodine handling and waste.

2.4. 2.5. Decant the DW and pour in 25% Lugol solution. Stain for 20 24 hours at room temperature. The 25% Lugol solution was made by diluting the below stock solution with DW prior to use: stock solution (100% Lugol solution); KI 10 g, $I_2$ 5 g, adjust to 100 ml with DW.

Note: Lugol solution is light sensitive, therefore storage of the stock solution and staining of the samples should be performed protected from the light.

Caution: Concerning iodine handling and waste, please follow the regulations of each country and institution.

## 3. MicroCT scanning

### 3.1 Stage Mounting

Preparation:

Make 0.5% agarose by dissolving 500 mg agarose in 100 ml DW in a 250 ml conical flask within a microwave (800 W, about 1-3 min.) minutes). Cool to about 30-40 °C at room temperature.

Caution: Do not overheat or completely seal the flask when heating, to prevent the agarose from boiling over.

3.1.1 【*Actina equina*】

3.1. Mounting large (>2 cm) samples using a 50-ml tube

3.1.1.1 Place the stained sample in a 60 mm Non-treated Dish dish with DW to wash off excessive staining solution from the surface.

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3.1.~~1.~~2.~~—~~ Gently pour 5 ml of 0.5% agarose ~~in~~into a 50-ml tube and harden the agarose on ice. Be careful not to make bubbles in the agarose.

3.1.~~1.~~3.~~ Harden the agarose on ice.~~

~~3.1.1.4.~~ Gently add 20 ml of 0.5% agarose ~~in~~into the 50-ml tube~~. Be careful not to make bubbles in the agarose.~~

~~3.1.1.5. Place~~ and place the specimen within the ~~0.5%~~ 0.5% agarose ~~with the distal end up~~ using forceps. Be careful not to ~~leave~~make bubbles in the agarose.~~ ~~

3.1.~~1.6.~~ 4. Adjust the position and orientation of the sample with forceps ~~before the agarose hardens.~~

~~3.1.1.7. Harden~~and harden the agarose on ice.

3.1.~~1.8.~~ 5. Place clay on the microCT mounting stage and set the 50-ml tube on the clay (Fig. ~~2B~~2A).

~~3.1.2【*Harmothoe* sp.】~~

3.2 Mounting small (<2 cm) samples using a 1000-µl micropipette 'blue' tip

3.~~1.~~2.1.~~—~~ Draw up 100 µl of 0.5% agarose into a 1000-µl micropipette 'blue' tip and harden the agarose on ice, making a plug in the tip~~. Make several of this plugged tip~~ (Fig. ~~2C~~2B-a).

3.~~1.2.~~2.~~—~~2. Decant the stained sample ~~in~~into a 60-mm ~~Non-treated Dish~~dish without using forceps. ~~The sample in the tube cannot be seen due to the colored staining solution, and the use of forceps at this step may damage the sample.~~

3.~~1.~~2.3.~~—~~ Gently transfer the sample using ring tweezers into ~~a~~another 60-mm ~~Non-treated Dish~~dish with DW to wash off excessive staining solution from the surface.

3.~~1.~~2.4.~~—~~ Add 1000 µl of either DW or 0.5% agarose into the plugged tip made at step 3.~~1.~~2.1 using a micropipette.~~ Be careful not to make bubbles in the agarose.~~

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3.~~1.~~2.5.~~—~~ Gently transfer the sample from the 60~~-~~mm ~~Dish~~dish into the DW or agarose in the plugged tip using ring tweezers. ~~Be careful not to make bubbles in the agarose. The sample was placed with its head downwards. Do not push the sample too far down the tip, to keep the chaetae standing in their natural state.~~

3.~~1.~~2.6.~~—~~ Gently adjust the position and orientation of the sample with a petiolate needle~~, avoiding damage to~~ or precision tweezers so that the sample~~. This step must be completed before~~ is stable between the walls of the tip. Be careful not to make bubbles in the agarose~~ hardens.~~

~~3.1.2.7.~~ . Harden the agarose on ice if agarose is used.

3.~~1.~~2.~~8.~~ ~~7.~~ Cut the tip off a new 1000~~-~~µl micropipette 'blue' tip ~~so that the remaining part is 5 cm long (Fig. 2C~~ (Fig. 2B-b, c~~),~~) and insert the tip of the plugged tip into the ~~5 cm~~new tip ~~(Fig. 2E-c).~~.

3.~~1.~~2.~~9.~~ ~~8.~~ Place clay on the microCT mounting stage and set the tips with the sample inside on the clay (Fig. ~~2E-c).~~

~~3.1.3 【*Xenoturbella japonica*】~~

~~3.1.3.1-3 Same as *Harmothoe* sp.~~

~~3.1.3.4. Add 1000 µl DW into the plugged tip using micropipette. Be careful not to make bubbles in the DW. The epidermis was starting to come off from the specimen in this study, and DW instead of agarose was used to prevent further damage.~~

~~3.1.3.5. Gently transfer the sample from the 60-mm Dish into the DW in the plugged tip using ring tweezers. The sample was placed with its anterior downwards. Be careful not to make bubbles in the DW.~~

~~3.1.3.6. Using a petiolate needle, gently push the sample downwards while adjusting its orientation so that the sample is stable between the walls of the tip (Fig. 2E-a,b). Avoid damage to the sample.~~

~~3.1.3.7. Cut the tip off a new 1000 µl micropipette 'blue' tip so that the remaining part is 6.5 cm long (Fig. 2C-b), and insert the tip of the plugged tip into the 6.5 cm tip (Fig. 2D,E-a,b).~~

~~3.1.3.8. Place clay on the microCT mounting stage and set the tips with the sample inside on the clay (Fig. 2D).~~2C, D). NOTE: The staining solution will start to wash off the sample once it is placed in DW, so proceed to the next scanning step promptly.

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~~3.2.~~ ~~4.~~ **MicroCT scanning**

~~ScanXmate-E090S105 (Comscantechno Co., Ltd., Japan), a relatively low-cost cone-beam microCT system was used in this study. This system employs L9421-02(Hamamatsu Photonics K.K) as its X-ray source, with a focal spot size of 7 μm (5 μm at 4 W) and tungsten as its anode target. The detector is a flat panel detector PaxScan1313DX (Varex Imaging Corporation). The active image matrix size of this system is 992 x 992 pixels. The internal structure and the operating coordinates of the stage is shown in Fig. 2A. Three types of scanning are possible with this system: 1) Normal Scan: scan the whole specimen in a single scan; 2) Multi-step Scan: scan parts of the specimen at high resolution and reconstruct the whole specimen afterwards; 3) Pinpoint Scan: scan only the necessary parts of the specimen at high resolution in a single scan. In this section, Normal Scan is explained using *A. equina*, Multi-step Scan using *Harmothoe* sp., and Pinpoint Scan using *X. japonica*.~~

~~3.2.1 Normal Scan of *A. equina*~~

~~3.2.1.1. After setting the sample on stage, turn on the X-ray beam at 80kV, 100μA.~~

~~3.2.1.2. Looking at the~~

4.1. Turn on the X-ray beam at 80 kV, 100 μA.

4.2. While observing the X-ray transmission image at the center of the screen (Fig. 3A), move the stage so that the whole sample can be seen~~. Adjust~~ by clicking on the X and Z axis button (Fig. 3A). Set the contrast ~~in~~of the ~~Fluoroscopic Image (Fig. 3A)~~image so that the internal structures ~~of the specimen~~ can be observed~~.~~ by adjusting the contrast conditions (Fig. 3A: Image contrast).

4.3.~~2.1.3.~~ Adjust the orientation of the sample by changing the angle of the tube/tip in the clay (Fig. 2B). Turn the stage 90 degrees by setting the rotation axis (Fig. 3A~~: Designation of axial~~) to 90 and clicking on the relative movement~~), and perform~~ button (Fig. 3A). Perform the same maneuver four times to complete a full rotation. NOTE: Manually turn off the X-ray beam each time you open the sample door, unless the system turns it off automatically.

~~3.2.1.~~

4.~~4.~~ Move the stage so that the sample is at the center of view~~.~~ by clicking on the Z axis button (Fig. 3A) and by manually adjusting the Y axis knob on the mounting stage

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(Fig. 3B). Turn the stage 90 degrees and do the same. Turn the stage 360 degrees and check that the sample is at the center of view from all directions.

~~3.2.1~~

4.5. Move the stage along the x-axis toward the X-ray beam source by clicking on the X axis button (Fig. ~~2A~~3A) to enlarge the sample so that it just fits in view (Fig. ~~3A lower right~~3C).

~~3.2.1~~

4.6. Turn the stage 360 degrees and check that the sample fits in view from all directions. ~~The mechanical magnifying rate, defined by the distance of the sample from the X-ray source, is determined at this step. The space resolution power during scanning, which is proportional to the magnifying rate, is also set here. In the case of Fig. 3A lower right panel, it is at 18.0μm/pixel.~~

~~3.2.1~~

4.7. Adjust the scanning conditions as ~~below:~~ shown in Table 1.

~~Voltage: 80kV, Current: 100μA~~

~~Number of integrations (average of integration): 2~~

~~Integration rate (frame rate): 8 fps~~

~~Bining mode: 1 x 1~~

~~Projection: 2000~~

~~Contrast of Fluoroscopic Image: MIN 0 , MAX 65535 , EDGE 0~~

~~3.2.1.8.~~

4.8. Start scanning. It will take about 10 minutes.

~~Note: If there are plans to use the sample for further experiments, promptly retrieve the sample from the mounted tip and transfer into storage solution (*e.g.* DW, 70% ethanol, depending on the following experiments). It is possible to store the sample in the hardened agarose for later re-scanning, but contractions will likely occur (see Fig. 10) and storage in agarose should be kept to a maximum of several days.~~

~~3.2.2 Multi-step Scan of *Harmothoe* sp.~~

~~3.2.2.1-4. Perform the same steps as Normal Scan 1-4.~~

~~3.2.2.5. Move the stage upwards along the z-axis so that the anterior tip of the specimen is at the center of view.~~

~~3.2.2.6. Move the stage along the x-axis toward the X-ray beam source to enlarge the sample so that its width just fits in view (Fig. 4C). Turn the stage 90 degrees and~~

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~~check that the sample fits in view (Fig. 4D). This position corresponds to Scan3 in Fig. 4A.~~

~~3.2.2.7. Lower the stage along the z-axis 800 pixels (Fig. 3A: Z axis position by pixel), and check that the sample fits in view by rotating the sample (Fig. 4A:Scan2, Fig. 4E). If parts of the sample cannot be seen in view, lower the magnification by moving the stage away from the X-ray source.~~

~~3.2.2.8. Lower the stage along the z-axis 800 pixels again, and do the same as the previous step (Fig. 4A:Scan1, Fig. 4F).~~

~~3.2.2.9. Check that the whole sample is included within the planned scanning area (Fig. 4A:Scan1-3). The space resolution power during scanning is determined through these steps. In the case of Fig. 4, it is at 6.5μm/pixel.~~

~~3.2.2.10. Adjust the scanning conditions to below:~~

~~Voltage: 80kV, Current: 100μA~~

~~Number of integrations (average of integration): 2~~

~~Integration rate (frame rate): 8 fps~~

~~Bining mode: 1 x 1~~

~~Projection: 2000~~

~~Number of Multi-steps: 3~~

~~Number of Slices_Y(height): 800~~

~~Contrast of Fluoroscopic Image: MIN 0 , MAX 65535 , EDGE 0~~

~~3.2.2.11. Start scanning. It will take about 26 minutes.~~

~~Note: If there are plans to use the sample for further experiments, promptly retrieve the sample from the mounted-tip and transfer into storage solution (*e.g.* DW, 70% ethanol, depending on the following experiments). It is possible to store the sample in the hardened agarose for later re-scanning, but contractions will likely occur (see Fig. 10) and storage in agarose should be kept to a maximum of several days.~~

~~3.2.3 Pinpoint Scan of *X. japonica* (scanning only the anterior part of the specimen)~~

~~3.2.3.1-4. Perform the same steps as Normal Scan 1-4.~~

~~3.2.3.5. Move the stage upwards along the z-axis so that the anterior tip of the specimen is at the center of view.~~

~~3.2.3.6. Move the stage along the x-axis toward the X-ray beam source to enlarge the sample so that the part to be observed is at the center of view. Parts that do not need to be observed can be excluded from the field of view, but the excluded parts may cause noises during scanning (hence, Pinpoint Scan is recommended especially for~~

~~observing distal parts of an animal). Therefore, it is advised to make the excluded parts as small as possible by adjusting the stage position, angle of specimen, or the magnifying rate. The *X. japonica* specimen was scanned at 2.8μm/pixel in this study.~~

~~3.2.3.7. Adjust the scanning conditions to below:~~

~~Voltage: 60kV, Current: 130μA~~

~~Number of integrations (average of integration): 2~~

~~Integration rate (frame rate): 8 fps~~

~~Bining mode: 1 x 1~~

~~Projection: 2000~~

~~3.2.3.9. Start scanning. It will take about 10 minutes.~~

~~4~~

**5. Image reconstruction**

~~4~~

5.1 ~~Reconstruction of *A. equina* Normal Scan~~

~~4.1.1. Raw data is saved as .raw files. Import~~. Start up the ~~raw data into a built-in reconstruction~~microCT system's accessory software ~~in~~(see table of materials) and open the ~~micro-CT system (*e.g.* coneCTexpress).~~scanned data.

~~4.1.2. Trim~~

5.2. Adjust differences in the rotation axis of the sample during scanning by clicking on the automatic shift value calculation button (Fig. 4A green square).

5.3. Adjust the orientation of the image by rotating the orange arrows (Fig. 4B). If the orientation was changed, repeat step 5.2.

5.4. Click on the area tab (Fig. 4C magenta square) and trim areas where samples are not present ~~(~~ (Fig. ~~3B:~~4C yellow square~~).~~).

~~4.1.3. Perform~~

5.5. Click on the reconfiguration ~~by setting~~tab (Fig. 4D magenta square) and set the filters as below to remove noise.

Ring artifact reduce filter: Median filter -3; Noise elimination filter: Average filter-1.

~~4.1.4.~~

5.6. Perform reconfiguration by clicking on the reconfiguration button (Fig. 4D green square).

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5.7. Adjust image brightness and contrast by setting the black and white values as ~~below (Fig. 3C). Note: Black~~black value 0, white value~~:~~ 250 (Fig. 4D blue square).

~~4.1.~~

5.~~—~~8. Save the reconstructed ~~Tiff~~TIFF image dataset~~.~~

~~4.1.6.—~~ as an 8-bit TIFF by clicking on the save button. Rename ~~tiff~~TIFF files as following: Date_sample_resolution (µm)_number.tiff.

~~4.2 Reconstruction of *Harmothoe* sp.Multi-step Scan (Fig. 5)~~

~~4.2.1. Raw data is saved as .raw files~~NOTE: The original microCT datasets from this study are available in ~~folders Scan1, 2, and 3. First, import the raw data of Scan1 into a built-in reconstruction software in the micro-CT system (*e.g.* coneCTexpress).~~

~~4.2.2. Set 'angle of rotation' so that~~ the ~~ventral side is to the top of the view (Fig. 5A,B).~~

~~4.2.3. Set the area of reconfiguration (Fig. 5C). Since the figures used here for the x- and y-axes will be used in all three scans, make sure to set the area in which the specimen fits in all three scans. For the z-axis in Scan1, set the upper limit depending on the sample and lower limit at 895 (Fig. 5C).~~

~~4.2.4. Perform reconfiguration by setting the filters as below to remove noise. Ring artifact reduce filter: Median filter -3; Noise elimination filter: Average filter-1.~~

~~4.2.5. Adjust image brightness and contrast by setting the black and white values as below (Fig. 5D. Black value 0; white value: 250).~~

~~4.2.6. Save the reconstructed Tiff image dataset in a new folder.~~

~~4.2.7. Import the raw data of Scan2.~~

~~4.2.8. Set the area of reconfiguration (Fig. 5E). Use the same numbers as Scan1 for the x- and y- axes, and 96-895 for the z-axis (Fig. 5E).~~

~~4.2.9. Save the reconstructed Tiff image dataset in a different folder from Scan1.~~

~~4.2.~~figshare repository, doi:10.~~Import the raw data of Scan3~~6084/m9.figshare.7670837[36].

~~4.2.11. Set the area of reconfiguration (Fig. 5F).~~ ~~Use the same numbers as Scan1 and Scan2 for the x- and y- axes. For the z-axis in Scan3, set the upper limit at 96 and the lower limit depending on the sample (Fig. 5F).~~

~~4.2.12. Save the reconstructed Tiff image dataset in a different folder from Scan1 or Scan2.~~

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~~4.2.13. Rename all tiff files (Scan1, 2, and 3) with a serial number as following: Date_sample_resolution (µm)_number.tiff. and save in a single folder.~~

~~4.3 Reconstruction of *X. japonica* Pinpoint Scan~~

~~-> Same as 4.1~~

~~**5. Specimen retrieval**~~

~~5.1 Remove the 1000µm tip or 50 ml tube with the specimen inside from the stage.~~

~~5.2 If the specimen was mounted in a 1000µm tip, cut off the plugged tip.~~

~~5.3 Turn the tip or tube upside down and decant the specimen in a 60mm Dish with DW inside. If the specimen in the tip does not come out, shake the tip gently, blow air into the tip from the narrow end, or insert a petiolate needle from the narrow end and push out the specimen.~~

~~5.4 If the specimen was mounted in agarose, carefully remove the agarose under a stereoscopic microscope using petiolate needle and precision tweezers.~~

~~5.5 Dehydrate in a graded ethanol series and store in 70% ethanol.~~

~~Note: To investigate the effects of long-term storage of stained samples, specimens of *A. equina* and *Harmothoe* sp. were placed in DW while still in the hardened agarose and stored at room temperature for 22 days. The samples were observed by microCT imaging after storage.~~

~~Note: DNA extraction was performed on pieces of the three samples (excised with razor blades) using DNeasy Blood & Tissue Kit (Qiagen) or Wizard Genomic DNA Purification Kit (Promega) according to the manufactures' instructions and PCR was performed using LCO1490 and HCO2198 described in Folmer *et al.*, 1994[26].~~

**6. Data analyses**

6.1. Start up the data analysis software (see table of materials) and enable importing of TIFF files by clicking on the Database icon (Fig. 5A magenta square) and turning off the box shown in Fig. 5B.

6.2. Click on the import icon (Fig. 5C magenta square), select the dataset saved at step 5, and click open.

6.3. Click the copy links button (Fig. 5D) to import the data.

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6.4. Display the 2D cross-section by clicking on the 2D viewer icon (Fig. 5C blue square).

6.5. Calibrate the dataset by clicking on the 3D viewer tab (Fig. 5E magenta square) and entering the resolution value at scanning, which was 0.018 in this study (Fig. 5F). ~~Data was analyzed using OsiriX (www.osirix-viewer.com), Imaris 9.2 (http://www.bitplane.com), and Adobe Premiere Pro CC according to Tsuda *et al.*, 2017[21] and Maeno & Tsuda 2018[27].~~

6.6. Click on the brightness/contrast icon (Fig. 5E green square). Adjust the brightness and contrast by moving the cursor inside the displayed 2D image and changing the window level and window width (Fig 5G).

6.7. Check other cross-section images by moving the scrollbar (Fig. 5G square).

6.8. Change the orientation of the cross-section by clicking on the orientation icon (Fig. 5E blue square) and check images at all orientations (Fig. 5H).

6.9. Click on the export icon to store cross-section images that you wish to save.

**REPRESENTATIVE RESULTS**

We ~~have~~ performed microCT imaging ~~for~~on *A. equina* (Anthozoa, Cnidaria), *Harmothoe* sp. (~~Polycheata~~Polychaeta, Annelida), and *X. japonica* (Xenoturbellida, Xenacoelomorpha) after staining the samples with 25% Lugol solution. The staining successfully enhanced the contrast of the internal structures in all specimens, enabling ~~the~~ observations of internal soft tissues (~~Figs~~Fig. 6~~-8~~). Together with past reports[6,7,~~14,~~16,~~20,25~~19,22-26,28,30-33], this shows that microCT can be used on various marine invertebrates for visualizing their morphology, including soft internal tissues. ~~In *A. equina*, we were able to visualize soft internal structures such as pharynx, siphonoglyphs, mesenteries, and mesenterial filaments, distinguish the digestive systems from the mesenteries, and show the relative position of these organs within the specimen (Fig. 6). A single reconstructed dataset was obtained from a *Harmothoe* sp. specimen more than 1 cm long using Multi-step Scan with our low-cost cone beam~~

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~~microCT system (Figs. 7,8). By comparing the images obtained with Pinpoint Scan and Multi-step Scan, one can apparently comprehend the clarity and potential of the Pinpoint Scan (Fig. 8). The parapodia could be observed in detail and a 3D reconstructed model of the jaw enabled its observations from all angles. Although the epidermis of the *X. japonica* sample was damaged, the internal structures were clearly visualized (Fig. 9), and the ventral glandular network extending from the frontal pore was visualized in a single image for the first time (Fig. 9C).~~Clear images were obtained even with the *X. japonica* specimen, whose epidermis was badly damaged (Fig. 6F, G), showing that this method is applicable to fragile specimens with external damage.

~~With~~ Scanning only the ~~aim~~region of ~~reconstructing~~ interest, in contrast to a wider area, greatly increased the clarity and resolution of the image (compare Fig. 6F and 6G). However, a single high-resolution dataset of a whole specimen~~, Multi-step Scan~~ was ~~performed on~~ reconstructed for *Harmothoe* sp. ~~(Figs. 4,5,7,8),~~(Fig. 6C) and *X. japonica* (Fig. ~~9A). This method reconstructs a single dataset~~6F) from multiple scans performed on different (but overlapping) parts of the specimen ~~and is useful for elongated samples.~~. The seams between each scan were inconspicuous in the reconstructed images ~~(Figs. 7,8,9A). Researchers have used helical CT systems, in which the X-ray source and the detector moves around the sample in a spiral, to reconstruct~~. Our study shows that single high-resolution images ~~of large and long samples. However, helical CT systems are generally highly expensive. The Multi-step Scan method described in this study enables less expensive~~ can be obtained with cone-beam microCT systems ~~with only small detectors to scan larger areas in the z-axis direction.~~. By scanning a larger area at high resolution, ~~the~~there is a smaller risk ~~for~~of overlooking small structures ~~becomes less when compared with Normal Scan.~~. Another ~~merit for this method~~advantage is that it ~~becomes~~is easier to ~~grasp~~locate the relative positions of structures that are situated far apart, such as the anterior and posterior tips of an elongated annelid.

~~Tissue contraction after fixation and staining was observed in this study. In the *A. equina* specimen, the diameter of its proximal end changed from 1.9 cm to 1.7cm at staining (Fig. 10A,B). Similarly, the body length of *X. japonica* shortened from 0.8cm to 0.7cm (Fig. 10D,E). Concerning *Harmothoe* sp., although bending of the body occurred after staining, no shortening of its body length was observed.~~

~~Further contraction was seen in the *A. equina* specimen after storage, with the diameter of its proximal end decreasing another 2 mm (Fig. 10A-C). Comparisons~~

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~~of microCT images revealed that internal tissues have also contracted notably. For *Harmothoe* sp., no apparent contraction was seen externally even after storage. However, microCT imaging revealed that muscles in the proboscis have apparently contracted, with the space between the muscles and the epidermis widening (Fig. 10F,G). Moreover, intense signals, apparently visible before storage at the dorsal epidermis, have disappeared after storage (Fig. 10F,G: white arrow heads).~~

~~DNA extraction and PCR failed to yield positive results, with no amplified fragments being observed in all samples. However, these samples were not handled with DNA extraction in mind (*e.g.* storage at room temperature for prolonged periods and fixation in formalin). There have been reports of successful DNA extraction from microCT scanned samples (summarized in Faulwetter *et al.*, 2013[14]), and the microCT scanning time in the present study was relatively short. Therefore, the negative data is most likely to be the result of sample handling, rather than the effects of microCT scanning on sample DNA.~~

## FIGURE AND TABLE LEGENDS

Figure 1: Marine invertebrate animals observed in this study. (A-C) ~~*Actina*~~*Actinia equina* (Anthozoa, Cnidaria). (A) Distal end of a live animal relaxed in 10% $MgCl_2$ ~~sea-water~~seawater. Distal (B) and proximal (C) ends after fixation in 70% ethanol. (D) Live~~-~~ ~~and~~ anesthetized *Harmothoe* sp. (Polychaeta, Annelida), dorsal view with anterior to the left. Most of the ~~elytron~~elytra were already missing ~~already~~at this stage, with only four remaining near the posterior end. (E) *Xenoturbella japonica* (Xenoturbellida, Xenacoelomorpha) fixed in 70% ethanol. Right view, with anterior to the ~~bottom. The~~top. Because of circumstances at collection, its epidermis is starting to come off. Scale bars: 3 mm.

Figure 2: Mounting samples on the ~~microCT~~microfocus X-ray computed tomography system. (A) ~~Schematic diagram of the internal structure of the system used in this study. (B)~~ Mounting samples in a 50~~-~~ml tube using clay. The orientation of the sample can be adjusted using the clay. (~~C~~B) Preparation of a 1000~~-~~µl micropipette 'blue' tip for mounting small samples. a: Tip with its end plugged with 100 µl of 0.5% agarose (diagonal lines). The samples are placed in this tip. The tip with the sample is inserted into another 1000~~-~~µl micropipette 'blue' tip (b, c) for mounting. b was used for ~~X.~~*Xenoturbella japonica*, and c was used for *Harmothoe* sp. (~~D~~C) Mounted *X. japonica*

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sample, overview (left) and close up (right). X-ray source can be seen to the right of the sample. (~~E~~D) Diagrams for mounting samples in ~~the~~a 1000~~-~~µl micropipette 'blue' tip. a: *X. japonica* sample in ~~DW~~distilled water. b: the sample is in contact with the tip wall (arrows), so that it does not move while scanning. c: *Harmothoe* sp. sample in 0.5% agarose.

Figure 3: ~~–~~Scanning samples on the microfocus X-ray computed tomography system. (A) Operating screen ~~of the microCT system used in this study. (A) Screen~~ during scanning~~. (B) Screen during image reconstruction~~ of the microfocus X-ray computed tomography system, showing an X-ray transmission image of an *Actinia equina* specimen. Adjust the contrast and brightness with the 'Image contrast' at the lower left. (B) View of the mounting stage showing the Y axis knob. (C) X-ray transmission image of the *A. equina* specimen after the mounting stage was moved closer to the X-ray beam source. Notice it is enlarged when compared to the image at the center of (A).

Figure 4: Operating screen of the image reconstruction system. (A) Screen for adjusting differences in the rotation axis of the sample during scanning, showing an *Actinia equina* specimen. Magenta square: shift tab; green square: automatic shift value calculation button. (B) Screen for adjusting the orientation of the image, with *Harmothoe* sp. shown. (C) Screen during the image reconstruction of *A. equina*, trimming the area outside the yellow square where no samples are present. ~~(C~~Magenta square: area tab. (D) Screen during image reconstruction, showing the reconstructed image of *A. ~~equina~~equina*. Magenta square: reconfiguration tab; green square: reconfiguration button; blue square: black and white value adjustment.

~~Figure 4: Multi-step Scan of *Harmothoe* sp. (A) Scanning of the sample. D1, D2, and D3 show the area that is scanned in Scan1, 2, and 3 respectively. The mounting stage is raised 800 pixels after each scan. Since the active image matrix size of this system is 992 x 992 pixels, 192 pixels overlap between each scan. (B) Image reconstruction. Since the top and bottom ends of each scan are distorted, 96 pixels are discarded from the overlapping parts of the scan. (C-F) Images during scanning. C,D: Image during Scan1 showing the anterior end of the *Harmothoe* sp. sample. E: Image during Scan2 showing the middle part of the sample. F: Image during Scan3 showing the posterior end.~~

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~~Figure 5: Operating screen during image reconstruction of *Harmothoe* sp. Multi-step Scan. (A) Import the Scan1 data. (B) Adjust the orientation by changing the angle of rotation. In this case, the dorsal side, which can be identified by the elytron, is to the bottom. (C) Check the data from all angles and trim the area where no samples are present (outside the yellow square). Concerning the z-axis, set the number on the right to 895. (D) Adjust the brightness and contrast by changing the 'Black value' and 'White value' (in this case, it is 0 and 250, respectively). Save the data as an 8 bit Tiff file after this step. (E) Perform A–D for Scan2 data, using the same configurations for the angle of rotation, black value, white value, x-axis and y-axis trimming area. For the z-axis trimming area, use 96 and 895. (F) Perform A–D for Scan3 data, using the same configurations. For the z-axis trimming area, set the number on the left to 96.~~

Figure 5: Operating screen of the image analysis system. (A) Preference window. The Database icon (magenta circle) is clicked to open the database file management window. (B) Database file management window. In this software, the box shown with an arrow needs to be off to enable importing TIFF files. (C) Menu and tool bars of the Database screen. Magenta square: import icon; blue square: 2D viewer icon. (D) Dataset import window. Magenta circle: copy links button. (E) Menu and tool bars of the 2D viewer screen. Magenta square: 3D viewer tab; green square: brightness/contrast icon; blue square: orientation icon. (F) Calibration setting window. Enter the desired resolution values within the columns in the magenta square. (G) Cross-section of an *Actinia equina* specimen displayed in the 2D viewer window for adjusting brightness and contrast. Magenta square: scrollbar for checking other cross-sections. (H) Cross-section of *A. equina* displayed in the 2D viewer window with a different orientation to (G).

Figure 6: Scanned and reconstructed images of ~~*Actina equina*.~~marine invertebrates. (A) Transverse ~~section at the green dotted line shown at the right top.~~ and (B) ~~Longitudinal section at the green dotted line shown at the right top.~~longitudinal sections of *Actinia equina*. The area inside the dotted yellow square in (B) is enlarged in the ~~left top~~ inset. ~~(C–E) 3D models constructed from scanned data. Turquoise blue; mesenteries and mesenterial filaments, red-brown; digestive organs (pharynx and siphonoglyphs).~~Abbreviations: dm~~:~~, pair of directive mesenteries~~;~~; m~~:~~, pair of perfect mesenteries~~;~~ mf~~:~~, mesenterial filament~~;~~ p~~:~~, pharynx~~;~~ si~~:~~, siphonoglyphs~~;~~ t~~:~~, tentacle~~;~~ arrows~~:~~, oral disc~~;~~ white arrowheads~~:~~, pedal disc~~;~~ black arrowheads~~:~~, sphincter muscle. Scale bars: A, B: 3 mm.

~~Figure 7: Scanned and reconstructed images of *Harmothoe* sp. (A) False color volume rendering image. Dorsal view with anterior to top. (B) Volume rendering image of the anterior end shown with yellow dotted square b in A. Top panel, dorsal view, bottom panel, frontal view. (C) Volume rendering image of the posterior end shown with yellow dotted square c in A. Top panel, dorsolateral view, bottom panel, ventrolateral view. (D) Volume rendering image of a left parapodia. (E) Volume rendering image of the upper and lower jaws, seen from the front (left column), frontal-left angle (middle column), and left (right column). (F~~ (C-E) *Harmothoe* sp. (C) Sagittal section of the anterior part. (~~G~~D, E) Transverse section at the dotted ~~line~~lines d and e in ~~F. (H) Sagittal section of the posterior part.~~(C). Abbreviations: aci~~:~~, aciculum~~,~~; acim~~:~~, acicular muscle~~,~~; coe~~:~~, coelom~~, dci: dorsal cirrus,~~; dlm~~:~~, dorsal longitudinal muscle~~, doc: dorsal chaetae, dvm: dorsal ventral muscle,~~; elp~~:~~, elytrophore~~, ely: elytron,~~; eye~~:~~, eye~~,~~; int~~:~~, intestine~~,~~; jaw~~:~~, jaw~~, lant: lateral antenna,~~; mant~~:~~, median antenna~~,~~; mo~~:~~, mouth~~, nop: notopodium, nup: neuropodium, palp: palp, par: parapodia, pha: pharynx, prob:~~; mp, muscles of proboscis~~, pros: prostomium, tci: tentacular cirri, vbv: ventral blood vessel, vci: ventral cirrus,~~; palp, palp; pha, pharynx; prob, proboscis; vlm~~:~~, ventral longitudinal muscle~~,~~; vnc~~:~~, ventral nerve cord.

~~Figure 8: Volume rendering image and transverse section movie of the whole body of *Harmothoe* sp. 6 sec to 16 sec: 3D volume rendering image. Top: left view, bottom: frontal view. 17 sec to 1min 42 sec: Transverse section movie generated from Multi-step Scan. The position of the section is shown with a moving green line on the sagittal section image at top. From 17 sec to 53 sec, transverse section movie of the anterior part of the specimen generated from Pinpoint Scan is shown at the left bottom. From 1 min 7 sec to 1 min 14 sec, a 3D model made using Imaris software showing the positions of major organs is present at right top.~~

~~Figure 9: Scanned and reconstructed images of~~ Scale bars: C: 1 mm; D, E: 0.3 mm. (F, G) *Xenoturbella japonica*. (~~A~~F) Sagittal section of the whole sample~~ reconstructed from a 2-step Multi-step Scan (4.2µm/pixel). (B.~~ (G) Sagittal section of the anterior part~~ *reconstructed* from a Pinpoint Scan (2.8µm/pixel). (C) False color volume rendering image of the anterior part seen from the ventral side, reconstructed from the Pinpoint Scan data.~~ bl~~:~~, basal lamina~~,~~; int~~:~~, intestine~~,~~; ml~~:~~, muscle layer~~,~~; mo~~:~~, mouth~~,~~; nn~~:~~, intraepidermal nerve net~~,~~; white arrow, statocyst; black ~~arrows:~~arrow, frontal pore~~,~~;

white arrowheads~~:~~, ventral glandular network~~, white arrows: statocyst,~~; black arrowheads~~:~~, oocytes.

~~Figure 10: Effects of preparation and storage on fixed samples. (A-C) Fixed *Actina equina*, seen from the proximal end. A: After fixation, before staining. B: Volume rendering image reconstructed from data obtained by scanning the sample after staining. C: Volume rendering image reconstructed from data obtained by scanning the sample 22 days after the first scanning. The sample was stored in hardened agarose at room temperature. (D,E) Fixed *Xenoturbella japonica*, right view with anterior to the bottom. D: After fixation, before staining. E: Volume rendering image reconstructed from data obtained by scanning the sample after staining. Scale bar: 1 mm. (F,G) Sagittal section of the anterior part of *Harmothoe* sp., left view. F: Image reconstructed from data obtained by scanning the sample after staining. G: Image reconstructed from data obtained by scanning the sample 22 days after the first scanning. The sample was stored in hardened agarose at room temperature. White arrowheads: these intense signals were obvious in F, but missing in G.~~ Scale ~~bar~~bars: F: 1 mm, G: 0.5 mm.

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Table 1: Sample preparation and scanning protocol for each specimen.

**DISCUSSION**

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Fixatives using formalin, such as the 10% (v/v) formalin solution in seawater used in this study, are known to preserve the morphology of diverse marine invertebrates and are often used for microCT imaging[18,24-26,28,30,33]. However, restrictions on the use of this chemical have become strict in some countries in recent years, and substitutes such as paraformaldehyde or glutaraldehyde may be used. If there are plans to extract DNA after scanning, it is better to avoid using formalin as a fixative, because it is known to fragment DNA. In this case, the use of fixatives that preserve DNA, such as 70% ethanol, is recommended. In this study, the cnidarian *A. equina* was fixed using 70% ethanol, and clear microCT images were obtained from the 70% ethanol-fixed samples (Fig. 6A, B).

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In a previous study ~~of performing~~that performed microCT scanning ~~on~~of various cnidarian taxa, many samples were dehydrated in 100% ethanol, and some

were ~~further~~ critical-point-dried prior to ~~scanning[18]~~scanning[24]. Although ~~the~~ soft internal organs such as tentacle clusters, muscles, and gonads were successfully observed in ~~the~~their study, dehydration and drying processes are known to result in major artefacts such as the deformation and ~~contractions~~contraction of soft ~~tissues[10,17]~~tissues[11,21]. In the present study, we were able to observe the internal structures of ~~a~~the cnidarian *A. equina* ~~using~~fixed in 70% ethanol and stained with 25% Lugol solution (Fig. ~~6~~6A, B). Our protocol, without any dehydration or drying steps, is ~~more~~ preferable, and should be performed ~~when~~whenever possible to reduce the risk of damage to the specimens and artefacts during scanning.

~~Although there were no dehydration or drying steps, contraction was observed in all three specimens after staining in the study. Contraction of the whole body was observed in *A. equina* and *X. japonica* specimens (Fig. 10A–E). Concerning *Harmothoe* sp., no shortening of its body length was observed. The differences in contraction is probably due to the differences in morphology of the samples. *A. equina* and *X. japonica* are both soft bodied with no external or internal skeletons, possessing a large space inside the body. On the other hand, *Harmothoe* sp. has a cuticle layer as an exoskeleton, preventing the body from extensive contractions. Contractions of samples stained with 25% Lugol solution have also been reported previously[8,28,29], and should be considered when using the solution for microCT.~~

~~Further contractions were seen in the *A. equina* and *Harmothoe* sp. specimens after 22 days storage at room temperature in hardened agarose (Fig. 10). For *Harmothoe* sp., no apparent contraction was seen externally, but microCT scanning revealed that muscles in the proboscis have apparently contracted, with creases appearing and the space between the muscles and the epidermis widening (Fig. 10F,G). This shows that during storage, organs with different composition of cells and ECMs will contract differently, resulting in the distortion of relative positions of organs within an organism. Intense signals, apparently visible before storage at the dorsal epidermis, have disappeared after 22 days (Fig. 10F,G: white arrow heads). The intense signals were found within a branching tubular structure, likely to be the dorsal blood vessel based on its position and morphology, with the signal itself possessing no obvious cellular structure (The dorsal blood vessel was observed after storage). Furthermore, although different in chemical composition, blood of vertebrates has been reported to be stained intensely with 25% Lugol solution[8]. Based on these information, we believe that the intense signals are the blood remaining within the~~

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~~dorsal blood vessels. As this unexpected disappearance of signals may occur in other organs and cells in other animals, and considering the inevitable contraction of soft tissues, we recommend that scanning should be performed as soon as possible after fixation and staining.~~

~~During specimen retrieval, it was difficult to completely remove the agarose from the *Harmothoe* sp. sample, especially from complex structure such as the parapodia or delicate structure such as the elytron. Mounting the sample in water and not agarose may solve this problem, but for samples with protruding structures (*e.g.* chaetae in *Harmothoe* sp. or tentacles in *A. equina*), these structures may move in the water during scanning, resulting in failure. Other methods, such as using low melting point agarose for mounting, need to be considered.~~

~~A previous study reported~~ Lugol solution, iodine solution, and phosphotungstic acid (PTA) are staining solutions that are often used on biological samples in microCT imaging[6,7,9,14,16,17,20,26,27,38]. From our experience of using various biological samples, Lugol solution provided the best results for many samples, with dark staining in a relatively short amount of time. Iodine solution yielded only very weak stains, and PTA required a long time for sufficient staining and the stained specimens showed strong contractions. Therefore, all specimens were stained with Lugol solution in this study. However, although Lugol solution is recommended, the appropriate staining solution differs between specimens, and we suggest that trials using other staining solutions be performed if there are enough specimens. Irrespective of the staining solution, samples do contract during staining[37,38], so it is important to keep the staining time short.

A critical step in microCT scanning is to mount the sample so as to prevent it from moving. In this study, this was performed in two steps, first using agarose as the direct mounting medium, and then using clay to mount the tube that contained the sample to the stage. For the first step, various low-density mounting media have been used in previous studies, including ethanol[6,17,20,25,30], agarose[9,29], and floral foam[15,22,31]. Agarose was selected in this study as it is a low-cost chemical that is accessible worldwide. A disadvantage of agarose is that it may be difficult to retrieve the sample from the hardened agarose after scanning but using low-melting-point agarose makes this retrieval step easier. For the second step, jaw clamps or screws are often used[6,9,17]. Clay was selected in this study as it enables fine adjustments in the orientation and angle of the sample. Caution is needed for experiments with long scanning times, as

the possibility of the sample moving is higher when using clay rather than jaw clamps or screws.

A previous study conducted microCT scanning ~~for~~on seven polychaete species ~~of polychaetes~~ with body lengths of 2-8 mm, smaller than the *Harmothoe* sp. used in this ~~study[14]~~study[16]. They were able to generate high-~~-~~resolution images, ~~showing~~and showed organs such as ~~the~~ vascular systems ~~or each~~and individual ~~chaetae~~chaeta clearer than in the present study. The ~~major~~main cause ~~for~~of this difference ~~is~~was not the protocol, but the specifications of the microCT systems used. The system used in the previous study was ~~'~~equipped with an 11-~~-~~megapixel ~~CCD~~charge-coupled device camera (4000 x 2672 pixel) with a maximum resolution of <~~-~~0.8 µm/~~pixel'[14]~~pixel[16]. The active image matrix size of the system used in this study ~~is~~was 992 x 992 pixels, with a maximum resolution of >~~-~~5 µm/pixel. ~~Thus~~Therefore, the spatial resolution of the ~~low-cost~~ microCT system used in this study ~~is~~was inferior to the high performance microCT system used in Faulwetter *et al.*, ~~2013[14]~~2013[16]. This difference was ~~especially~~particularly noticeable when scanning specimens smaller than 8 mm, in which we experienced a lack of resolution (data not shown). ~~On the other hand, since the~~However, because fewer data ~~acquired~~were obtained during scanning ~~is much smaller~~ in this study, the scanning time was much shorter than in the previous ~~study[14]~~study[16] (data: 992 x 992 and 4000 x 2672 ~~pixel~~pixels, respectively; scanning time: 10 to 26 minutes and 30 minutes to several hours, respectively). ~~The~~A short scanning time ~~lessens~~reduces the discoloration of the iodine staining, allowing the use of Lugol solution, which is a ~~great~~good staining solution with a high penetration rate, but ~~on the other hand known to diffuse~~ easily diffuses in ~~DW. The~~DW[34]. A short scanning time also ~~lowers~~decreases the possibility of the sample moving during scanning, which enabled the use of a simple mounting method using agarose or DW (Fig. ~~2).~~2). Longer scanning times also have the disadvantage of possible sample shrinkage blur in images. Several other mechanical and hardware problems that can occur during long scans have also been ~~reported[30]. Hence~~reported[39]. Therefore, when using microCT systems, it is important to accurately understand the specification of each system, and to choose the right system ~~depending on the~~in terms of specimen size or ~~the~~ research aim. In some cases, a ~~less expensive micro-CT~~microCT system with low resolution may be sufficient.

~~In the present study, we have applied microCT scanning on three phylogenetically distant marine invertebrate samples and explain in detail the different~~

~~steps in the protocol, from sample fixation, staining, scanning, image reconstruction, specimen retrieval, to data analyses. We believe this versatile system can be performed on not only marine invertebrates, but on a wide range of biological specimens. MicroCT enables researchers to observe the internal structures of precious samples, such as type specimens or rare samples as *X. japonica* in this study, without destroying the sample. Morphological research shall greatly progress by combining microCT, high-spec microCT, sectioning, tissue clearing techniques, and other methods depending on the characteristics of the sample and the objectives of the specific research.~~

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**DISCLOSURES**

The authors have nothing to disclose.

**REFERENCES**

1. Susaki, E. A., Tainaka, K., Perrin, D., Yukinaga, H., Kuno, A., Ueda, H. R. Advanced CUBIC protocols for whole-brain and whole-body clearing and imaging. ~~*Nat. Protoc.*~~*Nature Protocols.* **10**, 1709 – 1727, (2015).

2. Susaki, E. A., Ueda, H. R. Whole-body and whole-organ clearing and imaging techniques with single-cell resolution: toward organism-level systems biology in mammals. *Cell ~~Chem. Biol~~Chemical Biology*. **23**, 137 – 157, (2016).

3. Silvestri, L., Costantini, I., Sacconi, L., Pavone, F. S. Clearing of fixed tissue: a review from a microscopist's perspective. ~~*J. Biomed. Opt.*~~*Journal of Biomedical Optics.* **21**, 081205, (2016).

4. Greenbaum, A., *et al.* Bone CLARITY: clearing, imaging, and computational analysis of osteoprogenitors within intact bone marrow. ~~*Sci. Transl. Med.*~~*Science Translational Medicine.* **9**, eaah6518, (2017).

5. Konno, A., Okazaki, S. Aqueous-based tissue clearing in crustaceans. ~~*Zoolog. Lett*~~*Zoological Letters*. **4**, 13, (2018).

6. Metscher, B. D. MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. *BMC ~~Physiol~~Physiology*. **9**, 11, (2009a).

7. Metscher, B. D. MicroCT for developmental biology: a versatile tool for high-contrast 3D imaging at histological resolutions. ~~*Dev. Dyn.*~~*Developmental Dynamics.* **238** (3), 632 – 640, (2009b).

8. Degenhardt, K., Wright, A. C., Horng, D., Padmanabhan, A., Epstein, J. A. Rapid 3D phenotyping of cardiovascular development in mouse embryos by micro-CT with iodine staining. ~~*Circ. Cardiovasc.*~~*Circulation Cardiovascular Imaging.* **3** (3), 314 – 322, (2010).

9. Metscher, B. D. X-ray microtomographic imaging of intact vertebrate embryos. *Cold Spring Harbor Protocols* **12**, 1462 – 1471, (2011).

~~9.~~10. Boistel, R., Swoger, J., Kržič, U., Fernandez, V., Gillet, B., Reynaud, E. G. The future of three-dimensional microscopic imaging in marine biology. ~~*Mar. Ecol.*~~*Marine Ecology.* **32**, 438 – 452, (2011).

~~10.~~11. Mizutani, R., Suzuki, Y. X-ray microtomography in biology. *Micron*. **43**, 104 – 115,

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(2012).

~~11.~~12. Merkle, A. P., Gelb, J. The ascent of 3D X-ray microscopy in the laboratory. ~~*Microsc.*~~*Microscopy Today*, **21**, 10 – 15, (2013).

13. Ziegler, A., Menze, B. H. Accelerated acquisition, visualization, and analysis of zooanatomical data. In: Zander, J., Mosterman, P. J., eds. *Computation for humanity. Information technology to advance society.* CRC Press, Boca Raton, USA, 233 – 260, (2013).

~~12.~~14. Gignac, P. M., *et al*. Diffusible iodine-based contrast-enhanced computed tomography (diceCT): an emerging tool for rapid, high-resolution, 3-D imaging of metazoan soft tissues. ~~*J. Anat.*~~*Journal of Anatomy.* **228** (6), 889 – 909, (2016).

~~13.~~15. du Plessis, A., Broeckhoven, C., Guelpa, A., le Roux, S. G. Laboratory x-ray micro-computed tomography: a user guideline for biological samples. ~~*Gigascience*~~*GigaScience*. **6** (6), 1 – 11, (2017).

~~14.~~16. Faulwetter, S., Vasileiadou, A., Kouratoras, M., Dailianis, T., Arvanitidis, C. Micro-computed tomography: Introducing new dimensions in taxonomy. *ZooKeys*. **263**, 1 – 45, (2013).

17. Staedler, Y. M., Masson, D., Schonenberger, J. Plant tissues in 3D via X-ray tomography: simple contrasting methods allow high resolution imaging. *PLoS One.* **8** (9), e75295, (2013).

~~15.~~18. Fernández, R., Kvist, S., Lenihan, J., Giribet, G., Ziegler, A. Sine Systemate Chaos? A Versatile Tool for Earthworm Taxonomy: Non-Destructive Imaging of Freshly Fixed and Museum Specimens Using Micro-Computed Tomography. *PLoS One*. 9 (5), e96617, (2014).

~~16.~~19. Paterson, G. L. J., *et al*. The pros and cons of using micro-computed tomography in gross and microanatomical assessments of polychaetous annelids. *Memoirs of Museum Victoria*. **71**, 237 – 246, (2014).

20. Faulwetter, S., Dailianis, T., Vasileiadou, K., Kouratoras, M., Arvanitidis, C. Can micro-

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CT become an essential tool for the 21st century taxonomist? An evaluation using marine polychaetes. *Microscopy and Analysis.* **28**, S9 – S11, (2014).

~~17.~~21. Sombke, A., Lipke, E., Michalik, P., Uhl, G., Harzsch, S. Potential and limitations of X-ray micro-computed tomography in arthropod neuroanatomy: a methodological and comparative survey. ~~*J. Comp. Neurol.*~~*Journal of Comparative Neurology.* **523**, 1281 – 1295, (2015).

22. Landschoff, J., Plessis, A., Griffiths, C. L. A dataset describing brooding in three species of South African brittle stars, comprising seven high-resolution, micro X-ray computed tomography scans. *GigaScience*. **4** (1), 52, (2015).

23. Keiler, J., Richter, S., Wirkner, C. S. The anatomy of the king crab *Hapalogaster mertensii* Brandt, 1850 (Anomura: Paguroidea: Hapalogastridae) – new insights into the evolutionary transformation of hermit crabs into king crabs. *Contributions to Zoology*, **84** (2), 149 – 165, (2015).

~~18.~~24. Holst, S., Michalik, P., Noske, M., Krieger, J., Sötje, I. Potential of X-ray micro-computed tomography for soft-bodied and gelatinous cnidarians with emphasis on scyphozoan and cubozoan statoliths. ~~*J.*~~*Journal of* *Plankton* ~~*Res*~~*Research*. **38**, 1225 – 1242, (2016).

~~19.~~25. Moles, J., Wägele, H., Ballesteros, M., Pujals, Á., Uhl, G., Avila, C. The End of the Cold Loneliness: 3D Comparison between *Doto antarctica* and a New Sympatric Species of *Doto* (Heterobranchia: Nudibranchia). *PLoS One.* **11** (7), e0157941, (2016).

~~20.~~26. Nakano H., *et al*. A new species of *Xenoturbella* from the western Pacific Ocean and the evolution of *Xenoturbella*. *BMC* ~~*Evol. Biol*~~*Evolutionary Biology*. **17**, 245, (2017).

~~21.~~27. Tsuda, K., *et al.* KNOTTED1 Cofactors, BLH12 and BLH14, Regulate Internode Patterning and Vein Anastomosis in Maize. *Plant Cell.* **29** (5), 1105 – 1118, (2017).

28. Parapar, J., Candás, M., Cunha-Veira, X., Moreira, J. Exploring annelid anatomy using

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micro-computed tomography: A taxonomic approach. *Zoologischer Anzeiger*, **270**, 19 – 42, (2017).

~~22.~~29. Akkari, N., Ganske, A. S., Komerički, A., Metscher, B. New avatars for Myriapods: Complete 3D morphology of type specimens transcends conventional species description (Myriapoda, Chilopoda). *PLoS One*. **13** (7), e0200158, (2018).

30. Gusmao, L. C., Grajales, A., Rodriguez, E. Sea anemones through X-rays: visualization of two species of *Diadumene* (Cnidaria, Actiniaria) using micro-CT. *American Museum Novitates*. **3907** (2018).

31. Landschoff, J., Komai, T., du Plessis, A., Gouws, G., Griffiths, C. L. MicroCT imaging applied to description of a new species of *Pagurus Fabricius*, 1775 (Crustacea: Decapoda: Anomura: Paguridae), with selection of three-dimensional type data. *PLoS One*. **13** (9), p.e0203107, (2018).

32. Machado, F. M., Passos, F.D., Giribet, G. The use of micro-computed tomography as a minimally invasive tool for anatomical study of bivalves (Mollusca: Bivalvia). *Zoological Journal of the Linnean Society*. zly054, (2018).

33. Sasaki, T. *et al.* 3D visualization of calcified and non-calcified molluscan tissues using computed tomography. In: Endo, K., Kogure, T., Nagasawa, H., eds. *Biomineralization*. Springer, Singapore, 83 – 93 (2018).

~~23.1. Staedler, Y. M., Masson, D., Schonenberger, J. Plant tissues in 3D via X-ray tomography: simple contrasting methods allow high resolution imaging. *PLoS One* **8** (9), e75295, (2013).~~

~~24.1. Keiler, J., Richter, S., Wirkner, C. S. The anatomy of the king crab *Hapalogaster mertensii* Brandt, 1850 (Anomura: Paguroidea: Hapalogastridae) – new insights into the evolutionary transformation of hermit crabs into king crabs. *Contributions to Zool.* **84** (2), 149 – 165, (2015).~~

~~25.1. Parapar, J., Candás, M., Cunha-Veira, X., Moreira, J. Exploring annelid anatomy using micro-computed tomography: A taxonomic approach. *Zool. Anz.* **270**, 19 – 42, (2017).~~

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~~26. Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R. DNA primers for amplification of mitochondrial cytochrome c oxi- dase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnolog. **3**, 294 – 299, (1994).~~

~~27.~~34. Maeno, A., Tsuda, K. Micro-computed Tomography to Visualize Vascular Networks in Maize Stems. *Bio-protocol*. **8** (1), e2682, (2018)

35. Nakano, H., *et al*. Correction to: A new species of *Xenoturbella* from the western Pacific Ocean and the evolution of *Xenoturbella*. *BMC Evolutionary Biology*. **18**, 83, (2018).

36. Maeno, A., Kohtsuka, H., Takatani, K., Nakano, H. MicroCT files from 'Microfocus X-ray computed tomography (microCT) imaging of *Actinia equina* (Cnidaria), *Harmothoe* sp. (Annelida), and *Xenoturbella japonica* (Xenacoelomorpha)'. *figshare*. (2019). doi: 10.6084/m9.figshare.7670837

~~28.~~37. Vickerton, P., Jarvis, J., Jeffery, N. Concentration-dependent specimen shrinkage in iodine-enhanced microCT. ~~*J. Anat.*~~*Journal of Anatomy* **223** (2), 185 – 193, (2013).

~~29.~~38. Buytaert, J., Goyens, J., De Greef, D., Aerts, P., Dirckx, J. Volume shrinkage of bone, brain and muscle tissue in sample preparation for micro-CT and light sheet fluorescence microscopy (LSFM). ~~*Microsc. Microanal.*~~*Microscopy and Microanalysis* **20** (4), 1208 – 1217, (2014).

~~30.~~39. Sasov, A., Liu, X., Salmon, P. L. Compensation of mechanical inaccuracies in micro-CT and nano-CT. ~~Proc.~~*Proceedings of SPIE*. **7078**, 70781C, (2008).

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