Staining and resin embedding of whole *Daphnia* samples for micro-CT imaging

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Abstract

Micro-CT imaging is a powerful tool for generating high resolution, isotropic three-dimensional datasets of whole, small model organisms that are useful for qualitative and quantitative analysis. *D. magna,* one of the most common ecological model organisms used for toxicity testing and evaluation of environmental stressors, could benefit from this imaging method for enhancing whole-organism phenotype assessment. This protocol details the steps involved in *Daphnia* sample preparation for micro-CT imaging: euthanasia, fixation, staining, and resin embedding. The resulting 3D reconstructions of samples imaged using synchrotron micro-CT revealed histological (microanatomic) features of organ systems, tissues, and cells in the context of the entire organism at sub-micron resolution. 3D histology and renderings enabled by this imaging method could contribute to morphometric analysis of any tissue or organ system for both descriptive and hypothesis testing studies.

Introduction

Micro-computed tomography (micro-CT) is increasingly recognized as a valuable imaging technique for generating three-dimensional datasets that enable visualization, and qualitative and quantitative analysis of biological samples. Imaging of whole, intact samples enables detailed investigation of overall morphology and internal structures is especially useful for evaluation of microanatomy and phenotypes in various model organisms (1–5). *Daphnia magna* is one of the largest *Daphnia* species and is commonly used for toxicity testing and monitoring of environmental stressors. Studies using this crustacean model for the characterization of abnormalities or pathological change can greatly benefit from micro-CT imaging that allows full 3D examination of internal and external morphology at sub-micron resolution (6). However, this model is under-represented in micro-CT associated literature and there is a need for sample preparation protocol to promote reproducibility.

Proper sample preparation is the first step in the generation of high-quality 3D images. This protocol details the sample preparation of whole *D. magna* for micro-CT imaging that involves euthanasia, fixation, staining with metal and resin embedding. Metal staining is useful for micro-

CT imaging because the inherent contrast between different soft tissues in micro-CT images is low. Phosphotungstic acid (PTA), a heteropoly acid with chemical formula H_3PWO_{40}, is one of the most widely used contrast agent for micro-CT imaging because it provides superior contrast between adjacent tissues (7). However, PTA staining of invetebrates could take up to one week or longer (8–11). In this protocol, we show that higher concentration of PTA (3%) can be used to provide even staining of the whole *Daphnia* adults in 3 days. Commonly used PTA concentration of 0.3% works for staining small juveniles (instar 1-3) in 48 hours but does not provide uniform staining for the pregnant adults (instar 8 and older) in that timely manner. After staining, samples can be imaged in ethanol if immediate access to a micro-CT scanner is available or be dehydrated and embedded in resin (12). Our results illustrate the microanatomical details of whole *Daphnia* that would be useful for morphometric analysis. With this protocol, morphological and microanatomical visualization of *Daphnia* and other Cladocera species could be enhanced for both descriptive and hypothesis-testing studies.

Protocol

All liquid used for incubation, rinse, and infiltration should be at least 20X sample volume. All incubation, rinse, and infiltration steps are performed on a low-speed orbital shaker (Corning LSE) set to 55 revolutions per minute (RPM).

1. Fixation

    1. Transfer the *Daphnia* sample using bulb pipette containing the least amount of water into a glass vial filled with bicarbonate water to euthanize the *Daphnia*. As soon as the sample stops moving, transfer it into Bouin's solution. (Note: Tip of bulb pipette trimmed at a 45° angle such that the diameter is bigger than the size of the sample).
    2. Immediately, replace the Bouin's solution and fix the sample in fresh Bouin's solution at room temperature for 24 hours (hrs). (Note: replacing with fresh Bouin's is to remove any water carried over)

2. Staining

    1. Rinse the fixed sample with 1x phosphate buffered saline (PBS) pH 7.4 for 10 minutes (min), thrice.
    2. Submerge the sample in 35% ethyl alcohol (EtOH) for 15 min at room temperature with gentle agitation.
    3. Discard the 35% EtOH and submerge the sample in 50% EtOH for 15 min at room temperature with gentle agitation.
    4. Discard the 50% EtOH and submerge sample in PTA (in 70% EtOH) at room temperature with gentle agitation. Concentration of PTA and staining duration depend on the sample's age (Table 1). Replacement of PTA stain solution is highly recommended after 48 hrs, especially for samples with developing embryos in the brood chamber. (Note: stir pre-made PTA for 15 min before use to reduce deposition of PTA that appear as random bright spots in samples after reconstruction)

Table 1. Concentration of PTA and staining duration for different ages of *D. magna*

Age	Embryos in brood chamber	PTA concentration	Staining duration
Juvenile (instar 1-3)	no	0.3%	48 hrs
Juvenile (instar 4-7)	no	1%	48 hrs
Adult (instar 8 and older)	yes	3%	72 hrs or longer (with a PTA renewal at 48 hrs)

5. After staining, wash the sample in 70% EtOH for 5 min with gentle agitation, twice.
6. For adult sample to be scanned in 70% EtOH, transfer the sample using a trimmed bulb pipette or 1 ml pipette tip into a 200 µl micropipette tip that has been sealed with heat or clay. (Note: 10 µl micropipette tip can be used for smaller juvenile samples and a separate holder might be needed for mounting samples on the chuck for imaging).
7. Tap the sealed end gently to release any bubbles.
8. Using a long slender object with blunt end, GENTLY push the sample down the tip until it contacts the wall of the pipette tip to hold it in place, without squeezing or crushing the sample.
9. Seal the opening of the pipette tip with parafilm to avoid evaporation and the sample is ready to be scanned (Figure 1). (Note: PTA-stained samples generally can be stored up to a month. However, *Daphnia* samples in 70% EtOH should be scanned in 3 days because storing *Daphnia* samples in 70% EtOH will lead to ballooning artifact).

Figure 1. *D. magna* adult sample in 200 µl micropipette tip for imaging.

3. Dehydration and Resin Embedding (optional)

    1. Sample can be dehydrated and embedded in LR White acrylic resin for long-term storage and reacquisition or if immediate scanning is not available. Submerge the sample in 90%, 95%, 100% and 100% EtOH for 20 min each concentration at room temperature with gentle agitation.
    2. Prepare 1:1 v/v mixture of 100% EtOH and LR White acrylic resin. Submerge the samples in the 1:1 EtOH and LR White acrylic resin mixture overnight or at least 3 hrs at room temperature with gentle agitation.
    3. Submerge the sample in 100% LR White resin for 2 hrs at room temperature with gentle agitation.
    4. Replace the LR White resin with fresh 100% LR White resin and submerge for 1 hr at room temperature with gentle agitation.
    5. Cut a polyimide tubing of appropriate diameter to 30 mm length (Table 2)

    Table 2. Sizes of polyimide tubing and micropipette tip for different ages of *D. magna*

Age	Polyimide tubing inner diameter	Micropipette tip
Juvenile (instar 1-3)	0.0403”	200 µl (end of tip clipped off)
Juvenile (instar 4-7)	0.0808”	200 µl
Adult (instar 8 and older)	0.105”	1000 µl

    6. Attach the polyimide tubing to a micropipette tip so that it fits snugly (Figure 2). (Note: 200 µl micropipette tip for 0.0403” tubing and 1000 µl micropipette tip for 0.105” tubing. Clip off the end of 200 µl micropipette tip for the tubing to fit snugly.)

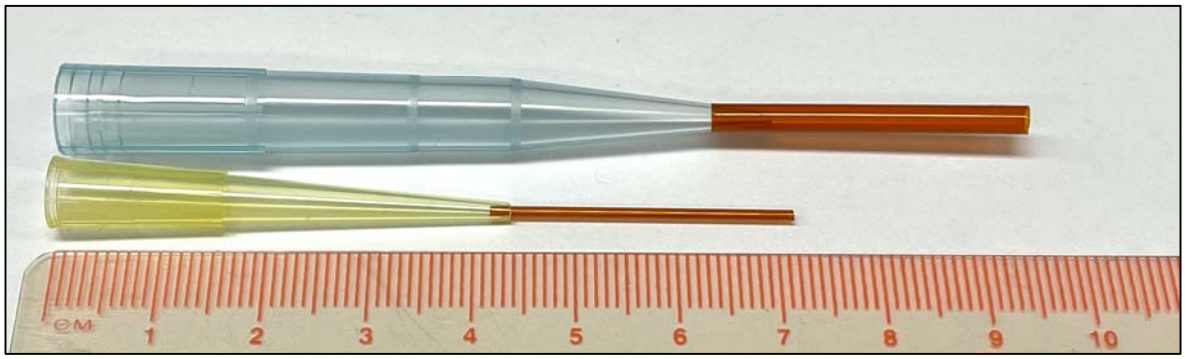

Figure 2. Polyimide tubing attached to micropipette tips for LR White resin embedding. The end of 200 µl micropipette tip was clipped off at 4.4 cm for 0.0403” polyimide tubing to fit snugly. 0.105” polyimide tubing can be attached snugly to the 1000 µl micropipette tip without clipping off the end.

    7. Attach the micropipette tip together with the polyimide tubing to a micropipette.

8. Transfer the sample to a small weigh boat or V-shaped solution basin and fully submerge the samples in fresh 100% LR White resin.
9. Position the tubing at the head of the sample and pipette resin to fill up half of the tubing before pipetting the specimen slowly into the tubing. (Pipette the sample into the tubing such that the sample is moving in the natural, forward direction to avoid backward movement that will damage extremities). Position the sample in the middle of the tubing and ensure the tubing above and below the specimen is filled with resin.
10. Immediately seal the open end tightly, using oil-based modeling clay
    a) Flatten the clay into 2mm-thick sheet.
    b) Stabilize the tubing with the index and middle fingers.
    c) Slowly push the clay into the tubing with thumb.
    d) Remove excess clay.
11. Remove the micropipette tip. Pull out the polyimide tube by gentle rotation and seal the other end of the tube with clay.
12. Place the tubing horizontally, with an end slightly elevated to prevent the formation of air bubble around the sample. Polymerize the resin for 24 hrs at 65 °C. Sample is ready for imaging (Figure 3). (NOTE: Horizontal placement avoids the sample movement during polymerization. If a small amount of air was trapped, the end with the air bubble may be elevated slightly to prevent air bubble movement toward the sample.)

Figure 3. Sample embedded in LR White acrylic resin using polyimide tubing. Removal of polyimide tubing is possible but not necessary for imaging.

4. Micro-CT Imaging and Reconstruction

Scans were performed using a custom benchtop system and synchrotron system at beamline 8.3.2 at the Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory. Custom benchtop system is equipped with an Indium Gallium liquid metal jet X-ray source (Excillum D2+) with a LuAG (Metal-laser) scintillator and secondary magnification. Source anode voltage was set to 70kV and 150W. 500 projections were taken of each sample.

Exposure time per projection was 1200ms. Samples were rotated with continuous motion over 220 degrees during each imaging session. Source to sample distance was 208 mm to avoid needing to use cone beam geometry during reconstruction, source to detector distance was 19 mm. The camera (Vieworks VP-151MC) was set to hardware SUM bin4 to boost signal. Reconstructions were performed using parallel geometry with the gridrec algorithm in Tomopy (13). Final image volumes achieved a voxel size of 2.8 μm^3.

Using synchrotron system, scans of samples were acquired at 20 keV, as a sequence of 150 ms projections. Depending on the diameter of samples, about 3000 projections were obtained over 180° for adult females. Additionally, 20 flat-field (gain) images (one at the beginning and one at the end of the acquisition) and 20 dark-field images were also acquired. Flat-field correction, stripe removal, and image reconstruction were performed using the open source TomoPy toolkit (13). Reconstructions resulted in isotropic voxel size of 0.52 μm^3.

Representative Results

The protocol described above details the sample preparation for whole *Daphnia* juveniles and adults for micro-CT imaging. Resulting 3D reconstructions reveal anatomic to micro-anatomic features in the context of the entire organism. Reconstructed voxel sizes of 0.5 µm by synchrotron micro-CT imaging would result in images which allow visualization of micro-anatomic features of organ systems (Figure 4A) and cellular structures, such as nucleoli of fat cells (Figure 4C), nucleoli (about 2 µm in diameter) of gut epithelial cells (Figure 4D) and developing cells in the embryo (Figure 4E). Details of thicker tissues or organs, such as the connections between compound eye, optic nerves, optic lobe, and cerebrum ganglia can also be visualized through customizing maximum intensity projection (MIP) thicknesses and angles (Figure 4B). Reconstructed voxel sizes of 2.8 µm from projections by benchtop micro-CT scanner would allow the visualization of anatomic features of organ systems (Supplemental Figure 1).

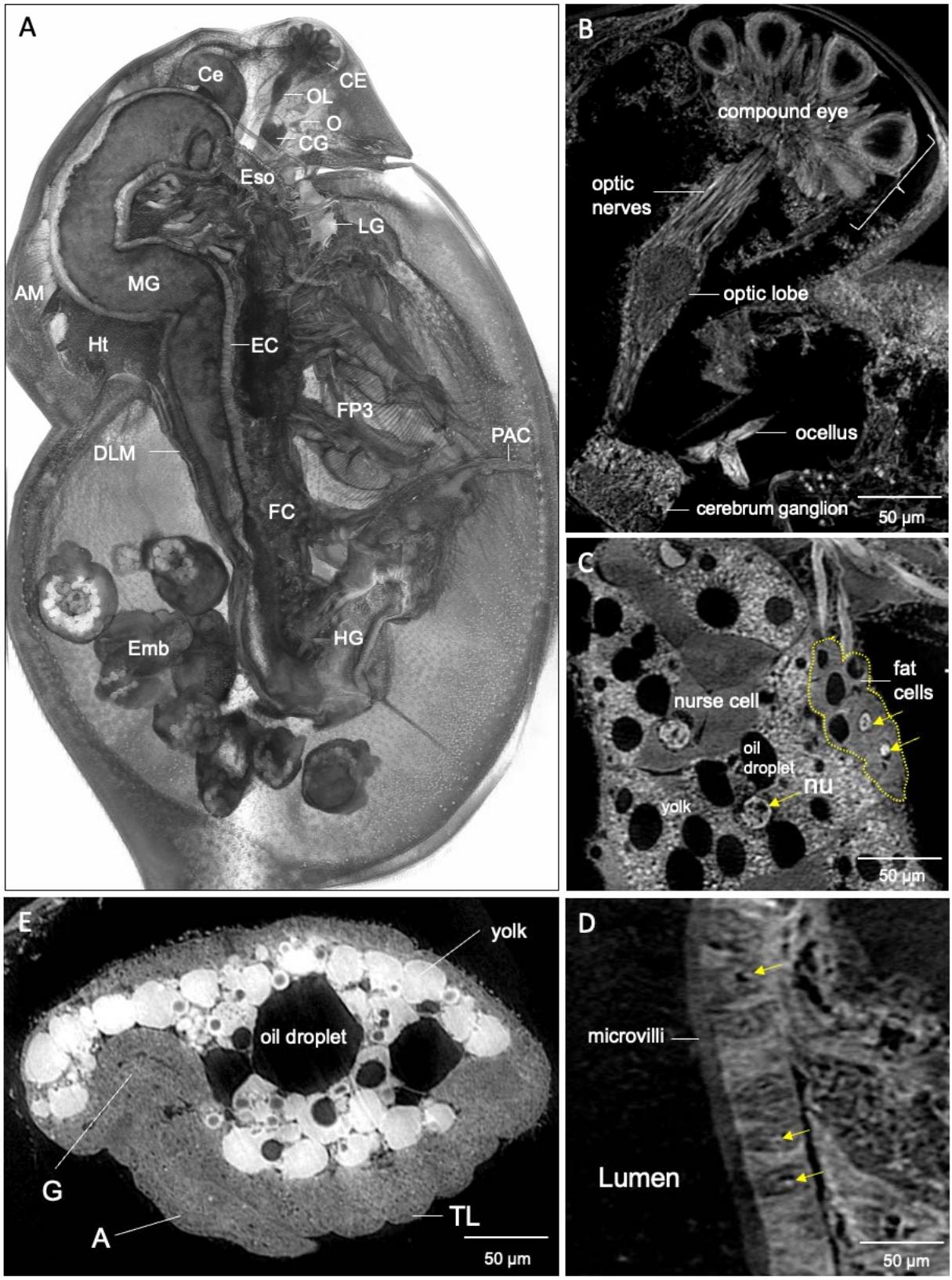

Figure 4. An adult female *D. magna* stained with 3% PTA illustrating contrast across different structures and cell types. (A) Volume rendering at mid-section of the sagittal plane

with various organs and cell types indicated. AM, antennal muscles; Ce, hepatic ceca; CE, compound eye; CG, cerebrum ganglia; DLM, dorsal longitudinal muscles; EC, gut epithelial cells, Emb, developing embryos; Eso, esophagus; FC, fat cells; FP3; filter plates on third pair of trunk limbs; HG, hindgut; Ht, heart; LG, labral glands; O, ocellus, OL; optic lobe; PAC, post-abdomen claws. Highlights of microanatomical features are such as: (B) Cellular details and connections between the compound eye, optic nerves, optic lobe, cerebrum ganglia and ocellus. (C) Details in the ovary showing nucleus (nu) of the oocyte, nurse cells, yolks, and oil droplets. Nucleoli (yellow arrows) in fat cells are also clearly visible. (D) Nucleoli (about 2 μm in diameter, indicated by yellow arrows) in the gut epithelial cells and the microvilli. (E) Details in the developing embryo showing the precursors of gut (G), swimming antenna (A) and trunk limbs (TL). (B) and (C) represent maximum intensity projections of 5μm thick sections while (D) and (E) represent individual slices of 0.5 μm in thickness.

Discussion

In this protocol, we present a time efficient and detailed way to prepare whole *Daphnia* samples for micro-CT imaging. Bouin's is the choice of fixative for whole *Daphnia* samples because paraformaldehyde and 10% neutral buffered formalin yield less consistent fixation as compared to Bouin's (14). Samples fixed overnight in paraformaldehyde and 10% neutral buffered formalin exhibited fixation artifact in which the carapace expanded, and the post-abdomen extended ventrally, causing the embryos to be dislodged from the brood chamber. Fixation of several arthropod taxa in Bouin's also provided better results in terms of tissue contrast when compared with ethanol and glutaraldehyde solution (15).

0.3-0.5% PTA is commonly used for staining samples for micro-CT imaging. 0.3% of PTA works for small/young *D. magna* juveniles but does not provide even staining of the adults after 72 hours of staining. Instead of longer incubation in 0.3% PTA, we demonstrate that 3% PTA could be used for even staining of adult samples in 72 hours that will yield good contrast after scanning. Adult samples carrying many developing embryos (>15) may need additional 24 hours for all the embryos to be evenly stained through. Renewal of PTA solution after 48 hours of incubation is important for achieving uniform staining. The optimal PTA concentrations and time efficient staining durations to achieve even contrast for samples of various ages had been summarized (Table 1).

This protocol provides steps to serially dehydrate and embed *Daphnia* samples in resin for long-term repository of samples and re-acquisition of data. Alternative methods are critical-point drying (15) and drying by chemical (hexamethyldisilazane) (16) and users are encouraged to evaluate each approach.

In conclusion, micro-CT imaging is a useful tool for morphological studies and our protocol will facilitate the deployment of micro-CT imaging to the investigation of *Daphnia* or other Cladocera in studies of taxonomy, ecology, anatomy and physiology, and toxicology.

Disclosures

The author declares no competing or financial interests.

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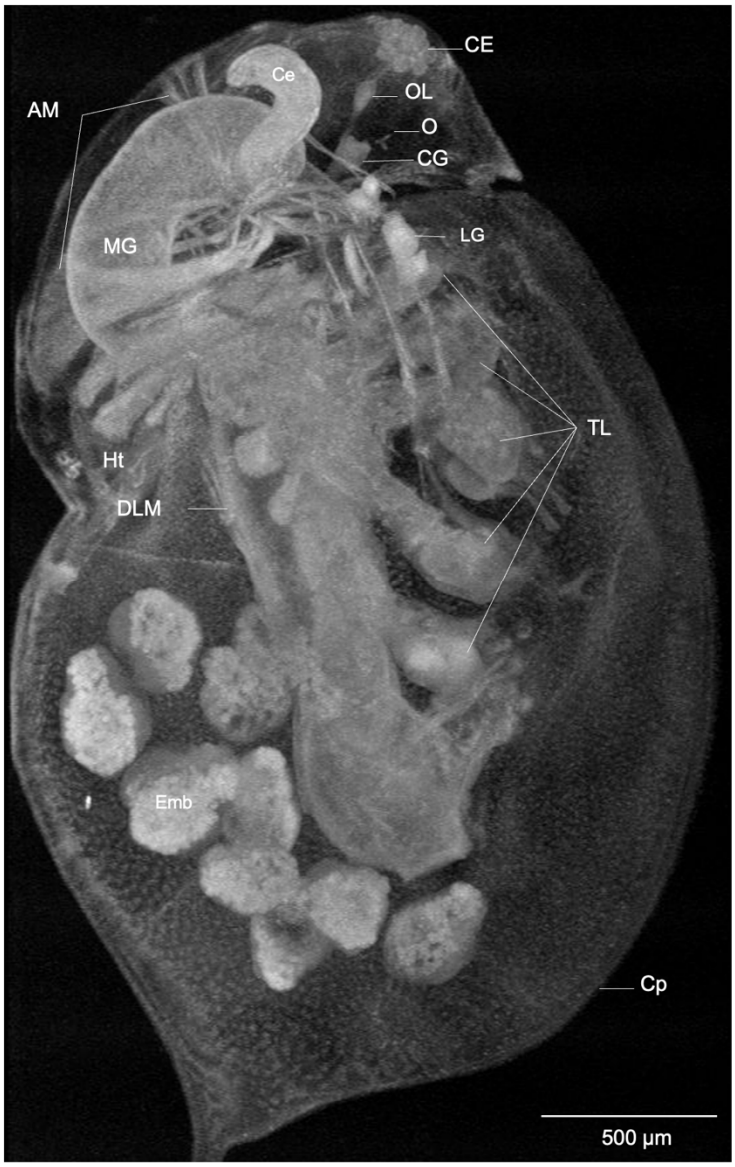
Supplemental Material



Supplemental Figure 1. Anatomic features of an adult female shown by scan from benchtop micro-CT scanner at 2.8 µm per pixel resolution. AM, antennal muscles; Ce, hepatic ceca; CE, compound eye; CG, cerebrum ganglia; DLM, dorsal longitudinal muscles; Emb, developing embryos; Ht, heart; LG, labral glands; O, ocellus, OL; optic lobe.