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PTA contrast enhancement and specimen preparation for micro-CT scanning of spiders and other small arthropods

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We use this protocol and it's working

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Disclaimer

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Abstract

Over the past decade, Micro-CT scanning has gained traction as one of the foremost imaging methods for studying arthropod morphology and internal anatomy. This method combines versatility, high data output, and relatively little impact on specimens, making it ideal for studying freshly collected specimens as well as material from natural history collections. In addition to the capabilities of the scanners, the selection of a staining method has a major impact on the quality of the data produced. This protocol is a modification of a PTA staining method published in 2022 (see references). Here, I further simplify the method by removing unnecessary steps, such as dehydration through an ethanol series and the use of rotators and vacuum chambers.

Image Attribution

Guidelines

Read the document in full before starting. Note that this staining protocol takes significantly more time than Iodine staining. Plan your project, specimen preparation, and scanning times well in advance.

Materials

See thematerial lists in the steps.

Safety warnings

This protocol uses ethanol and phosphotungstic acid. Please check the safety sheets and follow common rules for the use of these chemicals. Although I tested this protocol with good results on land snails, the acidic nature of PTA can negatively affect calcareous materials.

Before starting

5m

1 Prepare: 5m

1. [M] 70 % volume EtOH
2. [M] 1 % volume PTA (Phosphotungstic Acid) diluted in [M] 70 % volume EtOH
3. One small Petri dish
4. One 1.5ml Eppendorf tube
5. Fine tweezers
6. Small plastic pipettes

Specimen Preparation

10m

2 To optimize the penetration of the staining, it is recommended to remove the legs and abdomen of the specimen. This will allow the PTA solution to penetrate and stain the tissues evenly. 10m

2.1 If the specimen cannot be dissected (e.g. rare species or type material), it can remain intact, although the staining process will take longer.

Specimen Staining

2w 0d 0h 1m

3 Once the specimen has been fixed and prepared appropriately in 70% EtOH, it can be stained in the PTA solution. To do so, transfer the specimen to a 1.5ml Eppendorf tube (Fig. 1) and cover it with the 1% PTA in 70%EtOH solution. 1m

Figure 1. Small Linyphiid spider in PTA staining solution inside a 1.5ml Eppendorf tube.

3.1 The specimen should stay in the solution for several days, which will depend on the size and preparation of the specimen. For example, small non-sclerotised specimens (around 1 or 2mm) with removed legs can take between two days and a week to be properly stained. Larger specimens (between 0.5 and 1cm) can take 1-2 weeks to stain if the legs are removed fully. Heavily sclerotized specimens that have not been dissected or larger body sizes can take more than two weeks to fully stain. 2w

3.2 Older protocols have used vacuum pumps (see Rivera-Quiroz & Miller 2022) or further removed the cuticle of the specimen (see Penna-Gonçalves, et al. 2025) to expedite the penetration of PTA in the tissue. This usually leads either to uneven staining or damage of the internal tissues. Therefore, it is advisable to be patient and give enough time to the PTA to diffuse passively in the tissues.

Specimen mounting 6m

4 To facilitate the scanning and observation of the specimens, it is always advisable to mount the specimen in a way that stabilizes it and keeps it from moving or shifting during the scanning. To do so, prepare:

1. EtOH 70%

2. Petri Dish
3. Fine tweezers
4. Small plastic pipettes
5. Eppendorf tubes of various sizes
6. Cotton
7. Alcohol resistant paint markers (e.g. Edding 780)

4.1 Transfer the specimen from the PTA solution to a petri dish filled with 70%EtOH. This will help get rid of the excess PTA and function as a "washing step". No other washing steps are required. 1m

4.2 Place a tube of the appropriate size in the petri dish next to the specimen. The tube should be slightly wider than the specimen itself so it fits smoothly but does not create any distortion on the specimen or allow it to move laterally. The tube must be completely submerged in the ethanol to avoid the formation of air bubbles. The specimen can be gently placed inside the tube and then held in place with the help of the cotton (Fig. 2). This will prevent the specimen from shifting vertically during the scan. 5m

If you intend to prepare and scan large batches of specimens, it is critical to correctly label each tube to accurately identify the specimens. I suggest using alcohol resistant markers since normal markers are easily removed with ethanol.

Figure 2. The selection of the tube will depend on the size of the actual specimen and the capabilities of the scanner. A larger working distance allows more freedom in the width of the tube. A shorter working distance requires a smaller tube to get as close as possible to the specimen.

Staining Evaluation

15m

5 Since the PTA is completely transparent to the visual spectrum, and the specimen is not visibly altered by it (Fig. 3), it can be difficult to judge whether the specimen is properly stained or not. To check the status of the staining, I recommend using a desktop micro-CT scanner (e.g. Skyscan or Neoscan) to evaluate the tissue contrast under x-rays.

15m

Figure 3. Staining evaluation under visible light. The specimen on the left is unstained,
the one on the right has been stained for seven days. Note that the change in coloration is minimal, with just some slight decoloration in the abdomen. Despite this, the specimen on the left is fully visible under X-Ray.

5.1 To do so, the specimen can be placed inside the scanner in the same Eppendorf tube where it is being stained. This will allow observing if the straining of the specimen is even and complete, and if the contrast with the medium is sufficient for making meaningful observations of the internal anatomy (Fig. 4). This step usually takes less than five minutes per specimen and can save you several hours in preparation, mounting and scanning time.

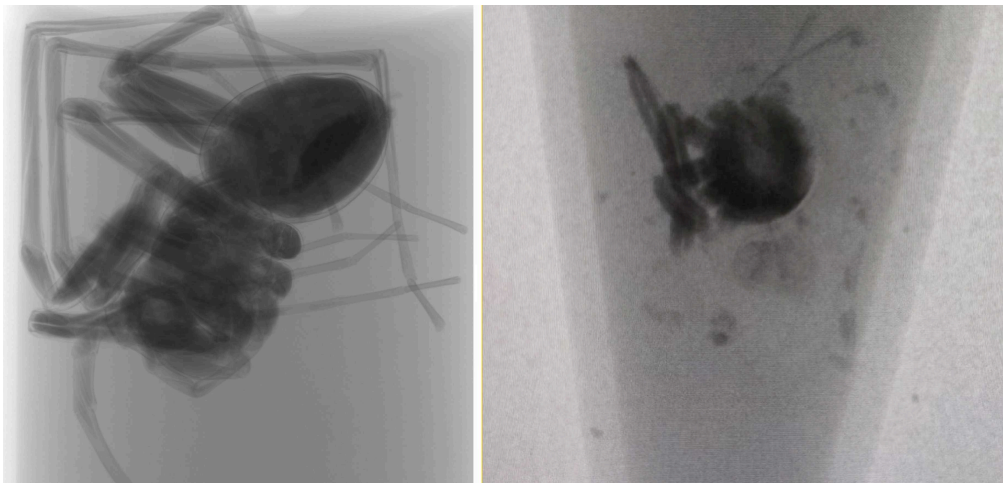

Figure 4. Staining evaluation under X-rays. The specimen on the left is fully stained. Note the darker and even coloration on the legs, cephalothorax and abdomen. The specimen on the right is not correctly stained yet and needs more time submerged in the PTA solution. The semi-transparent legs and abdomen indicate that the staining has not penetrated those tissues yet.

5.2 If the specimens are not evenly or fully stained, they can be left in the same PTA solution for a few more days. In the case of larger specimens, you might consider renewing the PTA solution to make sure the PTA has not been depleted.

5.3 If the specimens are fully stained, you can move on to the next step.

Specimen scanning

1h

6 The tube with the specimen can be placed directly in the scanner. The selection of an appropriate tube size in the previous step might have an impact on the magnification you can achieve (Fig. 5). This is especially important for table-top scanners that have a shorter working distance. In the case of larger scanners (e.g. Zeiss X-radia), the selection of the tube is not crucial. Still, it is critical to hold the specimen in place and avoid it from moving or shifting, or the entry of air that might create bubbles.

1h

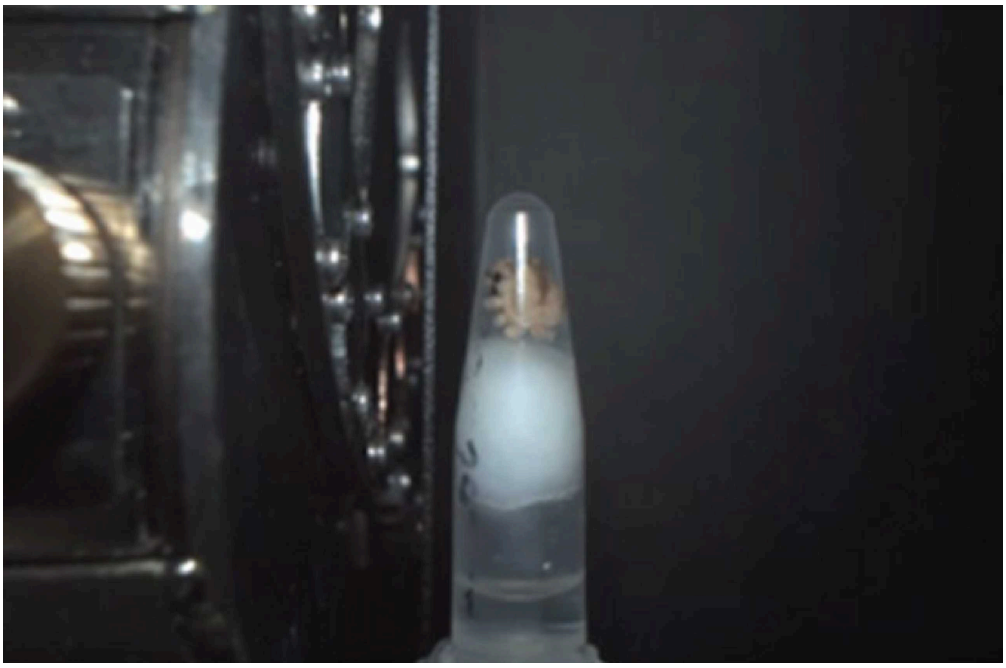

Figure 5. A tube closer to the same width of the specimen will allow the source/detector to get closer, taking full advantage of the magnification.

7 This protocol has been tested in a variety of samples from tiny spiders of the family Oonopidae and Tetrablemmidae to relatively large ones like *Araneus*, *Nephila* (Araneidae) or *Hogna* and *Lycosa* (Lycosidae). The scanning settings differ from scanner to scanner and depend on a variety of factors, including specimen size, magnification, and resolution, among others. Therefore, they are not covered here. Still, I include for reference some virtual slices and 3D renderings that have been obtained following this staining protocol (Fig. 6). Besides spiders, I have successfully used this same protocol with plant roots and land snails.

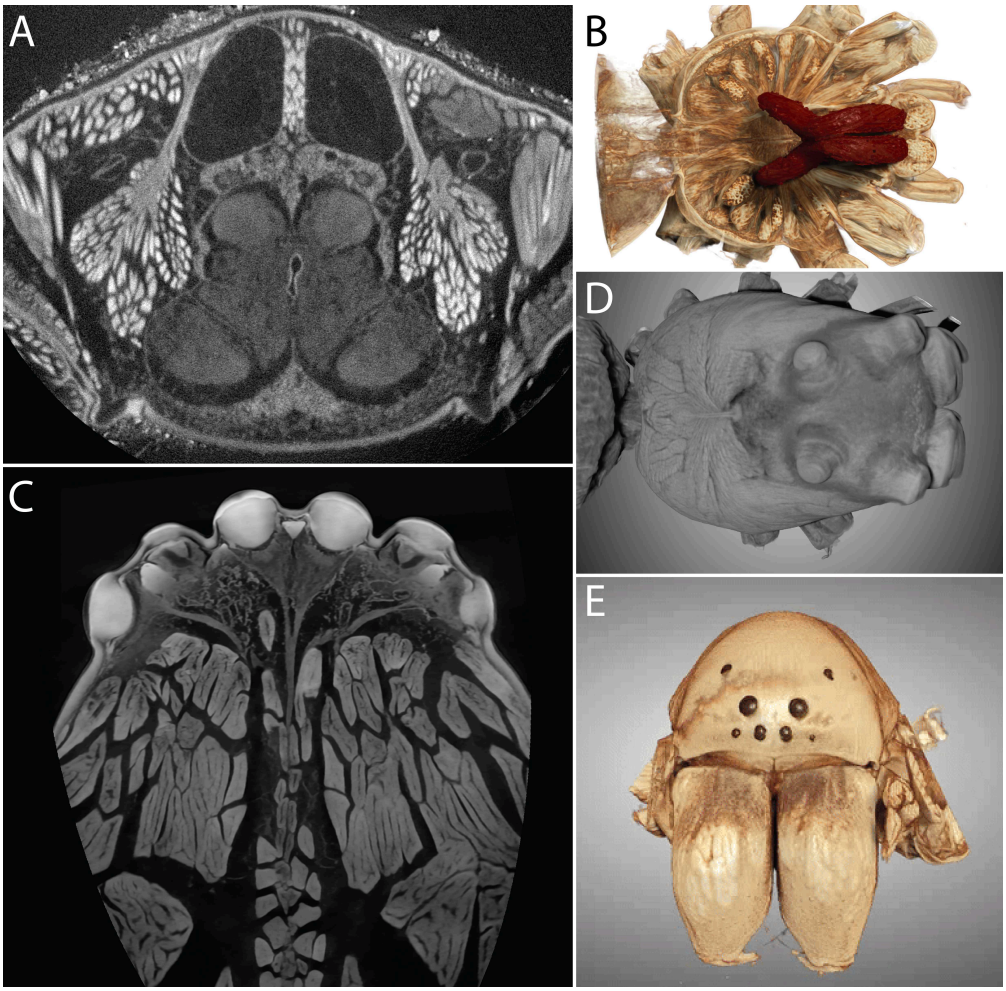

Figure 6. Virtual slices and 3D volume renderings of spider specimens for reference. A) Coronal slice of the cephalothorax focusing on the Central Nervous System. B) Axial view of the cephalothorax with emphasis on the venom glands. C) Axial view of the eyes, retinae and optic nerves. D) Volume rendering of the cephalothorax from a dorsal view. E) Volume rendering of the cephalothorax from an anterior view.

Final considerations

8 Although I have not personally tested thishere, Sumner-Rooney, *et al.* (2019) have shown that PTA staining and micro-CT scanning do not negatively affect DNA extraction, PCR amplification or sequencing, making this protocol compatible with the use of specimens for genetic examination.

Protocol references

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