Use of synchrotron tomography to image naturalistic anatomy in insects

John J. Socha and Francesco De Carlo

Advanced Photon Source, Argonne National Laboratory, Argonne, IL, USA 60439

ABSTRACT

Understanding the morphology of anatomical structures is a cornerstone of biology. For small animals, classical methods such as histology have provided a wealth of data, but such techniques can be problematic due to destruction of the sample. More importantly, fixation and physical slicing can cause deformation of anatomy, a critical limitation when precise three-dimensional data are required. Modern techniques such as confocal microscopy, MRI, and tabletop x-ray microCT provide effective non-invasive methods, but each of these tools each has limitations including sample size constraints, resolution limits, and difficulty visualizing soft tissue. Our research group at the Advanced Photon Source (Argonne National Laboratory) studies physiological processes in insects, focusing on the dynamics of breathing and feeding. To determine the size, shape, and relative location of internal anatomy in insects, we use synchrotron microtomography at the beamline 2-BM to image structures including tracheal tubes, muscles, and gut. Because obtaining naturalistic, undeformed anatomical information is a key component of our studies, we have developed methods to image fresh and non-fixed whole animals and tissues. Although motion artifacts remain a problem, we have successfully imaged multiple species including beetles, ants, fruit flies, and butterflies. Here we discuss advances in biological imaging and highlight key findings in insect morphology.

Keywords: synchrotron microtomography, anatomy, insect, tracheal system, muscle

1. INTRODUCTION

Knowledge of the size, shape, and relative location of internal anatomical structures is fundamental to studies in diverse fields of biology, including physiology, biomechanics, and development. For micro-scale anatomy, histology has provided a wealth of data for over centuries, but serial sectioning suffers three large drawbacks. First, due to fixation and manual cutting, the sample is deformed, potentially changing both the shape and position of tissues and organs. Second, the process is time-consuming, with durations on the order of days to weeks. Lastly, the sample is permanently destroyed, rendering it unavailable for other manipulations. Recent techniques such as MRI, micro-CT (μCT), and confocal microscopy can be non-destructive, and have been applied widely in the past decades. However, these methods also present restrictions to the study of internal 3D anatomy. For example, confocal microscopy samples are required to be small (roughly less than 300 μm) and transparent, and only tissue that fluoresces under laser illumination can be visualized. For a researcher seeking micron-scale resolution of soft tissue in millimeter-sized samples, none of these techniques is sufficient.

Synchrotron micro-CT (SR-μCT) has recently been shown to overcome these limitations in biological samples. Using beamline ID19 at the ESRF, Betz *et al.*[1] were able to distinguish muscles, ligaments, internal cuticle, and digestive organs in three-dimensional data sets of insects and mites. The demonstrated degree of detail, achieved with sub-micron resolution, was particularly impressive. For example, they were able to show fine structures in the digestive system of an oribatid mite less than one millimeter in total body size. However, despite the fact that SR-μCT is non-invasive, the insect samples showed a substantial degree of internal deformation. Because SRμ-CT requires that samples remain perfectly still while the raw projection images are collected, Betz and colleagues fixed the samples prior to imaging. Fixation greatly reduced the chance that internal tissues and organs would move during imaging, and thus maximized the success rate of sampling, but the process induced morphological changes including tissue disengagement and deformation. For example, ommatidia in the eyes of the beetle *Gyrophaena fasciata* can be seen to be discontinuous

Developments in X-Ray Tomography VI, edited by Stuart R. Stock,
Proc. of SPIE Vol. 7078, 70780A, (2008) · 0277-786X/08/$18 · doi: 10.1117/12.795210

from the outer eye, and empty spaces can be seen between muscles and the exoskeleton. Moreover, some structures such as tracheal tubes were massively deformed or appear to have disappeared entirely. Insect tracheal systems consist of tubes that range in size from hundreds to less than a single micrometer in diameter. Because they supply respiratory gases directly to the tissues, the tracheal tubes form a dense network that runs throughout the entire body of the living insect. The absence of tracheae in the tomographic datasets indicates that some aspect of the fixation process rendered the tubes unviewable.

Our research group has been studying respiration in living insects using synchrotron x-ray phase-contrast imaging[2-5]. In order to understand the biomechanics and anatomical origin of dynamic compression of the tracheae and other related physiological processes we have observed, it is necessary to determine the size, shape and position of internal structures in a natural, undeformed state. Here we describe preliminary results of our attempts to image non-fixed insects in three dimensions using SR-μCT at beamline 2-BM of the Advanced Photon Source. Specifically, we aimed to image tracheal tubes and muscles in their natural configuration. The primary challenge in using fresh insect specimens was to eliminate external and internal movements caused by drying and residual post-death biological activity.

2. MATERIALS AND METHODS

Insects were collected locally (beetle, *Platynus decentis*; ant, *Camponotus pennsylvanicus*; butterfly, *Pieris rapae*) or were lab-reared in colonies (fruit fly, *Drosophila melanogaster* wild type; desert locust, *Schistocerca gregaria*). Prior to experimentation, specimens were housed with *ad libitum* food and water.

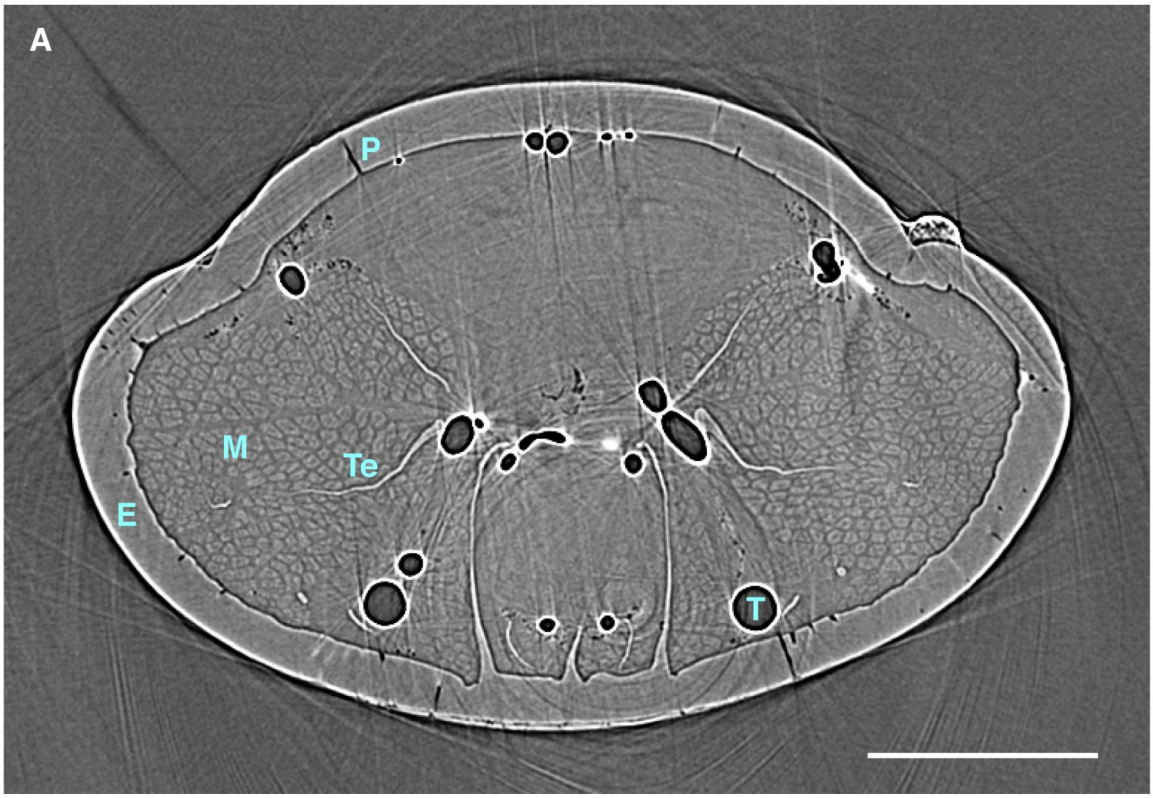
Specimens were sacrificed using ethyl acetate. Depending on the size of the insect, 10-50 μL of ethyl acetate was soaked in a cotton ball and placed with the insect in a sealed 15 or 50 mL polystyrene tube. After 30 minutes, the specimen was transferred to a second tube with a wetted cotton ball and allowed to equilibrate for two hours in a humid environment.

Specimens were imaged whole. To prevent desiccation and to stabilize the body, the specimen was mounted in a small polystyrene or polyimide tube whose diameter was chosen so that the specimen would fit snugly against the walls. For other parts of the body subject to shifting, cyanoacrylate or hot glue was used to prevent movement; additionally, some specimens were mounted head-down. Tubes were then sealed on the top using Prestik, a clay-like adhesive. All specimens except fruit flies were then transferred to a refrigerator and chilled at 6° C for 12 hours, which served to stabilize residual internal movements.

Synchrotron micro-CT image data were collected at beamline 2-BM at the Advanced Photon Source (Argonne National Laboratory). The sample-to-scintillator distance was 100 mm, and energies between 12-19 keV were used. Raw projections were taken in 0.125° steps over 180°. See Wang *et al.*[6] for further details of the beamline setup. Reconstructed slices were segmented using amira software (Visage Imaging, Germany).

3. RESULTS AND DISCUSSION

Undeformed internal soft tissue, including muscle, tracheae, and air sacs, was visualized successfully in three dimensions. Because samples were fresh specimens with no fixation and minimal manipulation, tissues could be seen in their natural configuration. For example, muscle bundles in a reconstructed slice of the head of the beetle *Platynus decentis* fill the space from the tentorium to the exoskeleton (Fig. 1). This visualization permits the precise calculation of the muscle cross-sectional area, a key to producing accurate models of force production. Another prominent example is the tracheal system: for the first time using SR-μCT, tracheal tubes and air sacs could be clearly identified in intact insects (Figs. 1-4). In *Platynus decentis*, tracheal cross-sectional shapes ranged from oval to round, with shape varying along the length of some tubes (Fig. 2). The smallest trachea that could be identified was 3.5 μm in diameter; smaller tubes (including the tracheoles) could not be resolved. In addition to analysis of shape, visualization and 3D rendering of the tracheal system permits the relatively easy analysis of connectivity and volume of the tracheal tubes. We are currently completing whole-body analyses of the tracheal systems of *Drosophila melanogaster* and the beetles *Platynus decentis* and *Pterostichus stygicus*.



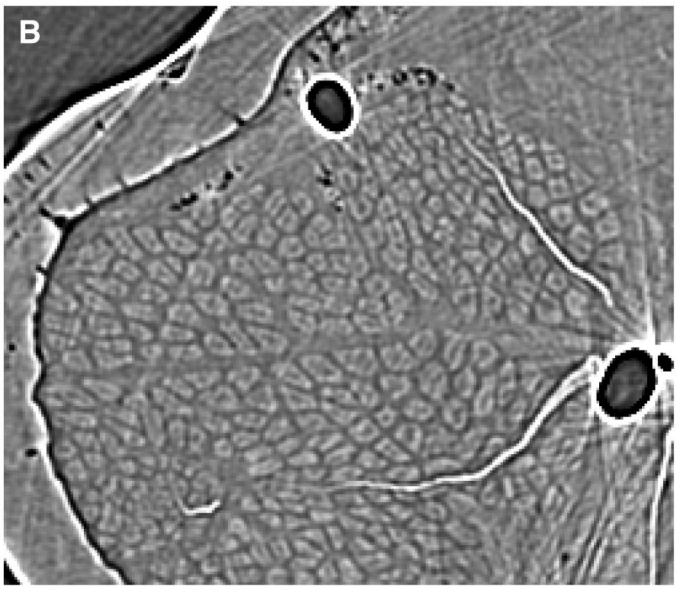


Fig. 1. A. Soft tissue details in the head of the beetle *Platynus decentis*. Dorsal is to the top. Examples of features that can be seen include exoskeleton depth (E), pores in cuticle wall (P), tentorium (Te), muscle bundles (M), and tracheal tubes (T), which appear as empty, circular structures. The small circular structures on the far right in (A) represent the posterior edge of the eye. Scale bar, 200 μm. B. Close-up view of the left side of the head, highlighting muscle tissue.

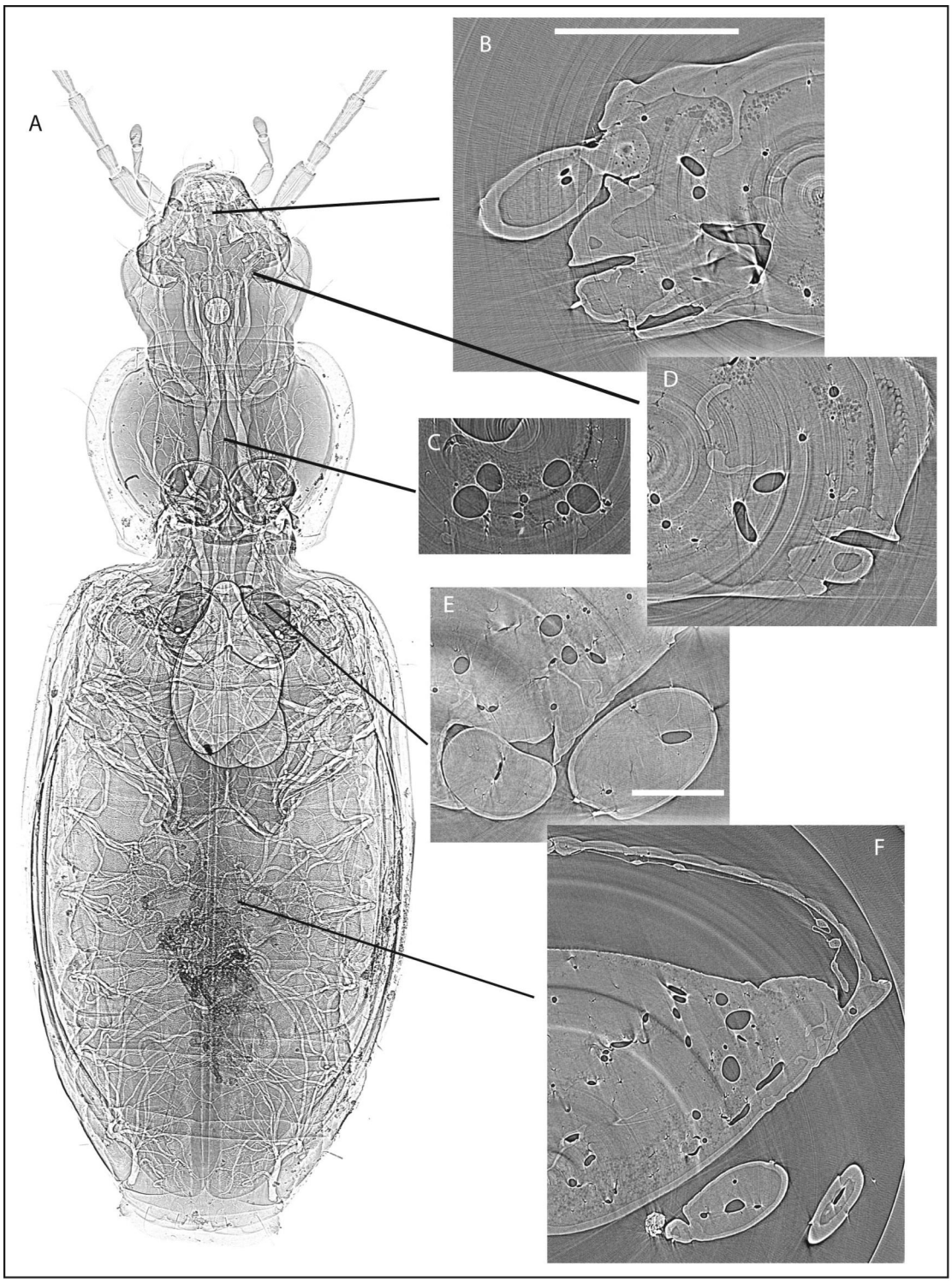


Fig. 2. Survey of tracheal cross-section shape throughout the body of the carabid beetle *Platynus decentis*. A. Composite scan of beetle in ventral view. Image is a tiled phase-contrast projection composite taken at 32-ID (APS). Scale bar, 1 mm. B.-F. Representative transverse tomographic slices depicting various cross-sectional shapes of tracheal tubes. As in Fig. 1, tracheae appear as empty, rounded structures. Note the wide range of cross-sectional shapes, ranging from oval to circular. Scale bar, 500 μm; bar in B also applies to C & D, bar in E also applies to F.

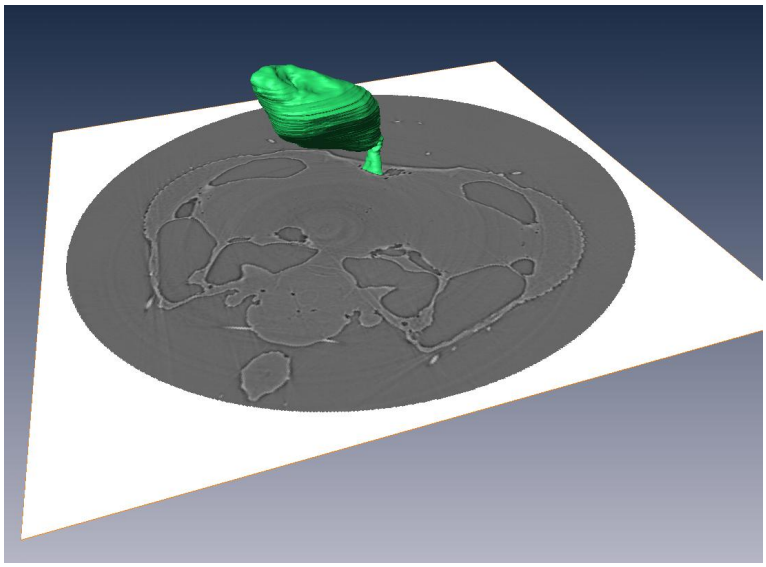


Fig. 3. Three-dimensional rendering of a single air sac in the head of an adult fruit fly (*Drosophila melanogaster*). Dorsal is to the upper right. Tomographic slice transects the eyes (sides) and proboscis (bottom).

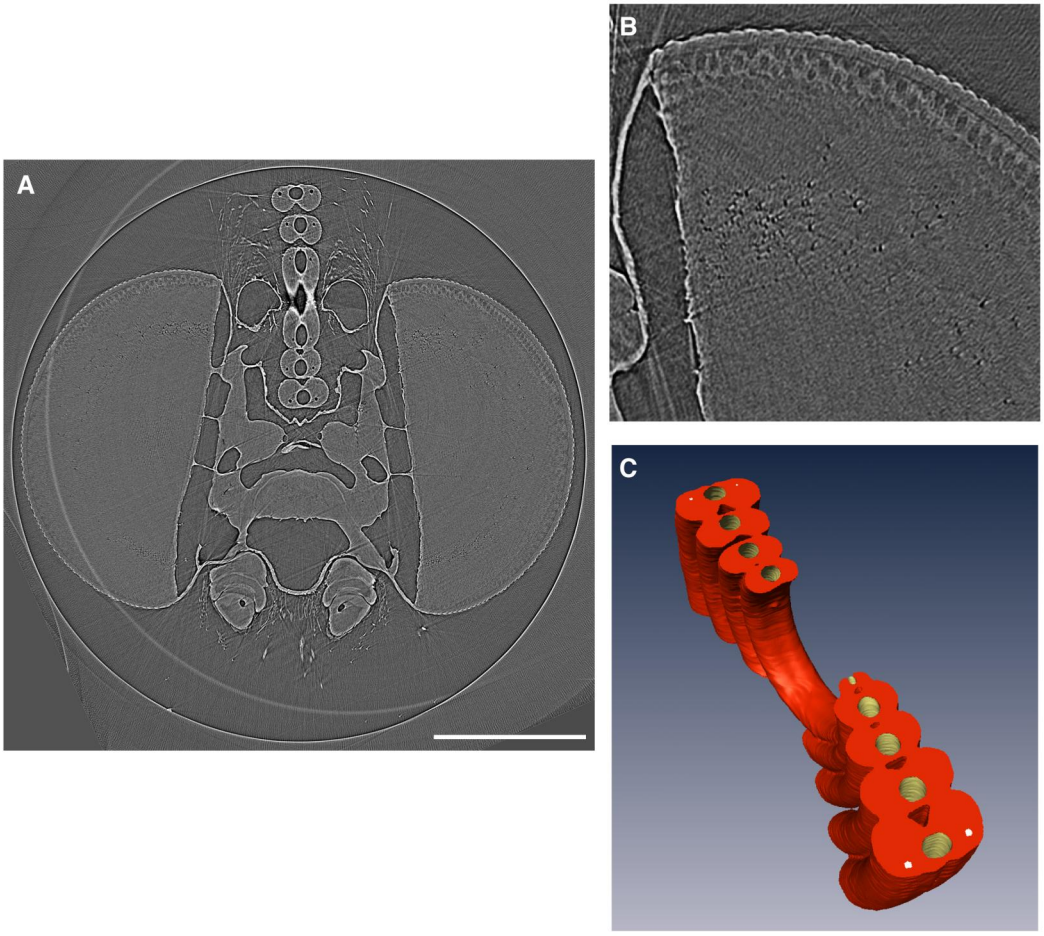


Fig. 4. A. Anatomy of the head of the cabbage white butterfly *Pieris rapae*. The eyes are large bulbous structures on the left and right. Air sacs are found medially throughout the central head. The coiled proboscis can be seen at the top. Scale bar, 500 µm. B. Close-up detail of the right eye showing ommatidia. C. Three-dimensional rendering of a portion of the coiled proboscis. The central feeding tube (yellow) is adjacent to two lateral tracheae, which are depicted only in the two outer segments.

Although we attempted to prepare and mount samples for maximum stability (given no fixation), some of the specimens showed evidence of internal or external movement. Externally, legs and antennae tended to move if not secured to the enclosing tube. Internally, of any tissues or organs, the tracheal tubes, air sacs, and digestive tract were most prone to movements. Blurring in the gut suggests that transport was taking place in the insect post-sacrifice. Tracheal structures tended to collapse (Fig 5). We are unsure what caused collapse, but we suspect that the success of imaging intact, inflated tracheae was related to differences in spiracular opening and closure between samples. If the insect's spiracles were closed post-sacrifice, it is possible that the still metabolically-active tissue depleted the oxygen within the tube or air sac. Because carbon dioxide is buffered by the tissues, it would not diffuse into the tubes; the net removal of gas would lower in the internal tracheal pressure to the point of collapse. We are currently exploring methods of chilling the sample during imaging to reduce the effect of internal movements.

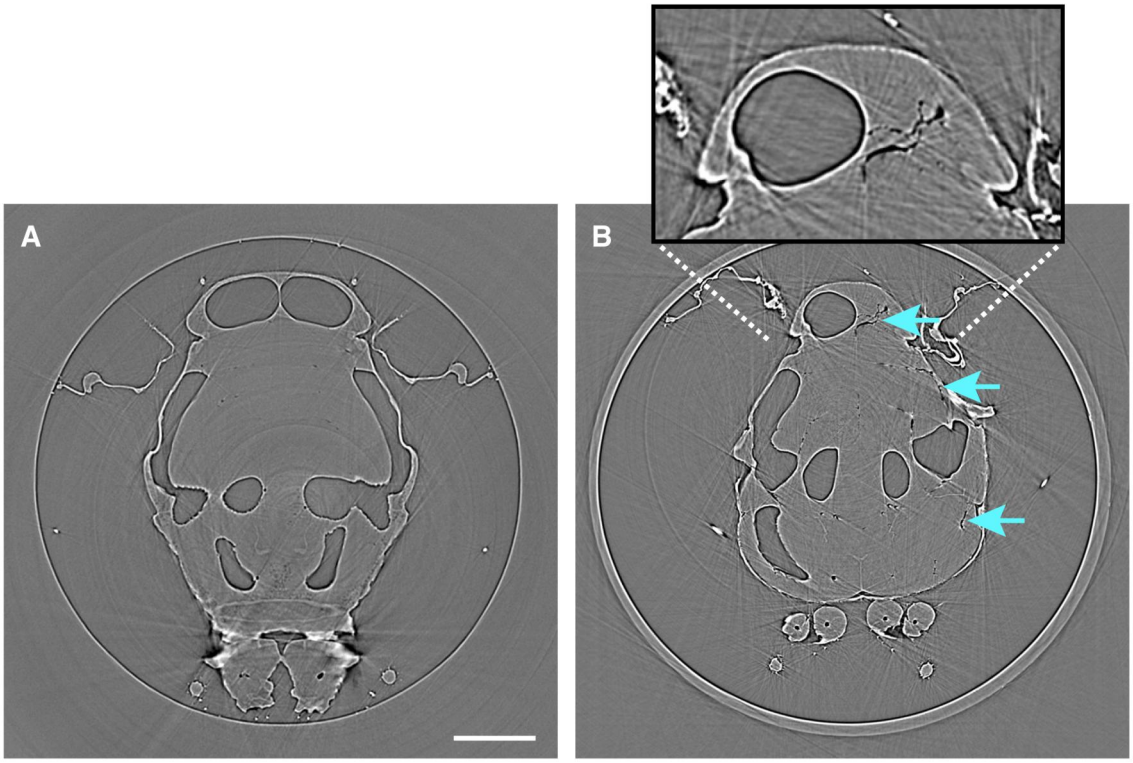


Fig. 5. Examples of fully inflated (A) and partially deflated (B) tracheal systems in the thorax of adult fruit flies (*Drosophila melanogaster*). A & B are tomographic slices from two different specimens. Dorsal is to the top; wings and legs can be seen at the top and bottom, respectively. The blue arrows in (B) point to air sacs that collapsed prior to imaging. In some specimens, air sacs or tracheal tubes collapsed during imaging (not shown).

Comparison with other techniques

The combination of minimal sample preparation (including no fixation or submersion in alcohol) with SR-μCT imaging yields perhaps the most non-invasive and naturalistic 3D anatomy currently feasible in intact insects. This study shows that such simple preparation can produce successful tomographic data. For perspective, we compare the visualization of tracheal structures with other techniques. Because tracheae consist of thin-walled tubes surrounded by hemolymph and are subject to collapse, these structures may represent a worst-case scenario for studies of internal insect anatomy.

Historically the shape of insect tracheal tubes has been determined using histology[7, 8]. However, the process of fixation and slicing can result in detachment of tissues and massive deformation of the tracheal tubes[9]. Recent studies have used non-destructive methods of determining 3D anatomy. NMR and MRI have both been used to study insects [10, 11]; neither has the necessary resolution to image most tracheal tubes. Wecker et al.[11] used NMR to investigate the internal anatomy

of the diving beetle *Dytiscus maginalis*. With a spatial resolution of approximately 60 µm, only the largest tracheal tubes were resolved in this study. Hart et al.[10] report images of an ant (*Dinoponera quadriceps*) and wasp (*Vespula vulgaris*) using MRI; however, tracheae could not be visualized. Hornschmeyer et al.[9] used a laboratory µCT system (Skyscan 1072) to image the head of the beetle *Priacma serrata*. Although they did not specifically aim to capture tracheae, their images (Figs. 4-7) show few if any tracheal tubes; more recent µCT systems may prove better.

Confocal microscopy and corrosion casting are two methods that can provide higher resolution of tracheal systems than possible with SR-µCT. However, the size limitations of confocal microscopy warrant that most insects would need to be sectioned for imaging. Corrosion casting, combined with SEM, produces excellent 3D detail of tracheae from the whole system level to the smallest tracheoles[12]. Meyer in fact reports tracheoles as small as 70 nm in diameter. But corrosion casting requires destructive digestion of the sample, and the remaining cast is subject to breakage at weak points such as the legs and antennae. Furthermore, the technique is time consuming (on the order of weeks), and based on the relative dearth of studies, may prove difficult to conduct in practice. In contrast, SR-µCT is extremely quick: reconstructed slices can be available within an hour of data collection. Overall SR-µCT has great potential to provide a rich source of data for studies of animal form and function.

ACKNOWLEDGEMENTS

We thank James Waters, You Zhou, John Sheppard, Xianghui Xiao, and Konstanin Ignatyev for help with data collection, and Wah-Keat Lee, Kamel Fezzaa, and Stewart Stock for discussion. Jon Harrison provided the grasshoppers. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Basic Energy Sciences, under Contract No. DE-AC02-06CH11357.

REFERENCES

[1] Betz, O., U. Wegst, D. Weide, M. Heethoff, L. Helfen, W.-K. Lee, and P. Cloetens. "Imaging applications of synchrotron x-ray phase-contrast microtomography in biological morphology and biomaterials science. I. General aspects of the technique and its advantages in the analysis of millimetre-sized arthropod structure." *Journal of Microscopy* 227, no. 1, 51-71 (2007).

[2] Socha, J. J., M. W. Westneat, J. F. Harrison, J. S. Waters, and W. K. Lee. "Real-time phase-contrast x-ray imaging: a new technique for the study of animal form and function." *BMC Biology* 5:6 (2007).

[3] Westneat, M. W., O. Betz, R. W. Blob, K. Fezzaa, W. J. Cooper, and W. K. Lee. "Tracheal respiration in insects visualized with synchrotron x-ray imaging." *Science* 299, no. 5606 558-60 (2003).

[4] Westneat, M.W., J.J. Socha, and W.K. Lee. "Advances in biological structure, function, and physiology using synchrotron x-ray imaging." *Annual Review of Physiology* 70 119-42 (2008).

[5] Socha, J. J., W. K. Lee, J. F. Harrison, J. S. Waters, K. Fezzaa, and M. W. Westneat. "Correlated patterns of tracheal compression and convective gas exchange in a carabid beetle." (*in review*).

[6] Wang, Y. X., F. De Carlo, D. C. Mancini, I. McNulty, B. Tieman, J. Bresnahan, I. Foster, J. Insley, P. Lane, G. von Laszewski, C. Kesselman, M. H. Su, and M. Thiebaux. "A high-throughput x-ray microtomography system at the Advanced Photon Source." *Review of Scientific Instruments* 72, no. 4 2062-68 (2001).

[7] Schmitz, A., and S. F. Perry. "Stereological determination of tracheal volume and diffusing capacity of the tracheal walls in the stick insect *Carausius morosus* (Phasmatodea, Lonchodidae)." *Physiol Biochem Zool* 72, no. 2 205-18 (1999).

[8] Hartung, D. K., S. D. Kirkton, and J. F. Harrison. "Ontogeny of tracheal system structure: a light and electron-microscopy study of the metathoracic femur of the american locust, *Schistocerca americana*." *Journal of Morphology* 262, no. 3 800-12 (2004).

[9] Hornschemeyer, T., R. G. Beutel, and F. Pasop. "Head structures of *Priacma serrata* Leconte (Coleptera, archostemata) inferred from x-ray tomography." *Journal of Morphology* 252, no. 3 298-314 (2002).

[10] Hart, A. G., R. W. Bowtell, W. ckenberger, T. Wenseleers, and F. L. W. Ratnieks. "Magnetic resonance imaging in entomology: a critical review." *Journal of Insect Science* 3, no. 5 1-9 (2003).

[11] Wecker, S., T. Hornschemeyer, and M. Hoehn. "Investigation of insect morphology by mri: assessment of spatial and temporal resolution." *Magnetic Resonance Imaging* 20, no. 1 105-11 (2002).

[12] Meyer, E.P. "Corrosion casts as a method for investigation of the insect tracheal system." *Cell & Tissue Research* 256, no. 1 1-6 (1988).