Penetrating view of nano-structures in *Aleochara verna* spermatheca and flagellum by hard X-ray microscopy*

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A penetrating view of the three-dimensional nanostructure of female spermatheca and male flagellum in the species *Aleochara verna* is obtained with 100-nm resolution using a hard X-ray microscope, which provides a fast noninvasive imaging technology for insect morphology. Through introducing Zernike phase contrast and heavy metal staining, images taken at 8 keV displayed sufficient contrast for observing nanoscale fine structures, such as the spermatheca cochleate duct and the subapex of the flagellum, which have some implications for the study of the sperm transfer process and genital evolution in insects. This work shows that both the spatial resolution and the contrast characteristic of hard X-ray microscopy are quite promising for insect morphology studies and, particularly, provide an attractive alternative to the destructive techniques used for investigating internal soft tissues.

Keywords: X-ray microscopy, computed tomography, synchrotron radiation source, morphology

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1. Introduction

Insects are the most species-rich group on the Earth and their origin and genital evolution are topics attracting the attention of evolutionary biologists.[1] Indeed, modern genital evolutionary studies now increasingly depend on delicate morphological unraveling and morph-diversity comparisons in micro-to-nanoscales.[2–4] Insect structural features, especially those with three-dimensional (3D) reconstructions from the macroscopic to the microscopic scales, have become more and more important in understanding insect origins and evolution. Classically, the investigation of the internal morphology of insects relies on the preparation of thin tissue sections. However, the risk of damaging sections can never be eliminated, and quick acquisition of 3D structural information by using such an approach is usually impractical.

Many microscopy techniques using different wavelengths of light have been developed in order to get the internal structure information. However, limitations still exist in these microscopy techniques. For example, optical microscopy has only low spatial resolution of ca. 200 nm, making it inapplicable in a great many potential applications and far from useful at nanoscale. Although electron microscopes have reached atomic resolution, they can only offer two-dimensional (2D) information from cross sections and very restricted 3D information due to the short penetration distance of the electrons. All these limitations persistently raise concerns about the prospects for efficient interior investigations. Fortunately, the development of microscopy using X-rays promises to overcome these difficulties and may benefit the insect metrology and evolutionary studies that prefer to capture an ideal visualization of the interior structure directly.

Hard X-ray microscopy is among the latest developments in the application of synchrotron radiation sources and the fabrication of optical elements that allow focusing of the X-rays to nanometer dimensions, which can bridge the gap between light microscopy and electron microscopy and provide a tool that is complementary to existing imaging techniques.[5–8] On one hand, using shorter wavelengths than visible light and appropriate photon optics, hard X-ray microscopy has already achieved 30 nm–50 nm resolution.[9–11] Moreover, when combined with tomographic procedures, hard X-ray microscopy is a powerful nondestructive 3D imaging technique, which offers unique opportunities to observe the interior 3D structures of complex, optically opaque samples.[12] Unlike electron microscopy, this means there is no need to section a sample with an ultramicrotome before imaging by hard X-ray microscopy. Moreover, because it has larger depth of focus and higher penetration power, hard X-ray microscopy and tomog-

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raphy have the potential to image large soft tissue with thickness up to several tens of micrometers, which is a unique advantage in biological sample imaging compared with soft X-ray microscopy. All the potential advantages made hard X-ray microscopy widely used in various research fields such as material science and environmental science.[13–15] However, there have been few relevant studies in the insect morphology field so far. So it is significant to develop the ability and methodology of imaging insect morphology with hard X-ray microscopy and tomography at nano resolution. In this work, we report the first attempt to use the hard X-ray microscopy to image and visualize the internal fine structure of insect genitalia.

Insects are an animal group with internal fertilization. In the reproductive season, a male copulates with a female and transfers sperm or a spermatophore to the latter. During this process, the male flagellum and the female spermatheca are the directly interacting structures in most insect species. Therefore, morphology studies of these structures are very important in understanding the sperm transfer process and genital evolution. These structures are usually membranous or subsclerotized, so it is impossible to view the morphology structures using traditional methods without causing damage.[4] However, 3D digital reconstructions with hard X-ray microscopy can be very important and helpful to the studies of insect anatomy, morphology, and functional biology. In this paper, the structures related to male–female copulation and interaction of the species *Aleochara verna*, are studied by using hard X-ray microscopy with nanoscale resolution. Through Zernike phase contrast, and fixation, and heavy metal stains, the powerful 3D images in visualizing the internal structure of the spermatheca and the flagellum were acquired, which are closely related to the mechanism of sperm transfer and thus are crucial in understanding it. This may be especially important in explaining the diversity of genitalia.

2. Material and methods

2.1. Sample preparation

The materials used in this study are specimens of *Aleochara verna* which were collected in the Olympic Forest Park, Beijing, China. The collection method was a free-searching method: to collect individual living beetles within a pile of half-rotten hay and maintain them in 70% alcohol. Couples of a male and a female in copulation were collected and quickly killed to preserve this state. All the specimens were fixed in Dubosque–Brazil solution for 2 days and then transferred to alcohol. The spermatheca itself and the spermatheca tube were dissected out and stained by 0.3% phosphotungstic acid (PTA) in 70% ethanol overnight, then dried in a critical point dryer (HCP-2). Finally, the dry specimens were mounted on the sample stage of a hard X-ray microscope.

2.2. BSRF X-ray microscope

The schematic optical layout of the BSRF X-ray microscope, as shown in Fig. 1, can be divided into the beam line and the X-ray microscope.[16] For the beam line, the X-ray generated from the wiggler is first vertically collimated by a bent flat mirror 1 located 22 m from the wiggler source, and then followed by a double crystal monochromator, which selects X-rays of energy 5 keV–11 keV and provides a monochromatic X-ray flux of 5×10^8 photons/s at 8 keV at the stored ring current of 250 mA. The focusing mirror 2 is similar to the flat mirror 1. A variable curvature will allow the X-ray to be horizontally focused at slit 2 at 42.8 m in the respect that the large illumination phase space can efficiently match the numerical aperture of the objective zone-plate.

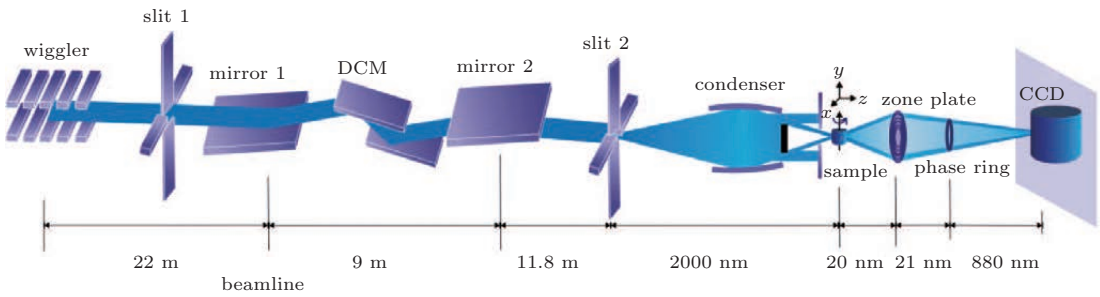

Fig. 1. (color online) The optical layout of the BSRF X-ray microscope.

The X-ray microscopy is quite similar to a visible light microscope. After the X-ray beam is monochromated and focused at slit 2, it is then focused onto the sample by a condenser consisting of an elliptically shaped capillary tube located 1.8 meters from the virtual source (slit 2). Hereafter, the X-ray image of a sample is magnified by the objective zone-plate and converted into a visual one on the CsI (Tl) scintillation screen. This visual image is further enlarged several times with a microscope objective lens and captured by a 1024×1024-charge-coupled device camera. In addition, a phase ring can be placed in the back focal plane of the objective zone plate to provide Zernike phase contrast, so that the hard X-ray microscope has the capability to investigate light materials. In the present microscope, the objective zoneplate

with 40-nm and 100-nm outermost -zone widths operated at 8 keV were used at BSRF, magnifying the images 45× and 11× for the high resolution and large field-of-view modes, respectively. Conjugated with 20× downstream optical magnification, the 2D and 3D resolutions shown in Fig. 2 can reach 30 nm and 100 nm for the high resolution and large field of view modes, respectively, providing an ideal tool for zoological insect morphology studies at the nanoscale.[16]

The 3D structure information of the spermatheca cochleate duct and the flagellum was obtained by using the BSRF hard X-ray microscope in the large field-of-view mode. In addition to the heavy metal stain, the Zernike phase contrast was introduced to improve image contrast, which is useful for biological sample in hard X-ray photon range. The total 161 sequential tomographic images of the spermatheca tube and the flagellum were automatically collected at 1° intervals from −80° to +80° at 8.0 keV. With an exposure time of 20 s per image, the total exposure time and the scan time were approximately 53 min and 10 min for each sample. By using the filtered back-projection algorithm, the 3D rendering and reconstructed slices of the spermatheca cochleate duct of the female and the flagellum of the male in the species *Aleochara verna* are shown in Fig. 3.

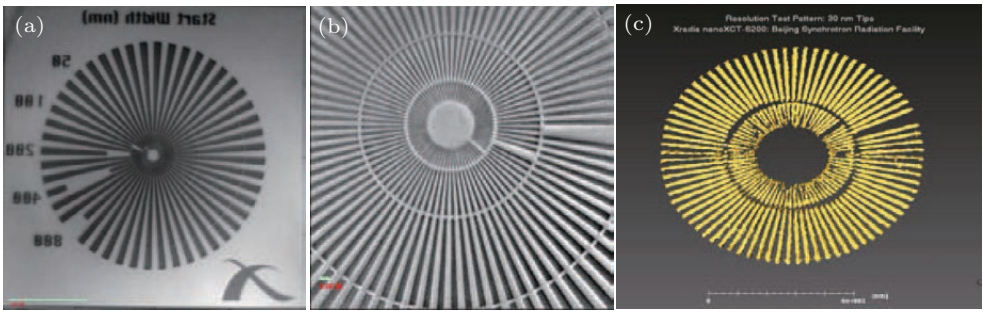

Fig. 2. (color online) The electroplated gold spoke patterns with 50-nm and 30-nm finest line widths were adapted to test the 2D and 3D resolution of the microscope. The visibly resolved 100-nm and 30-nm finest line widths imaged in the large field of view (FOV: 60 μm×60 μm) (a) and high resolution (FOV: 10 μm×10 μm) mode (b) indicates the achievement of theoretical 30 nm and 100 nm spatial resolution. (c) The 3D resolution was tested based on 141 sequential image frames taken in the high resolution mode with azimuth angle rotating from to −70° to 70°. It can reach 30 nm.

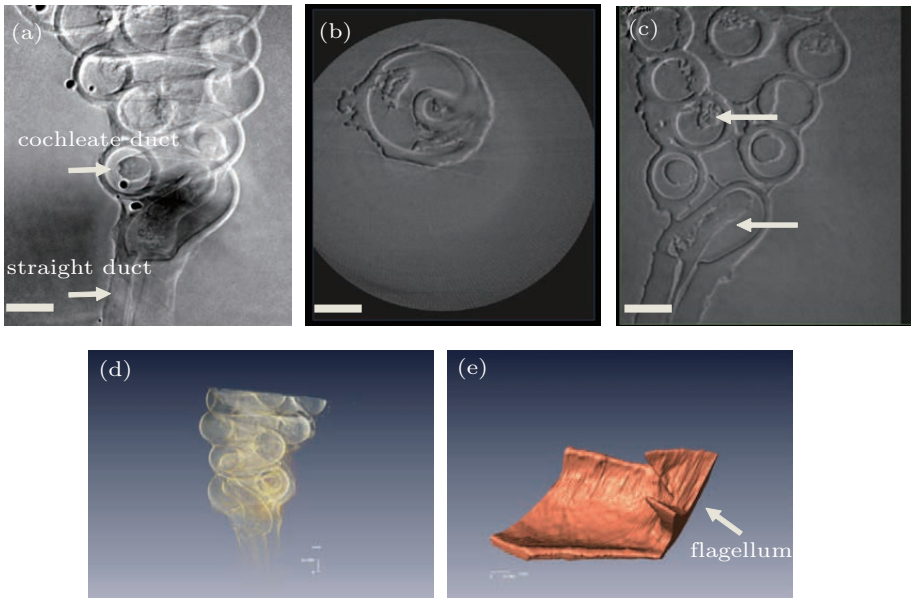

Fig. 3. (color online) The 3D structure of the female spermatheca cochleate and subapex of the male flagellum in the species Aleochara verna. (a) Projective image of the cochleate duct. The transverse slice (b), and coronal slice (c) of the cochleate duct, (d) 3D reconstruction of the cochleate duct, (e) 3D reconstruction of the subapex of the flagellum. The scale bar is 10 μm.

2.3. Results and discussion

The species *Aleochara verna* is a small beetle with body length of only 2.8 mm–3.0 mm, which belongs to the genus *Aleochara* (Coleoptera: Staphylinidae). All the species of the genus *Aleochara* are predators. The larva acts as a parasite in the fly pupa, while the adult lives in the down or below rotting

vegetation.[17] During oviposition season, beetles copulate to produce the next generation. Thereunto, the female external reproductive system consists of spermatheca, bursa copulatrix and vagina, and the male external reproductive system consists of the medial lobe, lateral lobe, endophallus and flagellum.[18] For the genus *Aleochara*, the spermatheca is a sperm storing and sperm selecting organ, which consists of three clearly defined divisions: a more or less spherical capsule, an enlarged chamber and a narrower duct.[19] In the species *Aleochara verna*, the duct can be divided into the cochleate duct and straight duct. Figure 3(a) presents the high contrast projective image of the cochleate duct of *Aleochara verna*, in which the observed intensity of each pixel contains both amplitude and phase information. It can be clearly seen that the image taken at 8 keV shows good contrast and the nanoscale spatial resolution already differentiates individual segments of the cochleate duct, and the tubular structures can be distinguished in Fig. 3(a); whereas, by using only a single 2D projection, the fine structure inside or outside of the tubular structures cannot be clearly recognized. The overlap of complex features in projections complicate image analysis. In order to distinguish the precise interior structures of the cochleate duct, the slice images and 3D imaging of the cochleate duct are thus necessary. Figures 3(b) and 3(c) show the slice images of the cochleate duct. The ring-shaped tubular structure can be clearly distinguished, and the triquetral structure marked by arrows in the duct is supposed to be the materials transferred by the male. The enlarged 3D reconstruction of the cochleate duct, without any loss of information, is displayed in Fig. 3(d). The clearly distinguished cochleate-shaped structure can be seen and it looks more slightly sclerotized and thinner than that of the straight duct. The 3D structure of the subapex of the flagellum was also reconstructed, as shown in Fig. 3(e). The apex of the flagellum is a scoop-shaped canal and gradually broadens in its anterior. The posterior of the canal is V-shaped and much narrower than the apex. As shown in Fig. 3(c), the materials transferred from the male extend anteriorly until in the cochleate duct. They are arranged cylinder-shaped in the first two turns, whereas the distribution density and diameter decrease toward the posterior. All the above information obtained by the BSRF hard X-ray microscope show more detail in nanoscale, which is very important for further genital evolution studies.

In the genus *Aleochara*, the spermatheca can be divided in three types. In the first type, the capsule and chamber are fused together and are connected with a sclerotized cochleate duct; in the second type, the capsule, chamber and duct are fused together; in the third type, the capsule is fused with the chamber. The spermatheca of *Aleochara verna* belongs to the first type.[20] According to the male–female interaction structures mentioned above, the sperm of the male apparently must move themselves to the female spermatheca, for no special transferring structure is found. Passing through a complex cochleate duct initially, sperm of good quality may be selected. However, species with spermatheca of the second type has different sperm transfer process. For example, for the species *Aleochara curtula,* the sperms transferred into the spermatheca capsule of the female through the elongation of a primary tube and a second tube,[21] which is supposed to be a result of sperm competition. Therefore, in the species of the genus *Aleochara*, the mechanisms of sperm transfer are not always the same, and it is impossible to explain the genital diversity with a single hypothesis. Although evoking lots of hypotheses (such as the lock-and-key, pleiotropy, sexual selection, sperm competition, cryptic female choice, antagonistic coevolution, etc.) to explain the genital evolution, most the studies deal with the pre-copulation, copulation or postcopulation period, not the whole male–female interaction process.[21,22] A detailed study with all the periods of one species is especially needed and more important. Further studies include different periods after copulation dealing within one species will be very useful and important.

In the present study, an important prerequisite for imaging insect morphology is to preserve the native state of biological samples during sample preparation. For chemical treatment, we used fixation and heavy metal staining agents, including Dubosque–Brazil solution and phosphotungstic acid, to improve the image contrast. Then gradual dehydration by critical point drying was employed at the end. These processes have already been proven to effectively improve contrast in transmission electron microscopy (TEM) through enhancing the electron density of biological samples. However, all these processes have some potential to change the structures of biological samples. For instance, drying can result in considerable shrinkage of the biological sample.[23–25] Admittedly, such sample preparation is a major disadvantage of our imaging method compared with soft X-ray microscopy, which can image cells close to their living state with the help of cryogenic sample stages. Cryofixation, which could preserve a biological sample's ultrastructures better than the normal fixation and drying, is a good alternative preparation method for future research. Moreover, specialized cryogenic sample stages, which have been used in soft X-ray tomography, may be the most effective tools to reduce structural changes, for using such stages can enable biological samples to be imaged in hydrated states and avoid the destructive drying process.[26,27]

In summary, we can see that hard X-ray microscopy provides a huge amount of useful experimental data. The slice results give a perfect description of the fine structure and distribution of sperm in the spermatheca cochleate duct, which is helpful to reveal the process of sperm transfer. By stacking sequences of X-ray microscope slices, the 3D recon-

struction technique provides an excellent view of the spermatheca cochleate duct of female and the flagellum of male in the species *Aleochara verna* and reveals structural details with nano resolution that were invisible to conventional microscopy. Moreover, BSRF X-ray microscopy can improve the density resolution of soft tissues greatly over the current attenuation-based imaging method, but it also has the potential for reducing radiation exposure because the signal is associated with the phase shift or refraction of individual photons rather than with the reduction of the photon flux. Therefore, all these advantages make BSRF X-ray microscopy a powerful new tool for studying insect morphology at nano resolution.

3. Conclusion

The complex procedures are necessary to obtain specimen cross sections for microtomy, and the use of optical micrographs are impractical for a 3D structural reconstruction of a specimen with nano resolution in a short period of time. We present here images based on experiments with BSRF X-ray microscopy, which require little sample preparation and relatively simple data post-processing, and overcome the limitations of conventional image technology like classic microscopy or TEM. We presented and discussed the internal 3D nano structure of the female spermatheca cochleate duct and the male flagellum in the species *Aleochara verna*, the reconstruction reveals more details of the male–female interaction structures in 3D, which may be very useful to provide clues to validate existing hypotheses about the diversity of genitalia. The present research in the paper also demonstrates that X-ray microscopy with high spatial resolution is necessary for the investigation of insect morphology. It will probably become a standard tool in many laboratories in the future, owing to the quantitative 3D data that it provides.

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