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Prof. Dr. Phillip Steindel
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Dear Prof. Steindel,

Thank you for your letter of April 16, 2019, concerning our previous manuscript (JoVE59251R1) entitled " Sample preparation method of scanning and transmission electron microscope for the appendages of woodboring beetle".

Your suggestions and the reviewers' comments were very helpful for revising and improving our study. We have revised the manuscript by taking into account all the comments made by you and the reviewers, and now as you suggested resubmit our revised version to the journal for your consideration.

In the following we give our replies to the questions (bold) raised by the reviewers and indicate how we revised the original manuscript.

We hope our manuscript is in-press before November 1st.

Here, we wish to express our heartfelt thanks to you and the three reviewers for your critical comments.

Looking forward to hearing from you soon.

Sincerely yours,
Peng-fei Lu

TITLE:

Sample Preparation of Woodboring Beetle Appendages for Electron Microscopy

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KEYWORDS:

insect, olfactory, gustatory, sensilla, ultrastructure, scanning electron microscope, transmission electron microscope, fluorescence microscope, chemical ecology

SUMMARY:

Scanning and transmission electron microscopy sample preparation protocols for the observation of the ultrastructure of insect sensilla are presented. Tween 20 was added to the fixative to avoid sample deformation in scanning electron microscopy. Fluorescence microscopy was helpful for improving slicing accuracy in transmission electron microscopy.

ABSTRACT:

This report describes sample preparation methods for scanning and transmission electron microscope observations of the woodboring beetle, *Chlorophorus caragana*. The methods are confirmed by preparing beetle appendages for both types of electron microscopy. The scanning electron microscopy (SEM) sample preparation protocol is based on sample chemical fixation, dehydration in a series of ethanol baths, drying, and sputter-coating. By adding Tween 20 (polyoxyethylene sorbitan laurate) to the fixative and the wash solution, the insect body surface of the woodboring beetle was clearer in SEM. This study's transmission electron microscopy (TEM) sample preparation involves a series of steps including fixation, ethanol dehydration, embedding in resin, positioning using fluorescence microscopy, sectioning, and staining. Fixation with Tween 20 enables easier penetration into the insect body wall to more easily fix tissues and organs in the body, thus yielding clear transmission electron microscope observations of insect sensilla

ultrastructures. The next step of this preparation is determining the positions of insect sensilla in the sample embedded in the resin block using fluorescence microscopy to increase the precision of target sensilla positioning. This improved slicing accuracy.

INTRODUCTION:

SEM is an important tool in many morphology studies because it shows surface structures[1-2]. TEM shows inner structures and can be used to study a wide range of biological structures at the nanometer scale[3-5].

Coleoptera is the largest group of insects, including about 182 families and 350,000 species. Most of the coleopteran insects, particularly the woodboring beetle, are serious pests in forests and fruit trees, causing devastating damage to plants[6]. At present, prevention and controlling the population of pests based on chemical ecology theory have received increasing attention[7]. Efficient, low-toxic, pollution-free pheromone control methods have become an effective technique[8]. Studying the sensilla morphology and ultrastructure of insects is an important part of insect chemical ecology research. SEM and TEM are important in studying insect morphology and internal anatomy. However, during the preparation of insect samples for electron microscopy, the objectivity and authenticity of the observation site may be affected[9]. In general, SEM sample preparation of insects requires cleaning, tissue fixation, dehydration, metathesis, drying, and sputter-coating[10]. Woodboring beetle appendages often have many fine long sensilla or bristles. Also, due to the complex environment in which they live, the body surface often has various pollutants. Some woodborers are not available from laboratory breeding but are collected directly in the field and then put into fixing fluid to ensure freshness and subsequently washed in the laboratory. If the sample is first fixed and then washed, it is obviously much more difficult to remove debris because glutaraldehyde strongly fixes it to the sample. Tween 20 is a surfactant[11-14] that plays an important role in the washing process, including reducing the surface tension of water and improving wettability. In this study, Tween 20 was added to the fixing solution and the PBS cleaning solution to reduce the surface tension of the liquid and prevent debris from accumulating on the body surface of the woodboring beetle to make the body surface cleaner in SEM.

Using TEM, sensilla on different organs of insects can be sliced to reveal the clear structures inside them, thus providing a basis for analyzing sensilla functions. When the subject insect, such as the woodboring beetle, is large and its body wall has a substantial degree of sclerotization, the fixative may not fully saturate the organ tissues inside the insect body. Tween 20 can enhance the dispersion and suspension capacity of the debris. In this study, Tween 20 was added to the fixative to enhance fixative fluid penetration into the insect body wall of the woodboring beetle, avoiding deformation and collapse of the epidermis[11-13]. In addition, using general slicing technology, it is difficult to accurately locate different types of sensilla, especially small sensilla[15]. Based on traditional TEM sample preparation, this study combined fluorescence microscopy and SEM to determine the position of insect sensilla in the embedded block, thus improving slicing accuracy.

PROTOCOL:

CAUTION: Consult the material safety data sheets of reagents before using them. Several of the chemicals used during sample preparation are toxic, mutagenic, carcinogenic, and/or reprotoxic. Use personal protective equipment (gloves, lab coat, full-length pants, and closed-toe shoes) and work under a fume hood while handling the samples.

1. SEM sample preparation and imaging

1.1. Sample fixation and cleaning

1.1.1. Working in an area where *C. caragana* live, attract adults into field traps baited with plant attractants, such as isophorone[16]. Preserve the clean bodies of adult *C. caragana* in 0.1 Mol/L phosphate-buffered saline (PBS, pH 7.2), 2.5% (wt/vol) glutaraldehyde (anhydrous EM grad), and 0.06% (vol/vol) Tween 20. Fix the sample at 4 °C for ~3 days.

1.1.2. Remove the bodies from the preservation liquid and rinse in the phosphate buffer. Using a stereomicroscope, remove the appendages and clean them ultrasonically (40 kHz) in a 0.1 Mol/L phosphate-buffered saline (pH 7.2) with 0.06% (vol/vol) Tween 20 (PBST). After cleaning for 100 s, transfer the sample to the microscope to check if it is clean. Under normal circumstances, clean for 400 s to ensure that the sample is clean enough to observe and is not damaged.

1.2. Sample dehydration, mounting, and drying

1.2.1. Dehydrate the samples by using 20 min successive treatments in 50%, 60%, 70%, 80%, 85%, 90%, 95%, 100%, and 100% (all vol/vol) ethanol. Under a stereomicroscope, use carbon double-sided adhesive tape to separately fix three observation surfaces (dorsal, ventral, and lateral) onto stubs. Note that all viewing surfaces must be kept clean and free of contamination. Place the sample stage in a Petri dish containing a silica gel desiccant for 48 h.

1.3. Sputter-coating and sample insertion

1.3.1. Using an ion sputtering instrument (see **Table of Materials**), rotate the **MAIN VALVE** to the **OPEN** position, remove the sample chamber cover, and put the sample into chamber. Turn the **POWER** switch on, and ensure the **READY** light is on.

1.3.2. Set the sputtering time as 45 s, and the coating thickness as 70.875 Å. Once the mechanical pump vacuum dial index drops below 7, press **DISCHARGE** and start spraying platinum.

1.3.3. At the end of the procedure, turn off the power supply and take the sample out of the chamber. The spray film thickness should be d = KIVt, where d = the thickness of the film (Å); K = a constant, determined by the sputtered metal and gas (e.g., K of air is 0.07); I = plasma flow (mA); V = voltage applied (kV); and t = time (seconds).

1.3.4. Insert the stub containing the sample onto the stage of the SEM. Make sure the sample stage with the sample stub has enough height to allow a good image. Open the SEM software and select a desired operating voltage, beginning at 20 kV.

2. TEM sample preparation and imaging

2.1. Cleaning, secondary fixation, and dehydration

2.1.1. After obtaining and fixing the sample as in section 1.1., remove adult *C. caragana* from the preservation liquid. Using a stereomicroscope, remove the appendages, wash the samples in PBST for 3 h, and then post-fix them in 1% (wt/vol) osmium tetroxide in PBS for 1 h at 25 °C.

2.1.2. Dehydrate the samples by using 20 min successive treatments in 50%, 60%, 70%, 80%, 85%, 90%, 95%, 100%, and 100% (all vol/vol) ethanol at room temperature.

2.2. Resin embedding and polymerization

2.2.1. Embed the samples in resin in a flat embedding mold. Ensure that the sample is at the bottom of the plate and placed as close as possible to the edge of the recessed groove. Label and then incubate the plate containing the sample at 60 °C for 72 h.

2.2.2. Remove the capsule from the incubator and verify that the resin has polymerized.

2.3. Sample sectioning and staining

2.3.1. Once the sample is solidified, place each resin block under a fluorescence microscope and photograph them under blue light. Move the microscope's fluorescent light source so it irradiates the sample from above. Make sure the sensilla in the resin block are clearly visible.

2.3.2. Photograph and measure distances to target the sensilla (**Figure 1**).

[Place **Figure 1** here]

2.3.3. Refer to the SEM image of the palps (**Figure 2A**) and roughly cut the resin block with a razor blade close to the target receptor (**Figure 2B**).

[Place **Figure 2** here]

2.3.4. Next, using blue-light fluorescence microscopy, photograph the roughly cut resin block, adjusting the light source from above so that the sensilla are observed clearly. Green light excited by the blue light will create good observation conditions.

2.3.5. When imaging, add the objective micrometer to the fluorescence microscope stage, and then measure the distance of the target using ImageJ (**Figure 2C**).

2.3.6. Cut 50–60 nm thick sections using an ultramicrotome until the target position has been reached. Use the fluorescence microscopy images to pinpoint the target receptor.

2.3.7. Mount the sections on Formvar-coated, 100-mesh copper grids and then double-stain with uranyl acetate and lead citrate.

2.3.7.1. First, add 3.75 g of uranyl acetate to 50 mL of 50% methanol. Stain the grids with a saturated solution of uranyl acetate filtered with a 0.45 µm syringe at room temperature for 10 min. Cover sections during staining to block light-induced precipitates.

2.3.7.2. Rinse 2x in 50% methanol then 2x in filtered degassed water.

2.3.7.3. Add 0.02 g of lead citrate to 10 mL of degassed distilled water in a centrifuge tube. Add 0.1 mL of 10 N sodium hydroxide, seal, and shake to dissolve. Briefly centrifuge before use. Place drops of the stain on the squares of the plastic Petri dishes, and let sit for 8 min.

NOTE: Staining must be done in a carbon dioxide free environment to prevent the formation of lead carbonate precipitates.

2.3.7.4. Rinse in degassed filtered water and dry[17].

2.3.8. Examine sections using a TEM at 80 kV.

REPRESENTATIVE RESULTS:

Using cleaning and fixative solution with Tween 20, a clearer SEM image was observed than that without Tween 20 (**Figure 3**). The Tween 20 fixing solution allowed the glutaraldehyde fixing solution to penetrate the tissue. Microtubule structures were clearly seen. The TEM image of the internal structure of the sample prepared without Tween 20 was blurry (**Figure 4**).

[Place **Figure 3** here]

[Place **Figure 4** here]

We used SEM to study the types and ultrastructures of sensilla on the palps of *C. caragana*, finding four types of sensilla including 10 subtypes: one Böhm's bristles (BB.), three sensilla chaetica (Ch.1-Ch.3), one digitiform sensilla (Dig.), and five sensilla twig basiconica (S.tb.1-S.tb.5) (**Table 1**). Sensilla identification and ultrastructure was based on their morphology and size[18-23]. Our sample preparation methods rendered clear images of the surfaces and internal ultrastructures of insect sensilla.

[Place **Table 1** here]

To examine the ultrastructure inside the sensilla on *C. caragana* palps, we used TEM. One

example of these studies was continuous cross-sectional views of the peg of the S.tb.1 on the maxillary palps. The views show that the dendritic sheath surrounded the outer dendritic segments and extended to the tip pore (**Figure 5A**-**D**). Seven unbranched outer dendritic segments existed inside the inner receptor lymph cavity, which was surrounded by an outer cavity (**Figure 5D**). The tubular body was separated by a dendritic sheath from other outer dendritic segments at each sensillar socket base (**Figure 5E**). In the ciliary region, we noted eight dendrites of different diameters, indicating the presence of eight bipolar neurons. Finally, the ciliary segment contained nine peripheral microtubule doublets (**Figure 5F**).

[Place **Figure 5** here]

FIGURE AND TABLE LEGENDS:

Figure 1: A fluorescent microscope photograph of a resin block enclosing the appendage of *Chlorophorus caragana*. (**A**) Antenna resin block. (**B**) Resin block at the end of the ovipositor. The arrow indicates the edge of the resin block. The dotted circle indicates the target sensilla.

Figure 2: Procedures of the precise sensilla location method. (**A**) The fourth subsegment of a maxillary palp of *Chlorophorus caragana*. The dotted circle shows the sensilla targeted by SEM. (**B**) The fourth subsegment of a maxillary palp of *C. caragana* viewed by fluorescence microscopy. The white arrow shows the roughly cut edge of the resin block and the dotted circle shows the target location. (**C**) The marked distance from the edge of the resin block to the maxillary palp target location (28 μm in this sample).

Figure 3. Locating the sensilla on a *Chlorophorus caragana* antenna under SEM. Comparison of SEM image with Tween 20 (**A**) and without Tween 20 (**B**), which shows that picture A is clearer than picture B in general.

Figure 4. *Chlorophorus caragana* viewed by TEM of sensilla twig basiconica on the labial palps. Comparison of TEM image with Tween 20 (**A**) and without Tween 20 (**B**). The microtubule structure of picture A is clear, while that of picture B is blurry.

Figure 5: TEM views of sensilla type 1 twig basiconica (S.tb.1) on a *Chlorophorus caragana* maxillary palp. (**A**) S.tb.1 with dotted lines marking regions close to the cross sections taken for panels **B**-**E**. (**B**) Cross section of finger-shaped protrusions showing scattered cuticula. (**C**) Cross section of the basal region of finger-shaped protrusions showing inner receptor lymph cavities without outer dendritic segments. (**D**) Cross section of the middle region of the peg showing the dendritic sheath dividing the sensillum-lymph cavity into both inner and outer cavities with seven outer dendritic segments in the inner cavity. (**E**) Basal region of the peg showing the tubular body surrounded by a dendritic sheath and separated from outer dendritic segments. A tormogen cell forms the outside of the dendritic sheath. (**F**) Cross section of the ciliary region showing eight dendrites of different diameters. Abbreviations: bb = basal body; cs = ciliary segment; CW = cuticular wall; DS = dendritic sheath; iRL = inner receptor lymph cavity; M = microtubule; Mi = microvilli; oD = outer dendritic segment; oRL = outer receptor lymph cavity; S.tb.1 = type 1 sensilla twig basiconica; TB = tubular body; TH = thecogen cell; TO = tormogen cell; TR = trichogen cell.

This figure has been adopted with permission from Zhang et al. 2018[30].

Table 1. Morphological characteristics of maxillary and labial palps sensilla of Chlorophorus caragana. BB. = Böhm's bristles; Ch.1–3 = type 1–3 sensilla chaetica; Dig. = digitiform sensilla; S.tb.1–5 = type 1–5 sensilla twig basiconica. [a]Values are means (± standard deviations) of the lengths and diameters of at least 30 sensilla of each type from both sexes, except for Ch. 3 (n = 24).

DISCUSSION:

In this article, we present a sample preparation scheme for scanning and transmission electron microscopy for the woodboring beetle. Using insect appendages as a representative study subject, we demonstrated several improvements over traditional sample preparation methods.

The liquid oil detached from the solid surface is emulsified into small droplets, which can be well dispersed and suspended in the washing medium to reduce redeposition on the surface of the object. The washing performance of the surfactant includes all the important characteristics such as wettability, permeability, emulsification, dispersibility, solubilization[11-14]. The effects of different detergents on the electron microscopic sample preparation of golden nematodes showed that Tween 20 had the best cleaning effect, followed by sodium bicarbonate, and distilled water[24]. In this study, we found that Tween 20 can be used to reduce the surface tension of the liquid and prevent debris from depositing on the body surface of the insect, especially for woodboring beetles collected directly in the field. Insect body surface was cleaner and more visible in SEM. Fixative with Tween 20 penetrated the insect body wall more easily, and subsequently better fixed tissues and organs in the body are observed in TEM. The advantages of surfactants in electron microscopy sample preparation have been extensively studied[24-31].

Also, we adopted a modified air-drying method for SEM sample preparation in which the dehydrated sample was placed in a Petri dish containing a silica gel desiccant that gradually evaporates the dehydrating agent. The biggest advantage of this method is that it is simple, easily maintained, no special equipment is required, and it keeps the microenvironment air dry. The natural drying method is a simple, practical, and effective method for seed, nut, and long-term preservation of insect specimens. Although the sample volume shrinks during the natural drying process, the basic morphology of the sample is retained[32]. In general, Coleoptera insects have relatively low water content, and their surface is surrounded by hard chitin walls. Thus, air-drying is suitable. However, this drying method is not suitable for the drying of tissues with a large water content, such as louse, mites, and larvae, because the surface tension will deform the sample during the drying process.

In order to observe and calculate the type and number of sensilla distributed across the surface of the appendage, dorsal, ventral, and lateral sides of the appendage must be considered. Some sensilla were few, small, and sometimes covered. Careful scanning and analysis from all angles is necessary to find those sensilla fully protruding the epidermis or arising from the depressions. Since many sensilla were relatively long and hair-like, the tip effect can be significant. Therefore, the electron microscope acceleration voltage must not be too high. We used 20 kV and found

that 5–20 kV was best.

In the TEM sample embedding, the sample should be close to the edge of the flat embedding mold groove in order to save time when roughening the resin block. The traditional TEM method for continuously cutting the resin is long, and it is usually blindly cut using an optical microscope[17,33]. To improve this method, we first explored an insect sensilla localization technique in resin-embedded blocks using fluorescence microscopy to view and measure the target distance to the cut. Compared with the traditional TEM trimming method, this technology can save sample preparation time and more accurately locate the target sensor. In the absence of measurement software, a scaled ruler can be placed in the field of view to roughly measure the target distance. The combination of an ultramicrotome with a fluorescence microscope provides clear observations of the cutting process, yielding accurate cuts of target sensilla and other appropriate subjects.

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DISCLOSURES:

We have no conflicts of interest to disclose.

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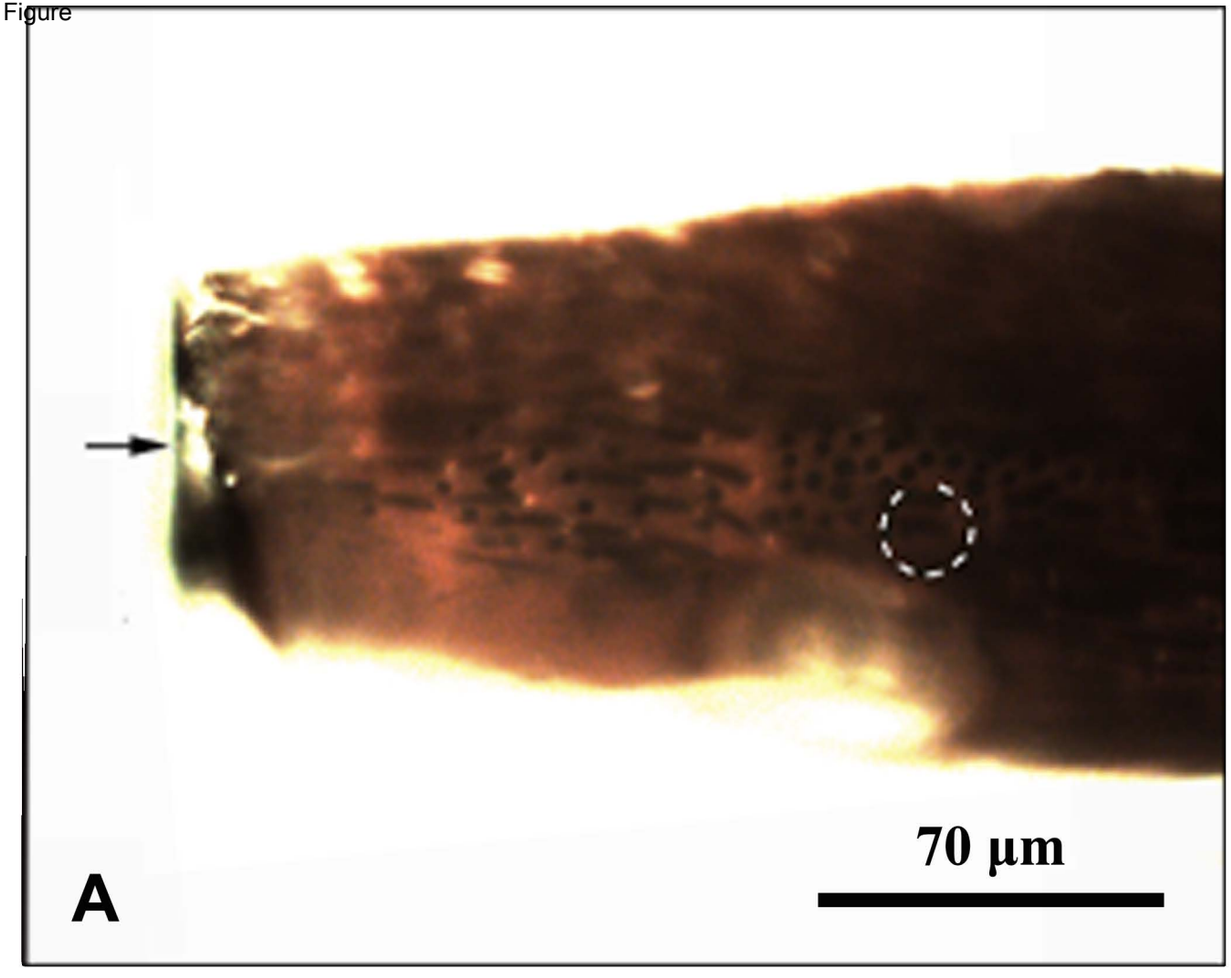
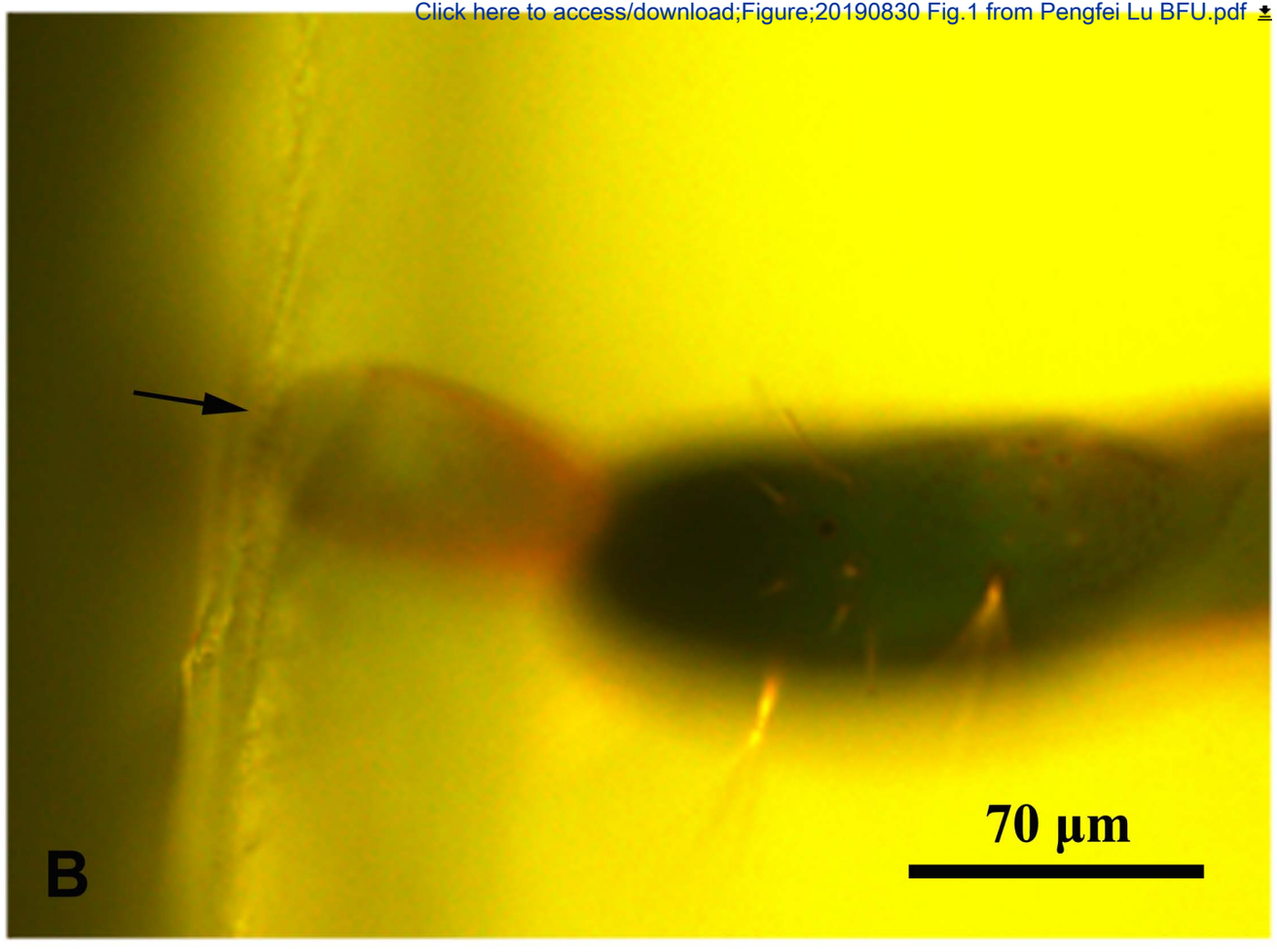
Figure





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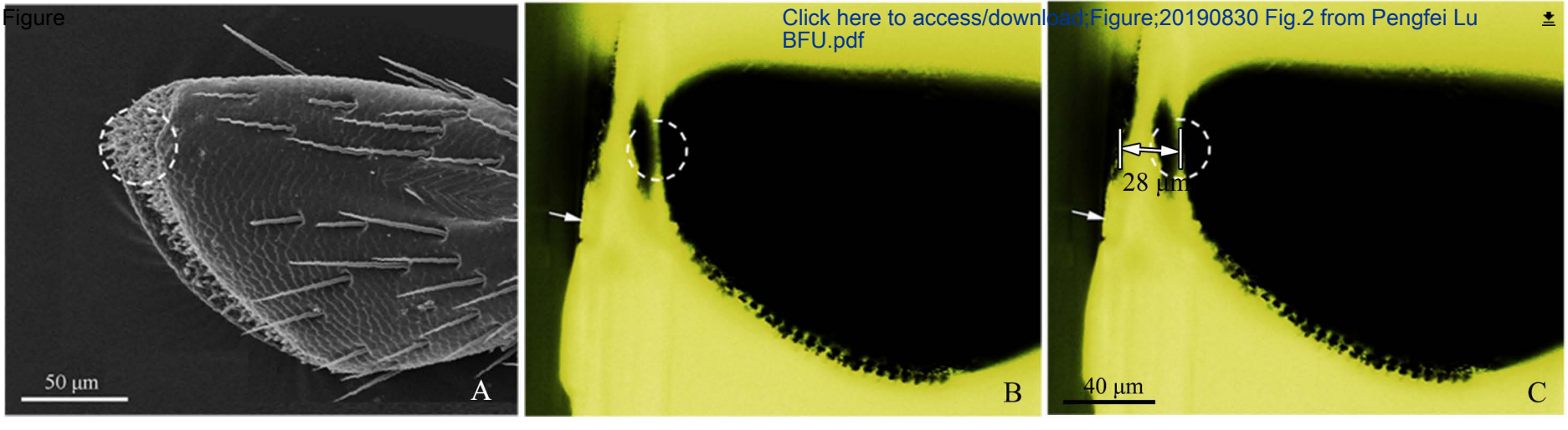

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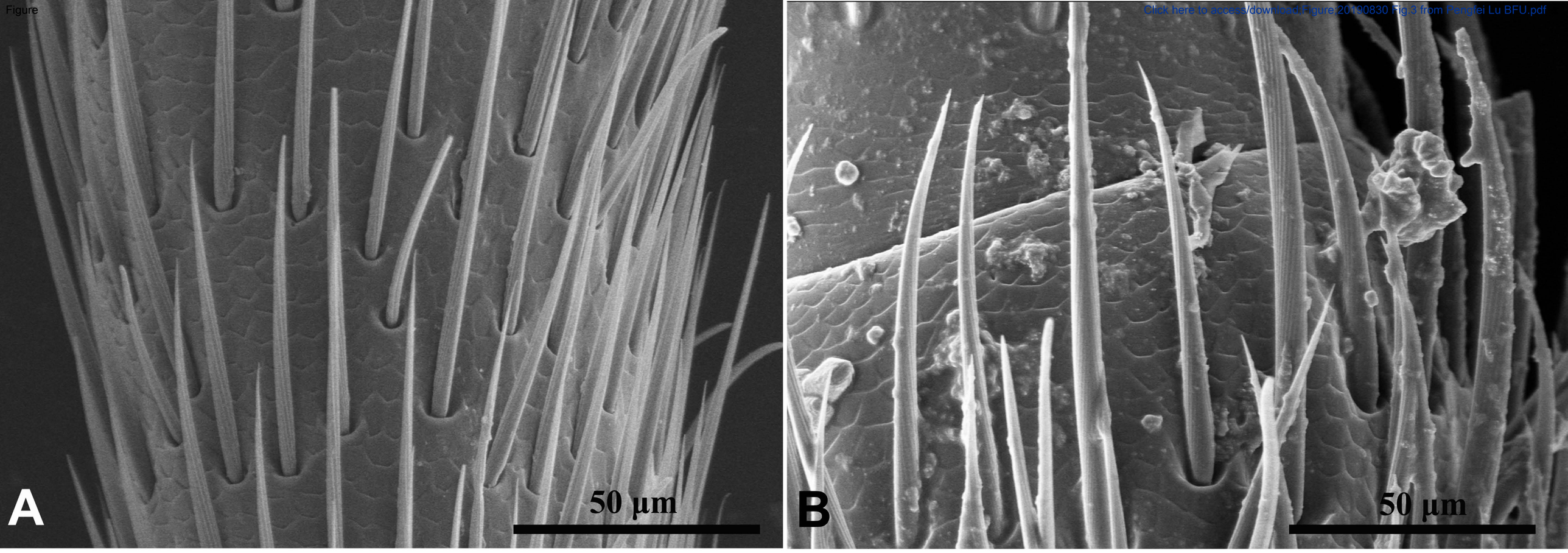
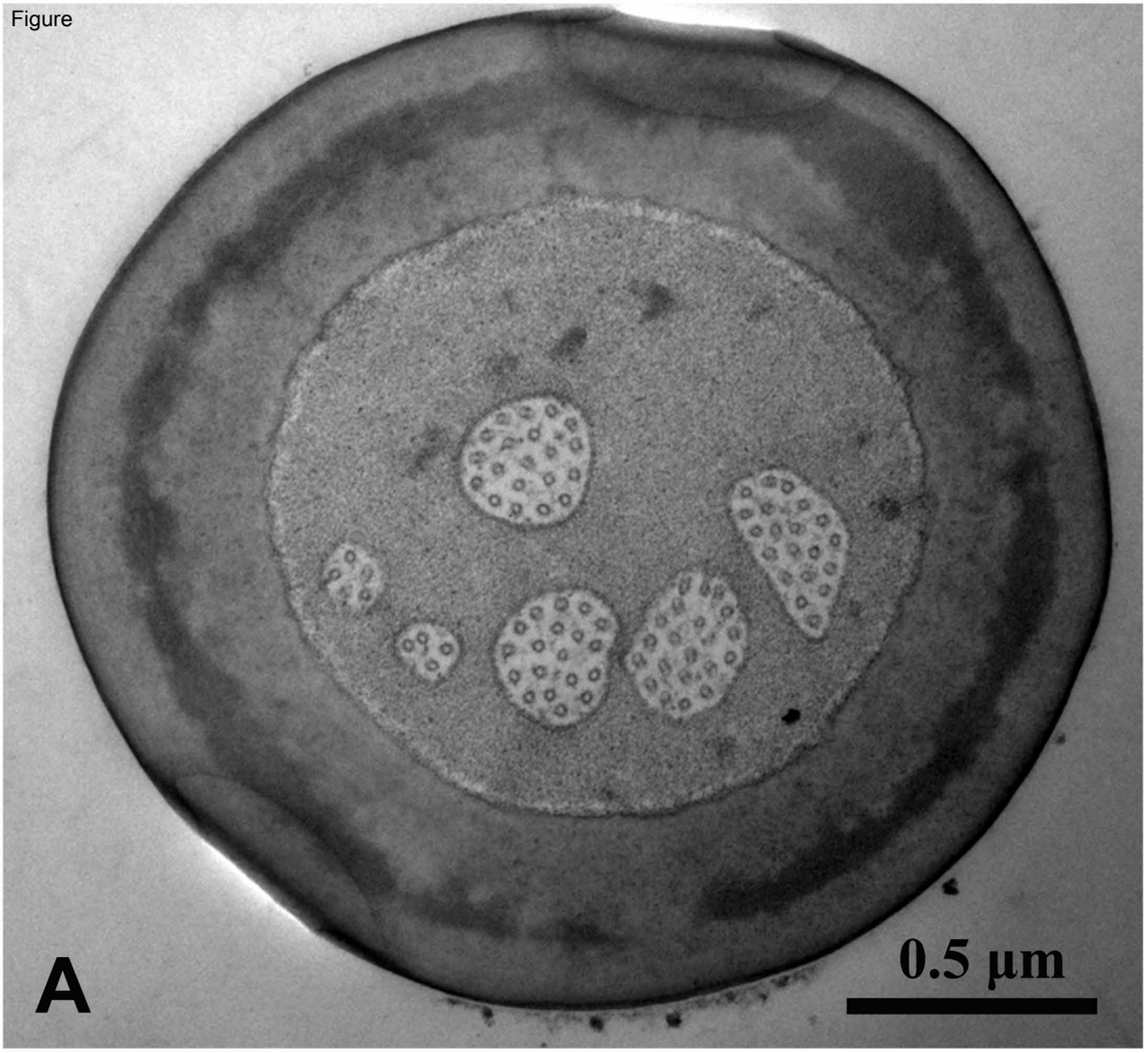
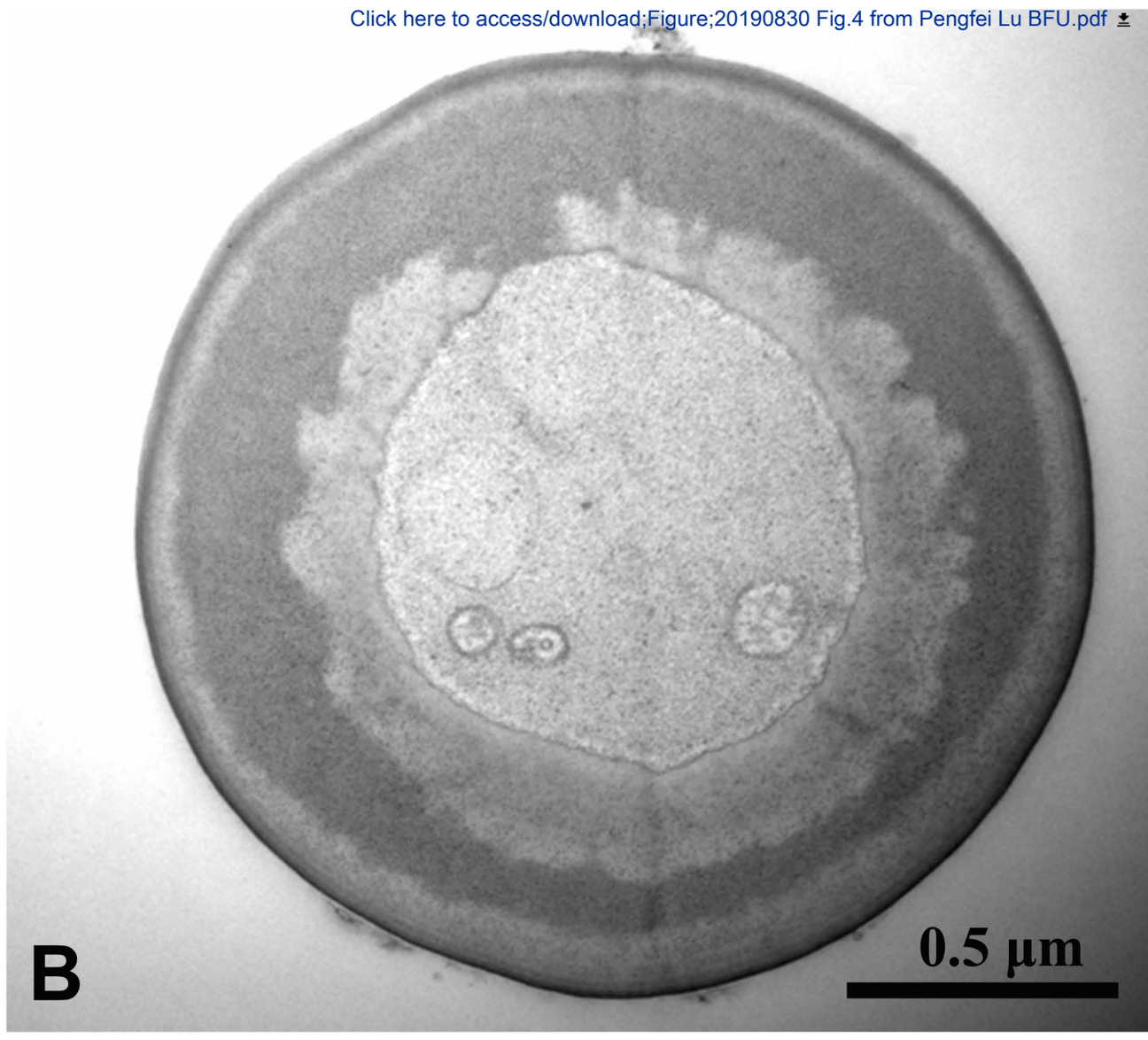


Figure





Figure

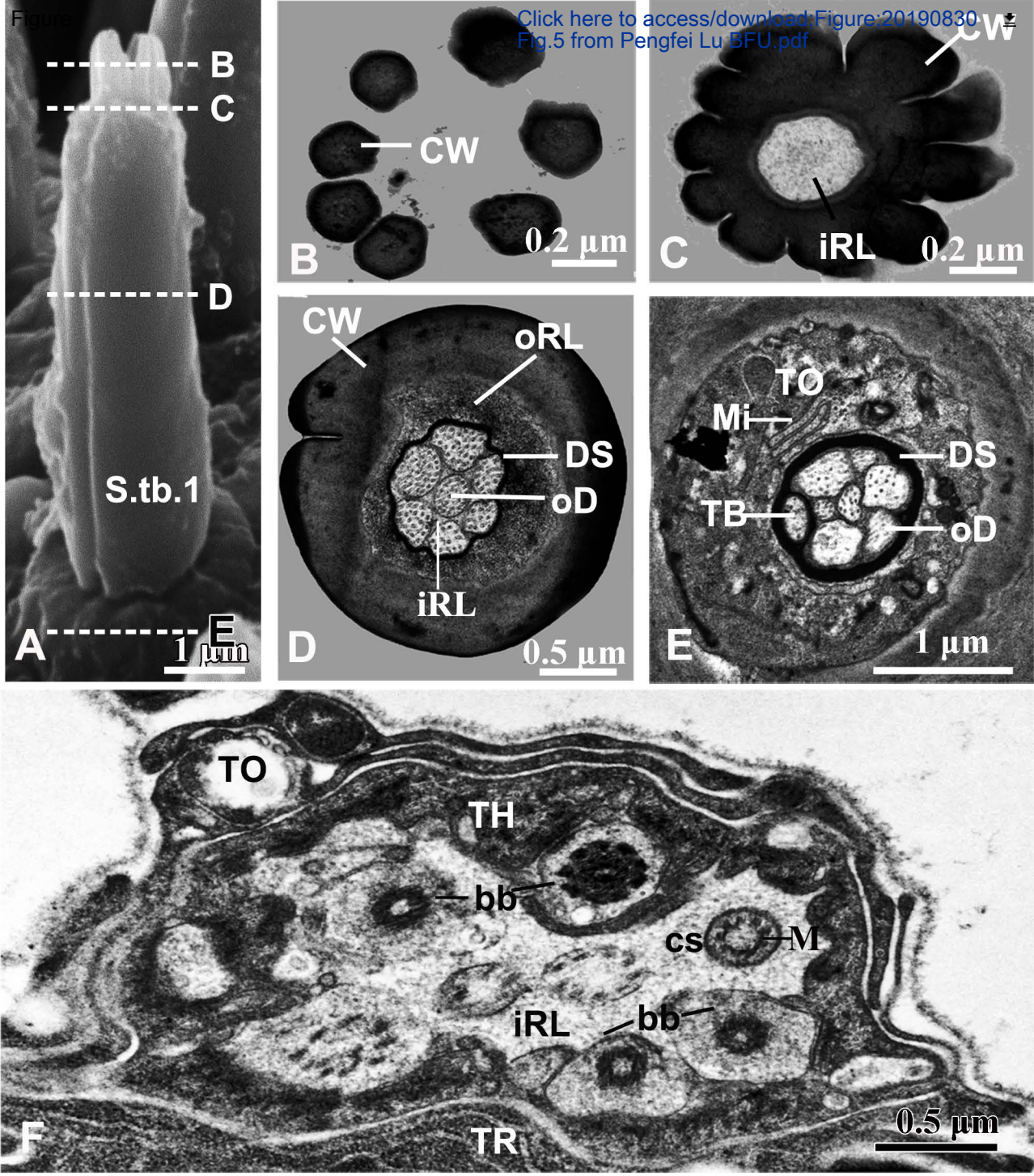


Table 1

Number	Type	Length (µm)[a]	Diameter at base (µm) [a]	Wall	Tip
1	BB.	5.18 ± 1.25	1.70 ± 0.47	Smooth	Sharp
2	Ch.1	38.59 ± 8.20	3.15 ± 0.84	Grooved	Sharp
3	Ch.2	81.54 ± 18.07	3.75 ± 0.88	Grooved	Sharp
4	Ch.3	282.06 ± 22.60	6.10 ± 0.70	Grooved	Sharp
5	Dig.	24.77 ± 2.98	1.24 ± 0.32	Smooth	Blunt
6	S.tb.1	6.51 ± 1.01	2.31 ± 0.25	Grooved	With protrusions
7	S.tb.2	5.91 ± 0.90	2.24 ± 0.30	Smooth	Blunt
8	S.tb.3	6.84 ± 0.98	1.96 ± 0.35	Smooth	With protrusions
9	S.tb.4	2.21 ± 0.59	2.86 ± 0.46	Grooved	With protrusions
10	S.tb.5	1.16 ± 0.29	1.05 ± 0.19	Smooth	Blunt

Socket	Cuticular pores	Distribution
Wide	No	maxillary palp, labial palp
Wide	No	maxillary palp, labial palp
Wide	No	maxillary palp, labial palp
Wide	No	labial palp
Wide	No	maxillary palp
Raised and tight	Tip pore	maxillary palp, labial palp
Raised and tight	Tip pore	maxillary palp, labial palp
Raised and tight	Tip pore	maxillary palp, labial palp
Raised	Tip pore	maxillary palp, labial palp
Raised and wide	Tip pore	maxillary palp, labial palp

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Anatomical lens	Chongqing Auto Optical limited liability company	1425277	
Carbon adhesive tape	SPI Supplies, Division of Structure	7311	
Carbon tetrachloride	Sigma	56-23-5	
Copper grids	GilderGrids	G300	
Disodium hydrogen phosphate	Sinopharm group chemical reage	10039-32-4	
Ethanol	J.T.Baker	64-17-5	
Flat embedding molds	Hyde Venture (Beijing) Biotechno	70900	
Fluorescence microscope	LEICA	DM2500	
Glutaraldehyde	Sigma-Aldrich	111-30-8	Anhydrous EM Grade
Isophorone	Sigma	78-59-1	
Lead citrate	Sigma	512-26-5	
Methanol	Sigma	67-56-1	
Monobasic sodium phosphate	Its group chemical reagent co., LT	7558-80-7	
Objective micrometer	Olympus	0-001-034	
Osmium tetroxide	Sigma	541-09-3	
Petri dish	Aldrich	1998	
Razor blade	Gillette		
Resin	Spurr	ERL4221	
Scalpel	Lianhui	GB/T19001-2008	
SEM	Hitachi	S-3400	
Silica gel desiccant	Suzhou Longhui Desiccant Co., Lt	112926-00-8	
Small brush	Martol	G1220	
Sodium hydroxide	Sigma	1310-73-2	
Sputter ion instrument	Hitachi Koki Co. Ltd., Tokyo, Japa	E-1010	
Stereo microscope	Leica	EZ4 HD	
TEM	Hitachi	H-7500	
Tween 20	Tianjin Damao Chemical Reagent	9005-64-5	
Ultramicrotome	Leica	UC6	
Ultrasonic cleaner	GT Sonic	GT-X1	
Uranyl acetate	Sigma	6159-44-0	

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Author(s): Yan-Rui Zhang, Li-Li Ren, Rong Wang, Peng-fei Lu, You-Qing Luo

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Department: School of Forestry

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Article Title: Improved Scanning and transmission electron microscope sample preparation methods demonstrated using the palps of Chlorophorus Caragana (Coleoptera: Cerambycidae)

Signature: Pengfei Lu Date: Oct. 15, 2018

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Editorial comments:

1. There are still some grammar and usage errors; please proofread, ideally by a fluent English speaker.

Answer: We have also revised our manuscript throughout the text according your suggestions. The revised manuscript has been edited extensively as to grammar, spelling, style and clarity by us as well as the native English speaker.

2. Protocol step 1.1: Please provide more detail on how to know if the sample is clean.

Answer: After cleaning for 100 s, transfer the sample to the microscope to check if it was clean. Under normal circumstances, clean for 400s to ensure that the sample was clean enough to observe and not damaged **(page 3, line 109-112)**.

3. 1.3: Please provide more information on these steps (e.g., what are the sputter instrument settings?).

Answer: We have added the detail on Sputter-coat and Sample Insertion **(page 3, line 124-132)**

4. Please cite the original source for Figure 5 within the legend.

Answer: Original source for Figure 5 has been cited within the legend **(page 6, line 248)**.

5. Please remove Table 1 from the manuscript and upload as a .xls/.xlsx file in your revision.

Answer: Table 1 has been removed from the manuscript and upload as a .xls/.xlsx file in our revision.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors describe a method to improve the cleanness of SEM samples and the structural preservation in TEM samples by adding the detergent Tween 20. Further, a method is described how the resin block can be optimally cut in order to find the structures of interest and ensure that they will be sectioned.

Major Concerns:

Question 1: The title of the manuscript differs from the title, which is given on the last side under "Title of new article". The latter title is better. I would include ".. palps of "the woodboring beetle" Chlorophorus… The abstract is appropriate. The "Summary" is superfluous since there is a more detailed "Abstract". A linguistic improvement of the manuscript is definitely necessary. Sometimes even the meaning is not clear.

Answer:

The study is applicable to most of woodboring beetle. "Summary" is a required part of manuscript. We have also revised our manuscript throughout the text according your suggestions. The revised manuscript has been edited extensively as to grammar, spelling, style and clarity by us as well as the native English speaker.

Question 2: The principle steps of the methods are described. Sometimes information is missing. I made detailed comments (see below). The advantages of the presented methods compared to comparable methods (e.g. use of other detergents) should be described.
Answer: Thank you very much for the good suggestion. We have added a citation **(page 6, line 376-378)** [24. Li, Y. Z., Zhong, G. Q. Screening of detergents and floating carriers for treating potato golden nematode cysts to improve the original appearance of electron microscopy. *Plant quarantine*. 8, 72-75 (1994).], which indicated the Tween 20 was best, compared with sodium bicarbonate and distilled water. We have also added the CK in the revised manuscript to compare the result with and without Tween 20 (Fig.3 for SEM and Fig.4 for TEM).

Question 3: In the introduction to the "Protocol" there is a general warning for the use of toxic substances. Add a short warning for the used toxic substances in the description of the protocol. Highlight critical steps.
Answer: I think the key step is to wear protective gear and work in the fume hood, which has been added in revised manuscript **(page 3, line 93-97)**.

Minor Concerns:
Question 4: Lines 52-55: Add that SEM shows surface structures, whereas TEM shows inner structures
Answer: Yes, the sentences has been added in **page 2 line 53-57**.

Question 5: Lines 56ff.: Add references, e.g. for the number of coleopterans and that most of them feed on plants.
Answer: A new citation was added **(page 2, line 60)** **[**6. Zhang, X. J., Sun, W., Zhang, J.,Zuo, T. T., Wang, Z. Q.,Zhao, H. W. Research progress of coleopteran insect species antennal sensilla. *Journal of Anhui Agricultural Sciences.* **41**,2932-2935 (2013).]

Question 6: Line 66: What is meant by "replacement"? – explain
Answer: Ethanol or acetone is usually used to dehydrate the material in SEM, and ethanol or acetone is then replaced with an intermediate, such as amyl acetate or ethyl acetate. The polarity of ethyl acetate is weak, which can be better replaced with co_2 during critical point drying, so that the surface morphology of biological samples can be maintained more completely. "replacement" has been changed into "metathesis" **(page 2, line 68).**

Question 7: Line 78: "Ossification" refers to bony structures. Maybe use "hardening" instead.

Answer: "replacement" has been changed into “sclerotization” **(page 2, line 83).**

Question 8: Line 93: What is meant with "Shooting"?
Answer: "Shooting" has been changed into “Imaging” **(page 3, line 99)**.

Question 9: Line 96: Give more information, e.g.: "For collecting the woodboring beetle, Chlorophorus caragana, work in an area… Which plant attractants can be used?
Answer: Isophorone was used as attractants in the field. [Zong, S. X., Liu, X. H., Cao, C. J., Luo, Y. Q., Ren, L. L., Zhang, H. Development of semiochemical attractants for monitoring and controlling *Chlorophorus caragana*. *Zeitschrift für Naturforschung*. **68**, 243 -252 (2013)] **(page 3, line 104)**.

Question 10: Line 97: What is the concentration of the used buffers? E.g. 0.1 M? It is impossible that there are 72 g of disodium hydrogen phosphate in 100 ml.
Answer: We have rephrased this sentence to “Preserve clean bodies of adult *C. caragana* in 0.1 mol l^{-1} phosphate-buffered saline (PBS, pH 7.2)” in the revised manuscript **(page 3, line 104-105**).

Question 11: Line 100: Why do you use carbon tetrachloride instead of phosphate buffer for washing out the fixative? It is a toxic substance and there are safety concerns. Is it really much better as washing solution?
Answer: We apologize for the mistake. We have even used carbon tetrachloride in the very beginning, but then quickly changed into phosphate buffer for washing out the fixative in our experiment, which has been corrected in the revised manuscript **(page 3, line 107**).

Question 12: Line 101: "Anatomical lense"? Magnifying glass?
Answer: we have revised the word into “stereomicroscope” **(page 3, line 108**).

Question 13: Line 109: Do you let the samples air-dry after the last step in 100% ethanol before they are attached to the stubs?
Answer: After the last step in 100% ethanol, the samples are firstly attached to the stubs and then are place in a petri dish containing a silica gel desiccant for 48 h **(page 3, line 119-120**).

Question 14: Line 110: .. carbon "double-sided" adhesive tape..
Answer: we have added “double-sided” **(page 3, line 117-118**).

Question 15: Line 118:..onto the the stage "of the SEM".
Answer: we have added “of the SEM” **(page 3, line 133**).

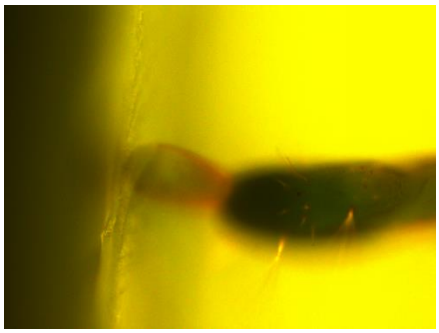
Question 16: Line 122: What do you mean with shooting?
Answer: we have revised the word to “imaging” **(page 4, line 139**).

Question 17: Line 126: Secondary fixation
Answer: we have revised the word to “Secondary fixation” **(page 4, line 143**).

Question 18: Line 127: *C. caragana* in italics.
Answer: *C. caragana* is italics **(page 4, line 145**).

Question 19: Line 134: "silicone embedded plate"?? Maybe you mean „flat embedding moulds" or "micromoulds"?
Answer: we have revised the word to “flat embedding moulds” **(page 4, line 153**).

Question 20: Line 142: Explain what is seen under UV irradiation. Auto fluorescence of the cuticle? Which color?
Answer: What is seen under UV irradiation is showed in the figure 1. Under the blue light mode of fluorescence microscope, light can pass through the resin block and see the sensor embedded in the block.



Question 21: Line 141: What is the thickness of the resin block? How can you put the block under a fluorescence microscope? Which objective do you use?
Answer: the thickness of resin block is 3mm. Place the resin block directly on the carrier of the fluorescence microscope. The objective magnifies more than 40 times until the sensilla can be seen clearly.

Question 22: Line 163: Cite a paper where these methods are described in more detail or give more information.
Answer: The detail are added in revised manuscript and a new citation was added **(page 5, line 183-191**) **[**17. Sumner, M. J. Epoxy resins for light and transmission electron microscopy. *Plant Microtechniques and Protocols*. 83–101(2015)].

Question 23: Lines 168/169: Include Fig. 4A or Fig. 4B. I am not convinced that the image without Tween shows identical structures to the other one. There is one clear microtubule in the lower part; maybe the other regions are not sectioned at levels with microtubules - provide more images to prove a bad structural preservation without Tween. Fig. 4A has much more contrast than Fig. 4B but this could be explained by a thicker section or digital image improvement. In case everything is identical for the two embedment, state this.

Answer: Transmission electron microscopy (TEM) images of transducers with Tween fixation show a clearer structure than those without Tween (Fig.4). Adding Tween only makes the fixing effect better (Fig.4A). Without tween, the structure will be relatively blurred, but the structure will not be missing (Fig.4B). Microtubules run through the dendrites, unless they do not exist at the tip. The two sections in Fig. 4 are not endings of sensilla. The slice thickness of the two pictures is same, both of them are 50 nm. Contrast adjustment of the image will improve the clarity, even if it is adjusted to a high contrast, samples without Tween will be relatively blurred.

Question 24: Lines 199, 204, 214: *C. caragana* in italics.
Answer: *C. caragana* in italics in legends.

Question 25: Lines 210, 212: Include the shown structure in the legend.
Answer: The shown structure are added in the legend **(page 6, line 239, 243**).

Question 26: Discussion
It is already known that the addition of detergents can help to clean the surfaces of samples for SEM. Comment on other detergents and their potential use. Why is Tween 20 the best? Did you try other detergents?
Answer: Thank you very much for the good suggestion. We have added a citation **(page 6, line 271)** [24. Li, Y. Z., Zhong, G. Q. Screening of detergents and floating carriers for treating potato golden nematode cysts to improve the original appearance of electron microscopy. *Plant quarantine*. 8, 72-75 (1994).], which indicated the Tween 20 was best, compared with sodium bicarbonate and distilled water. We have also added the CK in the revised manuscript to compare the result with and without Tween 20 (Fig.3 for SEM and Fig.4 for TEM).

Question 27: Line 232: Which "liquid oil", which "solid surface"? This is too general. You probably mean lipids and waxes from the epicuticle of the insect.
Answer: This is an introduction to the clean use of surfactants on solid surfaces. It is a wide range of uses, not specifically insects.

Question 28: Line 238: Sentence. You did not wash in the SEM.
Answer: "Insect body surface was washed more cleanly in SEM" has been added in the revised manuscript **(page 7, line 273**).

Question 29: Line 239: Also mention potential problems of the use of an detergent such as Tween 20. What about the preservation of membranes, which contain much lipid.
Answer: The concentration of Tween 20 in fixative solution was 0.06%, which was relatively low. No damage to insect body wall at present.

Question 30: Line 247: What are "insect needle inserts"?
Answer: "insect needle inserts" has been revised into "Insect specimen" **(page 7, line**

282).

Question 31: Line 250: Only plants have plastids. Beetles have a hard, sclerotized cuticle containing chitin.
Answer: “plastids” has been deleted.

Question 32: Line 256: What is meant with "borning" in the depression? Arising from a depression?
Answer: “arising from a depression” **(page 7, line 291**).

Question 33: Line 257: Explain the "tip effect".
Answer: In region with some prominent tips, or small particles, steeper slopes, as well as the intersection of multiple planes, image of SEM has a very high brightness, showing a white dot or white outline.

Question 34:Line 259: Which groove?
Answer: flat embedding moulds groove **(page 7, line 295**).

Question 35: Line 260: Why do you want to "repair" the resin? Do you mean, to remove the resin?
Answer: "repairing" has been changed into “**c**ontinuously cutting the resin” **(page 7, line 296**).

Question 36: Line 267: Is it necessary to use a fluorescence microscope? Why can the structures not be seen in a normal light microscope?
Answer: The sensor in resin block is more obvious under fluorescence microscope than under ordinary microscope.

Question 37: References: Check uniform style of citations. Don´t abbreviate the authors by "et al." Add general textbooks for electron microscopic techniques.
Answer: Style of citations has been revised carefully. Two texebooks for electron microscopic techniques were added in revised manuscript.
10. Zhou, W., Apkarian, R., Wang, Z. L., Joy, D. Fundamentals of Scanning Electron Microscopy (SEM). Scanning Microscopy for Nanotechnology. 1–40 (2006).
33. Graef, M. D. Introduction to Conventional Transmission Electron Microscopy. Cambridge University Press. 1-742 (2003).

List of materials
Question 38: What is meant with "Its group chemical reagent co.,"
Answer: Sinopharm group chemical reagent co., LTD.

Question 39: Tell grade of glutaraldehyde ("for electron microscopy")
Answer: Anhydrous EM Grade.

Question 40: Remove refrigerator
Answer: removed.

Reviewer #2:

The manuscript is interesting and the protocol for the use of Tween 20 to improve tissues fix and cuticle cleanly for TEM and SEM observations can be of large use for researchers working on insect sensory biology, because it is easy, clearly explained and proposes a solution to very common technical difficulties in this field of research. On the opposite, the method proposed to improve slicing accurancy during TEM preparation, by the use of fluorescence microscopy instead of optical microscopy, is not very clear, particularly it's efficiency is not supported by the images. Also TEM pictures from the study case, Clorophorus caragana appendages, could be clearer.

Major Concerns:
Question 1: in my opinion, it is not clear the sensilla localization technique in resin-embedded blocks by using fluorescence microscopy, instead of optical microscopy, to view and measure the target distance to the cut. The efficacy of the protocol is not clearly demostrated by the text and the figures (1-2). This part of the manuscript must be largely improved, maybe by the selection of better pictures (e.g. in fig. 1 no target sensilla are visible in the dotted circle) and a clearer comments of them, both in the text and in the figure captions.
Answer: The position of the target sensor can be inferred by referring to the scanning electron microscope images. It can be seen faintly under fluorescence microscope, but not under optical microscope at all, because the sensor is small. So it is valuable by using fluorescence microscopy, instead of optical microscopy.

Minor Concerns:
Question 2: In my opinion, while improvement due to the use of the surfactant in SEM and TEM specimens preparation is clearly demostrated by pictures in fig. 3-4, fig 5 can be improved. Indeed, quality of figures 5 a-e is good but the TEM image in Fig 5-f is not of the same level.
Answer: Thank you very much for the good suggestion. Fig. 5F shows the transverse section of the sensilla at the ciliary region below the epidermis.

Question 3: In addition, the authors should be more precise in the use of technical terms for describing the internal anatomy of sensilla and in general for entomological technical terms.
Answer: Entomological technical terms have been used including dendritic sheath, outer dendritic segments, inner receptor lymph cavity, tubular body, sensillar socket base, ciliary region, bipolar neurons, microtubule, maxillary palps, sensilla, Böhm’s bristles, sensilla chaetica, digitiform sensilla, sensilla twig basiconica.

Reviewer #3:
Manuscript Summary:
The article covers some techniques of preparation and processing specimens for the scanning and transmission electron microscopy. The most interesting parts address the issues of cleaning the specimen for SEM and enhancing the permeability of the cuticula for the TEM (in both cases, a surfactant is used with good results) and a exact locating of insect sensilla for TEM using fluorescent microscopy.

Major Concerns:
Question 1: Scanning and transmission electron microscopy are important techniques in biological research that often require many additional procedures to yield satisfying results. Those procedures are seldom covered in literature, so the information that the authors provide is undoubtedly valuable. However, I am not sure if the article quite fits the scope of the journal.
It covers a lot of techniques that are pretty well-known and do not deserve an extensive treatment in a video, since every qualified lab technician or experienced colleague could provide the same insights. The interesting points pertain to the cleaning of the beetle cuticula using Tween 20 (for SEM), enhancing the cuticula permeability for fixatives using Tween 20 (for TEM) and the exact locating of insect sensilla for TEM with fluorescence microscopy.
As for the usage of surfactant, it is not such a novelty or unknown technique (see e.g. Harrison, J. D. G. (2012). Cleaning and preparing adult beetles (Coleoptera) for light and scanning electron microscopy. African Entomology, 20(2), 395-401.) that would really need a special treatment in an article.
Or even if an article could possibly make sense, a video would most likely not, since the protocol step involving the surfactant treatment can be comprehensively (and much more briefly and less ressource-consumingly) described in simple text.
This makes me think that the authors should probably better try to submit the paper in some other journal dealing with electron microscopy, e.g. Micron.
However, the last point - locating the sensilla for TEM with fluorescence microscopy - might really be worth of explaining in a video. Unfortunately, the instructions for this process (section 2.4) that the authors provide in their protocol are very generic and also unclear due to poor language, so that I could not see if I cannot really grasp the instruction because of the language or because it is impossible to do without a video. The minimum of a major revision is needed i.a. to make clear why a video (and hence, a submission to JoVE) is indispensable and also to make the protocols (and text in general) more clear and to-the-point.
Answer: Aim of this study is to describe methods. Here we want to indicate the whole experimental steps and some changes to raise experiment effect for woodboring beetle, whose body wall is always difficulty to be washed clearly due to complex living environment. Fluorescence microscope provide clear observations of the cutting process, yielding accurate cuts of target sensilla and other appropriate

subjects, in particular, for some very small palpi tissues. Thank you for the citation [Harrison, J. D. G.(2012)], which has been cited in revised manuscript.
Minor Concerns:
Question 2: I already mentioned that the language of the article is very poor, unclear and hard to access. Almost every sentence needs a rephrasing to make the point clear, correct the scientific terminology, make the phrase logical or simply get rid of typos. I did not do a thorough copy-editing here since the guidelines for reviewers discourage to do this, but here just the several points that need to be addressed and occur throughout the article:
- the species name *Chlorophorus caragana*. When using it for the first time, the authors should provide the complete species name (including the describer's name(s) and description date). Also, "Chlorophorus caragana" is to be written in italics all the time.
Answer:
Thank you very much for the good suggestion. Complete species name "*Chlorophorus caragana* Xie &Wang (2012)" is used for the first time **(page 1, line 38)**. Species name is written in italics all the time.

Question 3: - the authors should used established terminology: "sputter-coating "instead of "gold spraying", "mounting" instead of "sticking" or "replacement" (although I am not sure what "replacement" in the line 67 at all means), "imaging" instead "shooting" etc. etc. "Sensilla" is a plural - in singular, "sensillum" should be used. Also, the authors tend to write "insect body wall" where "body surface" could be more correct.
Answer: Thank you very much for the good suggestion. Some established terminologies are used in revised manuscript. For example, "gold spraying" is change into "sputter-coating "; "sticking" is change into "mounting"; "shooting" is change into" imaging"; "replacement" is change into "metathesis". In most case, the "Sensilla" are mentioned with plural. "sensillum" is used in case of singular. To describe the body is clean or not, "body surface" could be more correct. If it specifically describes the penetration of the fixative solution, I think it is appropriate to use body wall.

Question 4: - in protocols, the authors sometimes switch between the 2nd and 3rd person-style. The should choose one style and adhere to it. - the text of the protocols would be more accessible if written as bullet points and not a long continuous text - thorough copy-editing is needed!
Answer: We have also revised our manuscript throughout the text according your suggestions. The revised manuscript has been edited extensively as to grammar, spelling, style and clarity by us as well as the native English speaker.

Question 5: Also, some comments on the special cases occuring in particular lines:
- Lines 58-60: „At present, prevention and control population of pests based on

chemical ecology theory have received increasing attention." Some literature references confirming this increasing attention are desirable.
Answer: New citation is added **(page 2, line 62)**. [7 Aldrich, J. R., Bartelt, R. J., Dickens, J. C., Knight, A. L., Light, D. M., Tumlinson, J. H. Insect chemical ecology research in the United States Department of Agriculture-Agricultural Research Service. *Pest Management Science*. 59, 777–787 (2003)]

Question 6: - Lines 60-61: „Efficient, low-toxic, pollution-free pheromone control methods have become an effective way." Again, some literature references confirming this for an unexperienced reader would be useful.
Answer: New citation is added **(page 2, line 63)**. **[**8 Thomas, C. B.， Marlin, E. R. Pheromone mating disruption: Novel, non-toxic control of the European corn borer. *Leopold Center*. 8, 57-60 (1999)]

Question 7: - Lines 66-67: „cleaning, tissue fixation, dehydration, replacement, drying, and gold spraying". Literature references to some general manual on SEM and TEM might be of use at the end of this sentence.
Answer: New citation is added **(page 2, line 69)**. **[**10 Zhou, W., Apkarian, R., Wang, Z. L., Joy, D. Fundamentals of Scanning Electron Microscopy (SEM). *Scanning Microscopy for Nanotechnology*. 1–40 (2006)]

Question 8: - Lines 69-71: „Tween 20 is a surfactant which plays an important role in the washing process, including reducing the surface tension of water and improving the wettability of water on the surface of the laundry." There is a literature reference number 12 after the part „Tween 20 is a surfactant" - but the previous reference number was 6 - why are 5 other references missing? They are well present in the reference list.
A comparison with the line 74 below shows that the „12" might be a typo and the correct reference number is „7".

Still, the title and abstract of the reference number 7 do not mention any Tween at all (unfortunately, I was not able to access the full text). The authors should revise and rectify this confusing citation, adressing the points I made.

If they do mean the reference number 12, it deals with Tween-80, not Tween-20, as its title says. Again, I was not able to access the full text of the cited article - but the authors should explain to the editor if the cited paper really deals with Tween-20 along with Tween-80 - or better choose a more appropriate reference treating the substance in question.

Last but not least, the whole sentence does not really say anything new for a person with a minimal scientific background - every such person knows what a surfactant is and what it does; also, it does not really influence the „wettability of water" - please consider major rephrasing here, pressing on more to the

importance of the substance in question and/or omitting information that can be presumed general knowledge.
Answer:
We apologize for confuse in citations. We have checked citation again.
Number 7 in origin manuscript (Number 9 in present revised manuscript) [Chen, X. F., Hu, M. Y. Studies on the specimen preparation techniques of scanning electron microscope of citrus rust mite. *Journal of Zhongkai Agrotechnical College.* **14**, 68-70 (2001)] is unrelated with Tween-20. The correct citation related with Tween-20 is Number 11-14 in present revised manuscript.

Question 9: - Line 78: „ossification" is incorrect, since insects do not have bones. The authors mean „sclerotization".
Answer: "ossification" is changed into "sclerotization". **(Page2, Line 83).**

Question 10: - Protocol part on sample fixation and cleaning (1.1.) - here it should be specified how the insects are killed.
Answer: We collect live insects in the field. There, and then directly put them into the fixing solution to ensure freshness. These insects will die in fixing solution.

Question 11: - Line 101, ultrasonic cleaning - the authors should specify the intensity of the ultrasonic signal. It is also important to know what equipment they used, since different models may deliver different results.
Answer: The GT-X1 type ultrasonic cleaning instrument was used (GT Sonic Company). The frequency of ultrasonic wave was 40 kHz. **(Page3, Line 108).**

Question 12: - Lines 104-105: „A control solution without Tween 20 was used to prove the improved methods were better." This sentence is to my mind not necessary in a protocol.
Answer: The sentence has been deleted.

Question 13: - Line 110-111: „separately fix 3 observation surfaces (back, ventral, and end surfaces) onto stubs". It is not clear what surfaces are meant here.
Answer: These have been changed into **"**dorsal ventral and lateral" **(Page3, Line 118).**

Question 14: - Lines 112-113 and line 116: mounting wet insect parts on stubs and then drying them over silica gel for 48 hours (this is how I understood the instructions till this point) - is there really no faster (and less material-consuming) alternative? Did the authors try critical point-drying or just simple air-drying (which could work pretty nice with such well-sclerotized insects as beetles)? Comparison with the line 116 („after air-drying") demonstrates that the did - so, the specimens are to be air-dried before silica gel? Or was these two alternative treatments and the auhtors think that silica gel performs better? The authors

should make clear all the steps of the procedure and also address the point of comparative performance of air-drying vs. silica gel-drying.
Answer:
This is an improved air-drying method. In order to prevent dust from falling into dehydrated samples, the samples were placed in capped Petri dishes, where some silica gel desiccants were placed. The moisture released by the samples was directly absorbed by silica gel desiccants. The biggest advantage of this method is that it is simple, easy to maintain, and it keeps the micro-environment air dry without special equipment. During the whole drying process, the silica gel desiccant will not contact the sample. We did not use the critical point drying method, because the critical point drying method requires special equipment, which is not available in our lab. In the study, air-dry is able to meet our requirements. We hope to apply critical point drying in the future.

Question 15: - Section 1.3. Generally, the instructions under the point 1.3. are either superfluous or not easy to follow for anyone who does not happen to have the same equipment as the authors, and they do not even specify what equipment they have. Instead of saying how many times and how long to sputter-coat the sample, the authors should either name the coat layer thickness that they consider desirable or give the model of the sputter-coater and details on the program/modus they used. Also, is is really indispensable to use gold, or are other metal mixtures (e.g. gold-palladium) possible?
Answer:
Thank you for the suggestion. We added the model and operation process of the ion sputtering instrument **(Page3, Line 124-132).** After discussing with the technicians of electron microscopy, we found that the platinum was used in the experiment.

Question 16: - Lines 143-144: „Enable the sensilla in the resin block to be clearly observed and photographed and measure distances to target the sensilla (Fig.1)." The instructions at this point are really quite hazy and it is also not very clear to me how the figure 1. contributes to better understanding of the photography and measurements.
Answer: Even under the fluorescence microscope, the antennal sensilla of small insects such as *Chlorophorus caragana* can hardly see a clear shape. It can only see a rough image like Figure 1. In contrast, the light source of the general microscope can not penetrate the resin block at all, and the effect of the observation is much worse. Figure 1 shows the sensilla observed under the fluorescence microscope. Ultimately, the position of the target sensor needs to be helped by means of the scanning electron microscope image, which is shown in Figure 2.

Question 17: - Lines 157-158: what exactly was the fluorescent microscopy measurement software that the authors used?
Answer: When imaging, the objective micrometer (DIV 0.01mm) was added to the fluorescence microscope stage, and then the distance of the target was measured by

ImageJ (U.S. National Institute of Health) software. The image ruler was made by Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA, USA).

Question 18: - Lines 176-177 and the table 1: the authors refere to 10 different sensilla types and sort them into types. A literature reference to a work establishing/employing the sensilla taxonomy that they adopt would be very useful here. It is also better in this case to refer to a work that as readily accessible as possible from all over the world.
Answer: New citations were added **(Page3, Line 206).**

Question 19: - Lines 182-183: The authors forgot to mention the digitiform sensillum in the table legend.
Answer: Digitiform sensillum is mentioned in the table legend.

Question 20: - Lines 210 and 212: what exactly is seen in the figures 3 and 4? What organ of the animal is that? Which parts of it are better seen in the specimens processed with Tween? Authors should refer to this points in the figure legends.
Answer:
Fig. 3. The sensilla locating on antenna of *C. caragana* under SEM. Comparison of SEM image with Tween 20 (A) and without Tween 20 (B), which showed that picture A is cleaner than picture B in general.
Fig. 4. *C. caragana* viewed by transmission electron microscopy of sensilla twig basiconica on the labial palps. Comparison of TEM image with Tween 20 (A) and without Tween 20 (B). Microtubule structure of picture A is clear, while that of picture B is blurred. **(Page6, Line 239-245).**

Question 21: - Lines 223-226: does it have a special meaning that some of the labels for the organelles or structures are written in lower- and other in uppercase letters?
Answer: No special meaning in lower- and other in uppercase letters.

Question 22: - Lines 240-241: auhtors cite references 12-18 as support for extensive studies on Tween 20, but the reference 12 deals with the usage of Tween 80 (again, judging only by its title and abstract, since I could not access the full text of the paper).
Answer: We apologize for confuse about the citation. We have checked citations again. The correct citation related with Tween-20 is Number 11-14 in present revised manuscript. Number in origin manuscript is deleted.

Question 23: - Lines 242-252. The authors think that air-drying or silica gel-drying is sufficient for beetles with their hard cuticula - a view that is justified. However, my personal experience shows that cuticula of the sensilla is

still very fine and its condition can be influenced by many things, such as fixation or preservation medium etc. Did the authors check if sensilla of C. caragana look the same after e.g. critical point-drying or chemical drying? If yes, they should compare the results in the discussion section; if not, they should conduct such an experiment to be sure - or do not speculate that air-drying does not influence the cuticula of the sensilla.
Answer: It is a pity that classical Critical Point Drying is not available now in our laboratory. We hope to apply the technique in the future and compare with each other. So we rephrased the sentence indicated "Air-dry is able to meet requirements in the study". **(Page7, Line 284-285).**

Question 24: - Line 247: what are „insect needle inserts"?
Answer: It has been changed into "insect specimens" **(Page7, Line 282).**

Question 25: - Line 250: „chitin plastid walls" is not correct, animals do not have plastids and the authors mean something else here.
Answer: "plastid" has been deleted.

Question 26: - Line 254: „the back, ventral and sides of the appendage" - please rephrase.
Answer: These have been changed into **"**dorsal ventral and lateral" **(Page3, Line 122), (Page7, Line 289).**

Question 27: - Line 256: „borning" - please correct.
Answer: "borning" has been changed into "arised from"**(Page7, Line 291).**

Question 28: - Line 257: „many sensilla were hairy" . Do the authors mean that the sensilla were trichoid, i.e. had the form of a hair - or did they carry hairs? Please make it clear.
Answer: These have been changed into **"**many sensilla were relatively long and hairlike" **(Page7, Line 492).**
The sensilla were relatively long and hairlike, but it was not the same as the sensillum trichodeum. When the electron beam strikes the sensor, it is easy to bend the sensor and the tip will be bright.

Question 29: - Lines 260-261: „The traditional TEM method for repairing resin is extensive, and it is usually blindly cut using an optical microscope." Do the authors mean „prepairing resin"? Also, the reference number 8 that the authors cite here in support of their sentence seems to deal only with properties of different surfactants (again, judging without having seen the full text) - if it really does not deal with TEM and resin preparation methods, the authors should better cite some other work.
Answer: "prepairing resin" means "continuously cutting the resin block with a razor

blade" **(Page7, Line 296).**
A new citation is added. [17. Sumner, M. J. Epoxy resins for light and transmission electron microscopy. *Plant Microtechniques and Protocols*. 83–101(2015)]

Question 30: - Lines 265-266: the authors suggest that a scaled ruler can be used to measure distance to the target sensillum. With sensilla normally being only several micrometers long, the reader is prone to have doubts if such a fine ruler might be used, achieves the necessary accuracy or exists at all. The authors should address this issue.
Answer: When imaging, the fluorescent microscope stage was added with objective micrometer (DIV 0.01mm), then the target distance was measured by ImageJ (U.S. National Institute of Health) software, and the image scale was made by Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA, USA.). The fine ruler can be used.

Question 31: - Lines 285-286: "JoVE Science Education Database. Analytical Chemistry. Scanning Electron Microscopy (SEM). Journal of visualized experiments: JoVE, Cambridge, MA. (2018)." This citation format is strange to me. Is it an article? then there should be volume and page numbers. Is it a website? Then the access date and URL is lacking.
Answer: The citation is neither an article nor a website, which has been deleted in revised manuscript.

Question 32: - Line 300, Reference 7 - The journal is referred to here as the „Journal of Zhangkai Aguotechaical College", which does seem a misspelling of „Agrotechnical College". An online search for this paper delivers a little confusing results: it is listed here (http://en.cnki.com.cn/Journal_en/D-D000-ZNJX-2001-02.htm) in the contents list of the „Journal of Zhongkai University of Agriculture and Technology" for 2001-02. However, a click on the article leads to the page http://en.cnki.com.cn/Article_en/CJFDTotal-ZNJX200102012.htm, where the journal is referred to as „Journal of Zhangkai Aguotechaical [sic!] College". My knowledge of Mandarin is vestigial and does not allow me to check which name of the journal is correct in this case; the authors should revise the citation, addressing the points I made.
Answer: We apologize for the confuse. The correct title of Journal should be "Journal of Zhongkai Agrotechnical College". It is a University-sponsored journal **(Page8, Line 341).**

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Licensed Content Title	Ultrastructure and distribution of sensilla on the maxillary and labial palps of Chlorophorus caragana (Coleoptera: Cerambycidae)
Licensed Content Author	Yan-Ru Zhang, Li-Li Ren, Lu Zhang, et al
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Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 6
Will you be translating?	No
Circulation	1000
Title of new article	Improved scanning and transmission electron microscope sample preparation methods demonstrated using the palps of Chlorophorus caragana
Publication the new article is in	Journal of visualized Experiments (JoVE)
Publisher of new article	Journal of visualized Experiments (JoVE)
Author of new article	Yan-Ru Zhang, Hai-Li Qiao, Li-Li Ren, Peng-Fei Lu, You-Qing Luo, Rong Wang
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