Proc. Arthropod. Embryol. Soc. Jpn. 50, 7–10 (2016)

ISSN 1341–1527

[SHORT COMMUNICATION]

Simple, Artifact-free SEM Observations of Insect Embryos: Application of the Nano-suit Method to Insect Embryology

Mari FUJITA[1, 5], Alexander BLANKE[2, 5], Shûhei NOMURA[3] and Ryuichiro MACHIDA[4, 5]

[1] *Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305–8572, Japan*
[2] *Medical and Biological Engineering Research Group, School of Engineering, University of Hull, Hull HU6 7RX, UK*
[3] *National Museum of Nature and Science, Tsukuba, Ibaraki 305–0005, Japan*
[4] *Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305–8572, Japan*
[5] *Current address: Sugadaira Montane Research Center, University of Tsukuba, Sugadaira Kogen, Ueda, Nagano 386–2204, Japan*
E-mail: fujita@sugadaira.tsukuba.ac.jp (MF)

Scanning electron microscopy (SEM) enables morphological observations with a high resolution, but unfavorable shrinkage and distortion of the materials are often associated with the preparation of biological samples. In insect embryological studies, which deal with fragile embryos, the situation is grave. The fixation, dehydration and drying are apt to lead to serious shrinkage of materials. In the later stages when the embryonic cuticle has already been secreted, the case is more serious (Fig. 2A, B). Separating from the surface of the embryo, the embryonic cuticle wrinkles up, which would be a major obstacle preventing determination of the correct morphology of the embryo. As a solution to this problem, Machida (2000a) proposed the use of low-vacuum SEM of uncoated specimens, which enables us to observe the surface structures of embryos through the wrinkled embryonic cuticle (Machida, 2000b). However, it does not always follow that this method is very effective for every case, and the most critical point is that the required low vacuum yields only inferior resolution.

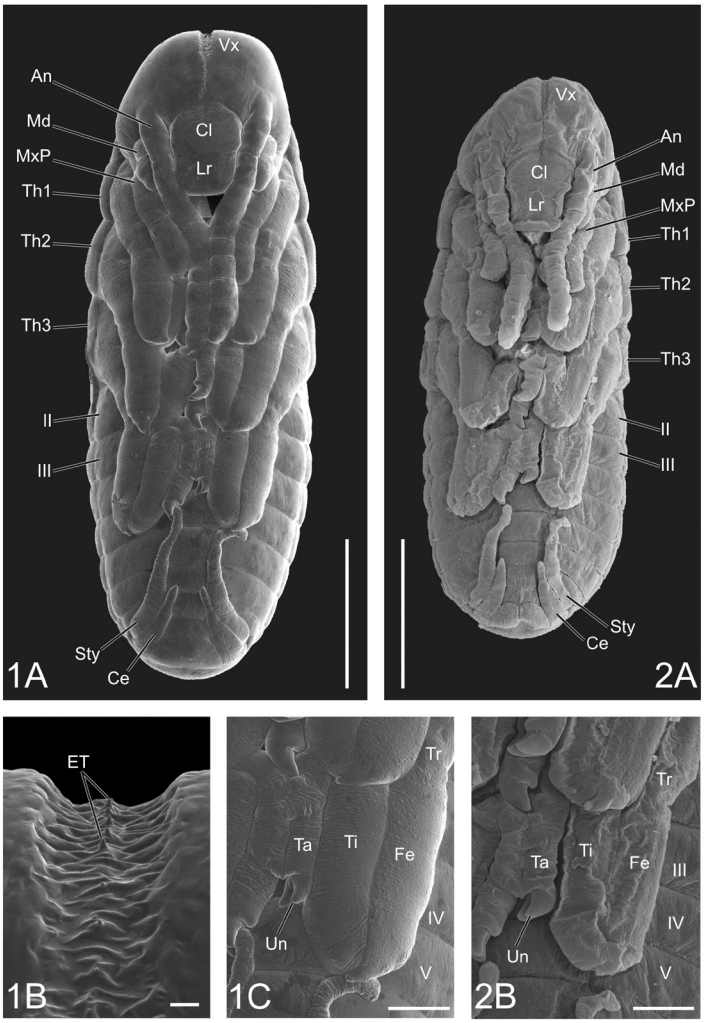
Chambers *et al*. (2003) reported that some insects such as dipteran larvae have been shown to possess special extracellular substances (ECSs) on their body surface. Takaku *et al*. (2013) found that the ECSs are polymerized by electron irradiation into an ultra-thin tough membrane named the "nano-suit", and that this nano-suit prevents the escape of evaporative substances from the specimens, even in high-vacuum conditions. They succeeded in biomimetically producing a nano-suit by electron irradiation to polyoxyethylene sorbitan monolaurate (Tween 20), which has high amphiphilic and biocompatible properties similar to ECSs. Specimens covered and protected by the nano-suit made from Tween 20, which acts as a flexible barrier to the passage of gases and liquids, can be observed under usual high-vacuum SEM with a high resolution being maintained, without any conventional treatments such as histological fixation, dehydration and drying (Ohta *et al*., 2014).

In this study, we applied the "nano-suit method" invented by Takaku *et al*. (2013) to insect embryology, using grown embryos of a corydiid cockroach, *Eucorydia yasumatsui* Asahina, in the later definitive dorsal closure stage, at which the larval cuticle is yet to be secreted with the surface of the embryo only covered with the fine embryonic cuticle. The procedure used is as follows.

1. Embryos dissected out of eggs in physiological saline were soaked in 1% Tween 20 solution dissolved in distilled water for 1 h.
2. Embryos taken out from the Tween 20 solution and excess solution were blotted with filter paper.
3. The embryos were mounted on a stub and observed with a scanning electron microscope (SEM), TOPCON SM-300, under a high vacuum at an accelerating voltage of 5 kV.

An example of an *E. yasumatsui* embryo observed using the nano-suit method is shown in Figure 1. The surface structure of the embryo was well preserved (Fig. 1A) in terms of the details (Fig. 1B, C). Figure 1B, which is a magnified image of the vertex, clearly demonstrates a row of pointed, tiny egg teeth on the vertex, this being the first finding of egg teeth in cockroaches. As shown in Figure 1C, which is a magnification around the thoracic legs, each structure is well preserved *in natura* (compare with Fig. 2B). Because the longer exposure to and higher intensity of an electron beam would lead to degradation of the nano-suit, the observation should be less than 1 h, and the accelerating voltage should be set lower than 5 kV.

After the observation using the nano-suit method, we processed the same embryo into the SEM specimen in the conventional methods: the specimen was detached from the stub (when the specimen is not easily detached, pour saline or distilled water on to it), fixed with Karnovsky's fixative (2% paraformaldehyde + 2.5% glutaraldehyde 0.1 M HCl-sodium



Figs. 1, 2 Scanning electron micrographs (SEMs) of a grown embryo of *Eucorydia yasumatsui* Asahina in the later definitive dorsal closure stage. The whole embryo images (Figs. 1A, 2A) were produced by merging separate photos using the software Adobe Photoshop CS2.

Fig. 1 A. An SEM of the embryo processed using the nano-suit method. B. Enlargement of the vertex, showing the egg teeth. C. Enlargement of a part of the thoracic region. See the text.

Fig. 2 A. An SEM of the same embryo as shown in Fig. 1A, processed in the conventional methods. B. Enlargement of a part of the thoracic region at approximately the same frame as shown in Fig. 1C. See the text.

An: antenna, Ce: cercus, Cl: clypeus, ET: egg tooth, Fe: femur, Lr: labrum, Md: mandible, MxP: maxillary palp, Sty: stylus, Ta: tarsus, Th1–3: pro-, meso- and metathorax, Ti: tibia, Tr: trochanter, Un: unguis, Vx: vertex, II-V: second to fifth abdominal segments. Bars = 1A, 2A: 500 μm; 1B: 10 μm; 1C, 2B: 100 μm.

cacodylate buffer solution, pH 7.2) and postfixed with 1% OsO_4 solution, dehydrated through a graded ethyl alcohol series, dried using a critical point dryer (tousimis Samdri-PVT-3D), stuck to the stub and coated with gold. Figure 2A and B are scanning electron micrographs (SEMs) of a specimen processed in the conventional methods. We found that the embryo was seriously contracted and distorted (Fig. 2A), compared with the SEM of the specimen processed using the nano-suit method (Fig. 1A): the embryo was contracted into *ca.* 85% in length. Figure 2B is an enlargement of Figure 2A, approximately the same frame as for Figure 1C. The embryonic cuticle wrinkled up due to separation form the surface of the embryo made it difficult to observe and grasp the correct surface structures of the appendages (Fig. 2B), the same as the case the embryos freshly dissected out of the eggs are directly processed in the SEM specimens (data not shown).

It was revealed that the nano-suit method has a great advantage over the conventional approach in the SEM observations of insect embryos, especially at the later stages; it guarantees 1) observations of high resolution, 2) with little contraction and distortion of the surface structures of the embryos, and 3) the requisite processing is very simple, namely, just immersing samples in the detergent Tween 20 solution for a short period. Furthermore, the nano-suit method has another remarkable advantage. Specimens used once for SEM observation can be reused for some histological observations because the embryos used usually survive after the SEM observation; even if they die due to the SEM observation, their tissues would remain undamaged. We present an example of this.

Figure 3A shows an SEM of an embryo observed using

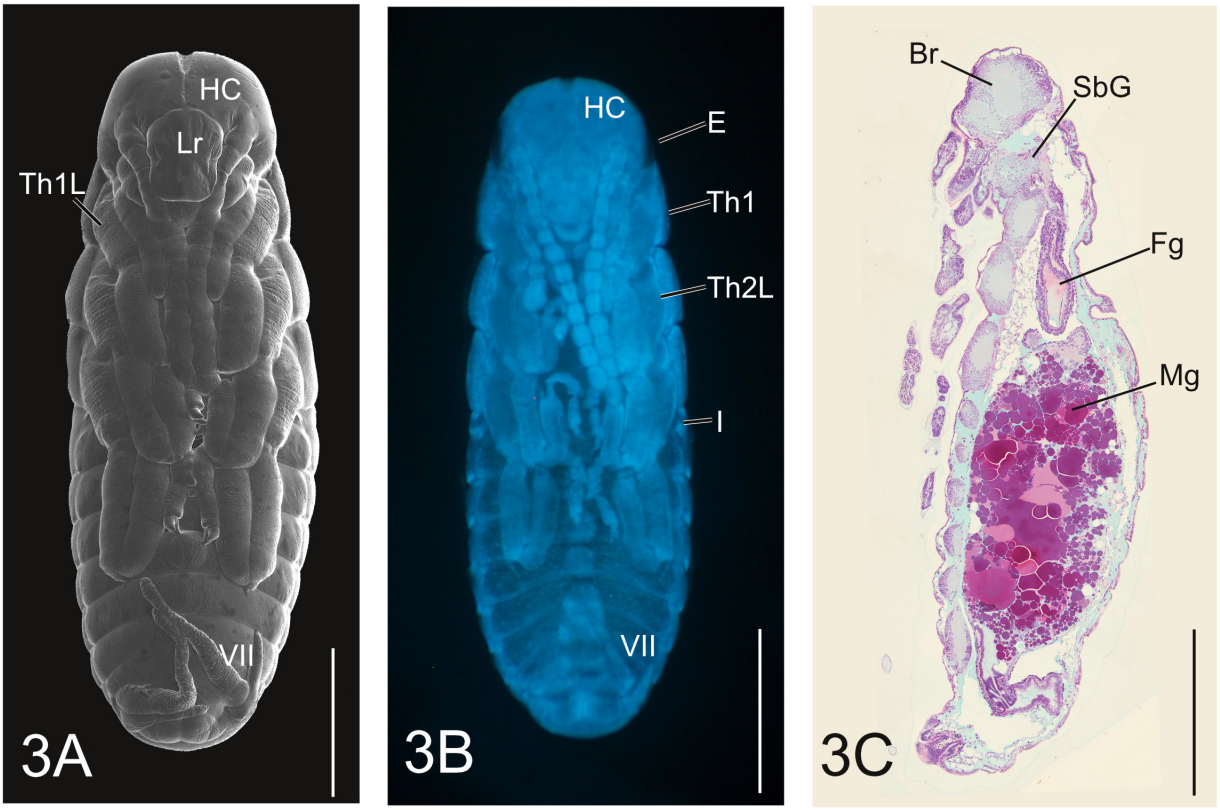


Fig. 3 Scanning electron micrography (SEM) and histological observations of a grown embryo of *Eucorydia yasumatsui* Asahina at the later definitive dorsal closure stage. A. SEM using the nano-suit method, which was produced by merging separate photos using the software Adobe Photoshop CS2. B. Fluorescence microscopy of the same embryo as shown in A, being stained with DAPI. C. A histological section of the same embryo as shown in A and B. See the text.
Br: brain, E: eye, Fg: foregut, HC: head capsule, Lr: labrum, Mg: midgut, SbG: suboesophageal ganglion, Th1: prothorax, Th1, 2L: pro- and mesothoracic leg, I, VII: first and seventh abdominal segments.
Bars = 500 μm.

the nano-suit method. After the SEM observation, the embryo was soaked in physiological saline, removed from the stub and then fixed with Karnovsky's fixative. Figure 3B shows the same embryo as in Figure 3A, which was stained with a DNA-specific fluorescent dye, DAPI (4',6-diamidino-2-phenylindole dihydrochloride, 10 μg/ml), and observed with a fluorescence stereomicroscope (LEICA MZ FLIII). Then, the embryo was processed into methacrylate resin (Kulzer Technovit 7100) sections according to Machida *et al*. (1994a, b), as shown in Figure 3C, which were stained with 1% Delafield's hematoxylin overnight, 0.5% eosin G for 1 h and 0.5% fast green FCF 100% ethyl alcohol solution for 1 min, and observed under a biological microscope (Nikon Optiphot-2).

As has been addressed, the nano-suit method for SEM observations was revealed to have great advantages in insect embryology. This method not only enables observations of high resolution with little contraction and distortion on the surface structures of embryos, but also after the SEM observation, the same embryos can be reused for routine histological analyses. This approach should become a standard method for scanning electron microscopy in this field.

Acknowledgments: We thank Prof. T. Hariyama and Dr. Y. Takaku of Hamamatsu University School of Medicine and Prof. M. Shimomura and Dr. Y. Hirai of Chitose Institute of Science and Technology for their kindly giving us valuable guidance and information of the nano-suit method. This study was supported by Grants-in-Aid from the JSPS (Scientific Research C: 2544020) to RM and from the University of Tsukuba — Deutscher Akademischer Austausch Dienst (DAAD) Partnership Program to RM and AB. This is a contribution from the Sugadaira Montane Research Center, University of Tsukuba.

References

Chambers, L., S. Woodrow, A.P. Brown, P.D. Harris, D. Phillips, M. Hall, J.C.T. Church and D.I. Pritchard (2003) Degradation of extracellular matrix components by defined proteinases from the greenbottle larva *Lucilia sericata* used for the clinical debridement of non-healing wounds. *British Journal of Dermatology*, **148**, 14–23.

Machida, R. (2000a) Usefulness of low-vacuum scanning electron microscopy in descriptive insect embryology. *Proceedings of Arthropodan Embryological Society of Japan*, **35**, 17–19.

Machida, R. (2000b) Serial homology of the mandible and maxilla in the jumping bristletail *Pedetontus unimaculatus* Machida, based on external embryology (Hexapoda: Archaeognatha, Machilidae). *Journal of Morphology*, **245**, 19–28.

Machida, R., T. Nagashima and H. Ando (1994a) Embryonic development of the jumping bristletail *Pedetontus unimaculatus* Machida, with special reference to embryonic membranes (Hexapoda: Microcoryphia, Machilidae). *Journal of Morphology*, **220**, 147–165.

Machida, R., T. Nagashima and T. Yokoyama (1994b) Mesoderm segregation of a jumping bristletail, *Pedetontus unimaculatus* Machida (Hexapoda, Microcoryphia), with a note on an automatic vacuum infiltrator. *Proceedings of Arthropodan Embryological Society of Japan*, **29**, 23–24. (in Japanese).

Ohta, I, Y. Takaku, H. Suzuki, D. Ishii, Y. Muranaka, M. Shimomura and T. Hariyama (2014) Dressing living organisms in a thin polymer membrane, the NanoSuit, for high-vacuum FE-SEM observation. *Microscopy*, **63**, 295–300.

Takaku, Y., H. Suzuki, I. Ohta, D. Ishii, Y. Muranaka, M. Shimomura and T. Hariyama (2013) A thin polymer membrane, nano-suit, enhancing survival across the continuum between air and high vacuum. *Proceedings of the National Academy of Science of the United States of America*, **110**, 7631–7635.