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**Roles of Actin Networks in Peristaltic Squeezing of Sperm Bundles in *Bombyx mori***

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## **Abstract**

Two types of sperm are produced in the silkworm, *Bombyx mori*. Nucleate eupyrene sperm is an ordinary sperm that contributes to fertilization, while anucleate apyrene sperm is considered to play important roles in assisting eupyrene sperm (Jamieson, 1987). At the very late stage of spermatogenesis, a phenomenon called ‘peristaltic squeezing’ occurs in both types of sperm, whereby cytoplasm of the eupyrene and nuclei of the apyrene sperm are discarded from the posterior end, forming matured sperm. In this study, rhodamine-phalloidin staining for actin is applied to sperm bundles. Before the start of peristaltic squeezing, actin filament networks are spread on the cyst cells and constrictions by the networks appear in several places of the bundles. Actin particles, which are later recognized as circlets, are localized within the bundles. Squeezing action by the networks occurs from the anterior region and transfers toward the posterior, eliminating cytoplasm together with circlets from the posterior end. It seems that actin filaments contribute to the peristaltic squeezing of the sperm bundles in *Bombyx mori*.

Key words: actin; apyrene sperm; eupyrene sperm; Lepidoptera; peristaltic squeezing; phalloidin

Silkworm, *Bombyx mori*, males produce dimorphic sperm, nucleate eupyrene sperm (eusperm) and anucleate apyrene sperm (parasperm) (Jamieson, 1987; Jamieson et al., 1999). Although both kinds of sperm are transferred to the female reproductive organs, only eupyrene sperm fertilize the eggs. In both types, a layer of cyst cells envelops 256 sperm cells after maturation division, later forming a sperm bundle. The marked difference between the two is that: (1) the eupyrene sperm is an ordinary sperm with a spearhead-shaped nucleus located in the anterior end, while the apyrene sperm loses its nucleus at the very late stage of spermatogenesis, (2) the length of the apyrene sperm is half that of the eupyrene sperm (Katsuno, 1978), (3) the centriole/basal body behaves quite differently (Yamashiki and Kawamura, 1997), and (4) the two types differentiate during distinctively different phases of metamorphosis: the eupyrene sperm in the larval stage and the apyrene sperm after spinning (ref. Tazima, 1964).

We previously demonstrated that peristaltic squeezing is a common phenomenon both in eupyrene and apyrene sperm bundles at the very late stage of spermatogenesis (Kawamura et al., 2000). Peristalsis starts from the anteriormost region of the bundle, transmitting toward the posterior. Cytoplasmic debris is discarded from the posterior end of eupyrene sperm bundles by this peristaltic action, while in apyrene sperm bundles the nuclei are eliminated, together with debris, by the action.

Previous experiments with cytochalasin D which suppressed peristaltic squeezing suggested that motility of actin filaments contributes to the peristaltic action of sperm bundles (Kawamura et al., 2000). In this study we visualize the behavior and dynamic changes of actin filaments in eupyrene and apyrene sperm bundles during peristaltic squeezing in *Bombyx* spermatogenesis.

## MATERIALS AND METHODS

The strains of the silkworm (*Bombyx mori*) used were the generation F<sub>1</sub> of the cross between an re9 (red egg:  $re/re$ ,  $+w^{-2}/+w^{-2}$ ) female and a Tw1 (white egg:  $+re/+re$ ,  $w^{-2}/w^{-2}$ ) male (Kawamura, 1978). Testes were excised from the 5th day pupae.

### **Indirect immunofluorescence staining for tubulin and rhodamine-phalloidin staining for actin filament**

We followed the methods described in Yamashiki and Kawamura (1997) with a slight modification. Sperm bundles from excised testes of pupae were smeared on a coverslip coated with 3-aminopropyl-triethoxy-silane (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.0) for 1h, and cold acetone for an additional 10 min. Mouse monoclonal antibody against human alpha-tubulin (Cederlane Laboratory, Hornby, Ont. Canada) and a cocktail of fluorescein isocyanate-conjugated goat antimouse IgG (MBL Co., Tokyo, Japan), Hoechst 33258 (Hoechst Co., Strasbourg, Germany), and rhodamine-conjugated phalloidin (#R-415; Molecular Probes Inc., Eugene, OR., U.S.A.) was applied to specimens on the coverslip. The coverslip was mounted on a slide glass with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA., U.S.A.). The stained sperm bundles were observed with a laser scanning confocal microscope (TCS SP2, Leica, Heidelberg, Germany) and captured images were processed by means of Adobe Photoshop version 6. In the images, the green color is for microtubules, blue is for nuclei and red is for actin filaments.

## RESULTS

### **Behavior of actin filaments in eupyrene sperm bundles**

Figure 1 displays eupyrene sperm bundles before (Fig. 1a-c) and after (Fig. 1d-f) the start of peristaltic squeezing. In order to show the behavior of actin filaments clearly, images (with the exception of green displayed in Fig. 1a) are displayed in two colors, red for actin filaments and blue for nuclei. Microtubules (green) of the flagella in Figure 1a fully extend to the final length (approx. 700 $\mu$ m), and the round spermatid nuclei are transferred to the anteriormost region. The nuclei are then transformed into a spearhead shape (Fig. 1b). Networks of actin filaments occur on the surface of the bundles, and bands of actin filaments surround the bundle as if they constrict the thick bundle in several places. Actin particles are accumulated around the head cyst cell and the region at one-tenth of the whole length from the apex of the bundle (Fig. 1a, c). After peristaltic squeezing starts anteriorly, actin particles previously located at the bottom of the head cyst cell extend beyond sperm nuclei (Fig. 1d). Thereafter, actin filaments of the networks show a depolymerized appearance, and begin to move toward the posterior together with actin particles. As peristaltic squeezing proceeds half way to the posterior end, the networks of the anterior half disappear and are replaced by longitudinal actin threads. The actin particles are now located in the middle part of the bundle (arrows in Fig. 1a, c-e). When actin networks reach the posterior end, they form a cap-like structure. Deeply stained actin threads appear in the posterior region (Fig. 1e). In the last stage of peristaltic squeezing (Fig. 1f), actin networks become inconspicuous, while actin filaments in the anterior region still remain. As the sperm bundle grows slim, the networks appear to be depolymerized into fragments. The cap-like structure at the posterior end is torn off and discarded from the posterior end of the sperm bundle (Fig. 1d-f). Actin particles that are replaced with circlets when they proceed to middle part of the bundle (arrow in Fig. 1e) are also eliminated together with cytoplasmic debris at completion of peristaltic squeezing (Fig. 3a). In most cases eighteen cyst cell nuclei may be counted in a sperm bundle throughout spermiogenesis.

### **Behavior of actin filaments in apyrene sperm bundles**

In an apyrene sperm bundle (Fig 2), sperm nuclei stay in the middle part, unlike those of a eupyrene sperm bundle. Actin filament networks on the surface of the bundle are observed after a cyst of spermatids transform into a bundle (Fig. 2a, b). The contractile

bands that constrict the bundle in the depression site of the wavy contour are also seen before peristaltic squeezing starts (Fig. 2b). Two rings of actin filaments begin to form in both anterior and posterior regions of the bundle (Fig. 2b). As the rings become conspicuous, actin filament networks are distributed between the two rings. A group of actin particles is present around the sperm nuclei in the middle of the bundle (arrows in Fig. 2a-c). A head cyst cell is not always located in the apex of the apyrene bundle, but it appears in the apex when peristaltic squeezing begins. The actin ring in the anterior end is replaced by an accumulation of actin filaments beneath the head cyst cell (Fig. 2d). As peristaltic squeezing proceeds, sperm nuclei together with the actin circlets (arrows in Figs. 2c, 3b) move toward the posterior. Actin networks grow narrower and filaments become thicker when faintly stained cap-like actin filaments appear in the posterior end (Fig. 2e). At the stage when sperm nuclei and actin circlets are discarded from the posterior end, the nucleus of the head cyst cell does not stay in the apex but slips a little sideways, while actin networks on the surface and cyst cell nuclei of the bundles still remain (Fig. 2f).

### **Circlets in eupyrene and apyrene sperm bundles**

In eupyrene sperm bundles, the actin particles that occur beneath the sperm nuclei (Fig. 1a) form circlets as they are pushed toward the posterior (Fig. 1c-e). The shape of circlets becomes distinct when they reach the middle part of the sperm bundle (Fig. 1e). Figure 3a shows the actin circlets ejected from the posterior end of a eupyrene sperm bundle together with debris. In apyrene sperm bundles, the actin circlets (Fig. 3b) are first recognized in the middle region of sperm bundles (arrows in Fig. 2a-c). The circlets in both types of sperm bundles move towards the posterior region as peristaltic squeezing proceeds and are finally discarded from the posterior end (Figs. 1d-f, 2f).

## DISCUSSION

In the last stage of spermatogenesis in the silkworm, *Bombyx mori*, peristaltic squeezing occurs in both eupyrene and apyrene sperm bundles (Kawamura et al., 2000). Cytoplasmic debris in the eupyrene sperm and nuclei in the apyrene sperm are discarded from the posterior end of the bundle by the squeezing action. They also found that the peristaltic squeezing is inhibited by cytochalasin D treatment that suppresses the motility of actin filaments.

Actin networks are definitely localized on the surface of eupyrene (Fig. 1) and apyrene sperm bundles (Fig. 2) of *Bombyx mori*. The wavy constrictions of the thick bundles previously observed by Kawamura et al. (2000) seem to be caused by contraction of actin filament bands. At the time of peristaltic squeezing, the actin networks that tightly bind sperm bundles constrict up sequentially from the anterior to the posterior of the bundle. The dynamism of actin filaments appears to be the main force behind peristaltic squeezing in eupyrene and apyrene sperm bundles in the silkworm.

Actin particles that form circlets occur anteriorly in eupyrene sperm bundles (Fig. 1a-c), and in the mid-region around apyrene nuclei (Fig. 2a-c). The particles move posteriorly as actin networks sequentially contract from the anterior end (Figs. 1d, e, 2d-f). It is doubtful that cytoplasmic debris of an individual eupyrene sperm or a nucleus of an individual apyrene sperm can be squeezed out only by the contractile action of actin filaments of surrounding cyst cells. If these actin particles transform into a circlet around the individual sperm, cytoplasm in a eupyrene sperm and a sperm nucleus in an apyrene sperm can be discarded by the movement toward the posterior. The actin circlets and actin fibers contained in the cytoplasmic debris (Fig. 3) strongly support these speculations. In *Drosophila melanogaster*, Hicks et al. (1999) proposed that a myosin ring in the plasma membrane of a sperm is responsible for individualization, whereby cytoplasmic contents are eliminated from a sperm bundle.

At initiation of peristaltic squeezing, actin filaments that are accumulated in the basement of the head cyst cell protrude into the anterior part of the sperm bundles. If these actin filaments tightly hold sperm nuclei, they can resist against the squeezing action and can stay in the anterior with the aid of an acrosome tubule-nucleus-basal body assembly anchored into the head cyst cell (Yamashiki and Kawamura, 1997).

Cell-to-cell contacts are of fundamentally importance to all multicellular organisms. In all solid tissues, actin filaments run along the edge of the cell as one of the components of adherent junctions (Gumbiner, 2000). Most cyst cell nuclei on the surface

of eupyrene and apyrene sperm bundles stay inside the framework of actin networks (Figs. 1a, b, 2b-d). This morphological situation strongly suggests that cyst cells produce the actin filaments that extend through cell-to-cell connections. The form of the systematic actin networks probably depends upon the arrangement of cyst cells. Figure 4 schematically illustrates the shape and arrangement of cyst cells on the surface of eupyrene sperm bundles. The actin networks displayed in Figure 1 coincide well with the borderlines of the connecting cyst cells. High temperature treatment of *Bombyx* pupae causes abnormal eupyrene and apyrene sperm bundles (Sahara and Kawamura, 2002). The experiments revealed that the bundles that lost normal arrangement of cyst cells could not elongate, but formed a spermatocyst like a thread-ball. The head cyst cell of the eupyrene and the apyrene sperm bundle seems to play the major role in initiating the morphological construction of sperm bundles. However, when and how a head cyst cell is selected from 18 cyst cells are still unknown.

At the end of mitosis, daughter cells are separated from each other by cytokinesis. The contractile ring composed of actin and myosin as major components appears at the division plane and generates a division furrow (Schroeder, 1968). Taking into account our previous result that not only cytochalasin D but also colcemid interrupts peristaltic squeezing (Kawamura et al., 2000), the mechanism of contraction of actin networks in sperm bundles may be assisted by microtubules like those of the contractile ring in dividing cells.

In higher insects, germline cysts containing spermatogonia, spermatocytes, or young spermatids are generally spherical or polyhedral (ref. Phillips, 1970). As the germ cells elongate, the cysts also elongate, and the spermatids align in parallel. In *in vitro* spermatocyte culture of *Drosophila* (Liebrich, 1981), elongated spermatocysts (sperm bundles) become slender as maturation progresses. The slenderizing process coincides with that of *B. mori* spermatogenesis (Kawamura et al., 2000). Elimination of irregular eupyrene nuclei in the silkworm is an important function of peristaltic squeezing (Kawamura et al., 2001). Insects other than *Bombyx* probably use the same mechanism to avoid irregular fertilization. The mechanism is still unclear how actin networks sequentially squeeze from anterior to posterior in sperm bundles. We are developing an *in vitro* culture system for *Bombyx* spermatocysts (Kawamura and Sahara, 2002). The cyst cell culture *in vitro* will be available to elucidate the mechanisms.

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## LITERATURE CITED

Gumbiner, BM. 2000. Regulation of cadherin adhesive activity. *J Cell Biol* 148:399-403.

Hicks JL, Deng W-M, Rogat AD, Miller KG, Bownes M. 1999. Class VI unconventional myosin is required for spermatogenesis in *Drosophila*. *Mol Bio Cell* 10:4341-4345.

Jamieson BGM. 1987. The ultrastructure and phylogeny of insect spermatozoa. Cambridge: Cambridge University Press. 320p.

Jamieson BGM, Dallai R, Afzelius BA. 1999. Insects: Their spermatozoa and phylogeny. New Hampshire: Scientific Publishers. 555p.

Katsuno S. 1978. Studies on eupyrene and apyrene spermatozoa in the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) VIII. The length of spermatozoa. *Appl Entomol Zool* 13:127-129.

Kawamura N. 1978. The early embryonic mitosis in normal and cooled eggs of the silkworm, *Bombyx mori*. *J Morphol* 158:57-72.

Kawamura N, Sahara K. 2002. *In vitro* cultivation of spermatocysts to matured sperm in the silkworm, *Bombyx mori*. *Dev Growth Differ* 44:in press.

Kawamura N, Yamashiki N, Saitoh H, Sahara K. 2000. Peristaltic squeezing of sperm bundles at the late stage of spermatogenesis in the silkworm, *Bombyx mori*. *J Morphol* 246:53-58.

Kawamura N, Yamashiki N, Saitoh H, Sahara K. 2001. Significance of peristaltic squeezing of sperm bundles in the silkworm, *Bombyx mori*: Elimination of irregular eupyrene sperm nuclei of the triploid. *Zygote* 9:159-166.

Liebrich, W. 1981. *In vitro* spermatogenesis in *Drosophila*. *Cell Tissue Res* 220:251-262.

Phillips D. 1970. Insect sperm: their structure and morphogenesis. *J Cell Biol* 44:243-277.

Sahara K, Kawamura N. 2002. Double copulation of a female with sterile diploid and polyploid males recovers fertility in *Bombyx mori*. *Zygote* 10:23-29.

Schroeder TE. 1968. Cytokinesis: filaments in the cleavage furrow. *Exp Cell Res* 53:272-276.

Tazima Y. 1964. The genetics of the silkworm. London: Logos Press. 253p.

Yamashiki N, Kawamura N. 1997. Behaviors of nucleus, basal bodies and microtubules during eupyrene and apyrene spermiogenesis in the silkworm, *Bombyx mori* (Lepidoptera). *Dev Growth Differ* 39:715-722.

## Figure legends

Fig. 1. *Bombyx mori*. Eupyrene sperm bundles displayed by confocal images with immunofluorescence staining for tubulin (green), with rhodamine-phalloidin for actin (red), and with Hoechst 33258 for nuclei (blue). **a**: An early eupyrene sperm bundle bearing round nuclei. **b**: The nuclei assume a spearhead shape. Green color for microtubules is eliminated to show actin filaments more clearly. **c**: A eupyrene sperm bundle shortly before the initiation of peristaltic squeezing. **d**: A eupyrene sperm bundle just after the start of squeezing action. **e**: Squeezing proceeds to the halfway point in a eupyrene sperm bundle. Note the transformation of actin bands and particles. **f**: Completion of peristaltic squeezing. Arrows (a, c-e) represent actin particles moving posteriorly as peristaltic squeezing proceeds. CCN, cyst cell nucleus; HCN, head cyst cell nucleus; SN, sperm nucleus. Scale bars, 80 $\mu$ m.

Fig. 2. *Bombyx mori*. Apyrene sperm bundles. **a**: A young apyrene sperm bundle. The nuclei are scattered in the bundle. **b**: An apyrene sperm bundle with the nuclei gathering in the middle region. **c**: An apyrene sperm bundle shortly before the initiation of peristaltic squeezing. **d**: Peristaltic squeezing initiates from the anterior of the bundle. **e**: Squeezing proceeds to the halfway point. **f**: Completion of peristaltic squeezing. The nuclei are eliminated from the posterior end. Arrows (a-c) represent actin particles. CCN, cyst cell nucleus; HCN, head cyst cell nucleus; SN, sperm nucleus. Scale bar, 40  $\mu$ m.

Fig. 3. *Bombyx mori*. Actin circlets and filaments displayed in gray tones. **a**: Circlets and fragments of the fibers in the cytoplasmic debris of a squeezed eupyrene sperm bundle. **b**: Circlets and filaments in the middle region of an apyrene sperm bundle before peristaltic squeezing. Scale bar, 20 $\mu$ m.

Fig. 4. *Bombyx mori*. Schematic illustration of the putative shape and arrangement of cyst cells on the surface of the mid-region of a eupyrene sperm bundle. CCN, cyst cell nucleus.

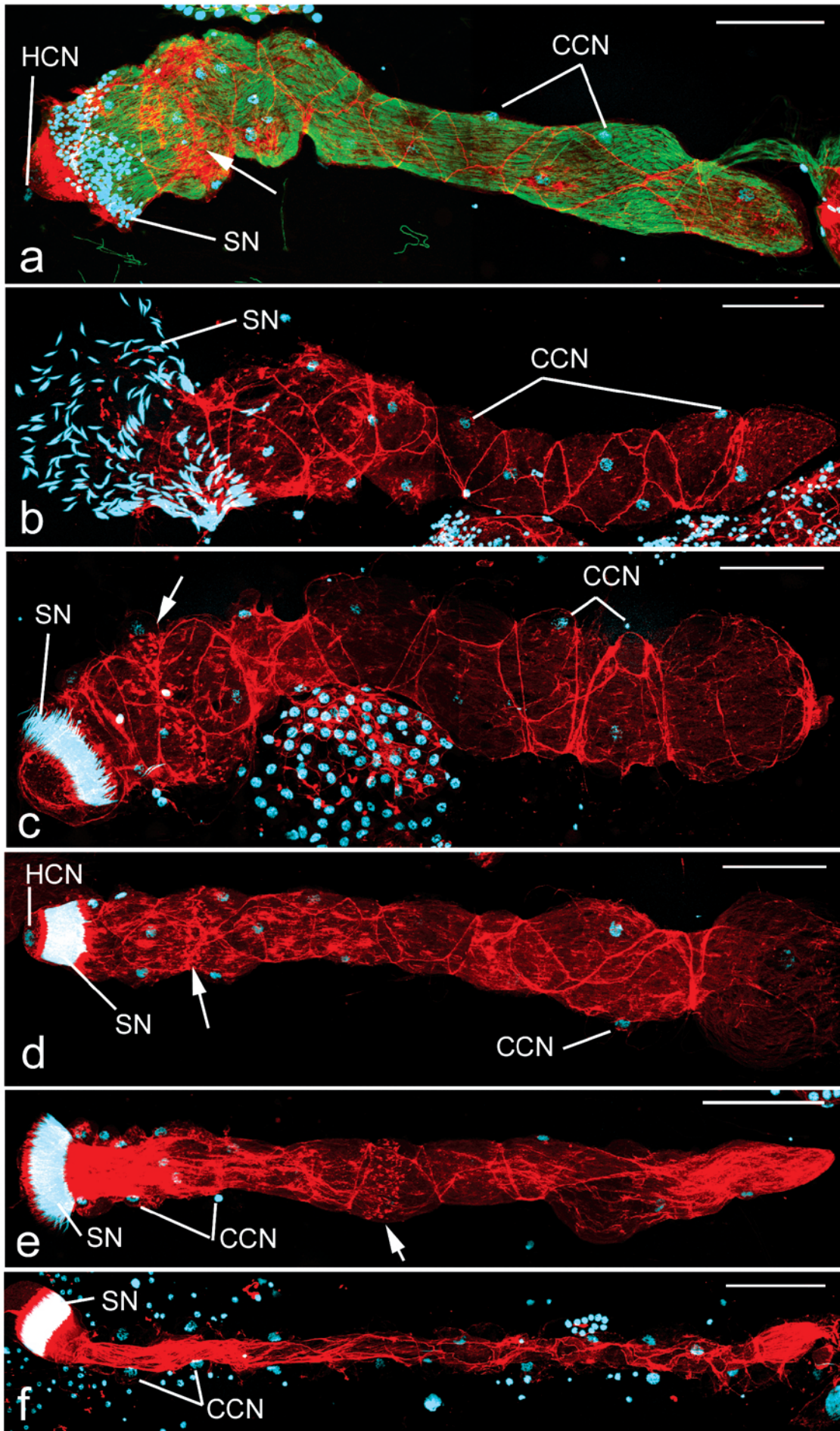


Fig.1. Sahara & Kawamura

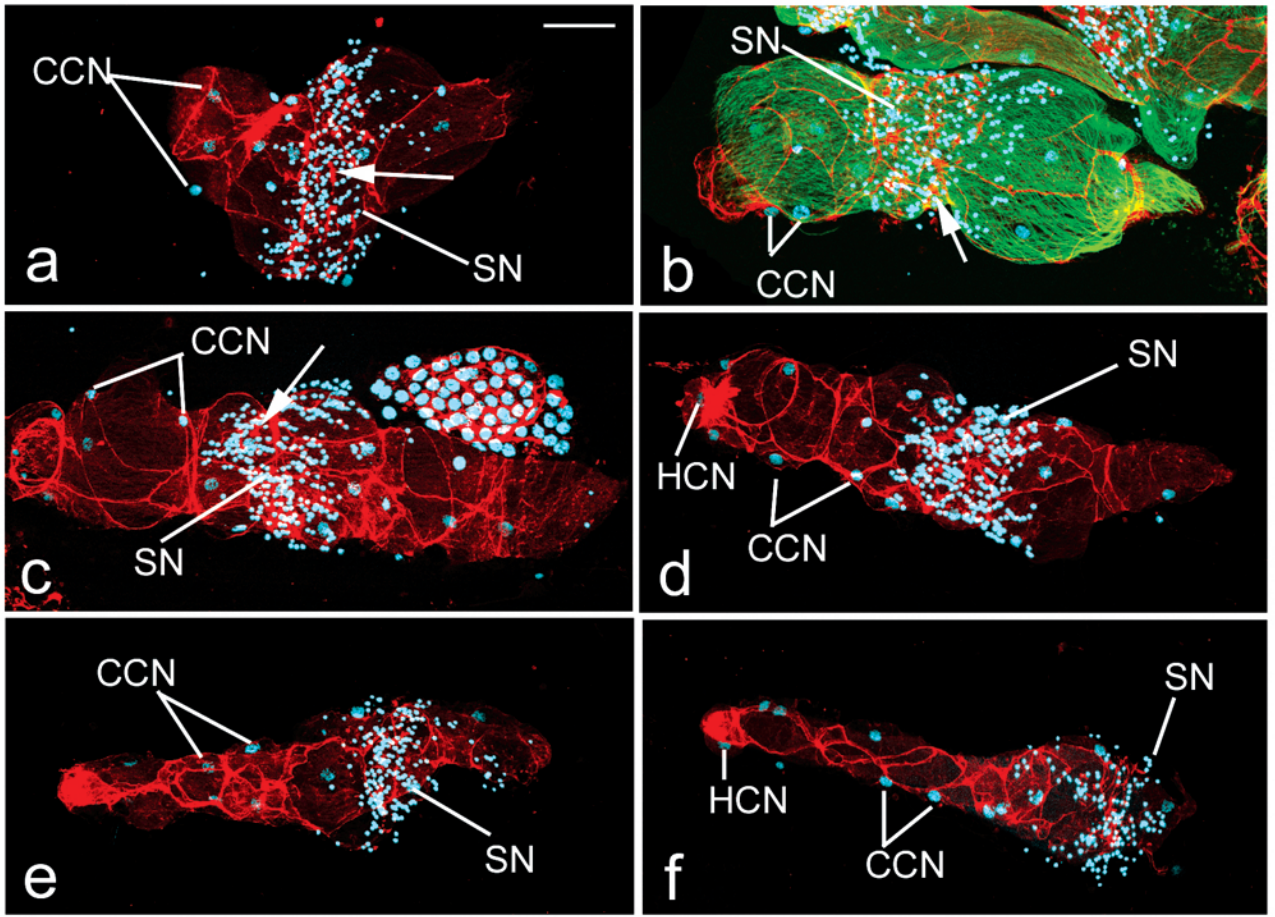


Fig.2. Sahara & Kawamua

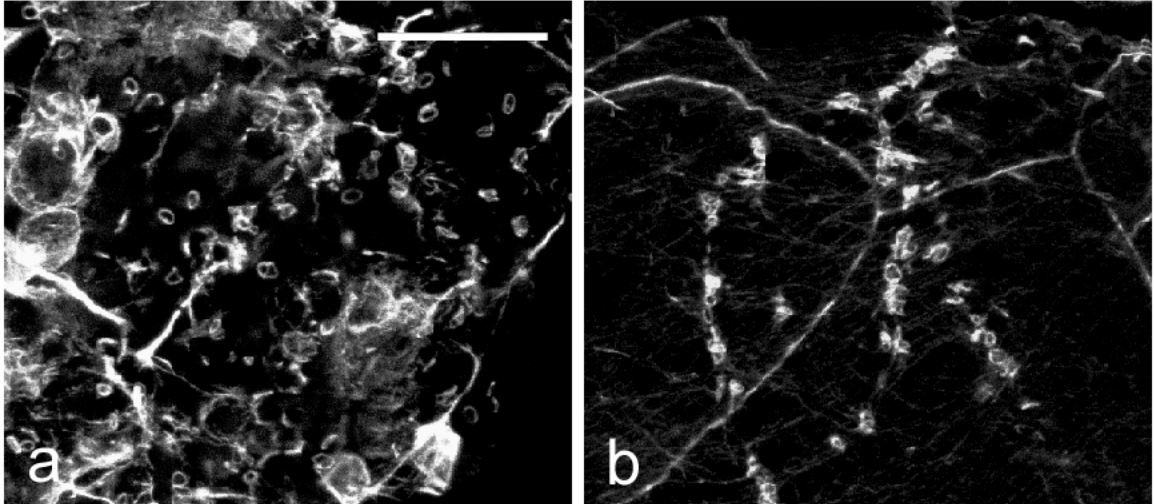


Fig. 3 Sahara & Kawamura

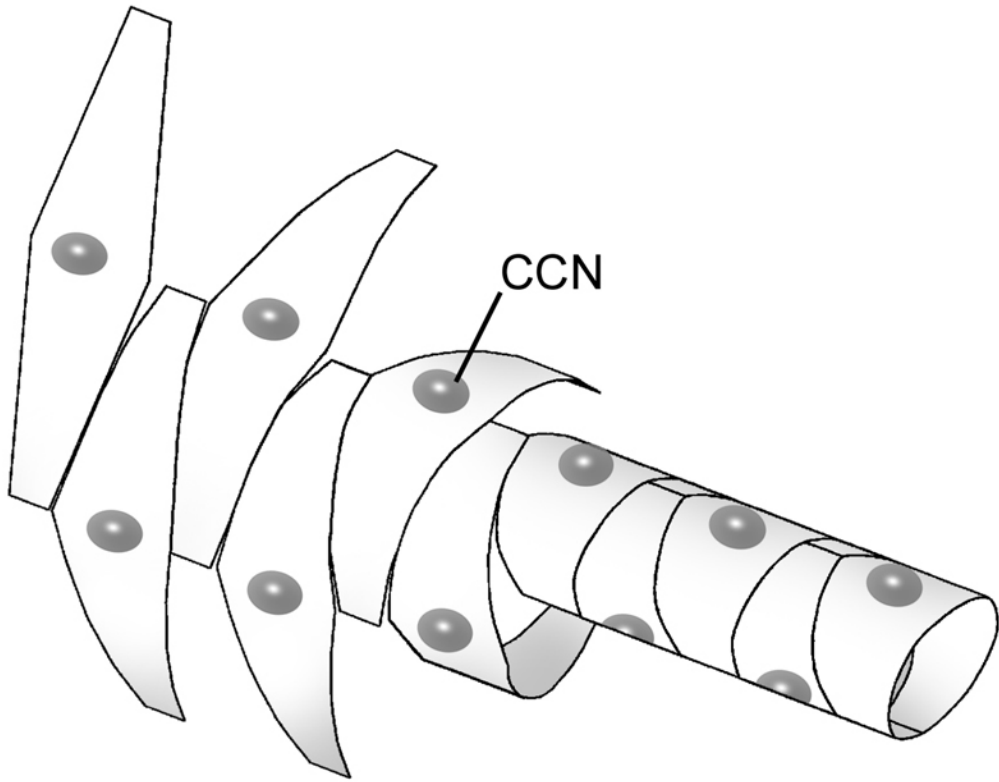


Fig. 4. Sahara & Kawamura  
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