1 Report

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3 Monoclonal antibodies that recognize symbiotic bacteria and hemocytes in the deep-sea
4 vesicomyid clam *Phreagena okutanii*

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Abstract

Vesicomyid clams, including the genus *Phreagena*, are dominant members of various deep-sea chemosynthesis-based animal communities. They harbor symbiotic sulfur-oxidizing bacteria in the epithelial cells of their gill tissue, and the bacteria are transmitted to the next generations via eggs. We created a monoclonal antibody (mAb) library against the gill of *P. okutanii* and observed mAb CokG-D1D3 to react to the symbiotic bacteria and three mAbs (CokG-Y1F7, CokG-J10D2, and CokG-J3G4) to bind to hemocytes of *P. okutanii*. The signals of mAb CokG-D1D3 were localized in the epithelial gill cells called bacteriocytes. We also observed a small number of clear signals of the antibody in the epithelial follicular cells of the ovary. The signals of mAb CokG-D1D3 almost exactly overlapped those of the anti-*E. coli* GroEL polyclonal antibody, while, in the gill, the signal areas of the latter seemed to be slightly wider than those of the former. Among the three mAbs against the hemocytes, mAbs CokG-Y1F7 and CokG-J10D2 reacted to a large fraction of the hemocyte populations, but mAb CokG-J3G4 reacted to a smaller fraction. mAb CokG-Y1F7 was observed to bind to the hemocytes distributed widely in the interstitial spaces of various tissues. These

43 monoclonal antibodies are expected to be useful for studying the interactions between

44 symbiotic bacteria and host cells and the distribution and functions of hemocytes in

45 deep-sea vesicomyid clams.

46

1. Introduction

Vesicomyid bivalves are one of the most abundant animals that inhabit deep-sea chemosynthesis-based ecosystems (Cavanaugh et al., 1981; Felbeck et al., 1981). They possess large gills with highly packed sulfur-oxidizing bacteria inside the epithelial cells; however, they have reduced gill ciliary groove, labial palps, and digestive tube (Boss and Turner, 1980; Cavanaugh, 1983: Fiala-Medioni and Metivier, 1986). The presence of many symbiotic bacteria in the bacteriocytes in the gill filament has been confirmed using *in situ* hybridization (Nakamura et al., 2013). The symbiotic bacteria are transmitted vertically to the offspring via eggs (Cary and Giovannoi, 1993; Ikuta et al., 2016). In the ovary, symbiotic bacterial cells have been observed in follicle cells and on egg cells by using transmission electronic microscopy and *in situ* hybridization (Ikuta et al., 2016). Genome analyses of the symbiotic bacteria in vesicomyid clams such as *Phreagena okutanii* Kojima and Ohta 1997 and *Calyptogena magnifica* Boss & Turner, 1980 have shown that the symbionts are composed of a single species: *Candidatus (Ca.)* Vesicomyosocius okutanii strain HA (Vok) and *Ca*. Ruthia magnifica

63 strain Cm (Rma) (Kuwahara et al., 2007; Newton et al., 2007). They have reduced
64 genomes and lack some genes that are essential for survival; however, they possess
65 genes for sulfur-oxidizing metabolism, carbon fixation, and synthesis of amino acids
66 and organic compounds (Kuwahara et al., 2007; Newton et al., 2007). The findings are
67 consistent with those of a previous stable isotope analysis that showed that the hosts
68 nutritionally depend on the organic compounds synthesized by the chemosynthetic
69 symbionts (Le Pennec et al., 1995). Thus, vesicomyid clams and their symbiotic
70 bacteria form an obligatory symbiotic system.
71 The clams are known to possess a unique oxygen transport system. Hemoglobin is
72 contained in the red hemocyte, which is thought to circulate in the body and transfer
73 oxygen to tissues (Suzuki et al., 2000). In general, the hemocytes function in
74 immunological defense against microbial invasion to maintain homeostasis (Bayne,
75 1983; Hine, 1999; Muroga and Takahashi, 2007; Takahashi and Muroga, 2008;
76 Donaghy et al., 2009, Tame et al., 2015). The symbiotic bacteria must escape from the
77 host defense system, including defense by the hemocytes. However, the mechanism
78 underlying the escape is still unclear.

To understand the molecular mechanisms underlying the maintenance of the symbiosis, tools for marking the protein components are necessary. We 0constructed a monoclonal antibody (mAb) library against the gill component of *P. okutanii* and reported an mAb that reacts against the mucus component secreted from epithelial cells in the gill (Nakamura et al., 2013: The mAb and hybridoma are available at the JAMSTEC Marine Biological Sample Database [http://www.godac.jamstec.go.jp/bio-sample/showDetail.do?menuId=6&sampleId=1090053148, IDs, 1090053149 and 1090053150; Accessed on 2017-11-04]). For studying the interaction between the symbiont and host, markings of the symbionts and hemocytes are mandatory. Here, we have reported four new mAbs: one mAb that reacts to the symbiotic bacteria and three mAbs that bind to the hemocytes.

2. Materials and Methods

An mAb library against the gills of *P. okutanii* was generated as described previously (Nakamura et al., 2013). Briefly, homogenized gills from *P. okutanii*

95 collected during the R/V Natsushima cruise (NT09-06 Leg1: April 24 to May 6, 2009)
96 by using the ROV *Hyper-Dolphin* (Dive#973) were used as antigens for inoculating
97 BALB/c mice. The mAb library was constructed using a conventional polyethylene
98 glycol method (Galfre and Milstein, 1981). Cloning of the hybridoma was performed
99 three times using the limited dilution method.
100 The binding activity of the mAbs in the supernatant of the hybridoma culture was
101 examined using indirect immunofluorescence microscopy with 4-μm-thin sections of
102 the gills or ovaries from adult clams or whole bodies of very small immature clams.
103 These clams were collected using the ROV *Hyper-Dolphin* during cruises of the R/V
104 Natsushima (NT09-06 Leg1, Dive#973: NT10-08, Dive#1125; NT11-09, Dive#1291
105 and 1293: NT13-07, Dive#1508 and 1511) and ROV *KAIKO* during the R/V Kairei
106 cruise (KR12-05, Dive#545) (Inoue 2010, 2011, Oguri 2012, Yoshida 2013). *P. okutanii*
107 was identified and distinguished from the sibling species *Phreagena soyoae* by partial
108 sequencing of the cytochrome c oxidase subunit 1 (*CO1*) gene (Harada et al., 2009).
109 The adult tissues and small clams were fixed with 4% paraformaldehyde in seawater for
110 18 h. The shells of immature clams were removed using decalcifying buffer (Wako Co.,

111 Tokyo), and three very small clams (length of the shell long axis, 16.0, 17.7, and 22.6
112 mm) were used to prepare the frozen sections. The adult tissues and the three small
113 clam bodies were frozen-embedded in optimal-cutting-temperature compound in liquid
114 nitrogen and sliced using a cryomicrotome (Microm HM550; Thermo Fisher Scientific
115 Inc., Tewksbury, MA). They reacted to the supernatant of the hybridoma culture at 4 °C
116 for 2 h. After washing with PBS (135 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and
117 1.8 mM KH_2PO_4, pH 8.0), they were incubated with Alexa Fluor 488-conjugated goat
118 anti-mouse IgG (Life Technologies Co. Ltd., Wyman, MA) diluted at 1:2000 at 4 °C for
119 2 h. Simultaneously, the sections reacted with anti-*E. coli* GroEL rabbit polyclonal
120 antibody (Sigma Aldrich Co. Ltd., St. Louis, MO) diluted at 1:1000, followed by
121 incubation with Alexa Fluor 647-conjugated anti-rabbit IgG antibodies (Life
122 Technologies Co. Ltd., Wyman, MA) diluted at 1:2000 at 4 °C for 2 h. The sections
123 were also stained with 6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-
124 Aldrich Co. Ltd., St. Louis, MO) at a concentration of 10 μg/ml at room temperature for
125 15 min. For morphological observation, 4-μm-thin sections were obtained from
126 paraformaldehyde-fixed and paraffin-embedded adult tissues or immature clam bodies.

Conventional hematoxylin and eosin (HE) staining was performed, and a microscope equipped with fluorescence optics (BZ-900; Keyence Co. Ltd., Osaka, Japan) was used for observation.

3. Results

3.1 mAb CokG-D1D3 reacted to the symbiotic bacteria in the gills and ovaries of *P. okutanii*

Newly established mAbs from the mAb library against *P. okutanii* gills are listed in Table 1. In a transverse section, the gill filament is composed of three parts: the inner zone containing many symbiont cells and two symbiont-lacking zones, the frontal zone and the junction area between the inner and the frontal zones (Ohishi et al., 2016; IZ, FZ, and J in Fig. 1a). Black particles indicating elemental sulfur were observed in the inner zone of a frozen section (Fig. 1b) (Ohishi et al., 2016). Strong signals for mAb CokG-D1D3 were detected in only the inner zone (Fig. 1c). No signal was detected in a negative control using PBS instead of primary antibodies. We concomitantly immunostained the

143 symbionts with the anti-*E. coli* GroEL antibody, which has been used to detect them (Fig.
144 1d and e; Nakamura et al., 2013; Ohishi et al., 2016). Signals of both the mAb and anti-
145 *E. coli* GroEL antibody were detected in almost the same area, indicating that this mAb
146 specifically reacts to the symbiont cells. However, their appearances were different. At
147 higher magnification, the mAb signals appeared as clear dots in the cytoplasm of the
148 epithelial cells, called bacteriocytes, of the inner zone (Fig. 2). However, the signals from
149 the anti-*E. coli* GroEL antibody were not clear but blurred (Fig. 2a, b, and d). In addition,
150 it is noteworthy that the signals of the anti-*E. coli* GroEL antibody were distributed a little
151 wider than those of the mAb: red color protruding slightly out of the area containing green
152 dot (white arrows in Fig. 2d).

153 Because the symbiotic bacteria are vertically transmitted via the female gonad, the
154 reactivity of mAb CokG-D1D3 to the ovary tissue was examined using frozen sections
155 from adult female clams. The follicle cells were observed as the epithelium lining the
156 ovarian cavity (yellow triangles in Fig. 3a), and various maturation stages of the oocytes
157 were in the cavities (white triangles in Fig. 3a). The dotted signals of the mAb were
158 clearly observed in the follicle cells, although the number of the dots in a cell (yellow

159 triangles in Fig. 3b and c) was much less than those in the gill (Fig. 2a and d). The
160 difference in signal appearances between the mAb and anti-*E. coli* GroEL antibody
161 detected in the gill was less conspicuous in the gonad than in the gill. We could detect no
162 signal from either mAb or anti-*E. coli* GroEL antibody in the oocytes (white triangles in
163 Fig. 3b–d). In some immature clams, we observed many ring-like or ellipsoidal structures
164 in the gonad tissue area at the base of the foot in the HE-stained paraffin-embedded whole
165 body (black triangles and a dotted ellipse in Fig. 4a), which seemed to be immature
166 follicle cells in the ovaries. The frozen sections from the three very small clam bodies
167 were also immunologically stained with mAb CokG-D1D3 or anti-*E. coli* GroEL
168 antibody. Both signals were observed in the ring-like or ellipsoidal shapes in the gonad
169 tissue area in the two immature clams (length of the shell long axis, 16.0 mm and 17.7
170 mm; white arrows and a dotted ellipse in Fig. 4b).
171
172 **3.2 mAbs CokG-Y1F7, CokG-J10D2, and CokG-J3G4 reacting to hemocyte-like**
173 **cells in *P. okutanii***
174 The signals of mAbs CokG-Y1F7, CokG-J10D2, and CokG-J3G4 reacted with

hemocyte-like cells inside the gill filament (Table 1). Since these cells were morphologically recognized as hemocytes, the mAbs were thought to react to the hemocytes. The mAbs CokG-Y1F7 and CokG-J10D2 were bound to a large proportion of hemocytes in the blood vein-like cavities (Fig. 5a and b), while mAb CokG-J3G4 reacted with a smaller fraction (Fig. 5c). Approximately 80% of the hemocytes in the images reacted positively with mAbs CokG-Y1F7 and CokG-J10D2 and 40% with mAb CokG-J3G4. The distribution of the signal-positive cells in the whole bodies of the immature clams was examined using mAb CokG-Y1F7 because its signal seemed to be the strongest. The signal-positive cells were found in the cavities of various tissues, such as the gill and mantle (Fig. 6); however, the signal was also detected on some parts of the mantle surface facing the shell (white arrow in Fig. 6). In the immature clams, the hemocyte-like cells were observed in a relatively large cavity at the base of the foot, which surrounds the intestine (Fig. 7a), although the cavity and hemocyte-like cells were not well-recognized in the adult clams (data not shown). The mAb CokG-Y1F7 bound to these cells which seemed to be free-moving cells, indicating that the cells in the cavity were hemocytes (Fig. 7b). Thus, mAb CokG-Y1F7 recognized the hemocytes in the

191 interstitial space of various tissues.

192

193 ## 4. Discussion

194 The dot-like signals of mAb CokG-D1D3 were abundantly found in the
195 bacteriocytes of the gill, while clear but smaller number of signals were detected in the
196 follicular cells of the ovary (Figs. 1–3). The dotted signals were found to be restricted to
197 the symbiosome of the bacteriocyte in the gill filament and follicle cell, where the signal
198 of the anti-GroEL antibody was also co-localized. Thus, the distribution and number of
199 signals by mAb CokG-D1D3 were consistent to those of the symbiotic bacteria. These
200 results indicate that the mAb binds to the symbiotic bacterium of *P. okutanii*, Vok,
201 although we failed to identify the target antigen by using western blot analysis. A recent
202 study has shown that approximately 400 cells of the symbiotic bacterium Vok were at the
203 vegetal pole of the eggs, but they were not found in the sections of other parts of the large
204 egg cells (Ikuta et al., 2016). This may explain why we could not find the signal for mAb
205 CokG-D1D3 in the sections of the oocytes. In the basic part of the foot of juvenile clams,
206 the symbiotic bacteria were located in circularly or elliptically arranged cells (Fig. 4).

207 Although further studies are required, this finding suggests that such small-sized clams
208 have ovarian follicle cells that already contain the symbionts. The metabolism of the
209 symbiont in the gill has been suggested to be different from that in the ovary (Ohishi et
210 al., 2016). This difference is understandable because sulfur oxidation metabolism is
211 important for maintaining the lives of both partners in the gill; however, its importance is
212 not clear in the follicle cells where the symbiont must be securely transmitted to the next
213 generation of the host. Ikuta et al. (2016) raised an important question: How are the
214 symbionts transferred from the outside surface of the egg to the inside of follicle cells,
215 oocytes, and gill epithelial cells during development. Although the target antigen is not
216 known, the symbiont-specific monoclonal antibody, mAb CokG-D1D3, could be useful
217 for tracing symbiotic bacteria during the development of the clam and/or regeneration
218 after the accidental or natural death of bacteriocytes. Studying the fate of symbiotic
219 bacteria during the life cycle of the host clam would be key in understanding how the
220 symbiosis is maintained.

221 The signals of mAb CokG-D1D3 and anti-*E. coli* GroEL antibody were almost co-
222 localized in the bacteriocytes in the gill epithelium and ovarian follicle cells (Figs. 2d and

3e). However, the signals of the latter formed blurred dots and seemed to expand slightly more than the former (Figs. 1 and 2). GroEL, a chaperon protein involved in protein folding, is known to be highly produced in the symbiotic bacterium *Buchnera aphidicola* (Ishikawa et al., 1984). GroEL in Vok is also highly expressed (Yoshida T, personal communication). The slightly expanded signal area detected by the anti-*E. coli* GroEL antibody might be due to the higher production of GroEL in Vok. Recently it has been shown that some intracellular pathogenic bacterial GroEL possesses multi-functions and is not always localized in the cytoplasm of the bacteria cells (Garduno et al., 2011). These questions need to be addressed in the future.

We observed that three mAbs (CokG-Y1F7, CokG-J10D2, and CokG-J3G4) reacted with the hemocytes. Two of them, CokG-Y1F7 and CokG-J10D2, were bound to a large fraction of the hemocytes, while the third one, CokG-J3G4, reacted to a smaller fraction. The results may indicate multiple populations in the hemocytes. Alternatively, the hemocytes may be at different developmental stages or immunological activation stages. The mAb CokG-Y1F7 bound to the hemocytes in the cavity at the base of the foot in an immature clam, where premature ovarian follicle cells containing symbiotic bacteria

239 seemed to be localized (Fig. 4). The mAb may be useful for detecting the distribution of
240 hemocytes in the open blood-vascular system at various developmental stages. Because
241 hemocytes of bivalves have been shown to play a crucial role in immunological defense
242 (Bayne, 1983; Hine, 1999; Muroga and Takahashi, 2007; Takahashi and Muroga, 2008;
243 Donaghy et al., 2009, Tame et al., 2015), it would be interesting to investigate whether
244 certain types of hemocytes are specifically localized in the symbiont-containing gill tissue
245 and how the symbiotic bacteria escape from the host defense system. The mAbs are
246 expected to be useful in addressing these immunological queries by recognizing the
247 symbiont cells and different types of hemocytes. It is important to establish additional
248 useful mAbs against various tissue cells or cell components of *P. okutanii* and characterize
249 them for further studies on symbiosis in deep-sea clams.
250

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258

Figure Legends

Fig. 1. Immunofluorescence micrographs of the monoclonal antibody (mAb) CokG-D1D3 binding to the gill filament of *Phreagena okutanii*. **a**, HE-stained paraffin-embedded transverse section of the gill. **b,** Bright-field micrographs of a frozen transverse section. **c and d,** Fluorescent images with mAb CokG-D1D3 (green) and anti-*E. coli* GroEL antibody (red). **e,** Triple merged fluorescent images of mAb CokG-D1D3 (green), anti-*E. coli* GroEL antibody (red), and DAPI (blue). Scale bars indicate 50 μm (**a**) and 20 μm (**b**–**d**). IZ, inner zone: FZ, frontal zone: J, junction area between IZ and FZ.

Fig. 2. Higher-magnification immunofluorescence micrograph of the monoclonal antibody (mAb) CokG-D1D3 binding to the gill filament of *Phreagena okutanii*. **a**–**c,** Fluorescent images with mAb CokG-D1D3 (**a**, green), anti-*E. coli* GroEL antibody (**b**, red), and DAPI (**c**, blue) of the same frozen transverse section of a gill filament. **d,** Triple merged fluorescent images of mAb CokG-D1D3 (green), anti-*E. coli* GroEL

275 antibody (red), and DAPI (blue). White arrows indicate the wider stained area with anti-

276 *E. coli* GroEL antibody than that with mAb CokG-D1D3. Scale bar, 40 μm.

277

278 Fig. 3. Immunofluorescence micrographs of the monoclonal antibody (mAb) CokG-

279 D1D3 bound to the ovary of *Phreagena okutanii*. **a**, HE-stained thin section of the

280 paraffin-embedded ovary. **b,** Bright-field micrographs of a frozen transverse section. **c**

281 and **d,** Fluorescent images with mAb CokG-D1D3 (green) and anti-*E. coli* GroEL

282 antibody (red). **e,** Triple merged fluorescent images of mAb CokG-D1D3 (green), anti-

283 *E. coli* GroEL antibody (red), and DAPI (blue). Scale bars indicate 100 μm (**a**) and 40

284 μm (**b–d**). Yellow triangles: follicular cells, white triangles: oocytes.

285

286 Fig. 4. Immunofluorescence micrographs of the monoclonal antibody (mAb) CokG-

287 D1D3 bound to an immature *Phreagena okutanii*. **a**, HE-stained cross-section of

288 paraffin-embedded whole immature clams. **b,** Triple merged fluorescent images of mAb

289 CokG-D1D3 (green), anti-*E. coli* GroEL antibody (red), and DAPI (blue) in a frozen

290 section of the whole immature clams. Scale bars indicate 500 μm (**a**) and 40 μm (**b**).

291 Black triangles and white triangles indicate immature ovary-like ellipsoidal structures. A
292 structure was surrounded by black or white dotted ellipses.
293
294 Fig. 5. Immunofluorescence micrographs of the monoclonal antibodies (mAbs) reacting
295 to hemocytes inside the gill filaments of *Phreagena okutanii*. **a,** Merged fluorescent
296 images of mAb CokG-Y1F7 (green) and DAPI (blue). **b,** Merged images of mAb
297 CokG-J10D2 (green), DAPI (blue), and bright-field micrographs. **c,** Merged images of
298 mAb CokG-J3G4 (green), DAPI (blue), and bright-field micrographs. Scale bars
299 indicate 40 μm (**a, b, and c**). Haze reduction function (Keyence fluorescence
300 microscope) was used for (**a**).
301
302 Fig. 6. Immunofluorescence micrographs of the monoclonal antibody (mAb) CokG-
303 Y1F7 in frozen whole sections from immature *Phreagena okutanii*. **a,** Bright-field
304 micrographs of the frozen cross-section. **b,** Fluorescent image of mAb CokG-Y1F7
305 (green). **c,** Merged fluorescent images of CokG-Y1F7 (green) and DAPI (blue) in the
306 mantle. White arrow indicates a part of the mantle binding to mAb CokG-Y1F7. Scale

307 bars indicate 50 μm (**a, b**) and 30 μm (**c**). Haze reduction function (Keyence

308 fluorescence microscope) was used for (**c**).

309

310 Fig. 7. Immunofluorescence micrographs of the monoclonal antibody (mAb) CokG-

311 Y1F7 bound to hemocyte-like cells in a cavity surrounding the intestine of immature

312 *Phreagena okutanii*. Paraffin-embedded section from an immature clam with a body

313 length of 20 mm was used. **a,** Bright-field micrographs of the section. **b,** Fluorescent

314 image of mAb CokG-Y1F7 (green). Scale bars indicate 30 μm.

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406 Table 1. List of the mAbs to *Phreagena okutanii*

407

mAb ID No.	Targets of mAb	JAMSTEC Database ID*	
		Antibody (supernatant)	Hybridoma cell
CokG-D1D3	Symbiotic bacteria	1090053151	1090053152
CokG-Y1F7	Hemocyte	1090053153	1090053154
CokG-J10D2	Hemocyte	1090053155	1090053156
CokG-J3G4	Hemocyte	1090053157	1090053158

408

409 *, mAb and hybridoma cell line are available. Please see Japan Agency for
410 Marine-Earth Science and Technology (JAMSTEC), Marine Biological Sample
411 Database
412 (http://www.godac.jamstec.go.jp/bio-sample/showDetail.do?menuId=6&sampleId=109
413 0053148, Accessed on 2017-11-04)

414

Fig. 1

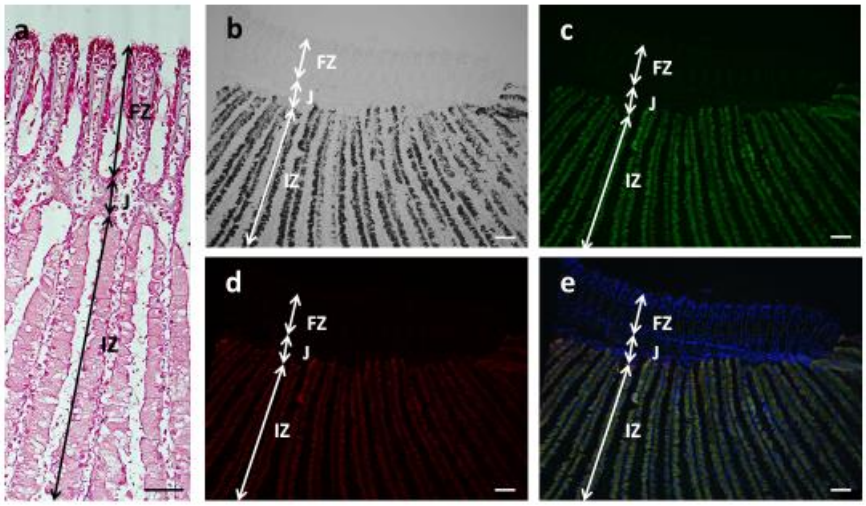

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Fig. 2

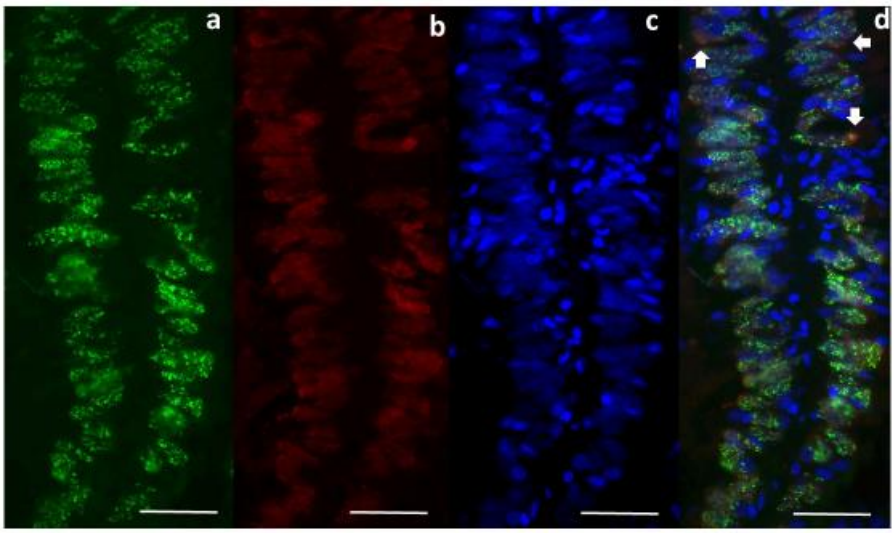

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Fig. 3

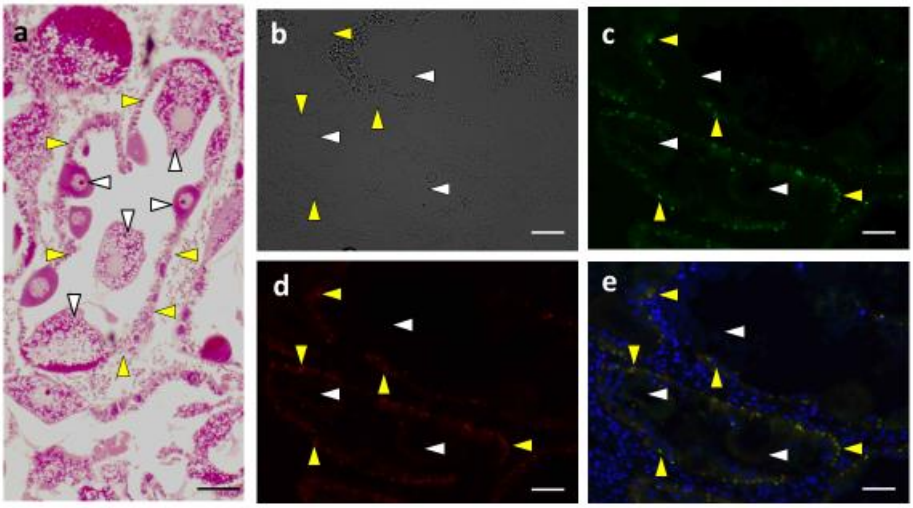

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Fig. 4

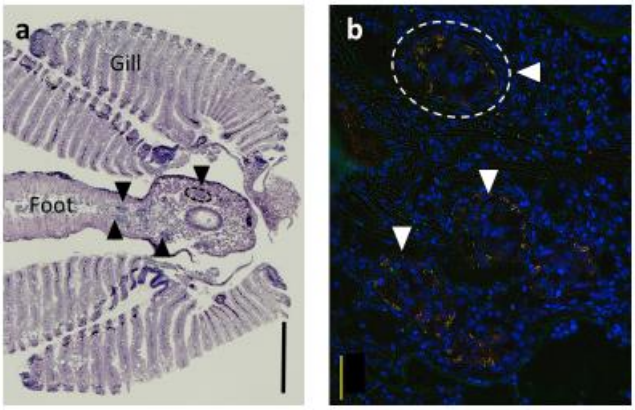

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Fig.5

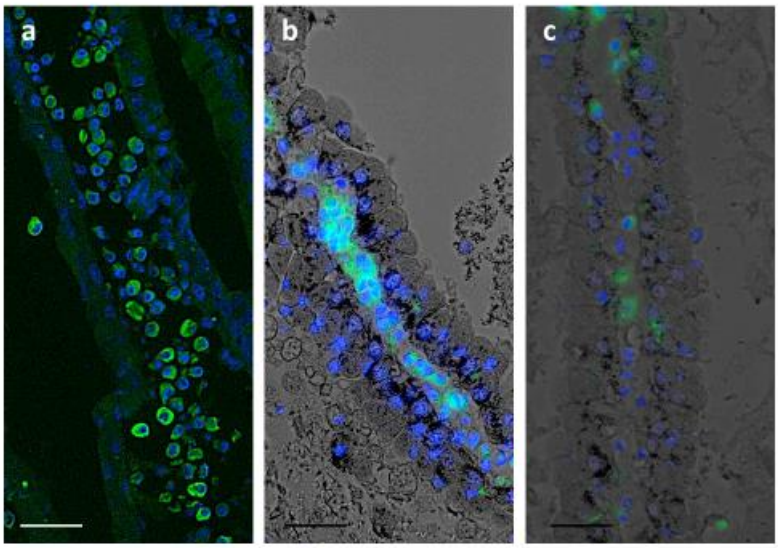

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Fig. 6

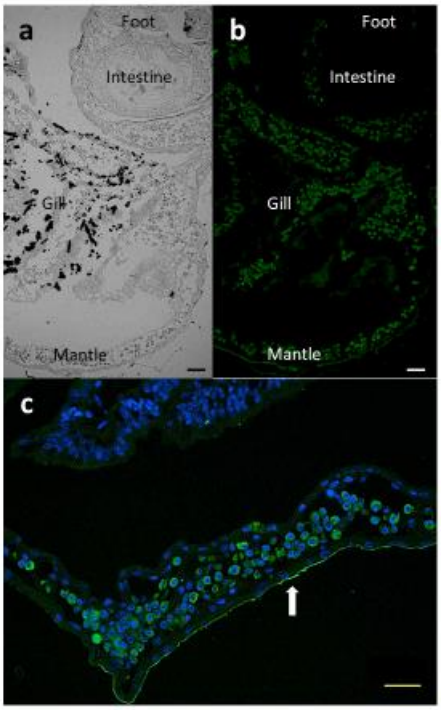

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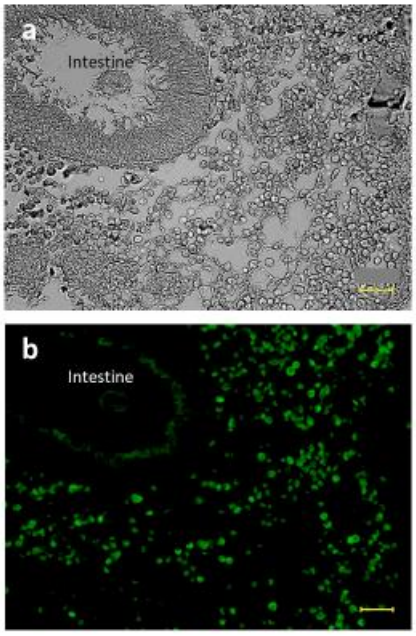
Fig.7



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