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Autolysis of *Chlorella variabilis* in starving *Paramecium bursaria* help the host cell survive against starvation stress

Running title: Trichocysts in paramecia with and without symbiotic algae

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Abstract

The endosymbiosis between *Paramecium bursaria* and *Chlorella* spp. is mutualistic. Symbiotic algae localize beneath the host *Paramecium* cell cortex compete for their attachment sites with preexisting organelle trichocysts. To examine the relationship between *P. bursaria* trichocysts and their symbiotic algae, algae-bearing or alga-free *P. bursaria* were starved for several days and the changes in the number of *Chlorella* sp. and presence or absence of trichocysts were evaluated. We conducted an indirect immunofluorescence microscopy with an anti-trichocyst monoclonal antibody against *P. bursaria* cells. Indirect

immunofluorescence microscopy demonstrated that under starvation and darkness conditions, the immunofluorescence of trichocysts in alga-free *P. bursaria* decreased much faster than that in the normal algae-bearing *P. bursaria*. In the latter case, our observations proposed the possibility that the nutrition obtained from symbiotic algal digestion may promote trichocysts synthesis. This algal digestion mechanism may permit host *P. bursaria* cells to survive for a longer time under starvation condition. To the best of our knowledge, this may be a new benefit that host *P. bursaria* gain from harboring symbiotic algae.

Keywords Algae · Ciliate · Endosymbiosis · Indirect immunofluorescence microscopy · Monoclonal antibody · Protist

Introduction

Numerous organisms are exposed to biotic and abiotic stresses including starvation, predation, and extremes in temperature, pH, or light, and ultraviolet irradiation. In nature, interactions between organisms and the environment create complex ecosystems. To endure environmental stress, certain organisms form symbiotic intracellular associations with algae [1-3]. Moreover, other organisms develop defense mechanism against predators [4]. Therefore, nutrition acquisition and defense systems are important survival factors in nature.

The endosymbiotic relationship between *Paramecium bursaria* and *Chlorella* spp. is mutualistic. Symbiotic algae excrete large amounts of maltose and supply it to the host cells [5-7]. In return, the algae are furnished with nitrogenous compounds and carbon dioxide by the host cells [8-11]. However, each symbiont can grow independently of the other. Alga-free *P. bursaria* can be reinfected with algal cells isolated from algae-bearing *P. bursaria* by engulfing the algal cells in digestive vacuoles (DVs) [12, 13]. The symbiotic associations between these eukaryotes are excellent models for eukaryotic cell evolution via secondary

endosymbiosis between protozoa (animal cells) and algae (plant cells). Recently, the genomic study of *P. bursaria* has progressed and provided us genetic basis for the establishment of endosymbiosis in this organism [14]. During reinfection, the algal cells that successfully reenter the endosymbiosis are individually enveloped in a symbiosome membrane (perialgal vacuole or PV membrane) derived from the host DV membrane [15, 16]. The algae translocate beneath the host cell cortex and anchor there at ~10-μm intervals [12]. The translocated algae initiate cell division and establish endosymbiosis [17, 18].

Thousands of trichocysts are embedded under the *Paramecium* cell cortex. In *P. caudatum* and *P. tetraurelia*, it is reported that their trichocysts act as defensive organelles against predators such as *Dileptus margaritifer* [4, 19], *Climacostomum virens* [20], *Echinospaerium nucleofilum* [21], and *Echinospaerium akamae* [21]. In *P. bursaria,* during the algal reinfection, the symbiotic algae appear to push the trichocysts aside to become fixed near the host cell cortex [22]. Thus, individual endosymbiotic algae may require the presence of trichocysts to be situated at their appropriate positions near the host cell cortex. To test this theory, trichocysts were removed by lysozyme exposure and the effect of this treatment on endosymbiotic algal localization was observed [22]. The algae successfully localized near the trichocyst-free host cell cortex. Moreover, the number of algae attached near the host cell cortex was greater in trichocyst-free than trichocyst-bearing cells when the paramecia were observed 3 h after mixing with algae [22]. Transmission electron microscopy (TEM) was used to detect acid phosphatase (AcPase) activity and showed that certain trichocysts near the host cell cortex were digested by host lysosomal fusion during algal reinfection. Therefore, symbiotic algae compete with preexisting trichocysts for attachment sites and can secure them beneath the host cell cortex [23]. Omura and Suzaki (2003) reported that the density of trichocysts in algae-bearing *P. bursaria* was lower than that in alga-free cells [24]. The same result was observed under indirect immunofluorescence microscopy in the presence of an

anti-trichocyst monoclonal antibody (mAb). Furthermore, we found that the trichocysts are not present in the area where the symbiotic alga localizes [23]. Gu et al. (2002) identified AcPase activity in the membranes of the trichocysts in starved algae-bearing *P*. *bursaria* [15]. Therefore, trichocysts may be decreased by culturing *P*. *bursaria* under starvation conditions. To examine the relationship between *P*. *bursaria* trichocysts and their symbiotic algae, algae-bearing or alga-free *P*. *bursaria* were starved for several days and the changes in the number of *Chlorella* sp. and presence or absence of trichocysts were evaluated. In this study, the trichocysts were visualized with a monoclonal antibody (mAb) against *Paramecium* trichocysts [23].

Materials and methods

Paramecium bursaria strains and cultures

Symbiotic *Chlorella* sp.-free (algae-removed) *P*. *bursaria* strain Yad1w was produced from *Chlorella* sp.-bearing *P*. *bursaria* strain Yad1g as described in a previous report [25]. The algae-bearing Yad1g1N strain was produced by infecting Yad1w with cloned symbiotic *Chlorella variabilis* strain 1N cells [23]. Both strains were cultured in red pea (*Pisum sativum*) extract culture medium [26] with modified Dryl's solution (MDS) [27] except that KH_2PO_4 substituted for $NaH_2PO_4 \cdot 2H_2O$. Then they were inoculated with a nonpathogenic strain of *Klebsiella pneumoniae* (strain 6081) 1 d before use [28]. Several hundred *Paramecium* cells were put into 2-mL culture medium aliquots in test tubes. Then 2-mL aliquots of fresh culture medium were added daily for 12 d. One day after final feeding, the cultures were in the early stationary phase. The cultivation of the algae-bearing *P*. *bursaria* strain and all experiments were performed at 25 ± 1°C under fluorescent lighting at 20–30 μmol photons m^{-2} s^{-1}. For the starvation experiment, *P*. *bursaria* were cultivated without feeding and lighting. Both *Paramecium* strains were provided by Yamaguchi University,

Japan, with support in part from the National Bio-Resource Project (NBRP) of the Japan Agency for Medical Research and Development (AMED) (http://nbrpcms.nig.ac.jp/paramecium/?lang=en).

Indirect immunofluorescence microscopy

Aliquots of the *Paramecium* cell cultures were air-dried on cover glasses (4.5 mm × 24 mm), fixed with 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 8.1 mM $NaHPO_4 \cdot 12H_2O$, and 1.47 mM KH_2PO_4; pH 7.2) for 10 min at 4 °C, washed with PBST (PBS containing 0.05% (w/v) Tween 20) and PBS for 10 min at 4 °C. The cells were incubated with mAb against *Paramecium* trichocysts [23] overnight at 4 °C then washed twice with PBS. The cells were incubated with Alexa Fluor 488 (AF488) goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) diluted 1,000-fold with PBS for 2 h at 25 ± 1 °C, washed twice with PBS, and observed under differential-interference contrast (DIC) and fluorescence microscopes (BX51; Olympus Corp., Tokyo, Japan) equipped with Olympus fluorescence mirror units U-FBNA (excitation 470 to 495 nm, emission 510 to 550 nm) for AF 488 and U-FGW (excitation 530 to 550 nm, emission 575 nm) for algal autofluorescence. Cell images were digitally captured with an Olympus DP73 camera system (Olympus Corp., Tokyo, Japan) and were analyzed with the Olympus CellSens Dimension software (Olympus) and with the Image J (NIH). Mean number of green algae per fixed host cell was counted under the DIC microscope using previously described method [18]. Professor Masahiro Fujishima (Yamaguchi University, Japan) gave us the monoclonal antibody against trichocysts.

Trichocyst discharge induced by saturated picric acid treatment

Starved *P*. *bursaria* cells were harvested by hand-operated centrifugation and suspended in 500 μL MDS. Then 500 μL saturated picric acid was mixed with the cell suspension to discharge trichocysts. The treated samples were observed under a DIC microscope.

Results and Discussion

Trichocysts of alga-free and algae-bearing *P*. *bursaria*

Figure 1 shows portions of the cell membranes of alga-free (a) and algae-bearing (b) *P*. *bursaria*. Numerous trichocysts (spindle-shaped) are observed in the cell membrane. The trichocysts of algae-bearing *P*. *bursaria* were pushed aside by green symbiotic algae enclosed in the PV membrane as reported by Kodama and Fujishima [22] (b, arrowheads). The PV membranes may be derived from DV membranes but neither the latter nor the crystals are localized beneath the cell cortex (a).

Indirect immunofluorescence microscopy of starved alga-free and algae-bearing *P*. *bursaria* using anti-trichocyst mAb

Alga-free paramecia are weakened by starvation resulting from the loss of their symbionts. Only a few alga-free cells survived for 20 d under starvation conditions (data not shown). In the present study, alga-free and algae-bearing *P*. *bursaria* were starved for 20 d and the changes in the immunofluorescence of trichocysts was observed using mAb. Trichocysts are colorless and must be observed under immunofluorescence microscopy.

Figure 2 shows alga-free *P*. *bursaria* cultured without feeding for 0 d (before starvation), 7 d, 10 d, and 20 d. The cells were then labeled with mAb. Before starvation (Fig. 2a), immunofluorescence appeared throughout the entire cell (Fig. 2b). Thus, numerous trichocysts were embedded in the cell cortex. After 7 d, the cells slightly shrank (Fig. 2c) and

an immunofluorescence-free gap began to appear (Fig. 2d). After 10 d, the cell size had reduced even further (Fig. 2e). Immunofluorescence showed that there were also comparatively fewer trichocysts (Fig. 2f). After 20 d, the cell was very small (Fig. 2g) and there was scant immunofluorescence (Fig. 2h). Therefore, starvation decreased the immunofluorescence of trichocysts in alga-free *P. bursaria*.

Figure 3 shows algae-bearing *P. bursaria* cultured without feeding for 0 d (before starvation), 7 d, 10 d, and 20 d. The cells were then labeled with mAb. Before starvation (Fig. 3a), there were hundreds of symbiotic algae throughout the cells (Fig. 3a, c). Immunofluorescence revealed no trichocysts in the cytopharynx or the areas where the symbiotic algae were localized (Fig. 3b). After 7 d, the number of symbiotic algae at the anterior cortex had slightly decreased due to algal digestion (Fig. 3d, f) likely resulting from starvation. Immunofluorescence was markedly increased (Fig. 3e). After 10 d, the number and cell diameters of the symbiotic algae had decreased considerably (Fig. 3g, i). The algal pigmentation became pale (Fig. 3g) and the autofluorescence weakened (Fig. 3i). On the other hand, immunofluorescence of trichocysts had increased (Fig. 3h). After 20 d, the symbiotic algal density had decreased even further (Fig. 3j, l). Immunofluorescence was observed in the areas free of symbiotic algae but was weaker than it was at 10 d cultivation (Fig. 3k). Therefore, the immunofluorescence of trichocysts increased with decreasing numbers of symbiotic algae. When the intracellular algae were almost entirely digested by starvation, the immunofluorescence of trichocysts also began to rise.

We demonstrated that the immunofluorescence of trichocysts had declined in starved, alga-free *P. bursaria* (Fig. 2). Thus, the trichocysts of *P. bursaria* are digested under these conditions and their relative abundance is influenced by the nutritional status of the host. In contrast, in the algae-bearing *P. bursaria*, the intracellular symbiotic algae were digested and the immunofluorescence of trichocysts increased even in the starvation (Fig. 3). Trichocysts

cannot adhere to sites beneath the host cell cortex where the symbiotic algae have localized. However, our results suggest that algal digestion by starved *P. bursaria* may introduce gaps at the algal attachment points on the host cell cortex. Moreover, *P. bursaria* may be able to synthesis trichocysts by using the nutrients derived from the digestion of the symbiotic algae. After almost all of the symbiotic algae are digested (Fig. 3j, l), the trichocysts themselves appear to undergo digestion (Fig. 3k).

Figure 4 shows the quantitative data from Figs. 2 and 3. Figure 4a shows statistical analyses on mean No. of *Paramecium* cell size (i.e. area of the fixed cell) of alga-free and algae-bearing *P. bursaria* cells cultured without feeding for 0 d (before starvation), 7 d, 10 d, and 20 d. With (black bar graphs) or without (white bar graphs) symbiotic algae, cell size of *P. bursaria* decreased according to the starvation days. After 20 d of starvation, the cell size of algae-bearing *P. bursaria* was significantly larger than that of alga-free cells. Cell size of algae-bearing cells shows that symbiotic algae help the host cell survive against starvation stress. Several metabolic interactions between the two species have been hypothesized as shown in previous literatures [5, 7-11], symbiotic *Chlorella* spps. seems to offer a distinct advantage to the host species, *P. bursaria*. Figure 4b shows the immunofluorescence intensity of trichocysts of alga-free and algae-bearing *P. bursaria* cells cultured without feeding for 0 d (before starvation), 7 d, 10 d, and 20 d. In alga-free *P. bursaria* cells (white bar graphs), immunofluorescence intensity decreased according to the starvation days. On the other hand, in alga-bearing *P. bursaria* cells (black bar graphs), in contrast to the decrease in the number of symbiotic algae, the immunofluorescence intensity became stronger until 10 d after the starvation. The immunofluorescence intensity weakened again at 20 d after the starvation. Figure 4c shows mean number of green algae per algae-bearing *P. bursaria* cell cultured without feeding for 0 d (before starvation), 7 d, 10 d, and 20 d. Before the starvation, about 600 of symbiotic algae were observed throughout the cells. The mean number of green algae

per host cell gradually decreased and became 288.8 and 177.8 at 7 d and 10 d after the starvation, respectively. At 20 d after the starvation, the mean number of green algae per host cell had become 61.4.

The following can be said from the above observation results. The symbiotic algae of *P*. *bursaria* tend to remain in the cell under constant light conditions. Under constant dark and starvation conditions, though, the host digests its algal symbionts and may use them as a nutrient source to synthesize trichocysts for protection against predators. To support this hypothesis, we have to provide more experimental evidence in the further study.

Trichocyst discharge in starved *P. bursaria* in response to saturated picric acid treatment

Saturated picric acid is a potent inducer of trichocyst discharge. Before and 10 d after starvation of alga-free and algae-bearing *P*. *bursaria*, trichocysts were discharged by picric acid treatment. Before starvation, trichocysts were discharged from whole alga-free cells (Fig. 5a). After 10 d, however, very few discharged trichocysts were observed (Fig. 5b). There were fewer trichocysts in algae-bearing than alga-free *P*. *bursaria* before starvation (Fig. 5c). After 10 d, the number of trichocysts had increased and they were discharged from the whole cells (Fig. 5d). These findings corroborate those obtained by indirect immunofluorescence microscopy as shown in Figs. 2 and 3. Therefore, it can be said that the intensity of immunofluorescence generally reflects the number of *Paramecium* cortical trichocysts.

It is clear that symbiotic algae of *P*. *bursaria* causes a decrease of host trichocysts in normal conditions from several results [23, 24]. Consequently, this may cause a reduction of effectiveness in predators' escaping. Berger [29] has proposed that the symbiotic algae of *P*. *bursaria* discourage *Didinium nasutum* predation by releasing repellent metabolites. Before that, Pollack [30] had reported that *D*. *nasutum* appears to prey wild-type (i.e., trichocysts

bearing) cells as easily as cells of trichocyst-defective mutants in *P. tetraurelia*. This means that *D. nasutum* may have effectively overcome the defense mechanism of *P. tetraurelia* [4]. Same results have been obtained in the predation experiments of alga-free and algae-bearing *P. bursaria* by *Didinium* sp. (Miyazaki and Kodama, unpubl. data). As model organisms, laboratory microcosm experiments using protists have been conducted traditionally and that are widely used to investigate general concepts in population biology, community ecology and evolutionary biology [31]. These observations made within the scope of laboratory microcosm experiments may be considered as being made in an artificial system. Additional research will need to be done to identify the benefits and disadvantages for *P. bursaria* to harbor symbiotic *Chlorella* spp. in the real ecosystem.

Conclusion

The present study confirmed that the unicellular green algal symbionts forming mutualistic associations with paramecia not only provide the host with photosynthate (carbon skeletons and metabolic energy) but also confer upon it tolerance to the starvation stress. When prey are deficient, paramecia will digest their algal symbionts and/or their endogenous trichocysts. It was suggested that algal autolysis in starving paramecia furnishes the energy and biomass the host needs for *de novo* trichocysts biosynthesis. This may be a new benefit that *P. bursaria* gain from harboring symbiotic algae.

Acknowledgements

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

Yuuki Kodama conceived and designed the experiments. Yuuki Kodama and Shoya Miyazaki performed the experiments. Yuuki Kodama wrote the manuscript.

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Figure legends

Fig. 1 Enlarged DIC images of the cell cortices of alga-free (**a**) and algae-bearing (**b**) *P. bursaria*. The symbiotic algae pushed the host trichocysts aside and became fixed beneath the

host cell cortex (**b**, *arrowhead*). D in **a** means digestive vacuole (DV) and C in **a** means crystals. Neither DV membranes nor crystals localized between the host trichocysts.

Fig. 2 Light microscopy of alga-free Yad1w cells cultivated under starvation conditions at 25 ± 1 °C. **a**, **c**, **e**, and **g**: DIC images. **b**, **d**, **f**, and **h**: immunofluorescence images. **a** and **b**: pre-culture. Immunofluorescence is observed in the whole cell (**b**). **c** and **d**: 7 d after cultivation. Immunofluorescence is reduced compared to pre-culture (**d**). **e** and **f**: 10 d after cultivation. *Paramecium* shrank as a result of starvation (**e**). Immunofluorescence has diminished further still (**f**). **g** and **h**: 20 d after cultivation. Immunofluorescence is very scant (**h**). Ma, macronucleus; Cy, cytopharynx. More than 100 cells were observed at each time point.

Fig. 3 Light microscopy of algae-bearing Yad1g1N cells cultivated under starvation conditions at 25 ± 1°C. **a**, **d**, **g**, and **j**: DIC images. **b**, **e**, **h**, and **k**: immunofluorescence images. **c**, **f**, **i**, and **l**: chlorophyll autofluorescence. **a**–**c**: pre-culture. Numerous symbiotic algae are visible (**a** and **c**). Immunofluorescence appears around symbiotic algae (**b**). **d**–**f**: 7 d after cultivation. Numerous symbiotic algae remain (**d** and **f**). Immunofluorescence is visible around the symbiotic alga and the fluorescence is stronger than it was at pre-culture (**e**). **g**–**i**: 10 d after cultivation. The number of symbiotic algae is dramatically reduced because the host was starved and digested the algae (**g** and **i**). On the other hand, the immunofluorescence of trichocysts were observed in the most of the cell cortex and trichocyst-free areas has decreased (**h**). **j**–**l**: 20 d after cultivation. Most of the algal cells have disappeared from the host cytoplasm (**j**). Digested algal chlorophyll autofluorescence persisted (**l**). Immunofluorescence is weaker than it was at 10 d (**k**). Ma, macronucleus; Cy, cytopharynx. More than 100 cells were observed at each time point.

Fig. 4 The quantitative data from Fig. 2 and 3. (**a**) shows statistical analyses on *Paramecium* cell size of alga-free (white bar graphs) and algae-bearing (black bar graphs) *P*. *bursaria* cells cultured without feeding for 0 d (before starvation), 7 d, 10 d, and 20 d. In both alga-free and algae-bearing *P*. *bursaria* cells, the cell size decreased according to the starvation days. (**b**) shows the immunofluorescence intensity of trichocysts of alga-free and algae-bearing *P*. *bursaria* cells cultured without feeding for 0 d (before starvation), 7 d, 10 d, and 20 d. In alga-free *P*. *bursaria* cells (white bar graphs), immunofluorescence intensity decreased according to the starvation days. In algae-bearing *P*. *bursaria* cells (black bar graphs), weak immunofluorescence intensity before starvation became stronger by the symbiotic algal digestion and then weakened again by the algal disappearance as detailed in the text. (**c**) shows mean number of green algae per algae-bearing *P*. *bursaria* cell cultured without feeding for 0 d (before starvation), 7 d, 10 d, and 20 d. Note that the number of symbiotic algae was decreased as cultivation days elapsed. In the all graphs, 5–8 *Paramecium* cells were observed at each day. Error bars show standard deviation (SD). Asterisks indicate significant differences (Two-sided Fisher's Exact Test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Fig. 5 Photomicrographs of alga-free (**a** and **b**) and algae-bearing (**c** and **d**) *P*. *bursaria* before (**a** and **c**) and 10 d after (**b** and **d**) cultivation under starvation conditions. In alga-free cells, trichocysts were discharged by saturated picric acid treatment (**a**). After cultivation, no trichocysts were observed (**b**). In algae-bearing cells, the number of trichocysts increased during cultivation under starvation conditions (**c** and **d**). Ma, macronucleus.

Figure 1

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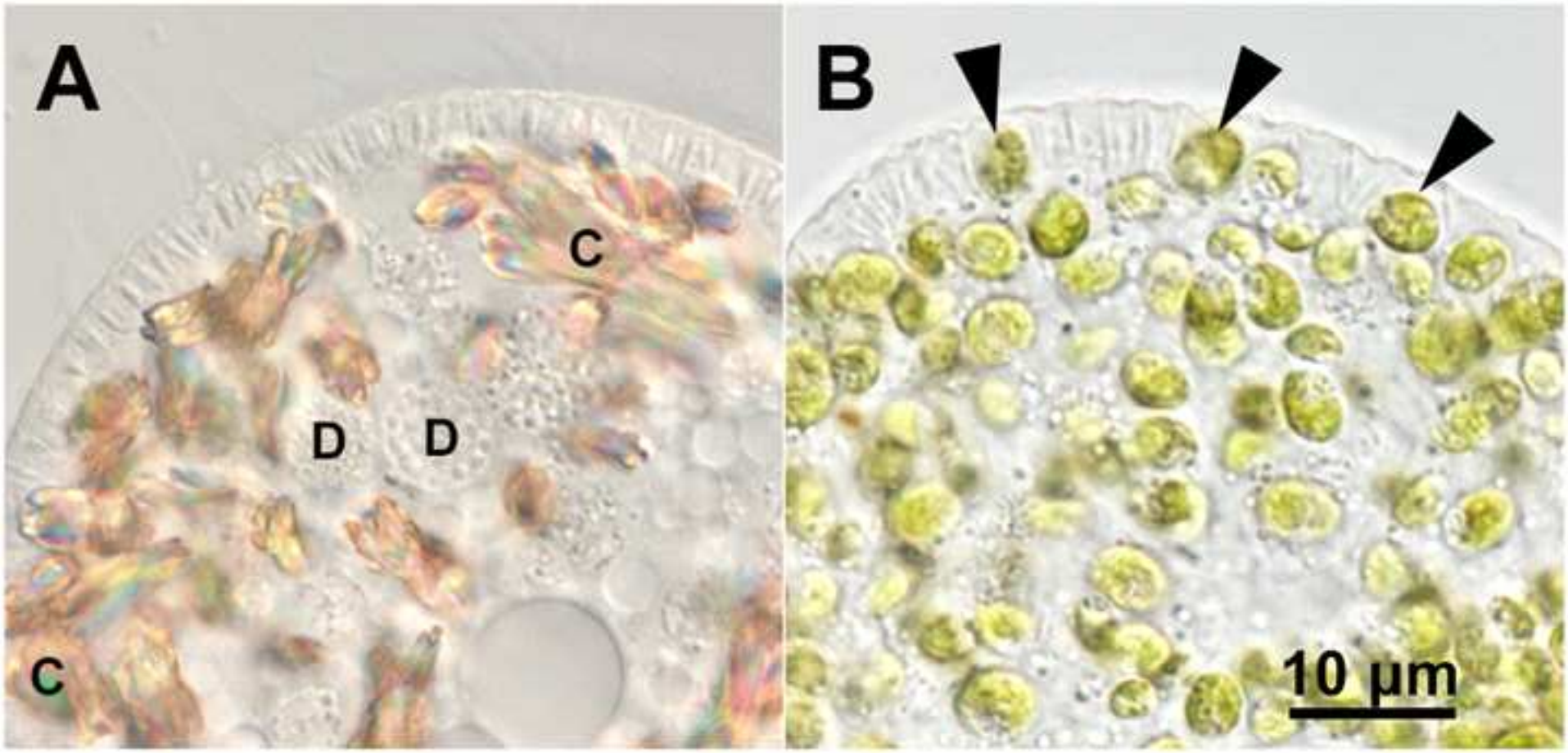

Figure 2

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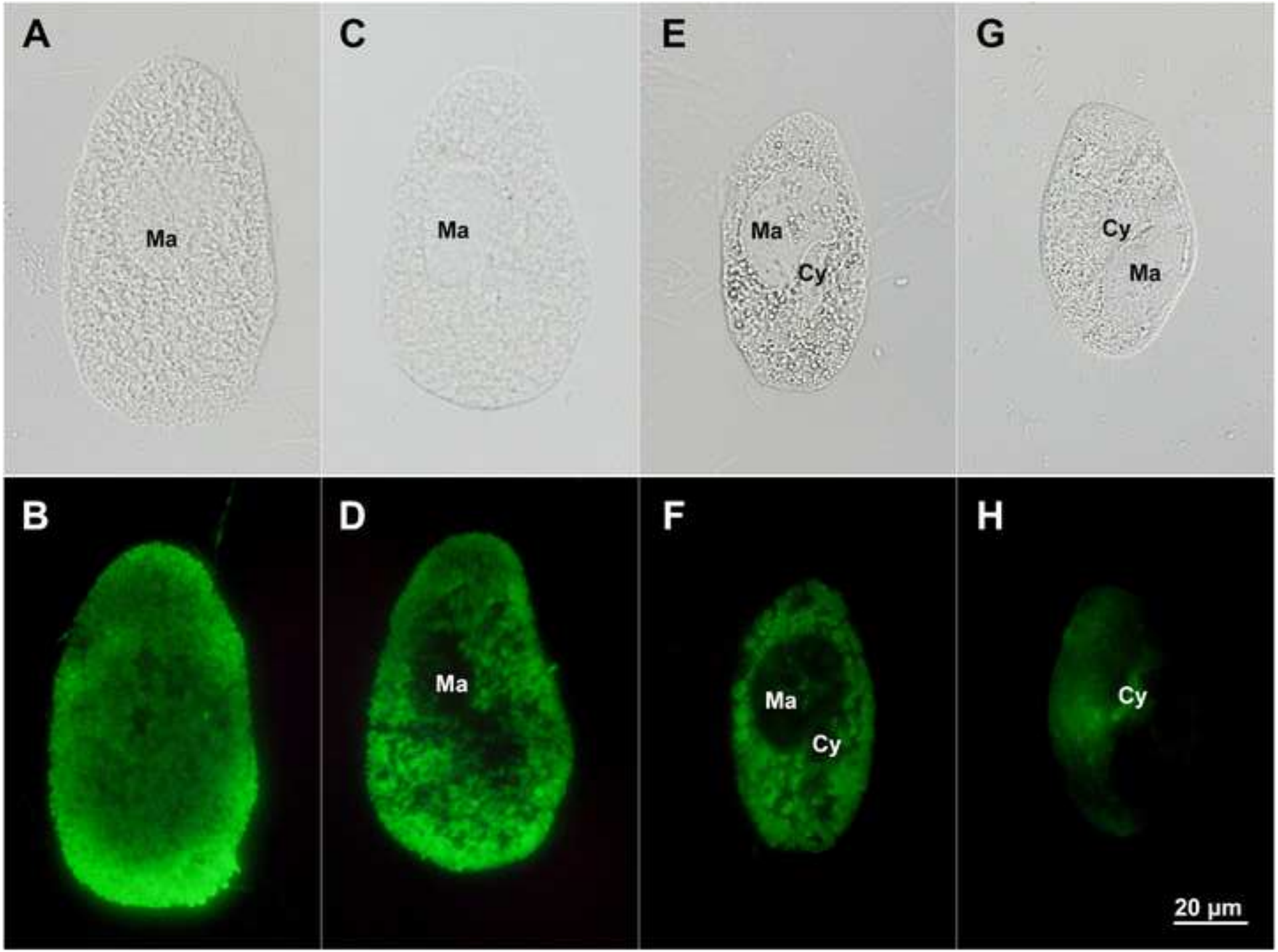
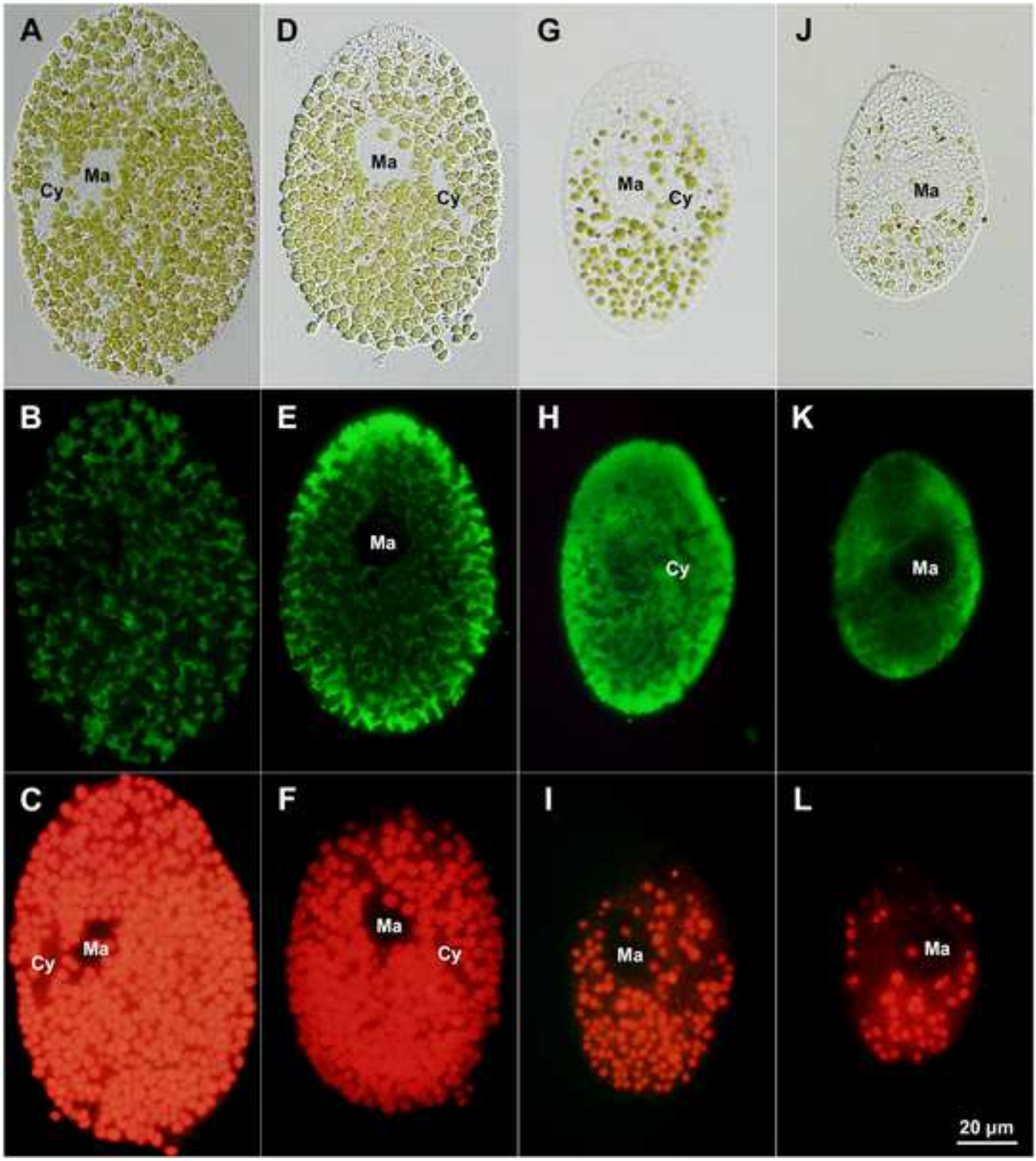


Figure 3

Click here to access/download;Figure;Fig3.tif



evicted new Figure 4

Click here to access/download;Figure;Fig4.tif

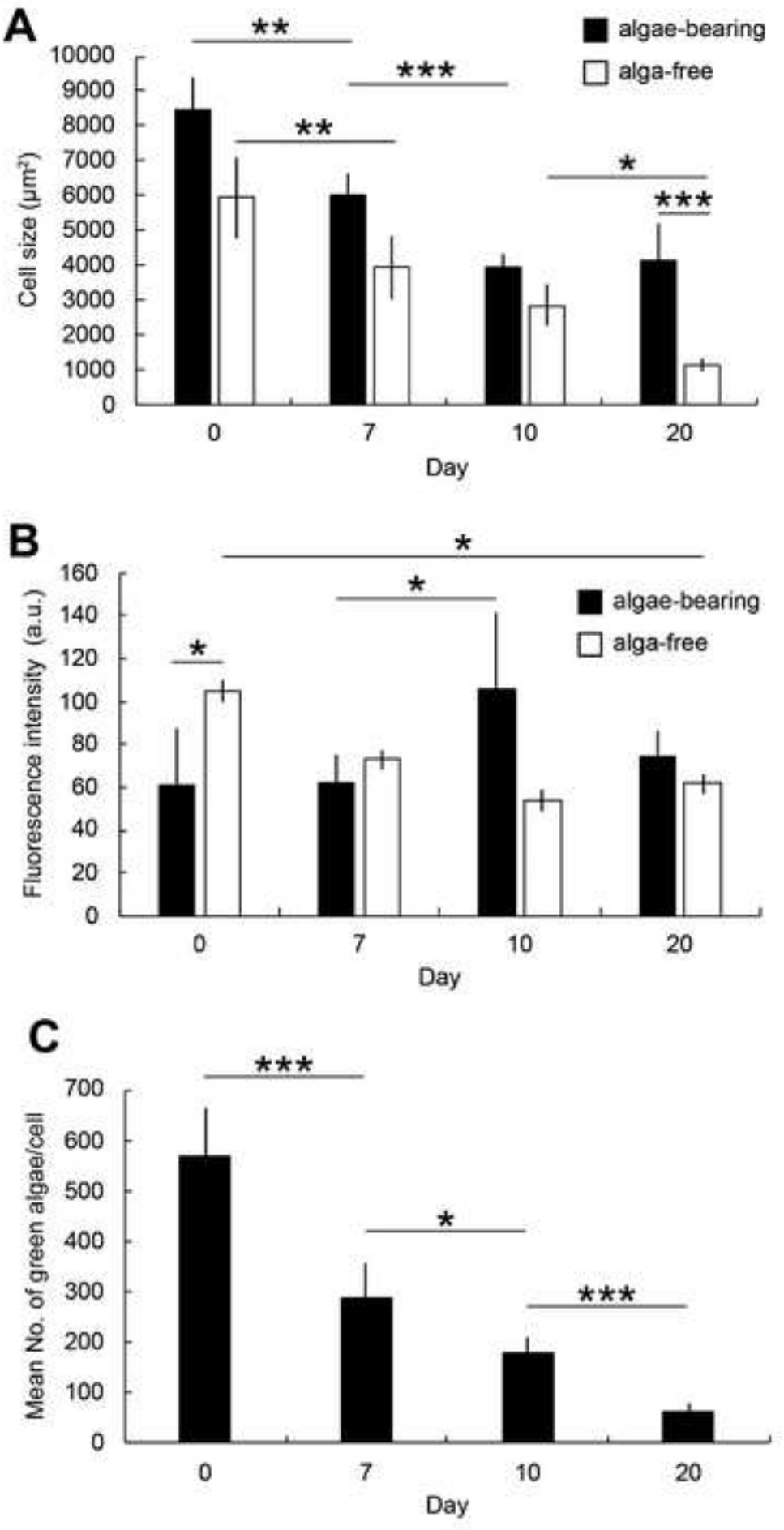


Figure 5

Click here to access/download;Figure;Fig. 5.tif