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Tunic Cell Morphology and Classification in Botryllid Ascidians[1]

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ABSTRACT—The morphology of tunic cells was investigated in ten botryllid ascidians, six *Botryllus* and four *Botrylloides* species, by means of light and electron microscopy. Three types of tunic cells were described, i.e. amoeboid, vacuo-granular and large granule tunic cells. Amoeboid tunic cells were irregularly shaped, and had many pseudopodia. They were found in the all species studied here. Vacuo-granular tunic cell had many vacuoles which contained round granules, and was found in four *Botryllus* and four *Botrylloides* species. The diameters of the granules varied amond species (ca. 1.4–0.8 μm). Large granule tunic cells were peculiar to *Botryllus scalaris*, and each of them contained a single large granule with lamellate substructure. Some tunic cells showed intermediate characteristics of amoeboid and vacuo-granular tunic cells. It seems that amoeboid tunic cells probably differentiate into vacuo-granular tunic cells in the tunic. There are thread-like bacteria in the tunic, and they often dangle around vacuo-granular tunic cells. There may be some interactions between tunic cells and the bacteria.

INTRODUCTION

The tunic is the outer covering of an ascidian, and is made of gelatinous, leathery or cartilaginous matrix, which contains cellulose-like fibers [1, 2]. The tunic has many functions, such as a protector for the soft body, and an adhesive to the substratum, a barrier against infection and, in some species, a site of self or non-self recognition in colony specificity. In the matrix of the tunic, free cells are found dispersively, and they are called tunic cells. These cells are thought to be concerned with some of the functions of the tunic.

Several species of the family Botryllidae (botryllid ascidians) have been used for the study on colony specificity, an occurrence of allo-recognition, which is manifested by the fusibility between contacting colonies [reviewed in 3]. In some of them allo-recognition seems to take place in the tunic, and tunic cells should have important roles for the recognition and following reactions. Moreover, our recent studies have suggested that only the tunic cells may be responsible for the early allo-recognition reaction in *Botrylloides violaceus* [4] and *Botrylloides fuscus* [Hirose *et al.*, in preparation].

Accordingly, the investigations of morphology and classification of tunic cells are necessary as a fundamental research in order to clarify the cellular reactions in colony specificity. However, in botryllid ascidians, *Botryllus schlosseri* is the only species which has been used for the study on tunic cells [5, 6]. The purpose of the present study is to examine the morphology of tunic cells in ten botryllid ascidians and to make a comprehensive classification of the tunic cells in botryllid ascidians.

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MATERIALS AND METHODS

Animals

We used four *Botryllus* (*Botryllus primigenus, B. scalaris, B. sexiens* and *B. schlosseri*), four *Botrylloides* (*Botrylloides fuscus, B. lentus, B. simodensis* and *B. violaceus*) and two undescribed

Botryllus which are tentatively called Pinkish *Botryllus* and Sikine *Botryllus* in this report. Sikine *Botryllus* was collected in the Sikine Island, one of Izu Islands (Tokyo Prefecture, Japan), and the other nine species were collected in Shimoda (Shizuoka Prefecture, Japan). The collected colonies were attached on slide glasses and reared in a culture box immersed in the Nabeta Bay in Shimoda. Colonial edges growing well were used for the specimens of the tunic. They were cut off from the colonies with a razor blade and were immediately fixed. As for *B. schlosseri,* colonies were also collected in Monterey and Woods Hole (U.S.A), the colonies from these two sites show different rejection reaction in colony specificity [7]. The collected colonies were immediately fixed and used only for light microscopical observation.

Light microscopy

The specimens collected in Shimoda were fixed in 2.5% glutaraldehyde-0.45 M sucrose-0.1 M cacodylate (pH 7.4). The specimens collected in Monterey and Woods Hole were fixed with 10% formalin in seawater. The fixed specimens were then dehydrated through a butanol series and embedded in paraffin. They were sectioned at 5 μm and stained with Congo red, Delafield's hematoxylin and eosin-orange G.

In *B. simodensis,* the motility of tunic cells was recorded by time lapse video cassette recorder AG-6010 (National, Japan). A part of the colonial edges was cut off with a razor blade, mounted with seawater, and observed by a light microscope equipped with a video camera WV-1800 (National, Japan). The recording speed was about one sixtieth of the actual speed.

The autonomous fluorescence of ascidian blood cells has been reported by Michibata *et al.* [8, 9] in solitary species, *Ascidia ahodori* and *A. sydneiensis samea.* In this study, we observed the living specimens of *B. simodensis* and *B. fuscus,* using a fluorescent microscope (Nikon FL).

Scanning electron microscopy (SEM)

In some of the species, the cut surface of the colonial margin was studied by the following procedure that was essentially the same as that reported by Armstrong [10]. The specimens embedded in paraffin were cut into pieces with a razor blade or a microtome blade. The paraffin was removed from the specimens in xylene (1 hr, 3 times), and then the specimens were cleared with absolute ethanol. After drying at critical point, the specimens were sputter-coated with gold palladium, and examined in Hitachi S-570 scanning electron microscope at 20 kV.

Transmission electron microscopy (TEM)

The specimens were prefixed in 2.5% glutaraldehyde-0.45 M sucrose-0.1 M cacodylate (pH 7.4), or 2.5% glutaraldehyde-2% NaCl-0.1 M Millonig's phosphate buffer (pH 7.4) for 2–3 hours. They were rinsed, and postfixed for 1.5 hours in the same buffer containing 1% osmium tetroxide. After dehydration through an ethanol series, the specimens were cleared with n-butyl glycidyl ether, and embedded in low viscosity epoxy resins [11]. Silver-gold sections were doubly stained with uranyl acetate and lead citrate; they were examined in Hitachi HS-9 transmission electron microscope at 75 kV.

RESULTS

Cell type, distribution and motility

Tunic cells are distinguished into three types; amoeboid, vacuo-granular and large granule tunic cells (Fig. 1). The amoeboid tunic cells are present in the all species studied here, and distribute throughout the tunic. Vacuo-granular tunic cells are present in all species, except for *B. primigenus* and *B. scalaris.* They are especially abundant near the cuticle covering the tunic matrix. Large granule tunic cells are found only in *B. scalaris,* and they are much smaller in number than amoeboid tunic cells. In *B. schlosseri,* there are not obvious differences of the tunic cell types and their morphology among the specimens collected in Shimoda, Monterey and Woods Hole.

The motility of both amoeboid and vacuo-granular tunic cells is recorded by a time lapse video cassette recorder in *B. simodensis.* They move in the tunic matrix at roughly about 10 μm/min (amoeboid tunic cell) and 2–3 μm/min (vacuo-granular tunic cell).

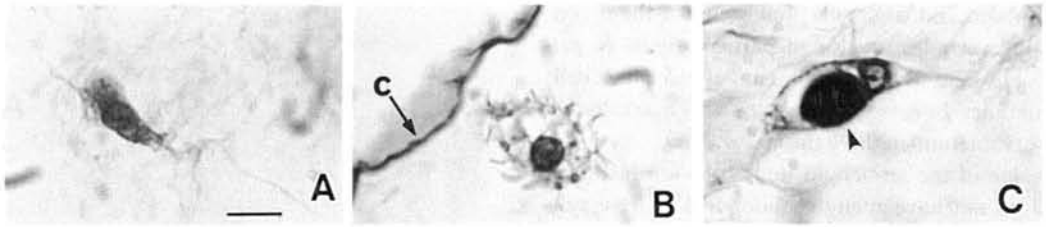
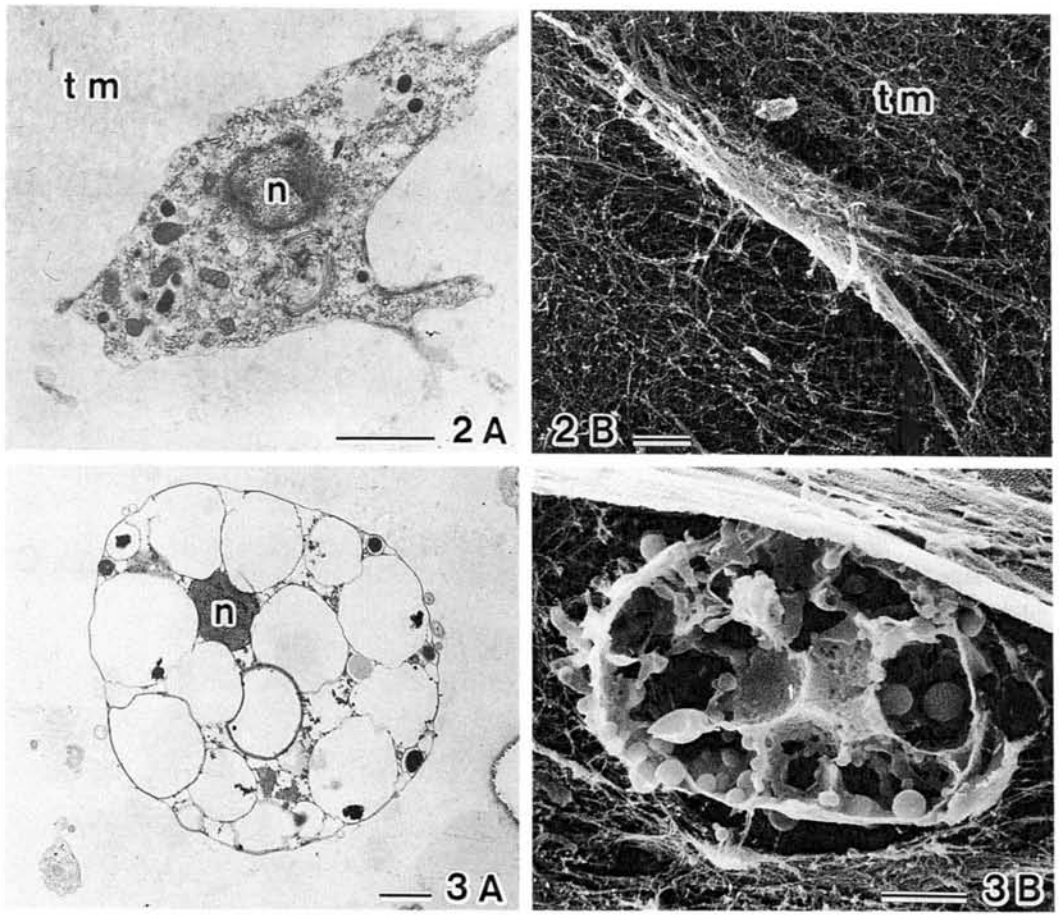


FIG. 1. Three types of tunic cells in paraffin sections. A: Amoeboid tunic cell in *B. simodensis*. B: Vacuo-granular tunic cell in *B. simodensis*. C: Large granule tunic cell in *B. scalaris*. Arrowhead indicates the large granule. c, cuticle of tunic. Scale bar, 5 μm.

In *B. simodensis* and *B. fuscus,* neither amoeboid nor vacuo-granular tunic cells emit autonomous fluorescence upon excitation with blue-violet light, while some of the blood cells emit autonomous fluorescence.

Amoeboid tunic cell

The cells are irregularly shaped, vary in size, and have a round nucleus and many pseudopodia (Fig. 2). Many of the amoeboid tunic cells are filled with



FIGS. 2, 3. Amoeboid tunic cell (FIG. 2) and vacuo-granular tunic cell (FIG. 3) in *B. simodensis*. A) Images of TEM. B) Images of SEM. n, nucleus; tm, tunic matrix. Scale bars, 2 μm.

cytoplasm and organella, but some of them containing vacuoles are found particularly in *B. primigenus* and *B. scalaris*. Amoeboid tunic cells sometimes have some granules which are barely observable under the light microscope.

Some of the amoeboid tunic cells are fusiform, and do not have many pseudopodia. These cells have a round or elliptical nucleus, and occasionally have a few vacuoles and/or some granules. These are usually found along the epithelium of blood vessels.

Vacuo-granular tunic cell

These cells are usually round or ovoid, ca. 10 μm in diameter, and a lot of filopodia radiate from the cell. These filopodia are easily observable in living specimens, but hardly in the specimens for SEM and TEM (Fig. 3). The round nucleus is usually positioned at the center of the cell. Most parts of the cell are occupied by many vacuoles, and there is a round electron-dense granule in some of the vacuoles. The size of the granules are different from species to species, e.g. ca. 1.4 μm in diameter (*B. lentus*), ca. 1.0 μm (*B. schlosseri*) and ca. 0.8 μm (*B. simodensis*).

In the all species which have vacuo-granular tunic cells, some tunic cells show intermediate characteristics of amoeboid and vacuo-granular tunic cells, which have homogeneous cytoplasm, vacuoles and granules (Fig. 4). The granules of the cells in intermediate stages are relatively larger and less dense than those of the fully vacuolated cells.

Large granule tunic cell

The large granule tunic cell contains a large granule well stained with hematoxylin, and the shape and size of the cell are similar to the amoeboid tunic cell (Fig. 5). The large granule shows lamellate substructure in an electron micrograph. This cell type is peculiar to *B. scalaris*.

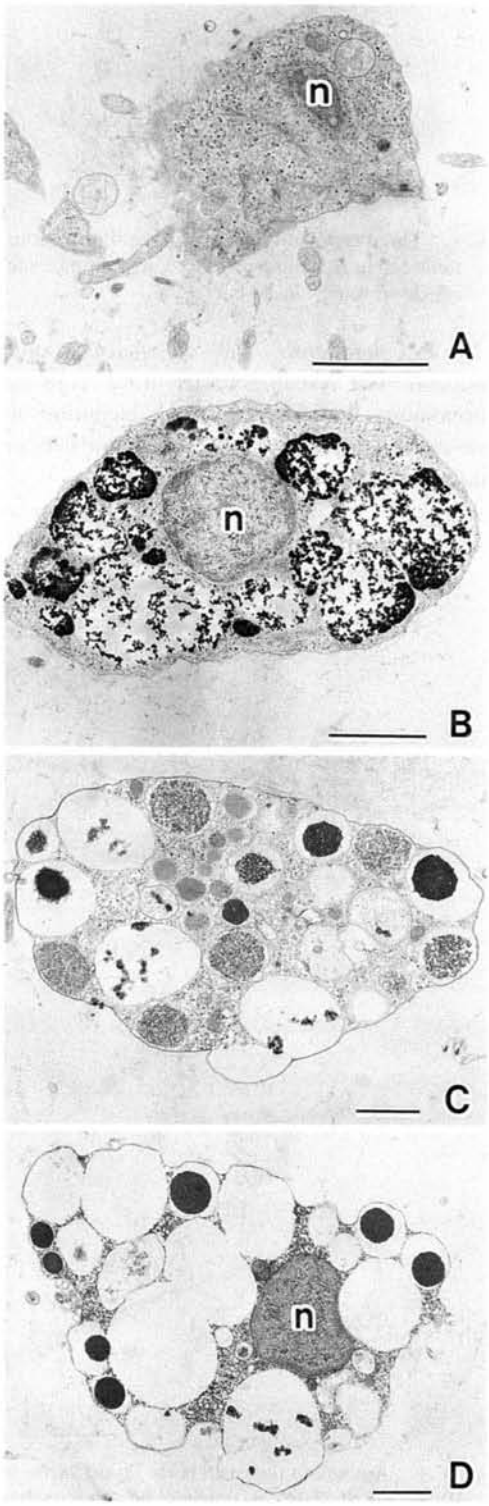


FIG. 4. Possible processes of tunic cell differentiation from amoeboid tunic cell to vacuo-granular tunic cell in *B. lentus*. A) Amoeboid tunic cell. B and C) Differentiating vacuo-granular tunic cell. D) Fully vacuolated vacuo-granular tunic cell. n, nucleus. Scales, 2 μm.

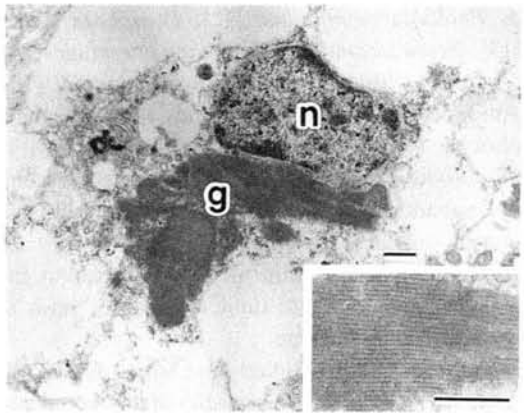


FIG. 5. Large granule tunic cell in *B. scalaris*. Inset, Striped structure of the granule. g, large granule; n, nucleus. Scales, 0.5 μm.

Bacteria in the tunic

There are thread-like bacteria in the tunic of the all species studied here, and other types of bacteria are rarely found (Fig. 6A). They abound in the tunic near the cuticle and colonial edge, but are never found in the vascular lumen. In a living tunic specimen, this bacterium glides in the tunic matrix like an arrow. These bacteria are occasionally found in an amoeboid tunic cell (Fig. 6B), and they often dangle around a vacuo-granular tunic cell (Fig. 6C). An electron dense structure is also found with the bacteria around a vacuo-granular tunic cells (Fig. 6D). It may be a disintegrated bacterium.

DISCUSSION

Three types of tunic cells are found in ten botryllid species. They are amoeboid, vacuo-granular and large granule tunic cells. Amoeboid tunic cells are found in all species, and large

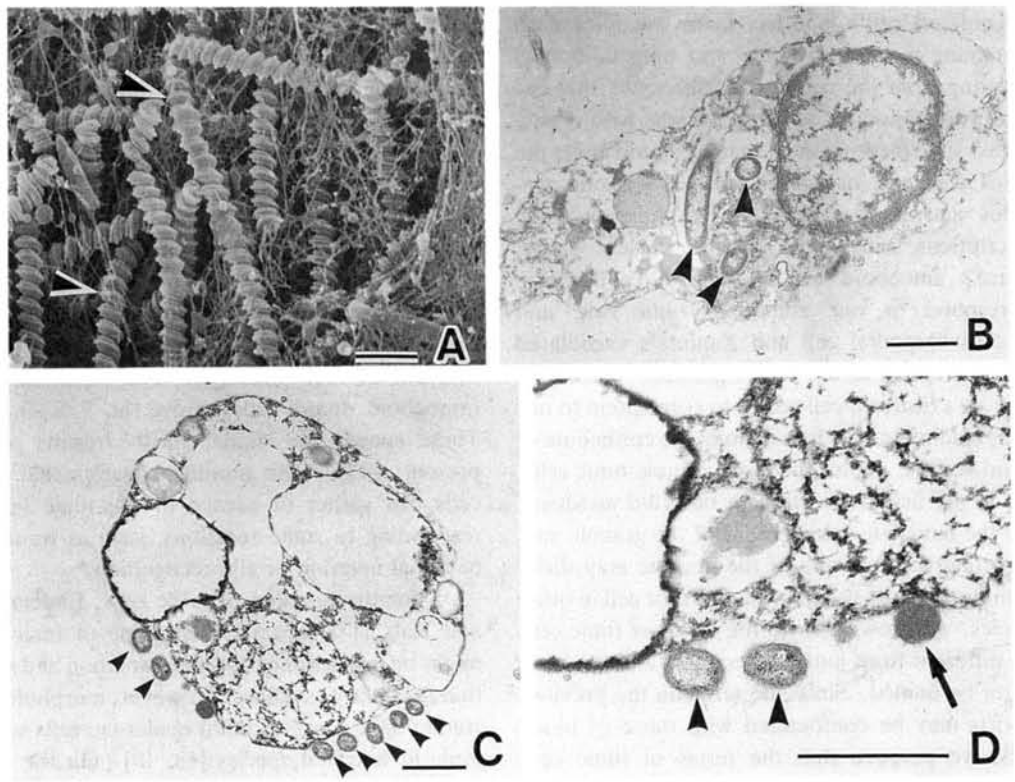


FIG. 6. Bacteria and tunic cells in *B. simodensis*. Arrowheads in each figure indicate bacteria. A) Bacteria in the tunic matrix (SEM). B) Bacteria in an amoeboid tunic cell. C) Bacteria dangling around a vacuo-granular tunic cell. D) Enlargement of C. Arrow indicates an electron dense structure which may be a disintegrated bacterium. Sacles, 1 μm.

granule tunic cells are peculiar to *Botryllus scalaris*. Vacuo-granular tunic cells can not be found only in *Botryllus primigenus* and *B. scalaris*, but they are possibly comparable to some of the amoeboid tunic cells with granules or vacuoles in these species.

Tunic cells in botryllid ascidians have been described only in *Botryllus schlosseri* [5, 6]. Izzard [5] has studied on the motility of tunic cells, and classified the tunic cells into two types from the observations under differential interference optics; "filopodial cell" that is characterized by long filopodia radiating from the cell body and the presence of large vacuoles in the cytoplasm, and "amoeboid cell" that moves by the eruption of hyaline pseudopodia and by cytoplasmic flow. On the other hand, Zaniolo [6] has made histological studies of the tunic, and classified the tunic cells into three types from the observations of histological sections stained by several methods; "vacuolated cell" that has many vacuoles each containing a round granule and long fiolopodia radiating from the cell body, "fibrocyte" that has small pseudopodia and large nucleus with a nucleolus, and "fusiform cell" that are found along the vessel wall and has ovoid nucleus without nucleolus and no pseudopodia. Comparing these descriptions with those of the present study, Izzard's amoeboid cell and Zaniolo's fibrocyte correspond to our amoeboid tunic cell, and Izzard's filopodial cell and Zaniolo's vacuolated cell correspond to our vacuo-granular tunic cells. Zaniolo's fusiform cell seems to correspond to our amoeboid tunic cells found along the epithelium of blood vessels. As for the large granule tunic cells, this is the first description in botryllid ascidians, and the lamellate substructure of the granule suggests that the contents of the granule may differ from those of the vacuo-granular tunic cell in other species. As shown above, the terms of tunic cells are different from author to author, and they had better be unified. Since the terms in the previous reports may be confounded with those of blood cells, we propose that the terms of tunic cells should be followed with the words "tunic cell", as those in the present study.

The morphology and classification of tunic cells have been described in some other ascidians, such as *Phallusia mammillata* [12], *Perophora viridis* [13], *Pyura stolonifera* [14], *Ciona intestinalis* [15], *Diplosoma listerianum* and *D. macdonaldi* [16]. Amoeboid tunic cell in botryllids resembles lymphocyte in *P. stolonifera* and filopodial cell in *Diplosoma,* and vacuo-granular tunic cell resembles vanadocyte in *P. mammilata* and *P. viridis* and morula cell in *P. stolonifera* and *C. intestinalis*. However, it is very difficult to discuss about the homology among these tunic cells on the basis of their morphology alone.

In the botryllids studied here, some tunic cells show intermediate characteristics of amoeboid and vacuo-granular tunic cells, and thus vacuo-granular tunic cells probably differentiate from amoeboid tunic cells in the tunic. Similar observations have been reported in *Pyura stolonifera* [14] as follows; the cells observed in the tunic were either lymphocytes, morula cells or cells of intermediate characteristics. On the other hand, it is uncertain whether the tunic cells have proliferative activities and where they originate from. The morphology of the amoeboid tunic cell suggests that the haemoblasts in blood cells or the epidermal cells are possible candidates of the origin of amoeboid tunic cells.

It was proved that the amoeboid and vacuo-granular tunic cells were migrating in the tunic matrix in this study. Izzard [5] has also reported on the motility of tunic cells in *B. schlosseri*; filopodial cells (vacuo-granular tunic cells) move at about 4 μm/min at their maximum and amoeboid cells (amoeboid tunic cells) move at 7–8 μm/min. These speeds are similar to the results of the present study. The motility suggests that tunic cells can gather or escape in the tunic matrix, responding to some reactions, such as traumata, bacterial invasion or allo-recognition.

As for the function of tunic cells, Endean [12] and Kalk [17] expected that some of tunic cells might be responsible for tunic formation and secretion in solitary ascidians. However, morphological studies have suggested that epidermal cells secrete tunic in botryllid species [18, 19]. On the other hand, Parrinello *et al.* [20–22] have shown that tunic cells are involved in "inflammatory-like response" to eliminate irritants injected in the tunic of *Ciona intestinalis*. We expect that tunic cells in

botryllid species are responsible for the defense against infections and for allo-recognition in colony specificity. In the tunic, there are thread-like bacteria, and it is uncertain whether they are symbiotic or pathogenic. Since they are also found in amoeboid tunic cells or dangle around vacuolated tunic cells, there may be some interactions between the tunic cells and the bacteria. In turn, it is worth mentioning that other types of bacteria are rarely found in the tunic. This indicates that the tunic possesses a defense system against bacterial infection. In botryllid ascidians, colony specificity is an occurrence of allo-recognition between colonies; when two conspecific colonies come into contact at their growing edges, they fuse into single colony sharing a common vascular system or reject with each other, depending on the genetic combinations. The tunic around the contact area is the site of the allogeneic rejection reaction. In the early stage of the rejection reactions in *Botrylloides fuscus,* vacuo-granular tunic cells often break down and discharge the contents of granules in the tunic matrix [Hirose, Unpublished]. This suggests that tunic cells are possibly involved in the allo-recognition and/or the induction of allo-specific rejection.

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