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Author(s)

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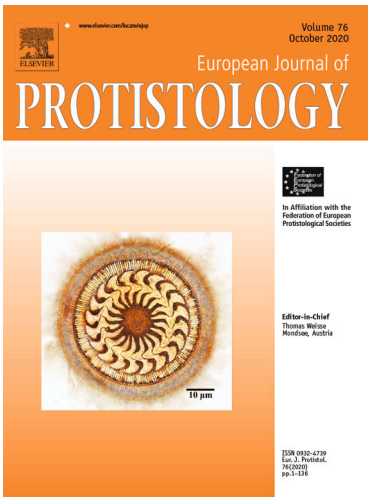

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SEM observation of non-fixed and water freeze-dried *Spirostomum ambiguum*

Hideki Ishida [a,*], Chika Matsumoto [a] , Maho Shimada [a] and Toshinobu Suzaki [b]

[a] Gradyate School of Natural Science and Technology, Shimane University, 1060 Nishikawatsu-cho, Matsue, 690-8504, Japan

[b] Department of Biology, Graduate School of Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan

*Corresponding author: Hideki Ishida

Institute of Agricultural and Life Sciences, Academic Assembly, Shimane University, 1060 Nishikawatsu-cho, Matsue, 690-8504, Japan

E-mail address: h-ishida@life.shimane-u.ac.jp

Abstract

We aimed to freeze-dry the ciliate *Spirostomum ambiguum*, obtained from water, without fixation and observe it using scanning electron microscopy (SEM). Living cells were placed on a specimen stub and frozen upon contact with a Cu block kept at either -80 °C or -100 °C. Samples were then freeze-dried and observed by SEM. In most cases, no damage to the specimen due to ice crystal formation was observed. Because of the instantaneous freezing, the metachronal wave of cilia on the body surface of the ciliate was well preserved. Approximately 70–80% of cells were preserved in the contracted state due to inevitable exposure to mechanical vibration immediately before freezing. The remaining samples were preserved in a fully-extended state. Morphometric measurements of the cell surface showed that in the extended state, ciliary rows were almost parallel to long axis of the cell, whereas in the contracted state, they were twisted in a left-handed helix at an angle of 45–65°. The distance between adjacent cilia along a ciliary row was 1.88 ± 0.43 μm in the extended state and 1.32 ± 0.41 μm in the contracted state (mean ± SD). However, the spacing between adjacent ciliary rows remained unchanged.

***Keywords**: Freeze-drying, ciliate, contraction, cilia*

Introduction

Protozoa with soft cells, such as ciliates, need to be dehydrated in order to be observed with a scanning electron microscope (SEM). Ciliates are protists with a delicate cell surface structure and numerous cilia. Thus, maintaining an intact surface appearance of these specimens during sample preparation is very difficult. Different sample processing techniques have been developed over time to preserve the surface structure of living ciliate cells, but none could guarantee artifact-free structures (Foissner, 2014). Since highly-contractile ciliates, such as *Spirostomum* and *Stentor*, can be easily induced to contract their cell bodies using chemical fixatives, treatment with high concentrations of divalent cation chelating agents is necessary to observe cells by SEM in their extended state; however, the effect of such pretreatment on the fine-structure of ciliates cannot be ignored (Huang and Pitelka, 1973; Ishida et al., 1996).

Water freeze-drying, as a sample preparation method for SEM, was first developed by Suzaki et al. (1978). This method takes advantage of the rapid freezing phenomenon caused by supercooling, and is an excellent method, since it does not form ice crystals that would interfere with visualization, and does not cause sample shrinkage. The current study aimed to investigate a sample preparation method, based on the water freeze-drying method without fixation, for the observation of freshwater ciliates with SEM, which could eliminate artifacts associated with fixation and dehydration. This method is possibly the only way to observe contractile ciliates in an intact state without pretreatment. In *Stentor*, the inter-ciliary distance along a ciliary row has been shown to shorten during cell shortening and widen during re-elongation, the change being a critical factor in explaining the mechanism of cell re-elongation by the

mutual sliding of microtubule bundles that exist in association with the ciliary bases (Huang and Pitelka, 1973). In *Spirostomum*, cell contraction and elongation have been inferred to be similar to that in *Stentor* (Lehman and Rebhun, 1971); however, the distance between cilia has been reported to be constant, regardless of cell contraction and elongation (Ishida et al., 1996). This discrepancy could be due to differences in sample preparation and electron microscopy methods. Therefore, the current study aimed to observe *Spirostomum* with SEM using the water freeze-drying method without fixation in order to minimize artifacts during sample preparation. Cell morphology and cilia spacing in the elongated and contracted states were then compared, and the mechanism of cell contraction in *Spirostomum* was discussed based on the results.

Material and Methods

Spirostomum ambiguum (strain GJ01) was originally collected from a pond in Hiroshima prefecture, Japan (34°23'31.0"N 132°18'52.5"E), and cultured at 22 °C in 0.01% Knop medium (0.24 mM $Ca(NO_3)_2$, 0.14 mM KNO_3, 0.06 mM $MgSO_4$, and 0.1 mM KH_2PO_4) with boiled wheat grain in a 9-cm Petri dish. The culture was kept in the dark, and a small amount of wheat infusion (adjusted to pH 7.0) was added once a week to the culture medium. Subculture was conducted at an interval of approximately 2–4 weeks.

For SEM observation, living cells were suspended in distilled water. *Spirostomum ambiguum*, used in this study, lives in freshwater ponds with very low solute concentrations; therefore, maintaining it in distilled water for up to 24 hours did not affect cell morphology or motility. A ring-shaped spacer made of silicone rubber,

with an inner diameter of 10 mm and a thickness of 1 mm, was placed on an aluminium sample stub, with a diameter of 15 mm and a height of 10 mm. Approximately 100 μL of the sample was placed in the spacer ring so that the sample suspension would rise slightly above the top surface of the spacer. The samples were then frozen by contacting a copper metal block (20 mm in diameter and 30 mm in height) pre-cooled to -80 °C (Fig. 1). In some experiments, a water-cooled freeze-dryer Aqua FD-6500 (SUN Technologies, Tokyo, Japan) was used, where the same preparation procedure was employed except that the precooling temperature of the metal block for freezing was set to -100 °C. The difference in freezing temperature had no effect on the observed results. According to the Aqua FD-6500 instruction manual, in this method, the water surrounding the sample is first frozen, creating a physical barrier around the unfrozen sample cells. Subsequently, the cell freezes, and the volume expansion during freezing increases the pressure inside the cell, preventing the formation of ice crystals there and allowing the cell to freeze without damage due to ice crystal formation. The frozen sample on the sample stub was allowed to stand for approximately 1 min to ensure temperature reduction. The Cu block was then removed from the top of the sample stub. In most cases, the frozen sample stayed on the sample stub; however, in some cases, it adhered to the side of the Cu block. In that case, the sample was abandoned and remade. The sample stubs were transferred to a freeze dryer (Vacuum Device VFD-21S, Mito, Japan) with the sample temperature set at 0 °C for freeze-drying. The dried samples were sputter-coated with Pt-Pd (Hitachi E-101, Tokyo, Japan) and observed by FE-SEM (Hitachi S-4800, Tokyo, Japan).

The distance between adjacent cilia in the same ciliary row and the distance between adjacent ciliary rows were measured from the obtained SEM images.

Measurements were taken in the central region of the cell for one cell in the elongated state and two cells in the shortened state. Preliminary measurements using 4-5 cells each showed little variation between cells.

Results

In this study, we successfully dried *Spirostomum ambiguum* in an unfixed state. The cells could be observed by SEM in their extended state, without any pretreatment. Most (70–80%) of the dried samples were in contracted state, since *Spirostomum* cells contract upon even very slight mechanical stimulus, such as vibration. In both the extended and contracted states, metachronal waves of the cilia were preserved, and the samples showed no evidence of ice crystal formation (Fig. 2). In the extended state, 50 to 60 rows of cilia ran almost parallel to the long axis of the cell, whereas in the contracted state, they formed a left helix with a 45 to 60° inclination to the long axis (Table 1 and Fig. 3A). When cell size was compared between live and dried cells, there was no significant difference in either cell length or width (Table S1).

Morphometric parameters of the cell surface were examined and compared between the extended and contracted states (Table 1). The distance between adjacent cilia along the same ciliary row was found to be reduced to approximately 70% in the contracted state than in the extended state (Table 1 and Fig. 3B). However, the distance between adjacent ciliary rows did not change (Table 1 and Fig. 3C). This measurement was performed only in the central part of the cell, indicating that, at least in this region, surface area of the cell was reduced by approximately 30% due to the shortening of inter-ciliary distance. Surface of the cell was not smooth; the area along a ciliary row

was found to be grooved and depressed, and cilia were found to grow from the base (Fig. 2C). The above results suggested that the apparent area of the cell may have decreased upon cell contraction as a result of deepening of the groove.

Discussion

Preparation of protist samples for SEM

In preparing samples for SEM, critical point drying or freeze drying, using t-butyl alcohol, is usually used after fixation and dehydration. Recently, chemical drying, using hexamethyldisilazane, has also been used (Rahman, 2021). During the process of chemical fixation and dehydration, deformation of the sample is inevitable. For example, the axopod of Heliozoa, which is a thin, long, and slender cell process, is easily shortened upon chemical fixation (Suzaki et al., 1980). Further, while it is possible to instantaneously stop and fix the ciliary beating of ciliates using Parducz's fixative solution (Foissner, 2014), it is difficult to dispose the waste liquid due to its mercury content; therefore, its use has been discouraged in recent years. Furthermore, there is no known method yet to fix ciliates (*Stentor, Spirostomum*, and *Condylostoma*) with highly contractile cell bodies while retaining their shape without any pretreatment. The contractile proboscis of the ciliate *Lacrymaria* and the stalk of *Vorticella* are also known to be readily contracted by chemical fixatives. In *Paramecium bursaria*, the process of dehydration has been shown to result in shrinkage artifacts (Song et al., 2017; Song and Suzaki, 2013). In order to prevent such fixation and dehydration artifacts, samples should preferably be dried under non-fixation and non-dehydration conditions; the freeze-drying method, using water, was developed for this purpose

(Suzaki et al., 1978). In this method, samples are rapidly supercooled in water and instantaneously frozen thereafter. We had previously reported SEM observations of the ciliates *Paramecium* and *Tetrahymena,* and of the heliozoan *Echinosphaerium* using this method (Suzaki et al., 1978, 1980). While this method does not require special equipment, it does require skill, and hence, has not been widely used. In recent years, equipment that automates the water freezing method has been developed, making sample preparation easy and highly reproducible. Using this method, microscopic organisms that live in freshwater can be directly frozen and dried without fixation.

Changes in the surface structure of Spirostomum during cell contraction

The arrangement of cilia on the cell surface of *Spirostomum ambiguum*, as revealed by this study, is schematically shown in Fig. 4. In the elongated cells, the ciliary rows were nearly parallel to the long axis of the cell (Fig. 4a), but after cell contraction they twisted in a left-handed helix (Fig. 4b). The distance between adjacent ciliary rows remained unchanged. In a previous study using *Spirostomum teres*, we had reported that the spacing between adjacent cilia along a ciliary row was not different between cells in the extended and contracted states (Ishida et al., 1996). However, the present results contradicted this and showed that spacing between the cilia of *Spirostomum ambiguum* shortens by about 30% as the cells contract. In the previous study, we had observed chemically fixed cells; therefore, shrinkage of the superficial structure may have possibly been due to fixation and drying artifacts. Another possibility is that there may have been an effect of the high concentration of chelating agents that were applied prior to fixation in order to prepare specimens of cells in

extended state, or it may have been due to differences in the species used. In fact, in the previous SEM image observed by chemical fixation and critical point drying (Fig. 2c in Ishida et al. 1996), there were fine wrinkles on the surface of the cells, whereas in the water freeze-dried image (Fig. 2C in this study), the surface layer appeared smooth. Since artifacts were minimized in the present observation, the results are even more reliable. However, the spacing between ciliary rows did not change with cell shortening. The retention of spacing between cilia rows may be characteristic of Spirostomum. In *Stentor*, a heterotrichous ciliate that exhibits a similar rapid cell contraction, ciliary rows have been found to shorten while remaining parallel to the long axis of the cell. In this case, spacing between cilia was shortened, although that between ciliary rows was widened (Huang and Pitelka, 1973).

Change in surface structure associated with cell contraction in *Spirostomum* was compared with that in *Stentor* (Table 2). The biggest difference between the two types of heterotrichous ciliates was that the surface structure of *Stentor* elongated and shortened only along the long axis of the cell, and the ciliary rows were always parallel to the long axis of the cell, whereas in *Spirostomum*, the ciliary rows formed a left-handed helix and the cell twisted during contraction. The myoneme of *Stentor* is a thick bundle arranged along the long axis of the cell and is tightly attached to the ciliary row (Huang and Pitelka, 1973). In contrast, the myoneme of *Spirostomum* does not form bundles, but is a fine meshwork, separated from the surface ciliary structures (Ettienne, 1970; Lehman and Rebhun, 1971). The structural differences in myoneme may give rise to oblique contraction of the cell surface in *Spirostomum*, resulting in helical cell contraction. As for the elongation of the cell, it has been suggested that in *Stentor,* the elongation of the entire cell is caused by active sliding of neighboring microtubule

sheets during cell elongation, and the average sliding distance of microtubule sheets during this process was approximately 2 μm (Huang and Pitelka, 1973). On the other hand, based on the observations in *Spirostomum*, in this study, while microtubule sheet sliding may occur in *Spirostomum*, the distance between microtubules was approximately 0.5 μm in the center of the cell (difference in the distance between cilia during elongation and shortening from Table 1). This suggested that, in *Spirostomum*, the mechanism of cell elongation may be different from the sliding motion of neighboring microtubule sheets. In this regard, the hypothesis (Yogosawa-Ohara et al., 1985) that conformational change of the basal body-associated filamentous structures generates the driving force for torsional movement of the entire surface structure cannot be discarded. Although the mechanism of cell elongation and the involvement of microtubules in *Spirostomum* cannot be conclusively stated, the morphometric results presented in this study will provide a morphological basis for future research.

Conclusions

In this study, a method was developed to freeze-dry the heterotrichous ciliate *Spirostomum ambiguum* from water without chemical fixation. The method allowed SEM observation of cells in both elongated and contracted states with reduced sample preparation artifacts. Morphometric measurements of the cell surface showed the distance between any two cilia along the ciliary rows to decrease by approximately 30% upon cell contraction. No change was observed in the distance between adjacent ciliary rows.

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Declarations of interest: none

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Figures

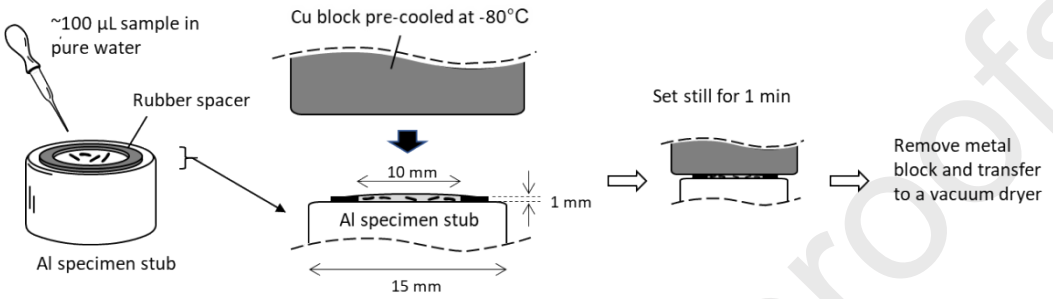

Fig. 1.

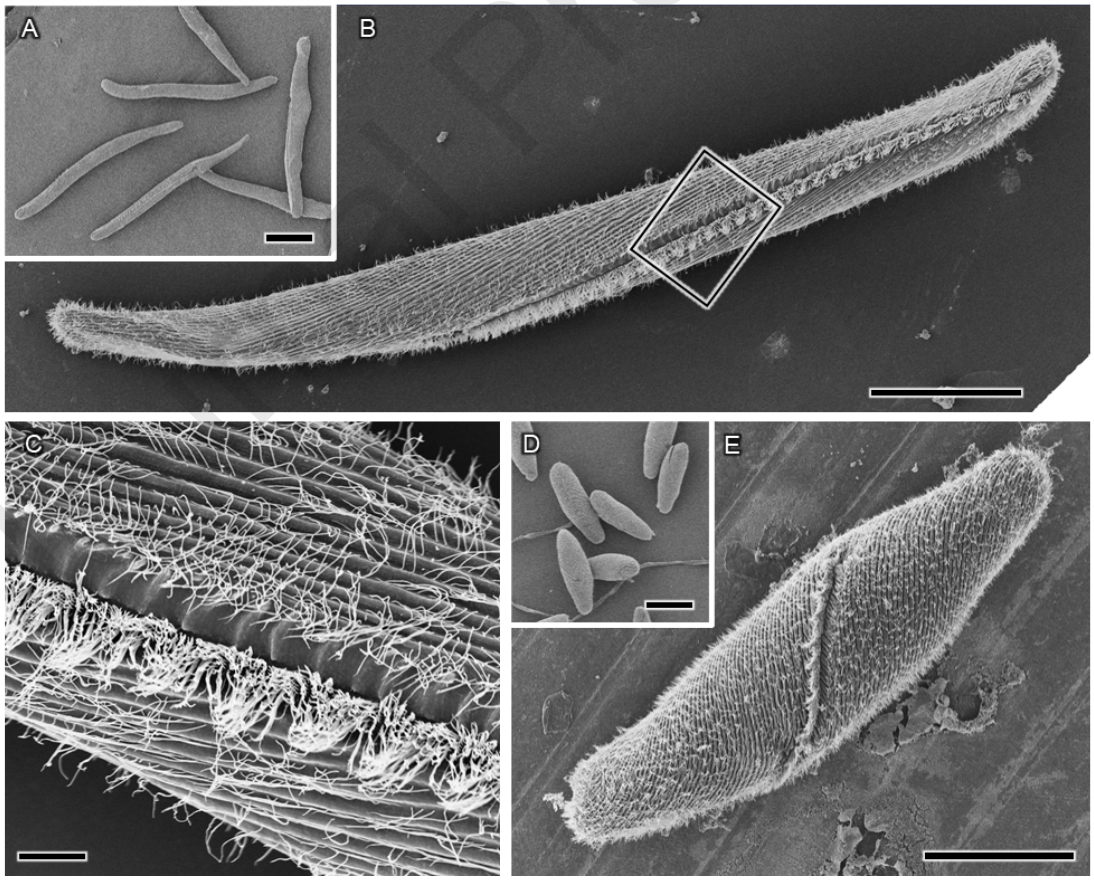

Fig. 2.

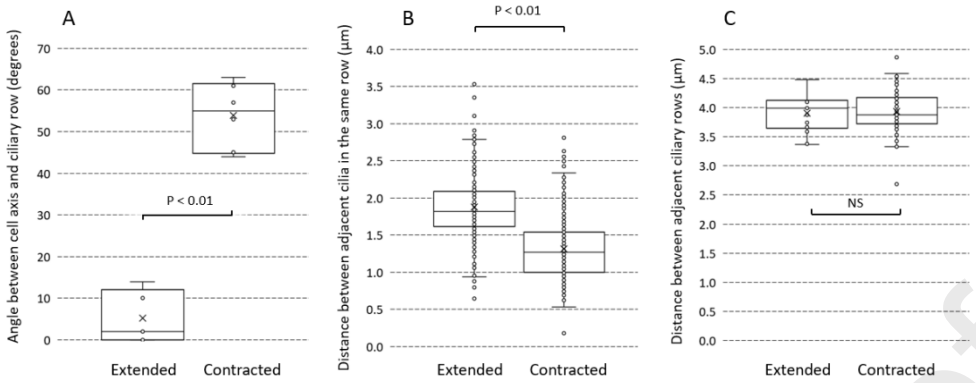

Fig. 3.

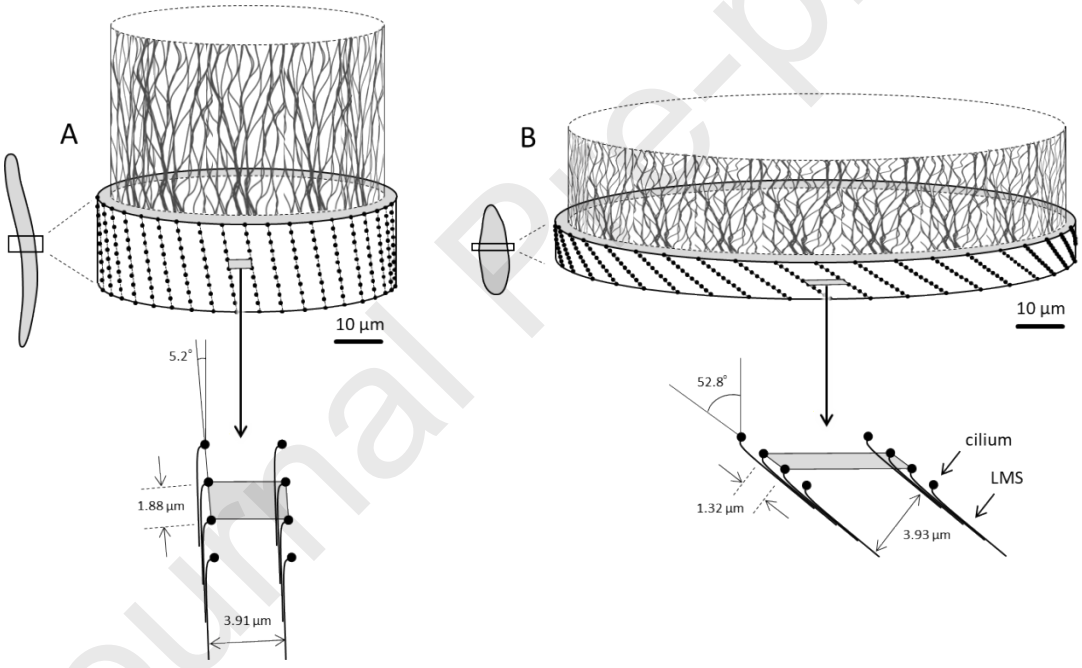

Fig. 4.

Figure captions

Fig. 1. Sample preparation for water freeze-drying. First, a ring-shaped silicon rubber spacer with an inner diameter of 10 mm and a thickness of 1 mm was placed on an Al sample stub for SEM. Next, approximately 100 μL of live ciliates suspended in distilled water was placed in the spacer so that the water surface was slightly raised. The sample suspension was frozen by pressing a Cu block, which had been previously cooled to -80 °C. After 1 min, the Cu block was removed. In most cases, the specimens stayed on the specimen stub below; however, sometimes they adhered to the Cu block above. If a frozen sample remained on the sample table, it was freeze-dried in a vacuum dryer, and observed by SEM after metal coating. If using the freeze-dryer Aqua FD-6500, sample preparation was performed by almost the same procedure as described above. However, in the FD-6500, the metal block for sample freezing was cooled to -100 °C.

Fig. 2. Scanning electron micrographs of *Spirostomum ambiguum*. A-C show cells in the extended state. A is a low-magnification picture, indicating that all cells in the region are in the extended state. C is a magnified image of the rectangular region in B, showing that the metachronal wave of cilia in the adoral zone of membranelles is well preserved due to rapid freezing. D and E are cells in the contracted state. D is a low-magnification picture, showing that all cells are preserved in the contracted state. In the contracted state, the ciliary rows, including the adoral zone of membranelles, form a left-handed spiral. The sample shown in A was prepared using Aqua FD-6500, and those in B-E were prepared manually. Bars represent 200 μm (A and D), 100 μm (B and E), and 10 μm (B and E).

Fig. 3. Comparison of morphological parameters between extended and contracted states of *Spirostomum ambiguum*. A. Angle of ciliary rows against the longitudinal axis of the cell. B. Distance between the adjacent ciliary bases in the same ciliary row. C. Distance between adjacent ciliary rows. NS indicates not significant ($p > 0.05$).

Fig. 4. Morphological features of the cell surface of *Spirostomum ambiguum* and changes during cell contraction. A is a schematic diagram showing the extended state while B shows the contracted state. A fragment of the central part of the cell is shown in magnified view. The small dots indicate the bases of cilia. In the extended state, the ciliary rows run almost parallel to the long axis of the cell (slightly to the left at an angle of 5.2°). In the contracted state, the ciliary rows were highly tilted with respect to the long axis of the cell (left-handed helices at an angle of 52.8°), and the spacing between cilia along the rows was shortened by approximately 30%. However, the spacing between adjacent rows of cilia did not change despite cell contraction. The basic unit, which has the shape of a parallelogram surrounded by four adjacent ciliary bases, is colored gray. A contractile myoneme (drawn from Lehman and Rebhun's (1971) electron micrographs) is also shown, located in a meshwork-like arrangement just below the cell surface, not in direct contact with the ciliary base or longitudinal microtubular sheets (LMS).

Table 1. Morphometric parameters of *Spirostomum ambiguum*

	Elongated cells	Contracted cells
Cell length (µm)	786.5 ± 54.7 (n=8)	386.6 ± 39.2 (n=15)
Cell width* (µm)	67.1 ± 4.9 (n=8)	116.1 ± 12.0 (n=15)
Angle between longitudinal cell axis and ciliary row (degrees)	5.2 ± 6.4 (n=5)	52.8 ± 8.0 (n=6)
Distance between adjacent cilia along the same ciliary row (µm)	1.88 ± 0.43 (n=201)	1.32 ± 0.41 (n=340)
Distance between adjacent ciliary rows (µm)	3.91 ± 0.30 (n=17)	3.93 ± 0.34 (n=89)

*Measured in the central part of cells

Table 2. Comparison of cell contraction between *Spirostomum* and *Stentor*

	Spirostomum	*Stentor*
Ciliary rows		
in extended state	Parallel to longitudinal cell axis	Parallel to longitudinal cell axis
in contracted state	Spiral (left-handed)	Parallel to longitudinal cell axis
Distance between ciliary rows	No change	Increased after contraction*
Myonemes	Interconnected meshwork of loose filaments without apparent association with other structures**	Densely packed filament bundles attached to the overlying microtubule ribbons*

*Huang and Pitelka, 1973

**Lehman and Rebhun, 1971

CRediT author statement

Hideki Ishida: Conceptualization, Methodology, Investigation. **Chika Matsumoto**: Data curation. **Maho Shimada**: Data curation. **Toshinobu Suzaki**: Writing- Reviewing and Editing,