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Siliceous scales in the centrohelid heliozoan *Raphidocystis contractilis* facilitate settlement to the substratum

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Running title: Adhesive function of scales in centrohelid heliozoan

Abstract

The centrohelid heliozoan *Raphidocystis contractilis* has hundreds of small scales on the surface of the cell body. To understand the biological functions of the scales, comparative examinations were conducted between wild-type and scale-deficient strains that has naturally lost scales after long-term cultivation. The scale-deficient strain exhibited decreased adhesion to the substratum and had a lower sedimentation rate in water than the wild-type strain, suggesting that the scale may have the ability to attach quickly and strongly to the substratum. Percoll density gradient centrifugation showed that the scale-deficient strain had a lower density than that of the wild-type strain. In the wild-type strain, more scales were observed in the higher specific gravity fractions. During the long-term culture of cells, only the cells suspended in the upper area of the flask were transferred to fresh medium. By repeating this procedure, we may have selected only cells that did not possess normal scales. In the natural environment, centrohelid heliozoans are easily flushed away if they cannot adhere strongly to the bottom. These results suggest that they use scales to ensure effective adhesion to the substratum.

Keywords*: Siliceous scales; axopodia; centrohelid heliozoa; electron microscopy; cell locomotion*

Introduction

Centrohelid heliozoa are unicellular eukaryotes with a centrosymmetric shape that have contractile pseudopodia, called axopodia, extending radially from the surface of their spherical cell bodies. They usually attach themselves to objects on the bottom of water and wait for prey to be trapped by the axopodia (Sakaguchi et al., 2002). *Raphidocystis contractilis* is a species belonging to the centrohelid heliozoa. This species is characterized by siliceous scales approximately 5 µm long and 2 µm wide with a rounded, swollen periphery reminiscent of the shape of a rubber boat (Sakaguchi et al., 2002). Centrohelid heliozoans have scales of various sizes, shapes, and patterns, and scale characteristics are often used to identify species (Kinoshita et al., 1995; Nicholl and Dürrschmidt, 1985; Zagumyonnyi et al., 2022). However, the function of siliceous scale structures is unclear. In general, siliceous structures are recognized as bioactive elements capable of causing a wide range of natural defenses (Knoll and Kotrc, 2015). However, it remains unclear how the siliceous structure exerts these protective functions (Nicholl et al., 1985).

In the present study, we obtained a strain of *R. contractilis* that lost its scales as a result of long-term culture in the laboratory. The cell surface of this strain was covered with fine needle-like structures, instead of siliceous scales. We examined the function of *R. contractilis* scales by comparing scale-deficient cells with wild-type cells.

Material and methods

Organism and culture method

Raphidocystis contractilis was originally collected in 1994 in a brackish pond (10% sea water) in Shukkeien Garden, Hiroshima, Japan (34°23’59.7” N, 132°28’05.4” E). Cloned cells were cultured monoxenically with *Chlorogonium capillatum* (NIES-3347) as the sole food source and maintained at 20 ± 1°C under constant illumination. The culture medium consisted of 46.2 mM NaCl, 0.9 mM KCl, 0.8 mM $CaCl_2$, 2.3 mM $MgCl_2$, 0.6 mM $NaHCO_3$, 0.74 mM sodium acetate, 0.01% polypeptone, 0.02% tryptone, 0.02% yeast extract, and 5 mM Tris-HCl (pH 7.8). Until 2010, numerous scales were present on the surface of the cells, but no scales were observed in 2020. This implies that the mutation occurred over a 10-year period and a scale-deficient strain was naturally created. The original culture containing scales was deposited in the National Institute for Environmental Studies (NIES) culture collection (NIES-2498). Thus, it was newly retrieved and used in this study as the wild-type strain. Before the experiment, cells were washed twice in 10% artificial seawater (10% ASW) by centrifugation (600 × *g*, 5 min).

Electron microscopy and elemental analysis

To prepare whole-mount samples, cell suspensions were placed on a grid mesh coated with Formvar, and excess water was removed using filter paper and air-dried. The samples were negatively stained with 1% uranyl acetate solution and observed using an H7100 transmission electron microscope (Hitachi, Tokyo, Japan). Elemental analyses were performed on the scales (wild-type) and needle-like structures (scale-deficient strain) using energy-dispersive X-ray spectroscopy (EDS, JED-2300, JEOL, Tokyo, Japan) at the Research Facility Center for Science and Technology, Kobe University. The elements analyzed were carbon, oxygen, silicon, and calcium.

Cell adhesiveness, sedimentation velocity, and Percoll density gradient centrifugation

In the experiment to determine the degree of cell adhesion, cells were first placed on a glass slide and sealed with a coverslip with a 1 mm thick rubber spacer. The entire setup was then flipped upside down and the cells were settled on top of the coverslip. After 1 h or 1 d, the device was inverted again, and immediately after that, the cells were observed under a microscope. Then, the number of cells that stayed on the coverslip and the number of cells that had fallen off were counted, and the percentage of cells that had adhered to the coverslip was calculated and used as an indicator of adhesiveness. Approximately 100 cells were examined at each determination. In an experiment to determine the free-fall velocity of cells in water, the cell suspension was first placed in a haematocrit capillary tube with an inner diameter of 1.1 to 1.2 mm and then closed at one end with Vaseline to prevent leakage. The capillary tube was then placed under an optical microscope with a horizontal optical axis and the free-fall velocity of the cells was measured by video recording. For the Percoll density gradient centrifugation, mixtures of Percoll solution (1.13 g/mL) and 10% ASW (ca. 1.00 g/mL) were made in 10% increments. Next, 1 mL of 100% Percoll was placed at the bottom of a 15 mL tube, and 1 mL each of 90%-10% Percoll solution was stacked on top of it, starting with the highest concentration. Finally, cells suspended in 10% ASW were placed on 10% Percoll and centrifuged for 30 min at 2,300 × *g*.

Motion analysis

Cells at the bottom of the plastic petri dish were video-recorded for 10 min for the wild-type and scale-deficient strains. The position of the cell center was then monitored at 10 s intervals using ImageJ to determine the trajectory of cell migration. To quantify the characteristics of

cell migration, the root mean square (RMS) displacement was calculated, as described by Textor et al. (2013).

Results

Cell surface structure of wild-type and scale-deficient strain

The cell surface of the wild strains of *R. contractilis* is covered with numerous elliptical disk-shaped scales that characterize the species. The scales were approximately 5–6 μm in long diameter and 2 μm in short diameter, with swollen margins resembling the shape of a rubber boat (Figs. 1A and B). By contrast, in the scale-deficient strain, the cell surface was covered with thin needle-like structures instead of scales. These structures were roughly 4–5 μm in length and resembled the electron-dense fibrous structures present centrally along the long axis of the scale in the wild-type strain (arrowheads in Figs. 1C and 1D). The other structures and sizes of the cells did not differ between the wild and scale-deficient strains. Despite these differences in the cell surface structures, there was no difference in cell growth (data not shown). Elemental analysis of the scale and needle structures was performed using X-ray spectroscopy (Fig. 2). The scales were shown to have a siliceous structure, with Si and O as the main components (Fig. 2A and pictures in the upper row in Fig. 2C). However, little Si signal was detected in the needle-like structures of the scale-deficient strain, indicating that they were organic materials composed mainly of C and O (Fig. 2B and lower pictures in Fig. 2C).

Cell adhesion to the glass substratum and free-fall velocities

The scale-bearing wild-type cells were observed to adhere more strongly to glass surfaces than scale-deficient cells. In this experiment, cells were placed on glass coverslips and turned upside down after 1 h or 1 d, and the percentage of cells still attached to the coverslip and not falling down was measured. In wild-type cells, approximately 90% of cells were attached to the substratum after 1 h, and majority of the cells were attached after 1 d. By contrast, in the scale-deficient strain, approximately half of the cells did not adhere to the substratum at either 1 h or 1 d (Fig. 3). The free-fall velocity of the cells in sealed capillary tubes was measured, and it was discovered that the cells fell more than seven times faster in the wild-type strain than those in the scale-deficient strain (Table 1). This result suggests that the presence of a scale increased the sedimentation rate of the cells.

Determination of specific gravity of cells

Since the free-sedimentation rate of the wild type strain with scales was faster than that of the scale-deficient strain, we tested the hypothesis that scales may serve as a weight or anchor for the cells. When discontinuous density gradient centrifugation with Percoll solution was performed, the wild-type cells were dispersed into fractions with Percoll concentrations ranging from 30–80%. Examination of each concentration portion using light microscopy revealed that the degree of scale coverage varied from cell to cell, with more scaled cells observed in the higher Percoll concentration fractions (Figs. 4A-E). The scale detached from the cells accumulated at the bottom of the tube (Fig. 4F), indicating that the specific gravity of the scale itself was higher than that of the Percoll solution (1.13 g/mL). By contrast, scale-deficient cells clustered at the border between 20 and 30% concentrations of Percoll solution (Fig. 4G), which corresponds to about 1.02 g/mL. These results suggest that scale plays a role in increasing the specific gravity of the cells.

Scale-mediated cell adhesion to the substratum

Since the presence of scales potentially enhances the adhesion of wild-type cells to the substratum, we observed the manner in which the cells adhered to the substratum. A series of differential interference micrographs were taken with the focal plane changed by 1.5 microns, and a 3D image constructed using Amira software showed that the cells were in contact with the coverslip surface via the scale and axopodial tips (Fig. 5).

Migratory movement of cells on the glass substratum

We observed that majority cells of the wild-type strain did not move significantly on the substratum (Fig. 6A). By contrast, scale-deficient cells on the substratum were observed to be more mobile than wild-type cells (Fig. 6B). To quantify cell locomotion, we measured the change in cell position every 10 s from the video images and plotted the resulting RMS distances against time. A linear relationship was observed for both the wild-type and scale-deficient strains. Thus, in both cell lines, the cell migration motion should be described as directed rather than being random. The slope of the graph indicates the speed of cell locomotion, which was 0.04 μm/s for wild-type cells and 0.39 μm/s for scale-deficient cells (Fig. 6C).

Discussion

Wild-type cells of *Raphidocystis contractilis* with scales are known to exhibit strong adhesion to a variety of substrates, including glass and plastic (Yoshimura et al., 2017). Cells adhere strongly to ordinary glass, glass surfaces coated with polycations to reverse the surface charge, polystyrene

Petri dishes, and Petri dishes with hydrophilic treatment. These results suggest that neither surface charging nor hydrophobic interactions are involved in scale-mediated adhesion to the substrate. Intermolecular forces such as van der Waals forces and hydrogen bonds may be involved in the adhesion between the scale and the substrate. Alternatively, there may be a sticky secretory substance similar to that observed in other organisms, such as diatoms (Chen et al., 2021), hydras (Seabra et al., 2022), and mussels (Lee et al., 2011; Waite, 2008) that adhere to the surface of the scale thereby adhering to the basal surface. Furthermore, as can be easily inferred from the shape of the scale, the cells may be held at the base by physically hooking onto the microscopic irregularities of the basal surface.

In this study, we discovered that cell adhesion to the basal surface was not permanent, and that cells slowly moved while adhering to the basal surface. In addition, the presence of scales potentially reduces the speed of the migratory movement of cells. On the other hand, cells lacking scales locomote at a fast rate on the substratum, but their adhesion to the substratum is low, and they easily detach from the surface. This indicates that the cells of the wild-type strain slowly expand their habitat without being swept away from the bottom surface, which may help them move even in the upstream direction against the flow. Even if they break away from the bottom for some reason, they are less likely to be swept downstream because the scale will help them descend to the bottom in a short period.

The shape of extracellular structures, such as scales, is an important factor in the classification of centrohelid heliozoans (Nicholl and Dürrschmidt, 1985). However, this study revealed that cells without scales can be produced spontaneously, although this is a phenomenon in a laboratory environment. This fact may indicate that the scale microstructure is not necessarily a good indicator of classification as already indicated, for example, by Drachko et al. (2020). To culture the cells in the laboratory, bacteria-free monoxenic cultures of cloned cells fed exclusively on *Chlorogonium capillatum* were performed. The process of pipetting cells floating at the top of a well-grown culture flask and transferring them to a new flask has continued for over 10 years. Since we discovered that cells with fewer scales were lighter and less likely to sink, this replanting process may have inadvertently selected for cells that did not have scales.

Biomineralization of silica has received much attention in recent years (Sumper and Brunner, 2008). Many groups of organisms are capable of crystallizing silica, including diatoms and sponges, but the detailed mechanism of crystallization is not well understood. For example, the siliceous cell wall (frustule) of diatoms is an essential structure for survival in many diatoms, so it is difficult to obtain strains deficient in frustule formation. In this respect, *Raphidocystis* would be a good model organism for studying the mechanism of silica crystallization since loss of scales does

not appear to affect cell growth or other cellular functions. Future studies on changes in gene expression in scale-deficient strains may help elucidate the molecular mechanism of silica crystallization.

In the present study we have shown that a function of the scale is to enhance adhesion to the substrate surface. Not all centrohelid heliozoans are strongly adhered to the water bottom, so this is probably not a trait shared by all organisms in this group. Thus, siliceous scales may have some other hitherto unknown function. One possibility is that by wearing a silica coating, they may be able to mimic themselves as sand grains and thereby circumvent the predator's chemical recognition function for the prey's surface molecules. Alternatively, there may be a protective effect against predators by increasing the mechanical strength of the cell surface, as shown in diatoms (Pančić et al., 2019), or against UV radiation (Aguirre et al., 2018).

Observations of cells lacking siliceous scales in centrohelid heliozoans have also been reported previously. For example, in the silicon-deficient medium, the cells of *Raphidiophrys Ambigua* completely lost their siliceous scales, but when returned to the medium containing normal silica, they made siliceous scales again (Patterson and Dürrschmidt, 1988). It has also been reported that *Raphidocystis pallida* lacked scales under enriched culture conditions (Drachko et al., 2022), suggesting that this may be because the amount of silica in the culture medium was insufficient for scale formation under these conditions. Alternatively, it was suggested that the unscaled cells of *R. pallida* may represent a special stage in the life cycle. In addition, different morphologies of cell cover structures were observed in *Raphidiophrys heterophryoidea* that could be transitional, presumably due to different stages of the life cycle and different feeding conditions (Drachko et al., 2020). On the other hand, in the present study we were able to obtain a cell line that exhibits a distinct phenotype of always having no scale. The cell line was always free of scale, which was neither due to low silica concentrations in the culture medium nor limited to any cell stage. Therefore, the scale-deficient strain is most likely the result of genetic mutation of some gene(s) related to scale formation as a result of long-term incubation in the laboratory. We are currently conducting transcriptomic and proteomic analyses comparing gene expressions in scale-deficient and wild strains to identify genes and their products that regulate scale formation.

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Table 1. Free-fall velocity of wild-type and scale-deficient cells in water (10% ASW)

Strain	**Velocity* (μm/s)**	**Sample number**
Wild-type	5.22 ± 1.89	12
Scale-deficient strain	0.70 ± 0.30**	12

*mean ± standard deviation

**significantly different from the wild-type (Welch's t-test, $p < 0.01$)

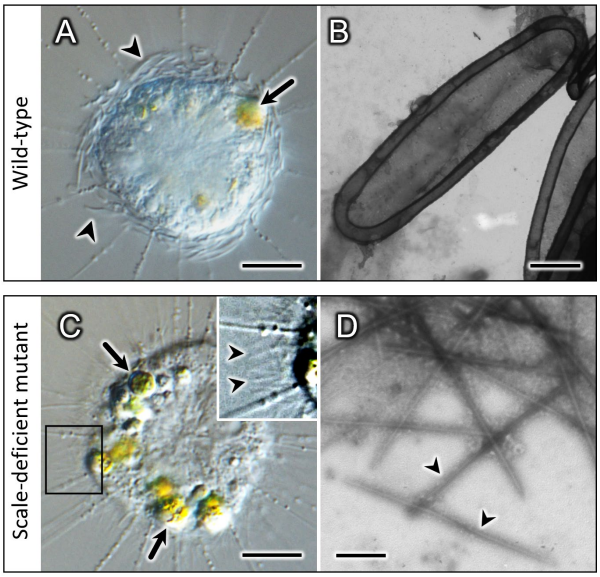

Fig. 1. Wild-type and scale-deficient strain cells of *Raphidocystis contractilis*. A and C show optical micrographs of the wild-type strain (A) and the scale-deficient strain (C). Arrows indicate phagocytic vesicles and arrowheads in A indicate scales found only in the wild strain. A magnified, contrast-enhanced photograph of the cell surface of the scale-deficient strain (indicated by the rectangle) is shown as an inset in C. In the scale-deficient strain, no scales are observed, but fine needle-like structures are seen covering the cell surface (arrowheads in the inset in C, and in D). B and D show transmission electron micrographs of negatively stained cell surface structures of the wild-type (B) and the scale-deficient strain (D). Scale bars: A and C: 5 µm; B and D: 1 µm.

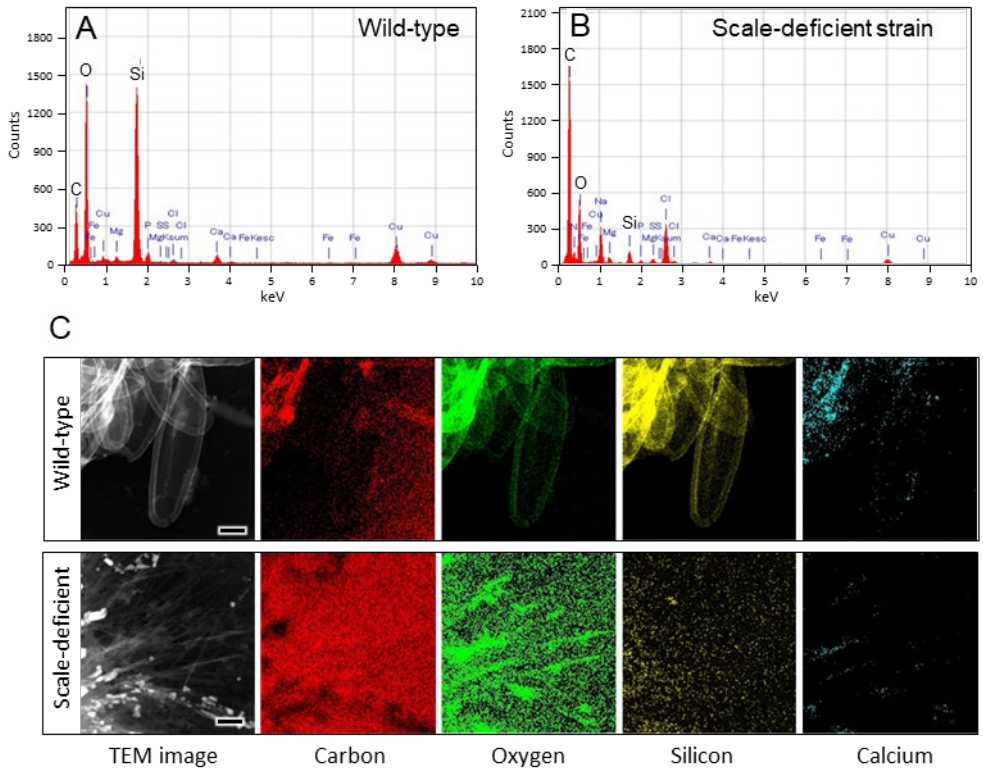

Fig. 2. Whole-mount specimens of the cell surface layers of *Raphidocystis contractilis* were examined using a transmission electron microscope (JSM-7100F, JEOL) equipped with an energy-dispersive X-ray spectroscope (EDS, JED-2300, JEOL). The spectra in A and B show the signals for the elements from the entire region shown in C for the wild and scale-deficient strains, respectively. C shows two-dimensional elemental mapping images of C, O, Si, and Ca for wild-type and scale-deficient cells; the leftmost pictures in C show transmission electron microscope images of the region where elemental analysis was performed. The scale of the wild strain showed silicon (Si) and oxygen (O) signals (A and upper column in C). On the other hand, little silicon signal was detected in the needle-like surface structure of the scale-deficient strain, where C and O were the major elements detected.

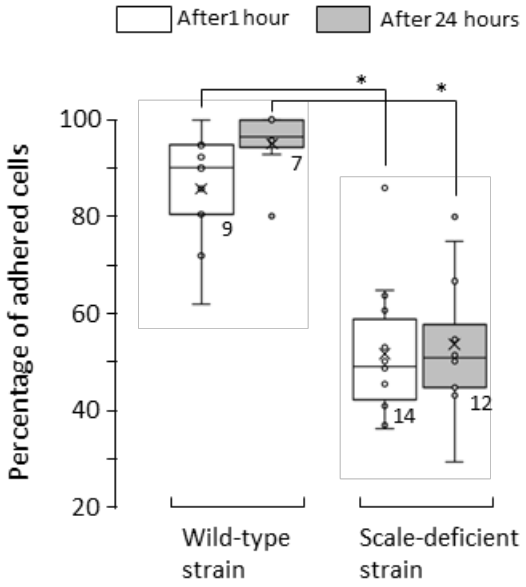

Fig. 3. Relationship between the presence of scales and the degree of cell adhesion to the surface of the coverslip. Wild-type or scale-deficient cells were placed on the coverslips and turned over after a certain time (1 h or 1 d). The percentage of cells adhering to the coverslip was then determined and shown as a box-and-whisker diagram. Both after 1 h and 1 d, the wild strain showed stronger adhesion (*: Welch's t-test, $p < 0.01$). The numbers below the boxes indicate the number of independent measurements. Individual data are indicated by open circles.

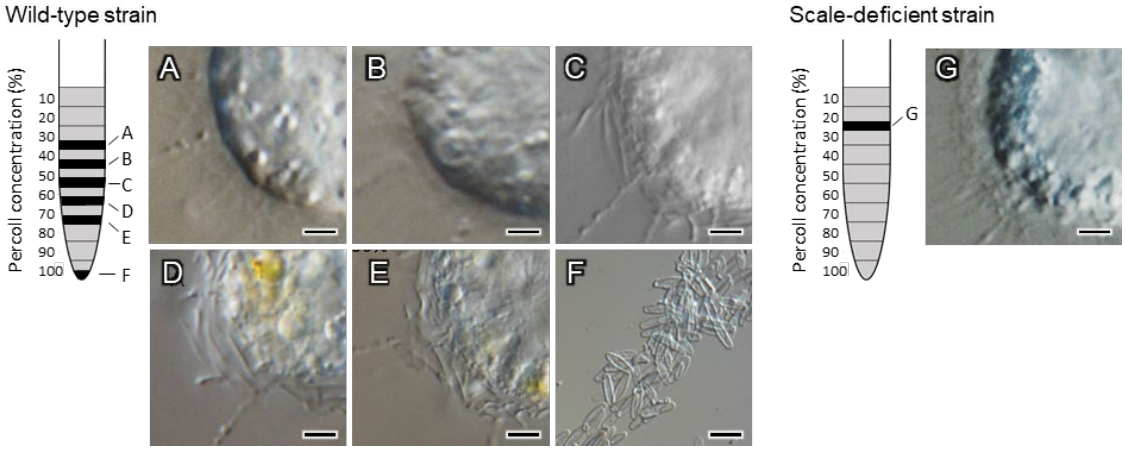

Fig. 4. Percoll density-gradient centrifugation of cells. Wild-type and scale-deficient cells were centrifuged in a discrete gradient of Percoll solution prepared by mixing 100% Percoll (density = 1.13 g/mL) and 10% ASW for 30 min at 2,300 × *g*. After centrifugation, wild-type cells were separated into interfaces of different Percoll concentrations, forming five layers (A–E, with pictures of cells corresponding to each layer shown). At the bottom of the tube, a pellet of scales detached from the cells was observed (F), indicating that the density of scales is higher than 1.13 g/mL. In contrast, scale-deficient cells accumulated at the interface alone between 20% and 30% Percoll layers (G). Scale bars: 2 µm.

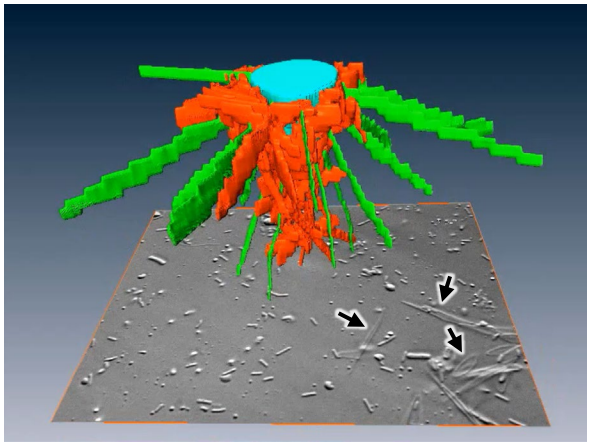

Fig. 5. A 3D image of a *Raphidocystis contractilis* wild-type cell showing its adhesion to the glass surface. The cell suspension was placed on a coverslip and allowed to stand for 1 d. The cells were then flipped upside down and consecutive photographs of the cells on the coverslip were taken with a differential interference microscope at 1.5 µm intervals from the adherent surface to the center of the cell body. The obtained photographs were reconstructed in 3D, in which orange shows scales, green shows axopodia, and light blue shows the cell body. For clarity, the lower half of the cell alone is depicted. As also shown in the reconstructed rotational movie (https://youtu.be/h30k9aFHcOk), the cell was attached to the coverslip using scales in addition to the tips of the axopodia. On the surface of the glass substratum, some scales were observed that had fallen off from the cell body and remained attached to the substratum (arrows).

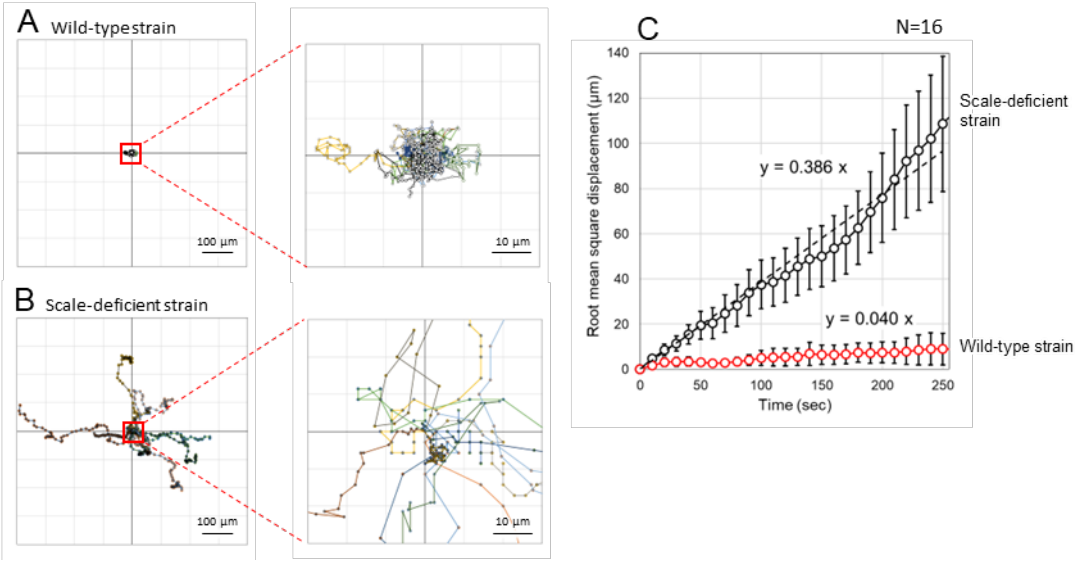

Fig. 6. The trajectory of the migratory movement of cells on the glass substratum traced at 10 s intervals for 10 min. The trajectories of 16 cells were superimposed using the cell position at the start of imaging as the origin. The central enlargement is shown on the right for wild-type (A) and scale-deficient strain (B), respectively. Wild-type cells show less movement than scale-deficient cells do. In C, root mean square (RMS) displacement of cells was plotted as a function of time. In both wild-type and scale-deficient cells, RMS displacement was linear, which is a characteristic of directed motion with cell migration velocities of 0.04 µm/s in the wild-type and 0.39 µm/s in the scale-deficient cells.