Morphological Analyses on the Siliceous Shell Formation Process and Search for Its Related Proteins in a Rhizarian Testate Amoeba *Paulinella chromatophora*

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Abstract

Paulinella chromatophora is a euglyphid testate amoeba. Most euglyphid species, including *P. chromatophora*, have a signature ovoid shell composed of siliceous scales. This shell is made by mother cell outside of itself and one of daughter cells moving into it during the cell division. It is a big mystery how a single cell can do such a complex task; forming siliceous scale and constructing a beautifully shaped new shell outside of the cell. *P. chromatophora* possesses a shell that is made of approximately 50 siliceous scales, each of which is different in size and shape. The scales are formed inside of mother cell and secreted out from the aperture of its shell. However, thus far, there are only a few studies on the detailed observation of shell formation process in *P. chromatophora* (Lauterborn 1895, Hoogenraad 1927, Kies 1974). The purpose of this study is to understand how they produce the siliceous scales in the mother cell and build up a new shell for a daughter cell.

To make detailed observations possible, I first established the new cultured strain (MYN1) of *P. chromatophora* that grows stably and quickly. This also enabled us to conduct biochemical experiment which needs a lot of cells.

In my transmission electron microscopic observations, I was able to confirm the reports from a previous study (Kies 1974) that the silica deposition occurred within the silica deposition vesicle (SDV), which was present just beneath the cell membrane at the posterior end of the cell, and

that scales were secreted out from its aperture, using cultured *P. chromatophora* cells (strain MYN1). In addition, I newly found that a small amount of silicon was detected from SDV containing premature scale that had not been deposited obvious silica yet, by qualitative elemental analysis using scanning transmission electron microscope. This indicates that the silica accumulation in the SDV is started at early phase of scale maturation process.

In the present study, I succeeded to observe the whole process of shell construction following a cultured single cell for the first time, using the light microscopic time-lapse recording. Siliceous scales, which were produced inside of the cell, were secreted out from its aperture. After the scale secretion that took more than 5 hours, the new shell began to take shape around its aperture. Scales were laid out one scale at a time using a thick specialized pseudopodium. This shell construction process took about 40 min from beginning of laying out scales to completion.

I also succeeded to observe all major stages of the shell construction under a transmission electron microscope (TEM). Mitochondria with tubular cristae, dense vesicles containing electron-dense substance and tubular membrane structures existed inside of the thick specialized pseudopodium during shell construction. Scales already laid out were attached each other with a cement-like substance. The scale that was about to be laid out was grasped by a branch of the thick pseudopodium and vesicles containing the electron-dense substance were observed near it. The scale that was considered to be laid out next step was grasped by the cell membrane at the

front edge of the thick pseudopodium. Other scales that would be laid out later steps were not grasped by the thick pseudopodium. These observations suggest that *P. chromatophora* might recognize each scale and lay out the scale to the specific position. It was revealed that *P. chromatophora* has a complicated unique shell construction process.

Silicic acid transporter (*SIT*) was firstly detected from diatoms. The SIT proteins in diatoms have 10 transmembrane helices and transport silicic acid with sodium ion as conjugate transport (Hildebrand 2008, Curnow et al. 2012). To search for SIT protein gene homologs in *P. chromatophora*, I conducted a transcriptome analysis and found sequences that have homology with diatom's SIT protein gene. The SIT of *P. chromatophora* was predicted to have only 5 transmembrane helices. Further investigation on the identification and comparison of SIT from other organisms is needed for understanding the evolution of silica-biomineralization.

In this study, scale production process inside of SDV was reconfirmed morphologically using clonal culture (strain MYN1) and was similar with other protists that produce siliceous structures (diatoms, chrysophytes and choanflagellates) and a gene for putative SIT, which transports silicic acid across a membrane previously identified in diatoms, chrysophytes belong to Stramenopile, was identified in *P. chromatophora* belongs to Rhizaria first. There were only a few studies on the unique shell formation in *P. chromatophora*. Success in observing whole shell construction process using light microscopic time-lapse recording and transmission electron microscope in this study provides a toehold for future studies for understanding when

and how *P. chromatophora* acquired the unique manner of shell construction and how it is regulated. These future studies will lead us to discover new functions and abilities of the cell.

Chapter 1: General Introduction

1-1. Origin of Silica-Biomineralization

Silicon is one of the most abundant elements on the earth and there are a lot of organisms that produce siliceous structures such as cell coverings and endoskeletons. This process of siliceous structure production is called the silica-biomineralization and the organisms that perform the silica-biomineralization are distributed in the different eukaryotic lineages (Fig. 1, Simpson and Volcani 1981, Preisig 1994). For example, gramineous plants deposit silica, which becomes to be called the plant opals (Yoshida et al. 1959), in the intercellular and intracellular structures, sponges, phylum Porifera produce siliceous spicules in a vesicle of the specialized cell called scleocyte (Garrone et al. 1981), and diatoms are well-known to produce siliceous cell-coverings called frustules. On the other hand, it is known that there are many rhizarian unicellular organisms that produce siliceous structures. Within the Rhizaria, the most members of Thaumatomonadidae produce siliceous scales to cover their cells (Karpov 1990, 1993, 2000, Karpov and Zhukov 1987, Moestrup 1982, Ota et al. 2012, Scoble and Cavalier-Smith 2014, Swale and Belcher 1974, 1975), the members of Ebriacea organisms have endoskeletons composed of several siliceous branched rods (Hargraves 2002), the members of radiolarians also have siliceous endoskeletons (Anderson 1976 a, b, c, Gamble 1909), and another lineage of Rhizaria, the euglyphids, produce pot-shaped shell composed of many siliceous scales (Ogden and Hedley 1980). However, the silica-biomineralization and the biogenesis of siliceous structures in the rhizarian organisms have not been

well-studied.

In the past decades, it has been suggested that the rhizarians and stramenopiles including diatoms form a monophyletic group (Adl et al. 2012). It has, therefore, become an interesting question whether the ability of silica-biomineralization was already present in the common ancestor of rhizarians and stramenopiles or each lineage acquired it independently. In addition, the rhizarian group of testate amoebae called the euglyphids has been suggested from a fossil record that it was the oldest group that had biomineralized siliceous shells (Porter et al. 2003). To understand the origin and the evolution of silica-biomineralizaion, studies on the silica-bomineralization in euglyphids are needed. However, there has been little study on the silica-biomineralization of euglyphids so far.

1-2. Diversity of Siliceous Structure Formation in Protists

Siliceous structures of diatoms, chrysophytes, choanoflagellates, rhizarian testate amoebae, silico-flagellates and radiolarians are produced commonly within the silicalemma-bounded silica deposition vesicle (SDV). It has been suggested that the silicalemma-bounded SDV has a role as a mold for the siliceous structure (Volcani 1981). It is still unclear, however, whether the silicalemma (SDV membrane) of all groups share same membrane features and functions or not. Even in the diatoms, which are well studied on the formation of siliceous frustule, there is no information about what nature the silicalemma has.

1-2-1. Frustule Formation in Diatoms

Diatoms are the most diversified group in the stramenopiles, and they have adapted to various environments from oceans to headwaters, and play an important role as primary producers (Nelson et al. 1995). Diatom frustules, which are often likened to a lunchbox, are composed of an epivalve (lunchbox cover), a hypovalve (box), and several zonate girdle bands that connect the epivalve and hypovalve (Round et al. 1990). The diatom cells usually divide into two cells asexually, and the cell division and frustule formation are well regulated. When a diatom cell divides, one of daughter cells become slightly smaller than the maternal cell, because newly formed valve always becomes the hypovalve, which has to be smaller than the epivalve. Before cell division, girdle band is elongated and cell grows up perpendicular to frustule. After nuclear division, the cytoplasm is divided in parallel with frustule and SDVs appear just beneath the cell membrane at the division plane. Like in other protists with siliceous structures, the frustule (hypovalve) formation also occurs within SDV in the cell. The place where the first SDV appears in the initial step of frustule formation is called "pattern center" that is close to microtubule organizing center (MTOC) and its position differs among species (Schmid and Volcani 1983). The SDV is elongated by the fusions of small vesicles, and silica deposition started from the pattern center (Chiappino and Volcani 1977). It is suggested that microtubule and actin filaments are major elements in the control of the silica morphogenesis by morphological observation and inhibitor experiments (Cohn et al. 1989, Schmid 1980, Tesson and Hildebrand 2010 a,

b). It is, therefore, suggested that microtubules and actin filaments must be involved in the formation of frustule.

The organic components that are related to siliceous frustule are known in diatoms (Hoagland et al. 1993, Pickett-Heaps et al. 1990). Later, the proteins and polyamine, called long chain polyamine (LCPA) (Kröger et al. 2000 a), silaffins (Kröger et al. 1999), silacidins (Wenzl et al. 2008) and cingulin (Scheffel et al. 2011), which are embedded within siliceous frustule, were identified (reviewed in Kroger and Poulsen 2008) and also proteins that are localized on siliceous structure had been discovered called frustulins (Kröger et al. 1996) and pleuralins (Kröger et al. 2000 b). It is considered that the regulation of these proteins enables to create elaborative siliceous frustule in diatoms.

1-2-2. Scale Formation of Chrysophytes

Chrysophytes are a group of biflagellate stramenopiles and have siliceous scales that cover the cells. The shape of the scale is usually discoidal and the genus *Mallomonas* have spine-like scales called bristle in addition to the normal discoid ones. Scales are partly overlapped each other at their rims. Scales and bristles are attached to each other and to a cell surface with a cement-like substance (Ludwig et al. 1996). Scales are doubled before cell division and two daughter cells inherit those scales equally (Lavau and Whetherbee 1994). The scale formation processes have been observed in the cells of *Synura petersenii* and *S. sphagnicola* under a transmission electron microscope (TEM), and it has been reported that silica

deposition starts within the SDV that is appressed to the chloroplast endoplasmic reticulum (Schnepf and Deichgräber 1969, Mignot and Brugerolle 1982). It has also been shown in a colorless chrysophyte flagellate, *Paraphysomonas* sp. that the silica deposition occurs within SDV that is appressed to the endoplasmic reticulum (Mignot and Brugerolle 1982).

1-2-3. Scale Formation in the Thaumatomonads

Thaumatomonads is one of flagellate groups that belong to the Imbricataea that includes the euglyphids. The cells of thaumatomonds bear two flagella and are covered with siliceous scales, which vary in shape from triangle to bobbin and disk-like structures depending on the species (Scoble and Cavalier-Smith 2014). This variety of scale forms is considered to have evolved from a simple plate-like scale that might have been present in the common ancestor of euglyphids and thaumatomonads (Scoble and Cavalier-Smith 2014). One of candidates for the ancestor of euglyphids and thaumtomonads is *Zoelucasa sablensis* that has simple disc-like siliceous scales like euglyphids, but also have two flagella like thaumatomonads (Nicholls 2012). However, the phylogentic position of *Z. sablensis* is still unknown, because its stable culture has not been established yet. Recently, it has been reported that a newly discovered thaumatomonad flagellate, *Esquamula lacrimiformis,* lacks siliceous scale. This has been explained that the scale has been lost secondarily, based on molecular phylogenetic evidences (Shiratori et al. 2012, Scoble and Cavalier-Smith 2014).

Scale formation in thaumatomonads also occurs in the putative SDV

that is appressed to the mitochondrion of which form can be changed by it (Karpov 1990, 1993, 2000, Karpov and Zhukov 1987, Moestrup 1982, Ota et al. 2012, Swale and Belcher 1974, 1975). Although it is known that siliceous scales that were produced near the mitochondria in other organisms, nothing is like the thaumatomonads in which the mitochondria is deformed by the attachment of SDV. The scales are secreted out and arranged on the surface of the own cell, like other organisms.

1-2-4. Shell Formation of Rhizarian Testate Amoebae

Testate amoebae that belong to Euglyphida (Imbricatea, Cercozoa, Rhizaria), have siliceous shell except one species (*Ovulinata parva*, Anderson et al. 1996, 1997). Their habitat is mainly from fresh water to brackish water and we can find them in lakes and soils. It is known that several species in the genus *Cyphoderia* and *Paulinella*, live in salt waters (Todorov et al. 2009, Johnson et al. 1988, Nicholls 2009). The siliceous shells of euglyphids are basically pot shape with one aperture and composed of small scales. Each scale is attached to the next scales with a cement-like substance. The form of the shell varies extensively among species, due to the differences in the scale shape and the position of aperture, which are used as taxonomic characteristics. It is known that scale shape is also diverse, tear-shaped, tear-shaped with spine, disc-like shape, rectangle and so on (Ogden and Hedley 1980).

Most members of the genus *Euglypha* have shells that are composed of two kinds of scales, one for the aperture that has many canine tooth-like

projections and the other with teardrop-shape for other parts of the shell. Some species have unique teardrop-shaped scales with spine (ex. *E. acanthophora*) or spines that come out from interspace between scales (ex. *E. filifera*) (Ogden and Hedley 1980).

Scale formation process is observed in *Assulina muscorum* (Anderson and Cowling 1994), *Euglypha rotunda* (Hedley and Ogden 1973), *Trinema lineare* (Hedley and Ogden 1974 b), *Paulinella chromatophora* (Kies 1974) and *Paulinella ovalis* (Johnson et al. 1988). These results suggest that the scales are produced within the putative silica deposition vesicle (SDV), as in the diatoms and other protists that produce siliceous structures. In the genus *Paulinella*, *P. chromatophora* and *P. ovalis*, a part of siliceous scale formation has been observed. The scales are formed within the putative SDV to which a row of microtubules was attached (Kies 1974, Johnson et al. 1988). It has also been reported in *P. ovalis* that the electron density of newly formed scales was low compared with maternal scales that contain manganese, iron and organic materials (Johnson et al. 1988).

Ovulinata parva, which is only eugliphid species that does not produce any siliceous structure, has been reported to form a sister relationship with the genus *Paulinella*. *O. parva*, therefore, was not considered as an ancestral euglyphid but regarded as euglyphids that lost the siliceous shell (Anderson et al. 1996, 1997, Howe et al. 2011).

1-3. Secretion and Arrangement of Mineralized Cell Covering in Protists

The secretion and arrangement of cell coverings is versatile in

protists that have mineralized scales. In most protists that have cell coverings, such as coccolithophores and chrysophytes, a cell secretes out and arranges the coverings around the surface of itself. The coccolithophores have calcareous plates called coccoliths as cell coverings. The coccolith secretion process has been observed in *Coccolithus pelagicus*, in which the coccolith is secreted out perpendicular to the plasma membrane, laid down and positioned on the surface of the cell (Taylor et al. 2007). The genus *Mallomonas* that belongs to the chrysophytes (Chrysophyceae/Synurophyceae) have siliceous bristles that are positioned at the posterior end of the cell surface separately from other scales. Bristles are formed at late interphase, secreted out from posterior end of a cell and re-orientated 180° in *Mallomonas splendes* (Beech et al. 1990). It is still unknown that how the cell can arrange scales in the precise positions in *Coccolithus pelagicus* and *Mallomonas splendes*.

Whereas the coccolithophores and chrysophytes secret scales around itself, it is known that rhizarian and amoebozoan testate amoebae create a new shell symmetrically (Hedley and Ogden 1973, 1974 a, b, Netzel 1972, Ogden 1979, 1989, Ogden and Hedley 1980). In the genus *Euglypha*, rhizarian testate amoebae, the scale formation (Hedley and Ogden 1973) and shell construction processes have been observed in *E. rotunda*, *E. acanthophora* and *E. strigosa* (Hedley and Ogden 1974 a, Netzel 1972). After scale secretion, the part of the cytoplasm comes out from the aperture, like a budding, and elongates into the secreted scales. At the same time, the scales are attached each other from the aperture side and the new shell is formed

(Hedley and Ogden 1974 a, Netzel 1972). It has been considered that microtubules and microfilaments are involved in the scale arrangement (Hedley and Ogden 1974 a).

1-4. *Paulinella chromatophora*

Paulinella chromatophora that belongs to Euglyphida (Imbricatea, Cercozoa, Rhizaria) has a shell which is constructed from a number of siliceous small scales. Amoeboid cell of *P. chromatophora* is always inside of its shell, put out filose pseudopodia from aperture of a shell and creep around the bottom. They are the only euglyphids that can grow under the autotrophic condition, because they have photosynthetic organelle called cyanelle or chromatophore (Fig. 2-A arrow, Hoogenraad 1927, Hoogenraad and De Groot 1927, Kies 1974, Kies and Kremer 1979, Lauterborn 1895). Recently, this photosynthetic organelle originated from a different cyanobacterium from that gave rise to the well-known plastids was revealed (Marin et al. 2005, Nakayama and Ishida 2009, Nowack et al. 2008). Therefore, *P. chromatophora* has been a focus of attention as a model of intermediate stage of integration of endosymbiont as an organelle during primary endosymbiosis.

The *P. chromatophora* shell is composed of approximately 50 siliceous scales that are precisely arranged in five rows (Fig. 2-B, Hoogenraad 1927, Hoogenraad and De Groot 1927, Kies 1974, Yoon et al. 2009). The scales are rectangular in shape, slightly curved longitudinally (Fig. 5, 6, Hoogenraad 1927, Hoogenraad and De Groot 1927, Kies 1974, Yoon

et al. 2009), and contain numerous large depressions on the concave side (Fig. 5-C, D arrowhead) and small holes on the convex side (Fig. 5-A, B, D arrows, Kies 1974, Yoon et al. 2009). The scales vary in size and shape depending on their position in the shell. The scales in the middle region are large, those near the aperture are small and distinctly shaped, and those near the round end are small and ornamented (Fig. 5-A double arrowhead, Kies 1974, Yoon et al. 2009).

Morphological studies on *P. chromatophora* are limited. There are only three published articles; species description in Lauterborn (1895), light microscopic observation in Hoogenraad (1927) and transmission electron microscopic observation in Kies (1974). These articles mentioned that *P. chromatophora* have a unique cell division process in which the mother cell constructs a new shell for one of daughter cells before cell division. Study on siliceous shell formation process has been neglected, though several researchers are interested in it, because a stable and well-grow culture of *P. chromatophora* has been absent.

1-5. Purpose of This Study

The shell formation process in euglyphids is really mysterious and interesting. There seems to be some highly regulated mechanisms. *P. chromatophora* is the only autotrophic organism in the euglyphids and should be able to establish a stable and well-grow culture. Structure of siliceous shell of *P. chromatophora* is featured as rectangular shape scales and these scales can be characterized by each morphologic character as

mentioned above. For further investigation, *P. chromatophora* can be a good material to know the shell formation process of euglyphids. Consequently, purpose of this study is to reveal shell formation process of *P. chromatophora* at both morphologically and molecular level.

Chapter 2: Observation of Siliceous Scale Production and Shell Construction Processes

2-1. Introduction

Siliceous structure formation usually occurs within the silica deposition vesicle (SDV) in protists and sponges. The scale formation of *P. chromatophora* also occurs within the SDV that is located just beneath the cell membrane and near the nucleus and the Golgi body. A single layer of microtubule's row is tightly associated with the SDV (Kies 1974). However, these information were obtained by gathering fragmentary observations from 21 individual cells in a natural sample (Kies 1974). Therefore it has not been confirmed that these features are constantly observable under clonal cultured condition and detailed process of scale formation is still unknown.

It has been said that *P. chromatophora* cell is covered with "siliceous shell", however, the chemical components of the shell has not been investigated. The presence of silicon in the SDV during the scale formation has not also been studied, although it has been said that the young SDV that looks empty contains organic materials (Kies 1974). As described in the chapter 1, the shell of *P. chromatophora* is composed of five sets of ten different scales, but the order of scale formation is still unknown. It would be interesting to know what order the scales with different sizes are formed in the SDV.

Lauterborn (1895) and Hoogenraad (1927) observed *P. chromatophora* cells with a newly formed empty shell whose aperture faced

the aperture of the old shell. They found that the empty shell was subsequently occupied by one of the two daughter cells. Through these fragmentary observations, they speculated that *P. chromatophora* produces a new shell before cell division and that a daughter cell moves into this shell after cell division (Hoogenraad 1927, Kies 1974, Lauterborn 1895). Other euglyphids also produce new shells for daughter cells before cell division (Hedley and Ogden 1974 a, b, Netzel 1972, Ogden 1979). However, the process of the mother cell's construction of an entirely new empty shell for a daughter cell before cell division remains to be elucidated. In this study, I used time-lapse video microscopy, fluorescent microscopy and electron microscopy to observe the scale formation and shell construction processes in a newly established culture (MYN1) of *P. chromatophora* that was isolated from a pond water.

2-2. Materials and Methods

2-2-1. Establishment of New Cultured Strains for *Paulinella chromatophora*

A water sample was collected from a pond in Tsukuba, Ibaraki, Japan (36.0485N, 140.1190E) on October 9, 2009. Several *P. chromatophora* cells were isolated from the sample with a micropipette, placed in a well of a microplate and cultivate the cells in AF-6 culture medium (Kato 1982) for a few days. Non-axenic cultured strains of *P. chromatophora* (MYN1, 2) from the primary culture sample were established using a single-cell isolation technique. The strains MYN1 and MYN2 were maintained in a modified

Waris-H+Si medium (McFadden and Melkonian 1986) where nutrients were reduced to one-half and Si was reduced to one-fifth of the concentration from the original formulation. The cultures were kept at 20°C under a 14 h light/10 h dark cycle. For further observations, the MYN1 strain was used.

2-2-2. Light Microscopy and Time-lapse Video Recording

The light microscopic examination of MYN1 cells was performed using a Zeiss Axio Imager.A2 (Carl Zeiss AG, Jena, Germany) equipped with differential interference contrast (DIC) optics. Images were taken with a Keyence VB-6010 CCD color camera (Keyence, Osaka, Japan). For time-lapse video microscopy, cells in both the crude sample and the MYN1 culture were used and video images were taken with an Olympus DP71 CCD color camera (Olympus, Tokyo, Japan) with intervals of 4, 10, and 30 s under an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan). In this observation, low light intensity and a high camera detection range were applied to reduce cell damage. Sequential images were edited and assembled into movie files using Windows Movie Maker (Microsoft, Washington, USA). The movie file was converted to an AVI format file using XMedia Recode (XMedia Recode, Germany) and edited using AviUtl (http://spring-fragrance.mints.ne.jp/aviutl/aviutl99m.zip).

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Figures

Figure 1.
Schematic phylogenetic tree showing the phylogenetic relationship of the eukaryotes (based on Adl et al. 2012). The red circles indicate the lineages containing siliceous structure producers.

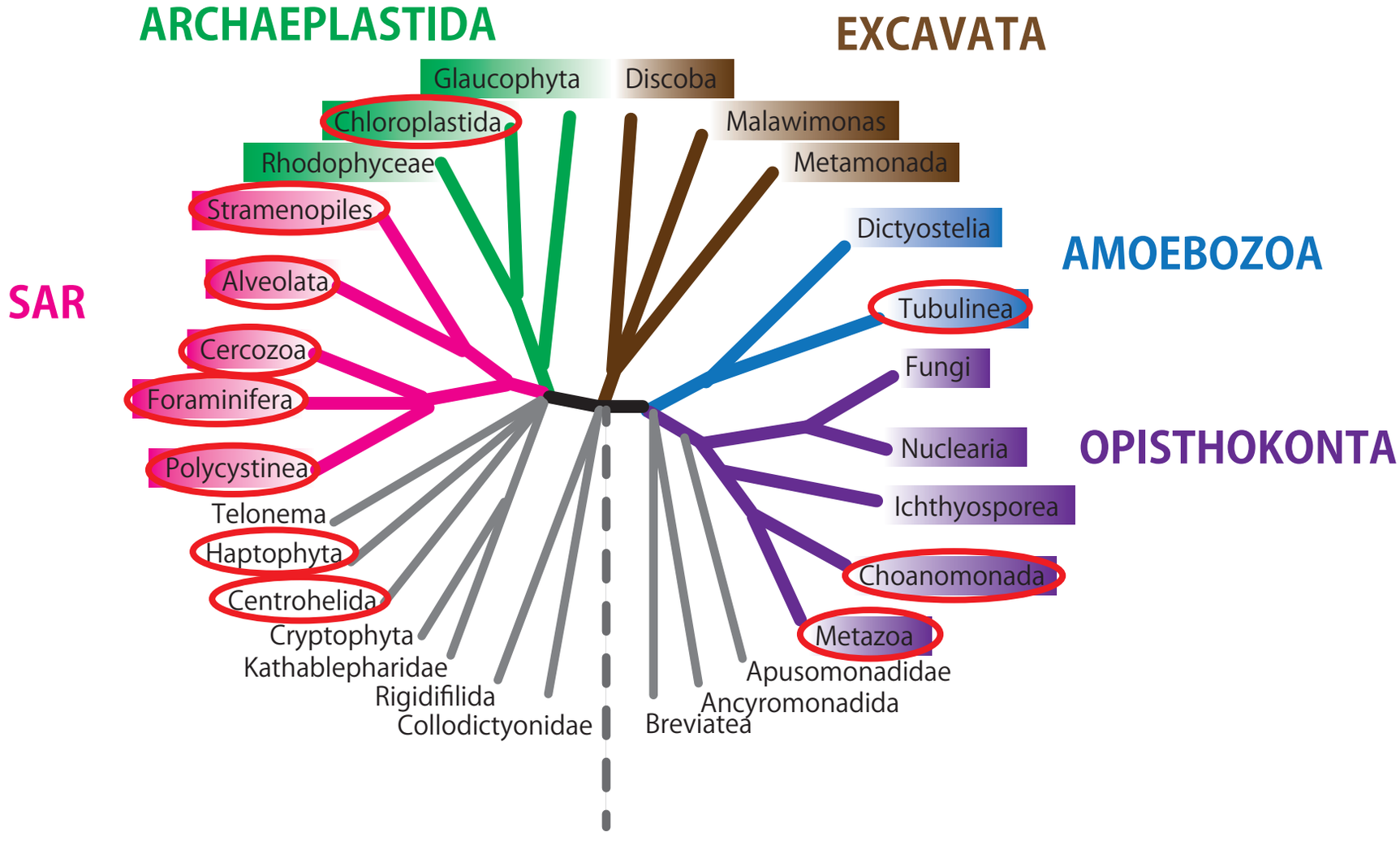

ARCHAEPLASTIDA
EXCAVATA
Glaucophyta
Discoba
Chloroplastida
Malawimonas
Rhodophyceae
Metamonada
Stramenopiles
Dictyostelia
AMOEBOZOA
SAR
Alveolata
Tubulinea
Cercozoa
Fungi
Foraminifera
Nucleariа
OPISTHOKONTA
Polycystinea
Ichthyosporea
Telonema
Haptophyta
Choanomonada
Centrohelida
Metazoa
Cryptophyta
Apusomonadidae
Kathablepharidae
Ancyromonadida
Rigidifilida
Breviatea
Collodictyonidae

Figure 2.

Morphology of *Paulinella chromatophora* (MYN1 strain). (**A**) Differential interference contrast (DIC) micrograph of a *P. chromatophora* cell showing an egg-shaped siliceous shell (arrowhead) and 2 blue-green chromatophores (arrows). (**B**) Scanning electron micrograph of a shell showing the regular arrangement of siliceous scales that differ in size and form: scales near the aperture (white arrowhead) are small, scales in the middle region are large, and scales near the round end (white arrow) are small and ornamented. Scale bars: 10 μm

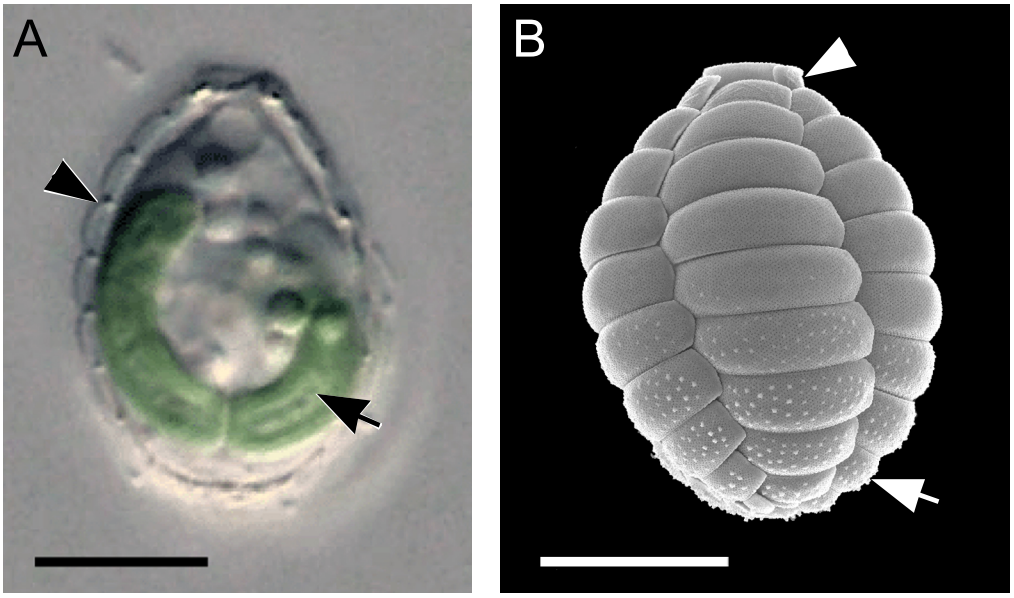

A
B

Figure 3.
Variety of *P. chromatophora* cell morphology. (A) Filose pseudopodia come out from the aperture (arrows). (B) Two *P. chromatophora* cells attached each other at their apertures. (C, D) Large colony of *P. chromatophora*. Scale bars: 5 μm in (A, B), 10 μm in (C, D).

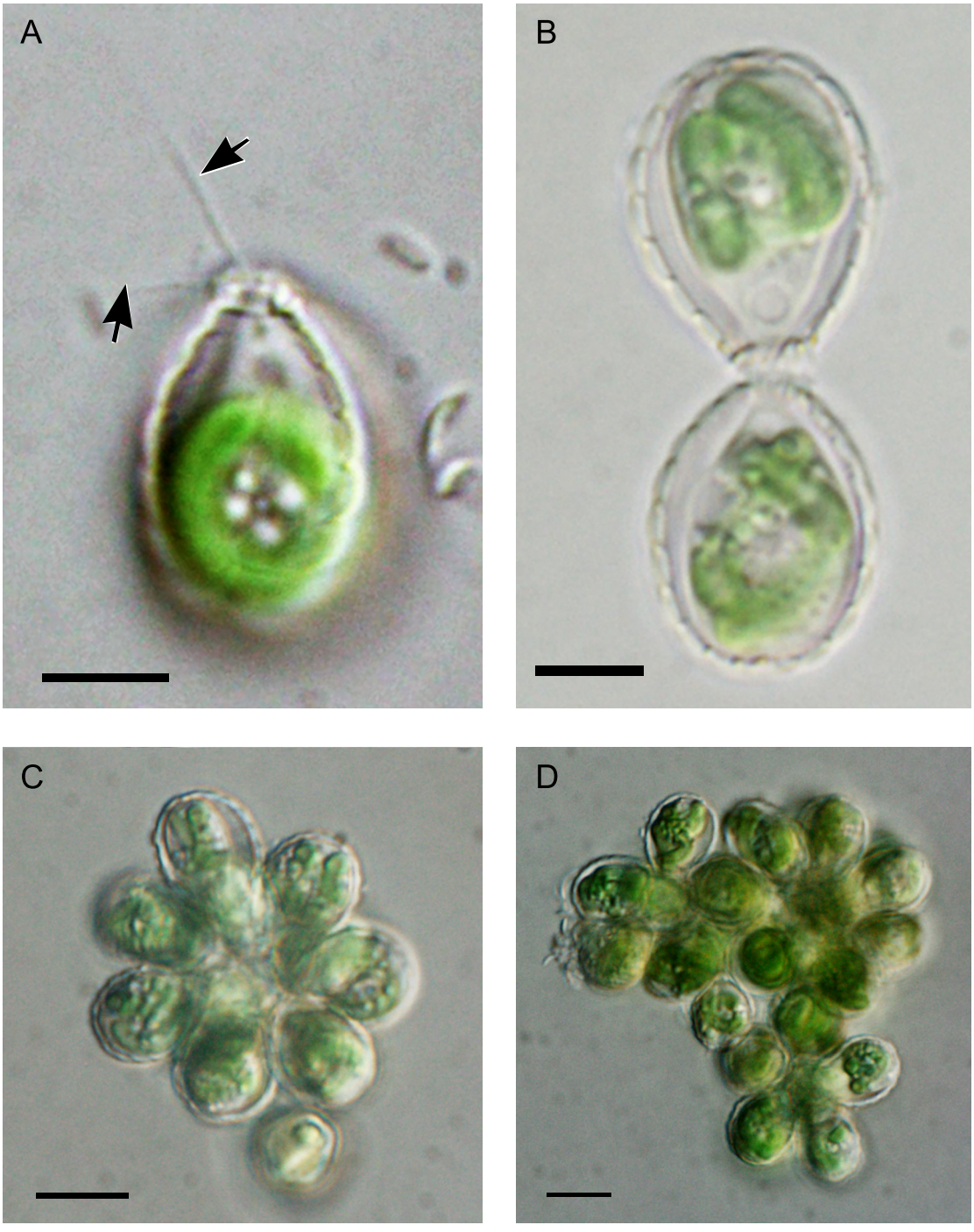

A
B
C
D

Figure 6.
Schematic image of a siliceous scale. The siliceous scale is composed of three layers, surface side, sponge like structure and reverse side. Silica is heavily deposited on the layers of surface and reverse sides.

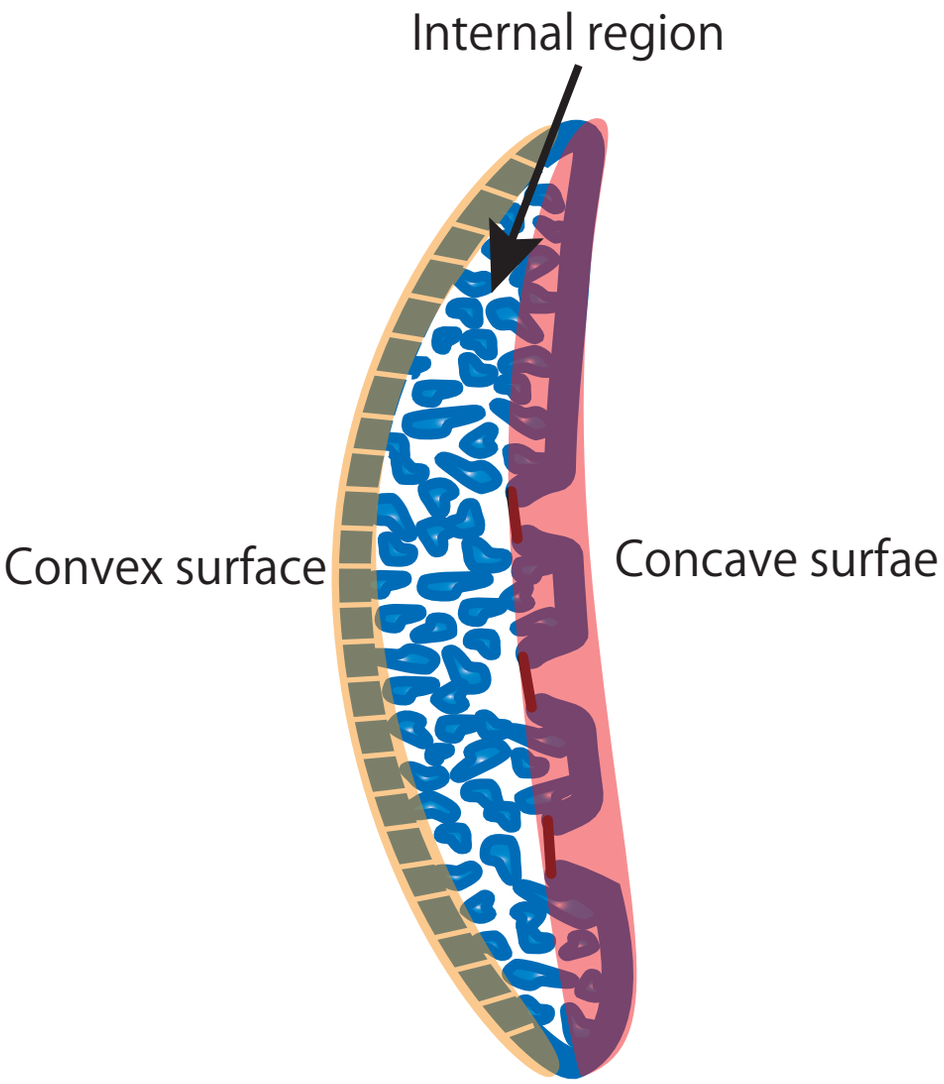

Internal region
Convex surface
Concave surfae

Figure 10.
Fluorescence micrographs of *P. chromatophora* cells stained by LysoTracker (A-2, B-2, C-2, D-2) and DIC images (A-1, B-1, C-1, D-1). Fluorescence images through 585 nm long-pass filters (A-2, B-2, C-2, D-2) show siliceous scales formed after the addition of LysoTracker (green). (A-1, A-2): A cell producing new scales in the cell. Newly formed scales were inside of the cell. (B-1, B-2): A cell secreting new scales. One of newly formed scales was secreted out from the aperture. (C-1, C-2): A cell with a newly constructed shell. The half of scales in the shell were stained. (D-1, D-2): Another cell with a newly constructed shell. Fewer scales in the shell were stained. These suggest newly formed scales are placed on the posterior side of a shell.

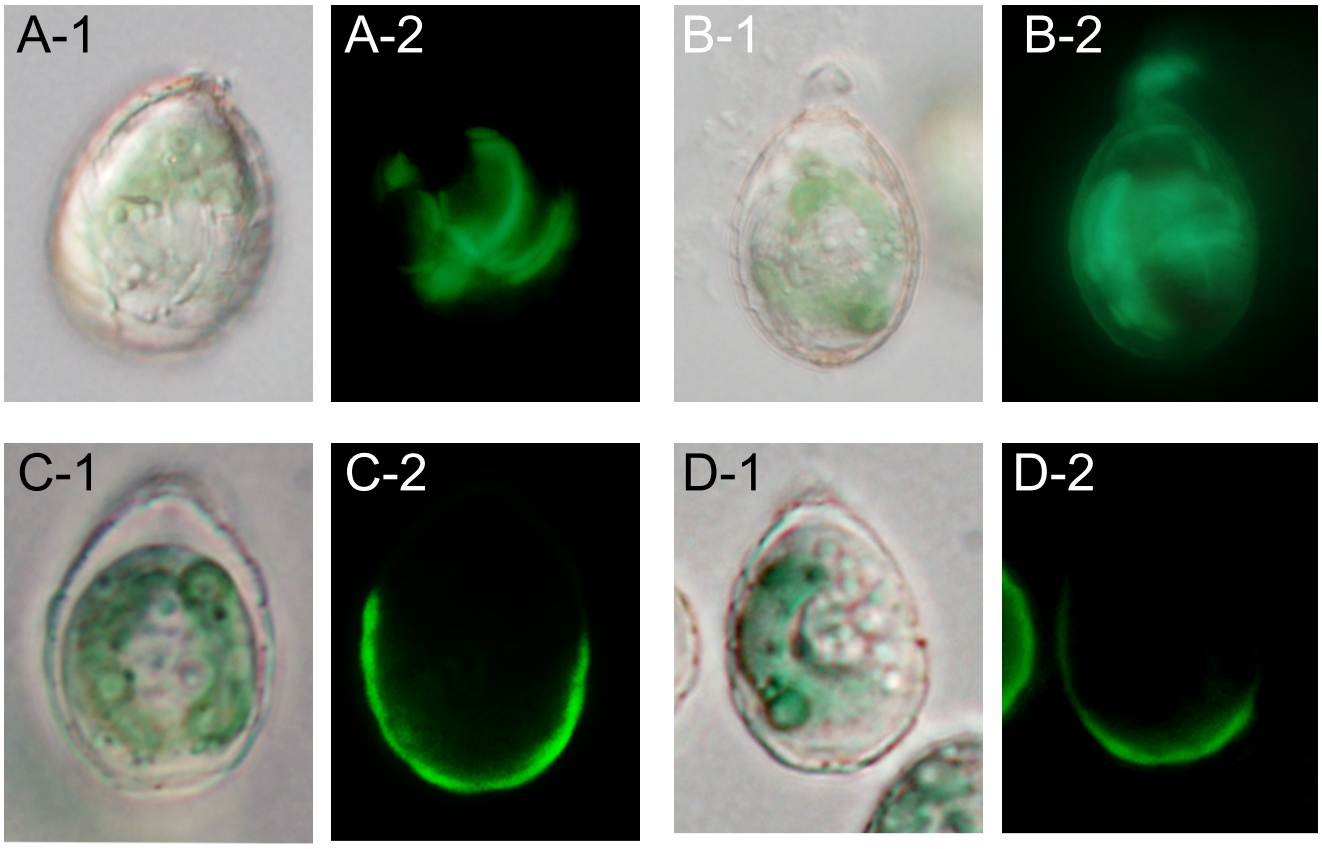

A-1
A-2
B-1
B-2
C-1
C-2
D-1
D-2

Figure 11.
Selected frame shots from a time-lapsed video clip showing the outline of the shell construction process. Assembly of the scales into a new shell took approximately 40 min. (A) A cell in an early stage of shell construction showing the mass of secreted scales (arrowhead) retained near the aperture (arrow). (B) A cell laying out scales into their correct positions using a thick pseudopodium (arrowhead). (C) A cell with a completed new empty shell showing the thick pseudopodium withdrawn into the mother shell. (D) A frame shot approximately 10 min after (C) showing a daughter cell (arrowhead) moving into the new shell constructed by the mother cell. Scale bar: 10 μm.

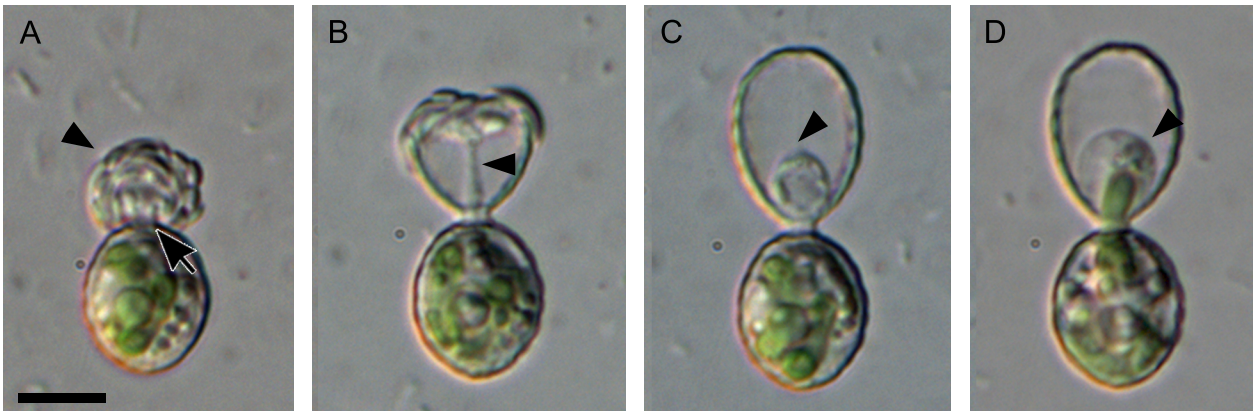

A
B
C
D

Figure 13.
Confocal fluorescent microscopic images of cells stained by the LysoTracker. (A-1): Early stage of scale secretion showing a few large scales held near the aperture. (A-2): A DIC image of A-1. (B-1): Late stage of scale secretion showing large scales arranged in lengthways. (B-2): A DIC image of B-1. Scale bars: 5 μm.

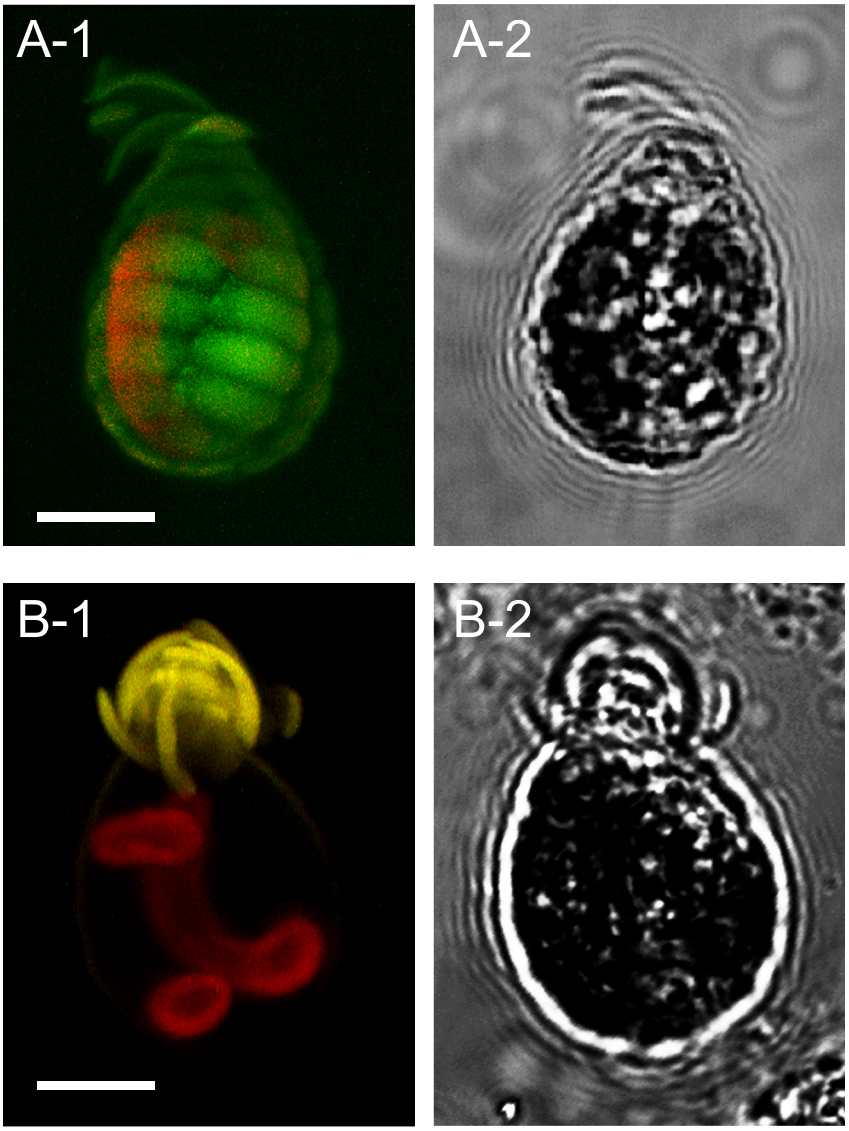

A-1
A-2
B-1
B-2

Figure 14.
Sequential frames extracted from a time-lapsed video clip showing a scale (arrowhead) in the mass of scales being flipped, re-oriented, and returned to the mass. The number at the lower right-hand corner of each frame indicates the time in seconds after frame A. Scale bar: 2 μm.

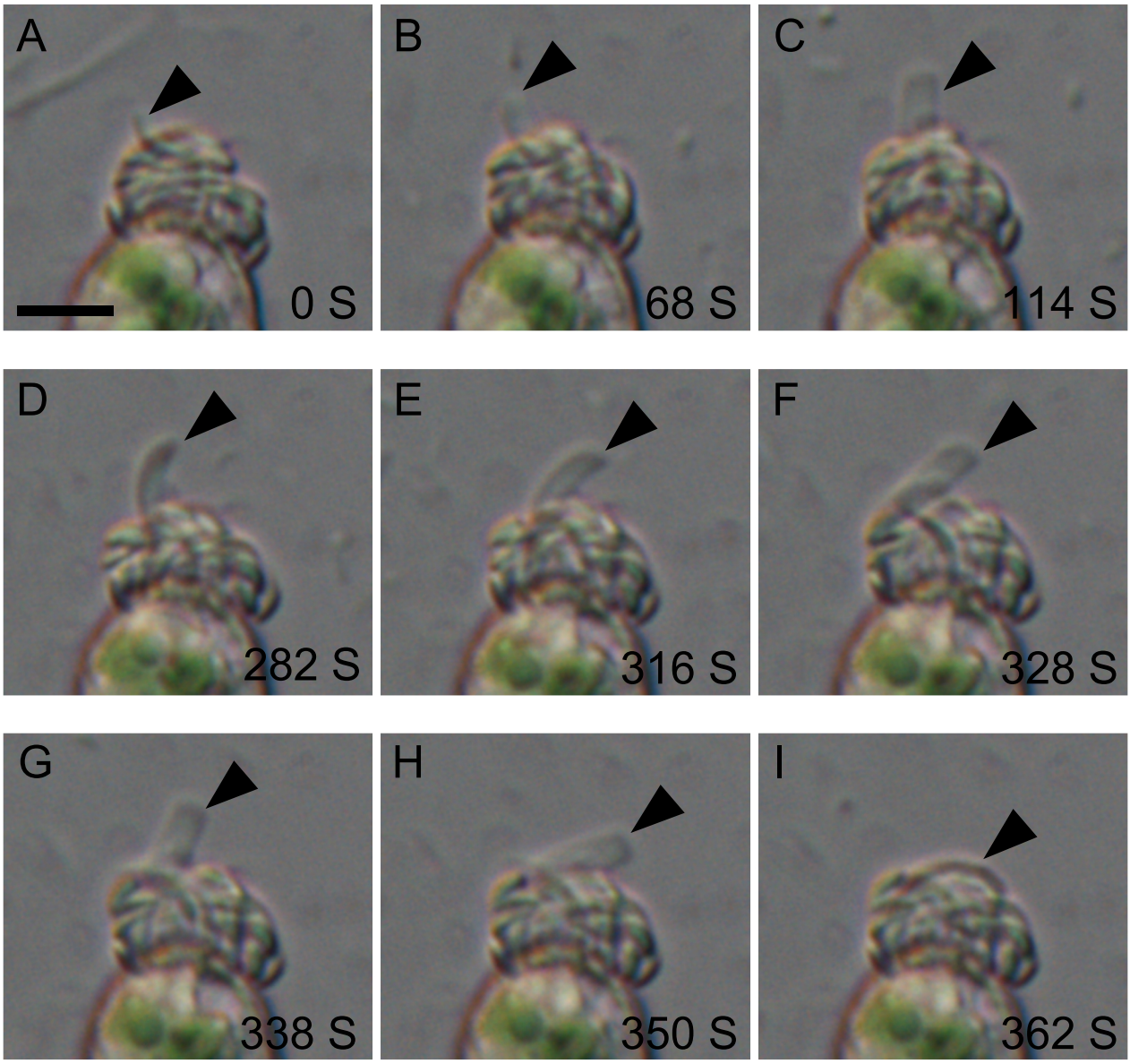

A
0 S
B
68 S
C
114 S
D
282 S
E
316 S
F
328 S
G
338 S
H
350 S
I
362 S

Figure 15.
Sequential frames extracted from a time-lapsed video clip showing shell construction process. Scales were laid out alternation from side to side. The white and black line and the number within arrowhead indicated that each layer of a shell.

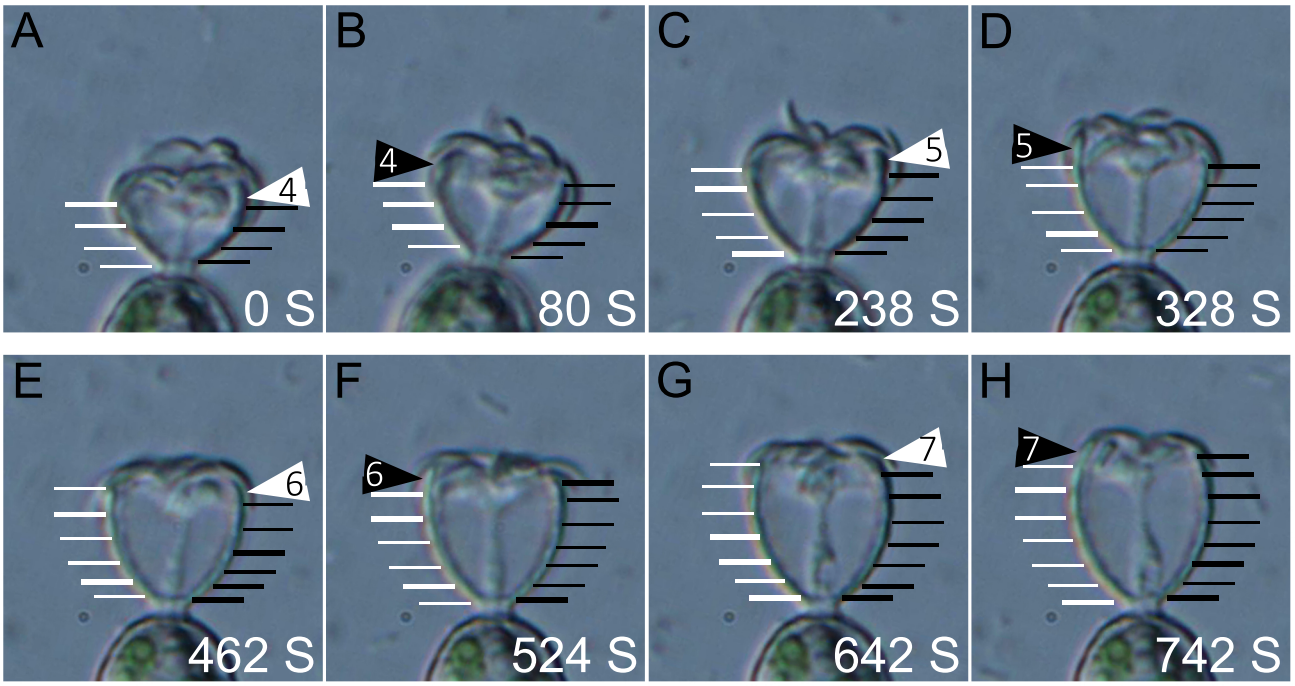

A
4
0 S
B
4
80 S
C
5
238 S
D
5
328 S
E
6
462 S
F
6
524 S
G
7
642 S
H
7
742 S

Figure 16.
Sequential frames extracted from a time-lapsed video clip showing one scale (arrowhead) being moved and laid out next to a scale that has already been assembled and another scale (arrow) being moved towards the next position. Scale bar: 1 μm.

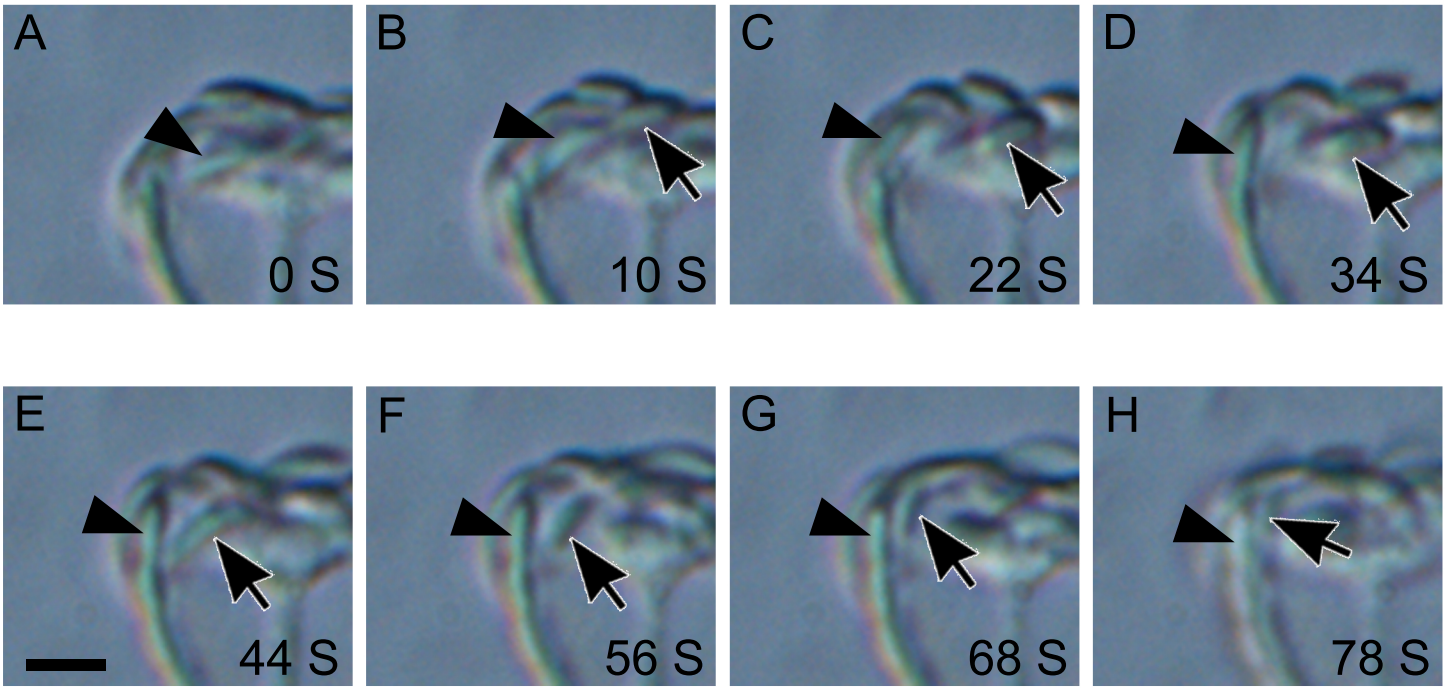
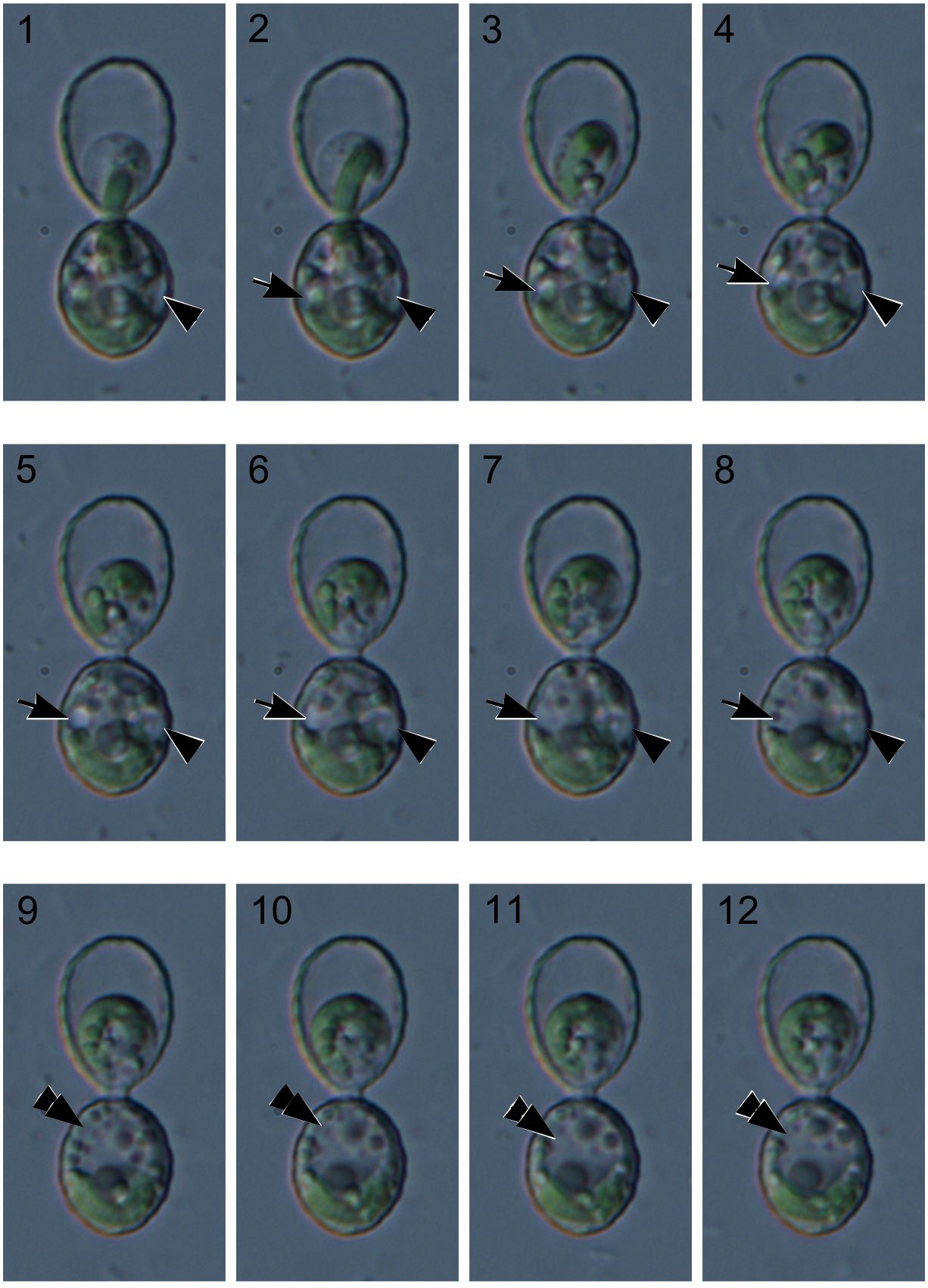

A
0 S
B
10 S
C
22 S
D
34 S
E
44 S
F
56 S
G
68 S
H
78 S

Figure 17.
Sequential frames extracted from a time-lapsed video clip showing vacuoles that are indicated arrows and arrowheads in the maternal cell. Two separated vacuoles are fused and became single large vacuole (double arrowhead).


1
2
3
4
5
6
7
8
9
10
11
12