First Direct Evidence for a Structurally Stable Adhesion Between the Perialgal Vacuole Membrane and Host Mitochondria in the *Paramecium-Chlorella* Endosymbiosis

Masahiro Fujishima * and Sho Nishiyama

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Article

First Direct Evidence for a Structurally Stable Adhesion Between the Perialgal Vacuole Membrane and Host Mitochondria in the *Paramecium-Chlorella* Endosymbiosis

Masahiro Fujishima [1,2,*] and Sho Nishiyama [3]

[1] Research Center for Thermotolerant Microbial Resources, Yamaguchi University, Yoshida 1677-1, Yamaguchi 753-8515, Japan

[2] Institute of Environmental Radioactivity, Fukushima University, Kanayagawa 1, Fukushima 960-1296, Japan

[3] Department of Biology and Chemistry, Faculty of Science, Yamaguchi University, Yoshida 1677-1, Yamaguchi 753-8512, Japan

* Correspondence: fujishim@yamaguchi-u.ac.jp

Abstract

Physical integration between endosymbiotic algae and host mitochondria is a recurring feature across photosynthetic symbioses, yet the structural nature of this association has remained unresolved. In the ciliate *Paramecium bursaria*, each endosymbiotic Chlorella cell is enclosed by a perialgal vacuole (PV) membrane consistently surrounded by host mitochondria, suggesting a conserved architecture for metabolic interaction. Although transmission electron microscopy has shown close membrane apposition, it has remained unclear whether this reflects incidental proximity or a reinforced adhesion. Here, we provide direct evidence that the PV membrane and host mitochondrial membrane form a stable physical association. Using discontinuous Percoll density-gradient centrifugation, we isolated intact units in which *Chlorella* and mitochondria co-sedimented, indicating that their association withstands mechanical disruption. By fluorescently labeling the PV and mitochondrial membranes with BODIPY FL C_5-ceramide complexed to BSA, together with a mitochondria-specific monoclonal antibody and DAPI, we visualized the PV membrane under light microscopy and demonstrated that the mitochondrial–PV membrane complex persists after homogenization and centrifugation. As expected from the membrane-insertion behavior of BC_5C, this fluorescent labeling revealed that the PV–mitochondrial membrane association is structurally reinforced rather than incidental, providing a mechanistic framework for understanding how *Chlorella* cells are stably positioned beneath the host cortex.

Keywords: endosymbiotic *Chlorella*; PV membrane; mitochondrial membrane; mitochondria–PV membrane complex; *Paramecium bursaria*; BODIPY FL C_5-ceramide; mitochondria-specific monoclonal antibody

1. Introduction

Photosynthetic endosymbiosis represents one of the most consequential innovations in eukaryotic evolution, enabling heterotrophic hosts to acquire photosynthetic capacity by incorporating phototrophic partners. The ciliate *Paramecium bursaria* is a well-established model for studying this process, as it harbors several hundred endosymbiotic *Chlorella* cells [1], each enclosed within a perialgal vacuole (PV) that physically separates the alga from the host cytoplasm. Although *P. bursaria* can survive without algae, the acquisition of photosynthetic symbionts confers substantial ecological advantages, including enhanced starvation tolerance [2–4], increased thermal [5] and

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hypoxic resistance [6], and access to photosynthetically derived maltose and oxygen [7–9]. Thus, the ability to maintain algae in a stable cortical position is a key adaptation that enhances host fitness.

After escaping from the host digestive vacuole (DV) by budding of the DV membrane, compatible algae migrate to the subcortical region and become immobilized. During this transition, the DV-derived membrane differentiates into the PV membrane, loses acid phosphatase activity, and prevents lysosomal fusion, thereby ensuring algal survival [10–13]. Immobilization beneath the cortex is essential not only for protection from digestion but also for reliable partitioning of algae into daughter cells during host division [14] and for shielding the symbionts from ultraviolet radiation [15]. In contrast, infection-incapable *Chlorella* species fail to establish this cortical attachment and are ultimately digested [16].

The consistent positioning of endosymbiotic algae near the host mitochondria just beneath the host cell surface is not unique to *P. bursaria*. Similar spatial arrangements have been documented in diverse protists [17] and other photosynthetic endosymbioses, such as coral–*Symbiodiniaceae* associations [18,19], sea anemones [20], giant clams (*Tridacna*) [21] and even kleptoplastic gastropods [22]. These observations suggest that the close apposition of host mitochondria to intracellular phototrophs may represent a recurrent architectural motif that facilitates metabolic exchange in photosynthetic symbioses.

In *P. bursaria*, transmission electron microscopy (TEM) studies have repeatedly shown that the PV membrane appears to be in direct contact with the outer membrane of host mitochondria [23–28]. Cryofixation analyses further revealed that mitochondria not only contact the PV membrane but also extend toward the algal cell wall, forming networks interconnected with other mitochondria and the host endoplasmic reticulum [29]. However, TEM alone cannot determine whether this association reflects incidental proximity or a structurally stable adhesion. This distinction is critical because a naturally occurring mutant isolated from the field exhibits defective cortical attachment of *Chlorella*, resulting in unstable inheritance of symbionts during host cell division and eventual loss of algae [14]. This phenotype provides strong evidence that PV–mitochondrion adhesion is essential for stable symbiont retention.

Physiological observations also indicate that this association is dynamically maintained. When living *P. bursaria* cells are subjected to centrifugal force, PV-enclosed algae detach from the cortex and accumulate at the posterior end of the cell, yet they reattach to their original subcortical positions within 15 min after centrifugation ceases [1]. This rapid recovery implies the presence of an active mechanism that restores PV–cortex association, but the structural basis of this process has remained unresolved.

A definitive test of whether the PV membrane and host mitochondria are truly adhered is to determine whether their association persists after mechanical disruption of host cells. If the two membranes are tightly attached rather than merely adjacent, they should remain connected even after homogenization and density-gradient centrifugation. In this study, we examined whether mitochondria remain associated with isolated PV-enclosed *Chlorella* cells following mechanical disruption by combining a newly developed isolation condition for symbiotic *Chlorella* cells from *P. bursaria* homogenates and a newly developed fluorescence labeling technique for the PV membrane and the host mitochondrial membrane. Using this combined approach, we provide direct evidence that the PV–mitochondrion association represents a physically stable membrane interaction rather than incidental proximity.

2. Materials and Methods

2.1. Strains and Cultures

The *Chlorella variabilis*-bearing (symbiotic) *Paramecium bursaria* strain Yad1g1N (syngen 1, mating type I) and the *Chlorella*-free (aposymbiotic) strain Yad1w were used in this study. The original Yad1g strain was collected from a pond at Yamaguchi University, Yoshida Campus, Japan,

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by Ayako Nishimura in 2004, and the aposymbiotic strain Yad1w was generated from Yad1g [12]. The symbiotic strain Yad1g1N was later established by infecting Yad1w cells with cloned symbiotic *C. variabilis* strain 1N [24]. The *C. variabilis* strain 1N was cloned by Dr. Miho Nakahara-Tsubota from *P. bursaria* strain OS1g (syngen 1, mating type I), originally collected by Dr. Isoji Miwa (Ibaraki University) from Itako City, Japan, in 2002.

Symbiotic and aposymbiotic paramecia were cultivated in glass test tubes (18 × 180 mm) containing modified Dryl's solution (MDS; KH_2PO_4 substituted for $NaH_2PO_4{\cdot}2H_2O$) [32,33] supplemented with 1.25% (w/v) fresh lettuce juice and 0.0001% (w/v) stigmasterol (Tama Biochemical Co., Ltd., Tokyo, Japan) at 25 ± 1 °C. The medium was inoculated with the non-pathogenic *Klebsiella pneumoniae* strain 6081 one day before use [33]. For routine culture, several hundred cells were inoculated into 2 mL of medium, and 2 mL of fresh medium was added daily for 12 days. One day after the final feeding, cultures reached early stationary phase, and cells at this stage were used for all experiments.

All strains were maintained in the Fujishima laboratory (Yamaguchi University, Japan) and subsequently deposited in the National BioResource Project *Paramecium* (NBRP–*Paramecium*, http://nbrpcms.nig.ac.jp/paramecium/).

2.2. Isolation of Symbiotic Algae Possessing PV Membranes and Mitochondria by Discontinuous Percoll Density-Gradient Centrifugation

Cultures of *P. bursaria* (approximately 600 mL) in early stationary phase were filtered through two layers of Kimwipes (Kimberly-Clark) to remove debris and centrifuged at 300 × g for 3 min at room temperature using an oil-test centrifuge (Kokusan H-210A) with 100-mL oil-separation glass tubes. The pellet was washed once with ice-chilled MDS under the same conditions, resuspended in 2.5 mL of ice-cold homogenization buffer (200 mM sucrose, 10 mM Na/K phosphate buffer, pH 6.5), and transferred to a pre-chilled 1-mL Teflon homogenizer. Cells were gently disrupted by five strokes of the pestle on ice.

The homogenate was layered onto a discontinuous Percoll (Cytiva, Uppsala, Sweden) gradient (2.5 mL of 75% (v/v) Percoll overlaid with 2.5 mL of 45% (v/v) Percoll) prepared in 12-mL centrifuge tubes (Nalgene 3110-0120PK) using stock isotonic Percoll (SIP; 100% (v/v) Percoll mixed with 2.5 M sucrose at 9:1). SIP was diluted with 250 mM sucrose to prepare the gradient. Centrifugation was performed at 600 × g for 25 min at 4 °C using a TS-7 swing rotor in an RS-18IV centrifuge (Tomy). The green *Chlorella*-containing band at the 75%/45% interface was collected with a Pasteur pipette, counted using a hemocytometer, and stored at 4 °C until use.

2.3. Routine Isolation of Symbiotic Chlorella

For routine isolation of symbiotic algae for infection experiments, cultures of *P. bursaria* (approximately 600 mL) were filtered through two layers of Kimwipes and concentrated using a 50-mL plastic centrifuge tube fitted with a 15-μm nylon mesh. Cells were washed with MDS on the mesh, concentrated to 1 mL, transferred to a 1-mL Teflon homogenizer, and disrupted by 10 strokes of the pestle on ice. The homogenate was passed through a new mesh to remove debris while allowing *Chlorella* cells to pass into the filtrate. The filtrate was centrifuged at 4,355 × g for 30 s at 25 ± 1 °C (TAITEC CR-12), washed three times with 1.5 mL of MDS, and stored at 4 °C in the dark until use.

2.4. Production of Monoclonal Antibodies Against P. bursaria Mitochondria

A mitochondrion-specific monoclonal antibody was produced by immunizing BALB/c mice (4–5 weeks old) with the symbiotic algae–mitochondria fraction obtained by discontinuous Percoll centrifugation. Mice received intraperitoneal injections of antigen three times at two-week intervals. For the first immunization, the antigen was mixed with an equal volume of BACTO Freund's complete adjuvant (Difco). For the second and third immunizations, the antigen was mixed with BACTO Freund's incomplete adjuvant (Difco). Hybridomas producing the desired antibody were

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screened by indirect immunofluorescence and limiting dilution [34]. The monoclonal antibody mAb-3G11E3F7 was used in this study.

Hybridoma production followed the institutional guidelines for animal use in research at Yamaguchi University.

2.5. Indirect Immunofluorescence Microscopy

P. bursaria cells in early stationary phase were air-dried on coverslips (4.5 × 24 mm, Matsunami), fixed with cold 4% (w/v) paraformaldehyde in PBS (137 mM NaCl, 2.68 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, pH 7.2) for 15 min, treated with cold PBST (PBS containing 0.05% (v/v) Tween-20) for 10 min, and washed twice with PBS. Cells were incubated with hybridoma culture supernatant containing the primary antibody (mAb-3G11E3F7) for 60 min at room temperature, washed twice with PBS, and incubated with Alexa Fluor 488–conjugated goat anti-mouse IgG (1:1000; Molecular Probes) for 60 min. After two PBS washes, cells were stained with 0.001% (w/v) DAPI for 5 min and washed again. Samples were examined using DIC and fluorescence microscopy (Olympus BX60). For isolated *Chlorella* enclosed by PV membranes and mitochondria, PBST washing after fixation was replaced with PBS washing.

2.6. Fluorescent Staining of the PV Membrane and Mitochondrial Membrane

The PV membrane surrounding symbiotic *Chlorella* and the mitochondrial membrane co-sedimented with the algae were stained with BODIPY FL C_5-ceramide complexed to BSA (BC_5C/BSA; Invitrogen B22650). A 50 μM stock solution was prepared in deionized water, stored at 4 °C, and diluted to 5 μM immediately before use. The diluted BC_5C/BSA was added to the isolated *Chlorella*–mitochondria fractions and incubated for 30 min at room temperature in the dark before fluorescence microscopy. When required, DAPI was added to a final concentration of 0.001% (w/v).

3. Results

3.1. Intracellular Distribution of Mitochondria

To visualize the intracellular distribution of mitochondria, we developed a monoclonal antibody (mAb-3G11E3F7) specific to *P. bursaria* mitochondria and performed indirect immunofluorescence microscopy combined with DAPI staining (Figure 1). Because *P. bursaria* cells were air-dried and fixed on coverslips, they became flattened during preparation (A, D). In aposymbiotic Yad1w cells, mitochondria were dispersed throughout the cytoplasm (B), whereas in symbiotic Yad1g1N cells, mitochondria were concentrated in the spaces between the endosymbiotic *Chlorella* cells (E). Mitochondrial DNA and the host nuclei exhibited DAPI fluorescence (C, F). The red signal in panel (F) represents chlorophyll autofluorescence from *Chlorella* chloroplasts.

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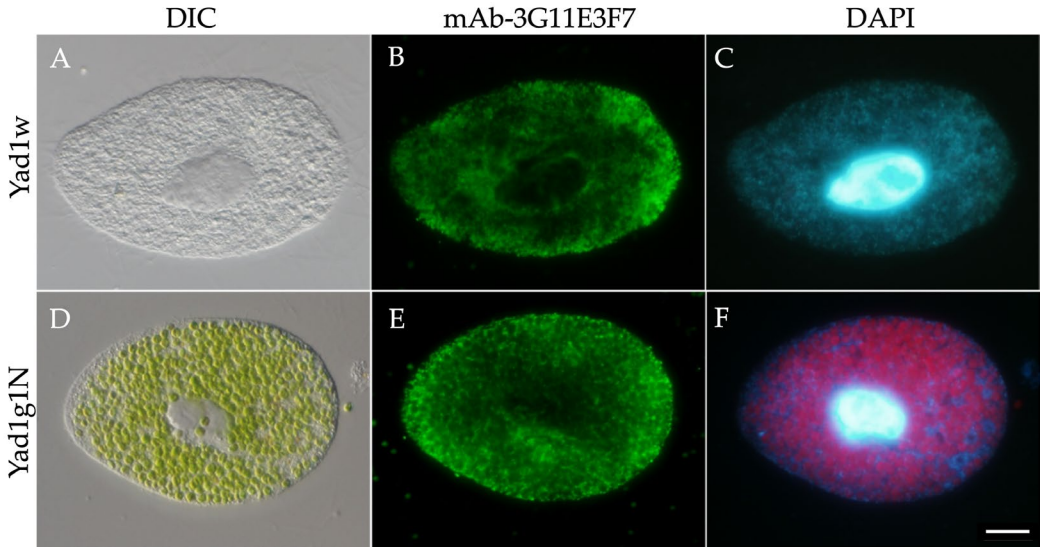

Figure 1. Indirect immunofluorescence staining using the anti-mitochondrial monoclonal antibody mAb-3G11E3F7. (**A–C**) Aposymbiotic Yad1w cell. (**D–F**) Symbiotic Yad1g1N cell. In Yad1w, mitochondria are dispersed throughout the cytoplasm (**B**). *I*n Yad1g1N, mitochondria are localized in the spaces between Chlorella cells (**E**). The left edge: anterior end of the cell. Images were acquired using 10× eyepieces and a 40× objective lens. Scale bar: 20 μm.

To confirm the detailed localization of mitochondria in symbiotic Yad1g1N cells, we performed high-magnification observations using a 100× oil-immersion objective (Figure 2). As shown in panel (A), endosymbiotic *Chlorella* cells were positioned near the host cell surface and were intimately surrounded by mitochondria (B). The particles labeled with mAb-3G11E3F7 were also labeled with DAPI (B–D), confirming that the antibody-labeled structures represent mitochondria.

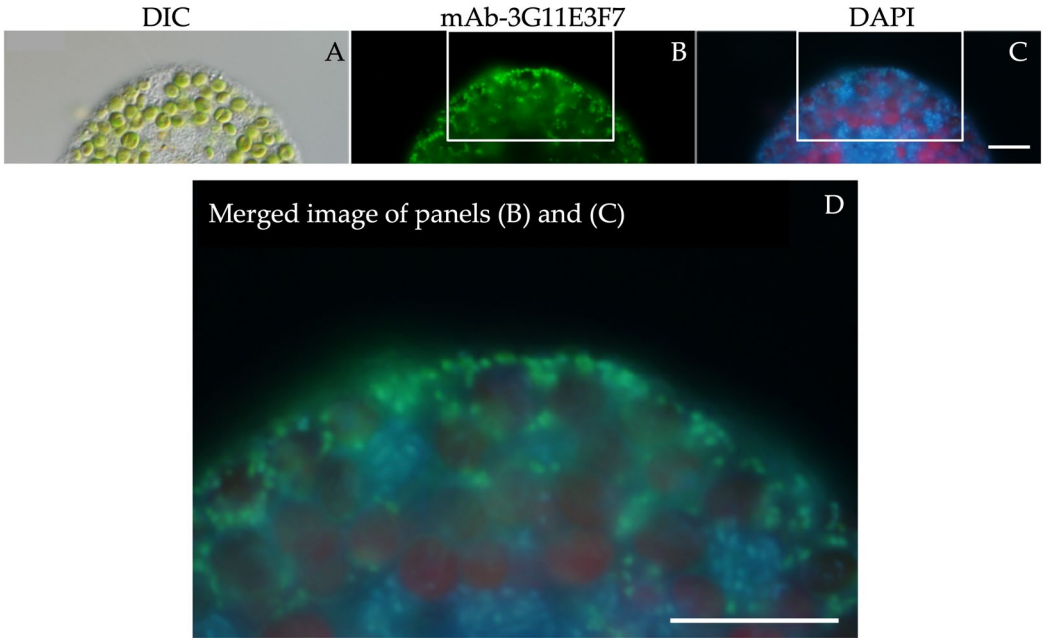

Figure 2. High-magnification indirect immunofluorescence staining of symbiotic Yad1g1N cell using mAb-3G11E3F7 and DAPI. (**A**) DIC image showing endosymbiotic *Chlorella* cells located near the host cell surface. (**B**) Alexa Fluor 488 immunofluorescence showing mitochondria surrounding the endosymbiotic algae. (**C**) DAPI fluorescence showing mitochondrial DNA (light blue) together with chlorophyll autofluorescence from *Chlorella* chloroplasts (red). (**D**) Merged image of the white-lined areas in panels (**B**) and (**C**). The mitochondrial

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immunofluorescence signal corresponds well with the mitochondrial DNA signal. Images were acquired using 10× eyepieces and a 100× objective lens. Scale bar: 10 μm.

3.2. Isolation of Symbiotic Algae Possessing PV Membranes and Mitochondria from Homogenates of Symbiotic P. bursaria

To determine whether the mitochondria surrounding *Chlorella* cells are firmly attached to the PV membrane rather than merely in close proximity, symbiotic *P. bursaria* cells were gently homogenized, and a *Chlorella*-enriched fraction was obtained using the discontinuous Percoll density-gradient centrifugation procedure described in Figure 3 and in the Materials and Methods section. After centrifugation, the green *Chlorella*-containing band at the 75%/45% interface was collected with a Pasteur pipette, the concentration of *Chlorella* cells was determined using a hemocytometer, and the sample was stored at 4 °C until use.

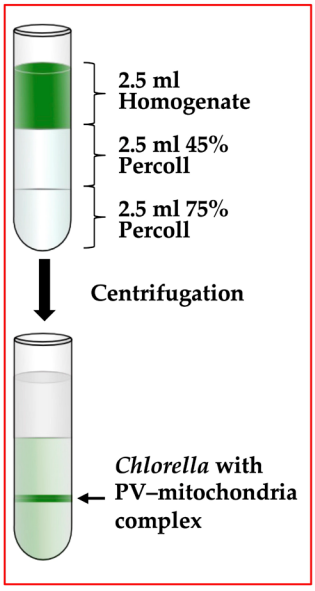

Figure 3. Isolation procedure for symbiotic *Chlorella* retaining PV membranes and associated host mitochondria. Symbiotic *P. bursaria* cells were gently homogenized and subjected to discontinuous Percoll density-gradient centrifugation. *Chlorella* cells enclosed by PV membranes and associated with host mitochondria were recovered from the interface between Percoll layers.

The isolated *Chlorella* fraction was examined by DIC and indirect immunofluorescence microscopy using mAb-3G11E3F7 together with DAPI staining (Figure 4). DIC images revealed small vesicular structures attached to the *Chlorella* cells (A, E; white arrows). In panel (A), the *Chlorella* cell on the right possessed one vesicle, whereas the cell on the left had none. The cell in panel (E) had two vesicles. These vesicles were labeled with mAb-3G11E3F7 (B, F) and also exhibited DAPI fluorescence (C, G, D, H), demonstrating that the antibody-labeled vesicles correspond to host mitochondria. However, panels (B) and (F) do not allow us to determine whether the antigen recognized by this monoclonal antibody resides on the mitochondrial outer membrane, the inner membrane, or another mitochondrial component. The antigen molecule recognized by this antibody has not yet been identified.

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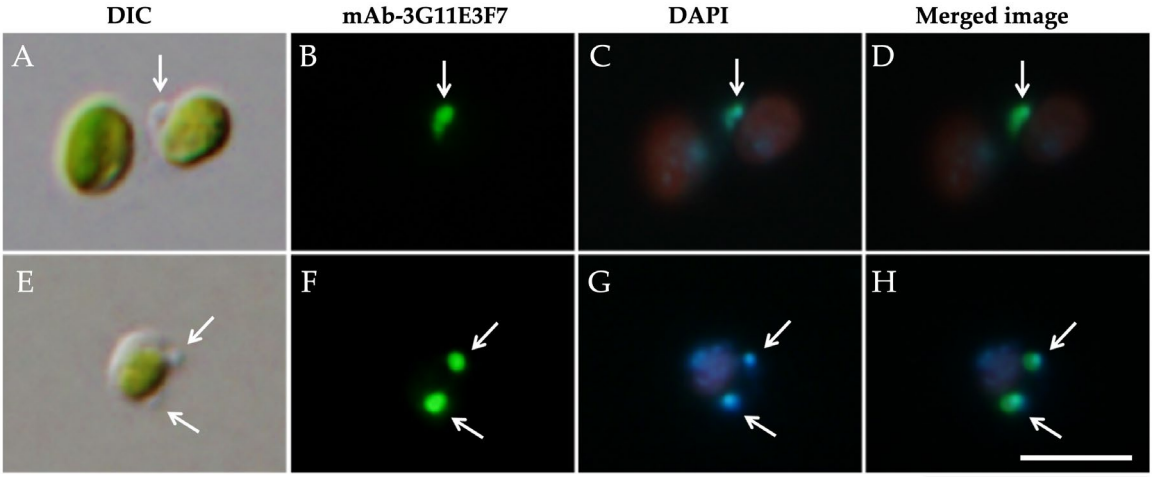

Figure 4. Indirect immunofluorescence staining with mAb-3G11E3F7 and DAPI of symbiotic *C. variabilis* strain 1N cells isolated by discontinuous Percoll density-gradient centrifugation. (**A**, **E**) DIC images showing small vesicular structures attached to the algal surface (arrows). (**B**, **F**) Immunofluorescence labeling with mAb-3G11E3F7.(**C**, **G**) DAPI fluorescence showing mitochondrial DNA. (**D**, **H**) Merged images confirming that the attached vesicular structures correspond to host mitochondria. These observations demonstrate that mitochondria remain associated with symbiotic *Chlorella*, and that this adhesion is sufficiently strong to withstand homogenization with a Teflon homogenizer and subsequent discontinuous Percoll density-gradient centrifugation. Red signals (**C**) and (**G**) represent chlorophyll autofluorescence from *Chlorella* chloroplasts. Images were acquired using 10× eyepieces and a 100× objective lens. Scale bar: 10 μm.

3.3. The Attachment Between Endosymbiotic Chlorella and Host Mitochondria Is Mediated by the PV Membrane

Incubating the *Chlorella* fraction isolated by discontinuous Percoll density-gradient centrifugation (Figure 3) with 5 μM BC_5C/BSA for 30 min at room temperature in the dark enabled fluorescent labeling of both the PV membrane surrounding the endosymbiotic *Chlorella* cell and the mitochondrial membrane. Although the PV membrane has previously been observed by electron microscopy, this study provides the first fluorescent visualization of the PV membrane under light microscopy. BC_5C/BSA is widely used as a fluorescent probe for labeling the Golgi apparatus and tracing sphingolipid trafficking in eukaryotic cells [35,36]. Although typically used for this purpose, we found—as expected—that BC_5C/BSA binds to both the PV membrane and the mitochondrial membrane, enabling fluorescent visualization of these membranes.

Figure 5 shows that BC_5C/BSA labels both the PV membrane and the mitochondrial membrane. Symbiotic *Chlorella* cells obtained by discontinuous Percoll density-gradient centrifugation were observed by DIC (A, E), BC_5C/BSA fluorescence (B, F), and DAPI staining (C, G). Panels (D) and (H) show merged images of BC_5C/BSA and DAPI staining. DIC images reveal two small structures attached to *Chlorella* cells (solid arrows) and one structure not attached (wavy arrows). These small structures correspond to mitochondria, as indicated by their DAPI labeling (C, G). Mitochondria bound only to *Chlorella* cells labeled with BC_5C/BSA and did not bind to unlabeled cells. Furthermore, the mitochondrion indicated by the wavy arrow in panel (B) exhibited ring-shaped fluorescence, demonstrating that BC_5C/BSA labels the mitochondrial membrane. Because the distance between the PV membrane and the *Chlorella* cell wall is very small [27], the presence or absence of the PV membrane cannot be determined from DIC images alone (A, E). However, BC_5C/BSA clearly labeled the outer surface of the algae (B, F).

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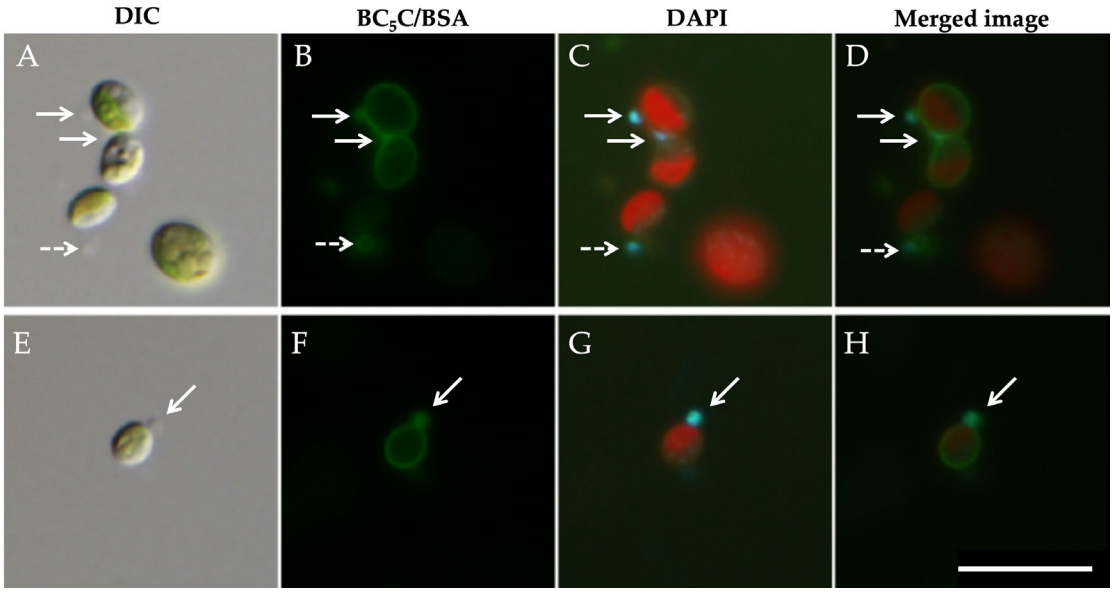

Figure 5. Fluorescence labeling of the PV membrane and host mitochondrial membrane using BC_5C/BSA. Symbiotic *Chlorella* cells isolated by discontinuous Percoll density-gradient centrifugation were observed by DIC (**A**, E), BC_5C/BSA fluorescence (**B**, **F**), and DAPI staining (**C**, **G**). Panels (**D)** and (**H)** show merged images of BC_5C/BSA and DAPI staining. DIC images show small structures attached to *Chlorella* cells (solid arrows) and one structure not attached (wavy arrow). These structures correspond to mitochondria, as confirmed by DAPI staining. Mitochondria bind only to *Chlorella* cells labeled with BC_5C/BSA and do not bind to unlabeled cells. BC_5C/BSA labels the outer surface of the algae (**B**, **F**). Because the only structure surrounding the outside of the *Chlorella* cell wall is the PV membrane, these observations strongly suggest that adhesion between mitochondria and *Chlorella* is mediated by interactions between the mitochondrial membrane and the PV membrane. The red signal (**C**, **G**) represents chlorophyll autofluorescence from *Chlorella* chloroplasts. Approximately 77% of isolated *Chlorella* cells (n=100) retained PV membranes immediately after isolation, and approximately 65% of PV-positive *Chlorella* cells (n=31) had attached mitochondria. Images were acquired using 10× eyepieces and a 100× objective lens. Scale bar: 10 μm.

BC_5C/BSA contains BSA, which has a molecular weight of approximately 66 kDa and a molecular diameter of approximately 7 nm in its unbound form [37]. The pore size of plant and algal cell walls is typically approximately 3–5 nm [38], indicating that BSA is unlikely to pass through these pores. Molecules of this size are also generally unable to permeate the plasma membrane [39]. However, the interaction between BC_5C and BSA is weak. When BC_5C/BSA approaches the plasma membrane, BC_5C likely dissociates from BSA because of its higher affinity for the hydrophobic core of the membrane and subsequently associates with the membrane. The fluorescent labeling observed in panels (B) and (F) indicates that BC_5C is localized to the PV membrane and the mitochondrial outer membrane. These observations suggest that adhesion between *Chlorella* and mitochondria is maintained through the association between the PV membrane and the mitochondrial outer membrane. In contrast, *Chlorella* cells that were not labeled with BC_5C/BSA retained normal morphology, indicating that BC_5C associates with the PV membrane but not with the *Chlorella* cell wall. If BC_5C/BSA or BC_5C were able to penetrate the cell wall, labeling of the *Chlorella* plasma membrane and other internal membranes would be expected; however, such labeling was not observed.

Approximately 77% of *Chlorella* cells (n = 100) retained their PV membranes immediately after isolation, and about 65% of BC_5C/BSA-positive *Chlorella* cells (n = 31) had attached mitochondria. Up to three mitochondria were observed to attach to a single PV-enclosed *Chlorella* cell. The two *Chlorella* cells shown at the top of panels (A–D) appear to be connected by a single mitochondrion that attaches to the PV membranes of both cells. However, it remains unclear whether this represents two PV-enclosed *Chlorella* cells, each bearing one mitochondrion and positioned in close proximity, or a single

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mitochondrion attached to a PV-enclosed *Chlorella* cell undergoing binary fission. The red signal (C, G) represents chlorophyll autofluorescence from *Chlorella* chloroplasts.

3.4. Stability of PV Membranes After Isolation by Discontinuous Percoll Density-Gradient Centrifugation

To quantify the stability of PV membranes after isolation, the *Chlorella* fraction obtained by discontinuous Percoll density-gradient centrifugation was incubated with BC_5C/BSA from day 0 to day 7. At day 0, 77.1 ± 4.9% of isolated algae retained PV membranes. This proportion decreased to 51.8 ± 3.1% on day 1, 31.0 ± 5.2% on day 2, 17.9 ± 3.2% on day 3, 7.5 ± 1.7% on day 4, 3.0 ± 0.5% on day 5, 1.1 ± 0.2% on day 6, and 0.4 ± 0.1% on day 7 (Figure 6). One hundred cells were examined each day.

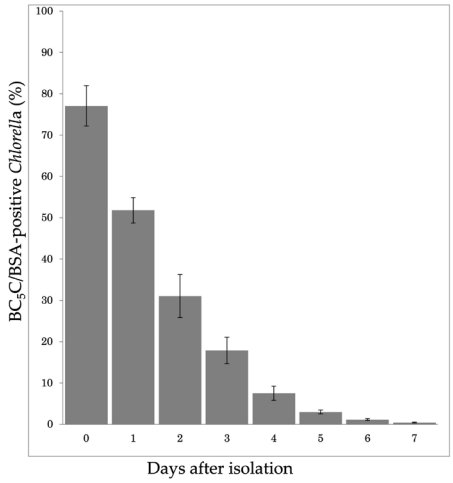

Figure 6. Time-dependent loss of PV membranes in *Chlorella* cells isolated by discontinuous Percoll density-gradient centrifugation. The isolated *Chlorella* fraction was stored at 4 °C, and the proportion of cells retaining PV membranes was assessed daily by BC_5C/BSA fluorescence labeling. One hundred cells were examined each day. The percentage of PV-retaining cells decreased progressively over time. Error bars represent 90% confidence limits.

Representative BC_5C/BSA labeling images from days 0, 3, and 7 are shown in Figure 7. By day 3, both the number of BC_5C/BSA-labeled algae and the fluorescence intensity had decreased markedly (D–F). By day 7, BC_5C/BSA-labeled *Chlorella* cells had almost completely disappeared (G–I). In panel (B), several small, strongly fluorescent dots are visible on the BC_5C/BSA-labeled PV membrane. These correspond to the mitochondria observed in Figure 5 (B, F). A cluster consisting of three to four *Chlorella* cells is present, and enlarged views of the three-cell cluster indicated by an arrow in panel (B) are shown in the lower right corner. Three mitochondria are attached to the PV membrane of the uppermost *Chlorella* cell in this cluster. It remains unclear whether this cluster formed within the cytoplasm of *P. bursaria*, during isolation of the *Chlorella* fraction, or during preparation of the microscopic specimen.

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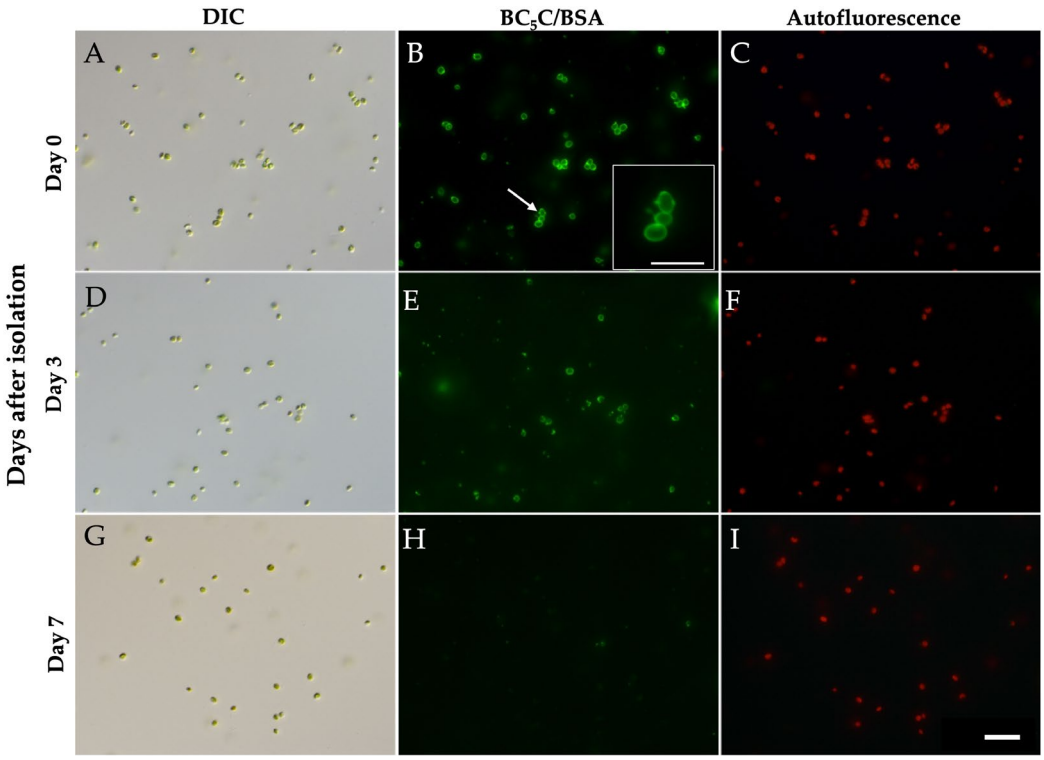

Figure 7. Representative BC_5C/BSA fluorescence images showing progressive loss of PV membranes after isolation by discontinuous Percoll density-gradient centrifugation. (**A–C**) Depending on days after isolation, both the number of PV-positive cells and fluorescence intensity decreased. In panel (B), bright fluorescent puncta on PV membranes correspond to associated mitochondria. An enlarged view of the three-*Chlorella* cell cluster indicated by an arrow in panel (**B**) is shown in the lower-right corner. Panels (**C**), (**F**), and (**I**) show chlorophyll autofluorescence. Images were acquired using 10× eyepieces and a 40× objective lens. Scale bars: 10 μm in I and 20 μm in B.

Figure 8 shows BC_5C/BSA labeling images at days 0 and 7 after isolation of symbiotic *Chlorella variabilis* strain 1N cells using the routine isolation method without discontinuous Percoll density-gradient centrifugation. At day 0, approximately 33% of the cells (n = 103) retained PV membranes, and BC_5C/BSA-labeled *Chlorella* cells had almost completely disappeared by day 7. Compared with cells isolated using discontinuous Percoll density-gradient centrifugation, both the proportion of BC5C/BSA-labeled cells and the proportion of PVs with attached mitochondria were lower.

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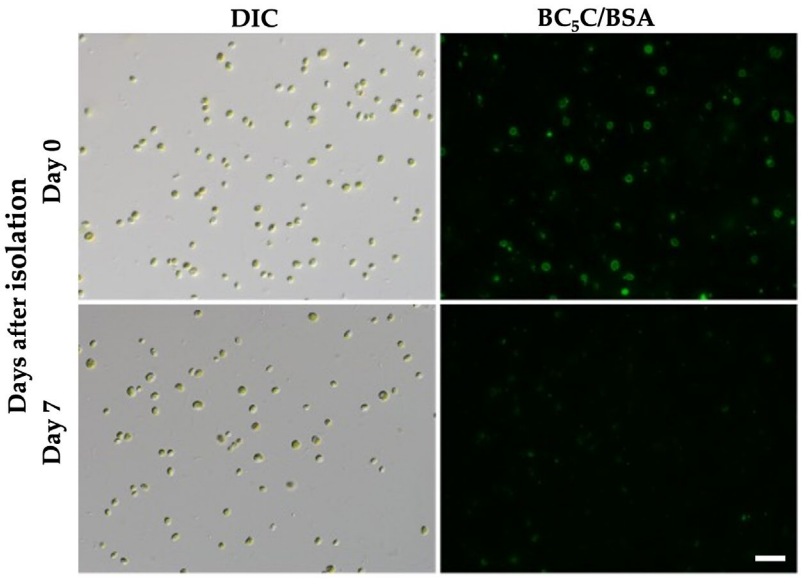

Figure 8. Reduced retention of PV membranes in *Chlorella* cells isolated without density-gradient centrifugation. Symbiotic *P. bursaria* cells were isolated using routine isolation method as shown in Materials and Methods section, and labeled with BC_5C/BSA. At day 0, approximately 33% of cells retained PV membranes (n=103). By day 7, PV membrane labeling was rarely observed. Images were acquired using 10× eyepieces and a 40× objective lens. Scale bar: 20 μm.

4. Discussion

The present study provides direct evidence that the PV membrane surrounding endosymbiotic *Chlorella* in *P. bursaria* forms a stable physical association with the host mitochondrial outer membrane. By combining discontinuous Percoll density-gradient centrifugation, fluorescent labeling with BC_5C/BSA, and a mitochondria-specific monoclonal antibody, we demonstrate that the PV–mitochondrial membrane association persists even after homogenization and centrifugation, indicating that the interaction is mechanically robust rather than a fragile or transient contact. The consistent localization of mitochondria around the PV membrane has long been interpreted as a hallmark of the *P. bursaria–Chlorella* symbiosis. Electron microscopy study has described mitochondria forming a "cage-like" structure around each alga [29], suggesting a specialized architecture for metabolic exchange. However, electron microscopy alone cannot determine whether the membranes are physically attached or merely adjacent. Our findings bridge this gap by showing that isolated *Chlorella* cells frequently retain attached mitochondria, and that this association withstands mechanical disruption. The co-sedimentation of *Chlorella* and mitochondria during density-gradient centrifugation further supports the presence of a stable membrane–membrane interaction.

A key advance of this study is the successful fluorescent visualization of the PV membrane using BODIPY FL C_5-ceramide complexed to BSA (BC_5C/BSA). Although BC5C/BSA is widely used to label the Golgi apparatus and trace sphingolipid trafficking [36,40], its ability to label the PV membrane and mitochondrial membrane in isolated symbiotic units had not been previously demonstrated. As expected from the membrane-insertion behavior of BC_5C, the fluorescent probe dissociated from BSA and inserted into the lipid bilayers of both the PV membrane and the mitochondrial outer membrane, enabling clear visualization of both structures under light microscopy. This represents the first optical method for observing the PV membrane, which previously could only be visualized by electron microscopy. Iwamoto and Allen [41] demonstrated in *Paramecium multimicronucleatum* (a species unable to maintain algae) that BODIPY-ceramide is internalized from the plasma membrane into the cytoplasm in an ATP-dependent manner, and that depletion of ATP causes BODIPY-ceramide to accumulate in the plasma membrane. This finding is consistent with our interpretation that BODIPY

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FL C_5-ceramide incorporated into the PV membrane surrounding isolated *Chlorella* cells, as well as into the host mitochondrial membrane attached to the PV membrane, remains in these membranes because the isolated organelles lack the intracellular transport machinery required for further trafficking of the probe. To our knowledge, this is the first report demonstrating fluorescent labeling of the plasma-membrane–derived unit membranes of isolated organelles using BODIPY FL C_5-ceramide. This approach enables optical visualization of the PV membrane, which previously could only be observed by electron microscopy. The ability of BC_5C to insert into the PV membrane but not into the *Chlorella* cell wall provides important insight into the structural properties of the PV. The pore size of algal cell walls (3–5 nm) is too small to permit the passage of BSA (7 nm), and BC_5C alone does not penetrate the cell wall or internal membranes. Thus, BC_5C labeling specifically marks the PV membrane rather than the algal cell membrane or internal organelles. This specificity allowed us to distinguish the PV membrane from the algal cell wall, which is difficult to resolve by DIC microscopy alone due to their close proximity.

The presence of mitochondria attached to BC_5C-labeled PV membranes strongly suggests that the PV membrane and mitochondrial outer membrane interact directly. The ring-shaped BC_5C fluorescence observed around mitochondria indicates that BC_5C inserts into the mitochondrial outer membrane, consistent with its known affinity for lipid bilayers. The fact that mitochondria were observed only on BC_5C-positive *Chlorella* cells further supports the conclusion that mitochondrial attachment depends on the presence of an intact PV membrane. *Chlorella* cells lacking PV membranes did not exhibit mitochondrial attachment, indicating that the PV membrane is essential for maintaining the association. The stability of the PV membrane after isolation provides additional insight into the nature of the PV–mitochondrial interaction. Approximately 77% of isolated *Chlorella* cells retained PV membranes immediately after isolation, and 65% of these PV-positive cells had attached mitochondria. The proportion of PV-positive cells decreased gradually over seven days, consistent with the expected degradation of membrane structures outside the host cytoplasm. The decline in BC_5C labeling intensity over time further reflects the gradual loss of membrane integrity. These observations suggest that the PV membrane is relatively stable immediately after isolation but becomes progressively destabilized in the absence of host cellular support.

The presence of multiple mitochondria attached to a single PV-enclosed *Chlorella* cell, and the occasional observation of mitochondria appearing to bridge two adjacent *Chlorella* cells, raises intriguing questions about the dynamics of mitochondrial positioning. It is unclear whether such configurations arise within the host cytoplasm or during the isolation process. However, the consistent presence of mitochondria at specific positions around the PV membrane suggests that mitochondrial attachment is not random but may reflect a regulated process. The possibility that mitochondria attach to specific domains of the PV membrane, perhaps enriched in particular lipids or proteins, warrants further investigation. The mechanistic basis of the PV–mitochondrial membrane association remains to be elucidated. One possibility is that protein complexes span the PV membrane and mitochondrial outer membrane, forming a physical tether analogous to mitochondria–ER contact sites shown in *Toxoplasma gondii* [42,43]. Alternatively, specific lipid compositions of the PV membrane may promote adhesion to mitochondrial membranes. The PV membrane is derived from the host digestive vacuole (DV) but is modified during infection process [12], potentially acquiring unique properties that facilitate mitochondrial attachment. Identifying the molecular components responsible for this interaction will be an important direction for future research.

The functional significance of the PV–mitochondrial association is also an important question. Mitochondria positioned around the PV membrane may facilitate efficient exchange of metabolites between the host and endosymbiont. For example, mitochondria could supply ATP or reducing equivalents to the alga, or receive metabolites produced by the alga. The close apposition of membranes may also enable signaling interactions or contribute to the regulation of equal distribution of algae to the host daughter cells. Understanding the stability and efficiency of the PV–

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mitochondrial association is expected to help elucidate the conditions necessary for environmental adaptation and acquisition of photosynthetic capacity in cell evolution.

This study provides the first direct evidence that the PV membrane and host mitochondrial outer membrane form a stable physical association in *P. bursaria*. As expected from the membrane-insertion properties of BC_5C, fluorescent labeling enabled optical visualization of both membranes, revealing a structurally reinforced interaction that persists outside the host cytoplasm. These findings establish a mechanistic framework for understanding how endosymbiotic *Chlorella* cells are stably positioned beneath the host cortex and introduce a new experimental approach for studying membrane interactions in photosynthetic symbioses.

5. Conclusions

This study demonstrates that the PV membrane and the host mitochondrial outer membrane form a stable physical association that persists even after homogenization and centrifugation. As expected from the membrane-insertion properties of BC_5C, fluorescent labeling enabled direct visualization of both membranes under light microscopy, providing the first optical method for observing the PV membrane and mitochondria surrounding endosymbiotic *Chlorella*. These findings establish a mechanistic basis for the stable positioning of endosymbionts beneath the host cortex and introduce a new experimental approach for studying membrane interactions in photosynthetic symbioses.

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Abbreviations

The following abbreviations are used in this manuscript:

PV	Perialgal Vacuole
TEM	Transmission Electron Microscope
DV	Digestive Vacuole
AcPase	Acid Phosphatase Activity
ER	Endoplasmic Reticulum
MDS	Modified Dryl's Solution

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NBRP	National BioResource Project
SIP	Stock Isotonic Percoll
PBS	Phosphate-Buffered Saline
PBST	PBS-Containing 0.05% (v/v) Tween 20
DAPI	4′,6-DiAmidino-2-PhenolIndole
DIC	Differential Interference Contrast
BC_5C/BSA	BODIPY FL C_5-ceramide complexed to BSA
BSA	Bovine Serum Albumin

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