The University of South Bohemia in České Budějovice

Faculty of Science

3D SBF-SEM analysis of inner organisation of unicellular protist cell

Bachelor Thesis

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České Budějovice 2023

Salburg. A., 2023: 3D SBF-SEM analysis of inner organisation of unicellular protist cell. Bc. Thesis, in English. 45-p., Faculty of science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

A culture of a unicellular protist *Diplonema japonicum* was imaged with a Serial Blockface Scanning Electron Microscope and the resulting images were further processed with the Microscopy Image Browser program. From the obtained images the whole ultrastructure of various cells was analysed and the relationship between the cells mitochondria and endosymbionts was investigated. In addition a 3D model based on the resulting images of a *D. japonicum* cell is constructed to visualise potential endosymbionts residing inside the mitochondria.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 17/08/2023

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Audra Salburg

Acknowledgments

First and foremost, I want to thank my supervisor RNDr. Jiří Týč, Ph.D. for guiding me with my research/writing process, helping me whenever I needed it and for being patient with me. Secondly I want to thank Daria Tashyrevia, Ph.D. and Prof. RNDr. Julius Lukeš, CSc. for allowing me to work on their research on diplonemids and giving input whenever it was needed.

I also want to thank the Laboratory of Electron Microscopy for being welcoming to me and providing all the necessary materials to work on this thesis. Following that I want to thank my colleague and friend Tamara who worked on her research at the same time as me and made the laboratory work more fun.

Special thanks goes to my parents who supported me throughout my entire study and life. Finally I want to thank my friends I made during this study program as we could always rely on each other.

Abstract

Spatial organisation of unicellular protists cells is one of not fully answered questions. Volume electron microscopy, such as SBF-SEM can help us to reveal the details of not only the inner organisation of the individual cells, but also their relationships with their endosymbionts.

Diplonemids are regarded as one of the most species rich and abundant marine protists. Despite their large presence in the oceans little information about this unicellular group and their role in the marine ecosystem is known. Out of the few named and described Diplonemids only three species were found to have endosymbionts and *Diplonema japonicum* is the only one to harbour two different species of intracellular bacteria so far.

Endosymbiosis is a relationship between two different organisms, where one of the partners lives inside the other. The interactions can be beneficial to the host and even lead to the acquisition of new capabilities or organelles. However, it can also be exploitative as the endosymbiont can steal various resources from the cell and act pathogenic towards their host.

In the case of Diplonemids it is unclear if this relationship is beneficial or harmful to the host cells. In the case of *D. japonicum* the endosymbionts seem to be in contact with the mitochondria and in some cases even appear to be inside the organelle. So far only one other case of an endosymbiont residing inside the mitochondria has been reported which makes the mitochondria a relatively rare and unexplored ecological niche for endosymbionts. Previous imaging techniques only show the cross section of the *Diplonema japonicum* cell and due to the limiting 2D perspective there was no definitive proof of the endosymbionts residing inside the organelle.

With the help of 3D analysis by SBF-SEM and programmes such as Microscopy Image Browser and Amira this thesis determines that endosymbionts are actually inside of the mitochondria and also how common these interactions are. Various characteristics of the *Diplonema japonicum* cells and that of the endosymbionts are compared to investigate if different traits and conditions affect the endosymbiont-mitochondria interaction.

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Abbreviations

Acetyl-CoA: Acetyl coenzyme A

ADP: Adenosine diphosphate

ATP: Adenosine triphosphate

B: Bacteria

CIA: Cytosolic Iron-Sulphur protein assembly

Cy: Cytoplasm

DNA: Deoxyribonucleic acid

DSPD: Deep sea pelagic diplonemid

FS: Freeze Substitution

ISC: Iron Sulphur cluster assembly

NADH: Nicotinamide adenine dinucleotide + hydrogen

MIB: Microscopy Image Browser

Mt: Mitochondria

rRNA: ribosomal Ribonucleic acid

SBF-SEM- Serial Block Face Scanning Electron Microscopy

SEM: Scanning Electron Microscopy

TCA: Tricarboxylic acid

TEM: Transmission Electron Microscopy

1. Introduction

1.1. Endosymbiosis

Endosymbiosis is defined as a relationship between two living organisms, where one of the organisms, the symbiont, lives inside the host partner (Wernegreen, 2012). The host, typically an eukaryote, harbours the endosymbiont due to their larger size and is able to provide a nutrient rich environment and protection for their symbiotic partner. The endosymbiont, a pro- or eukaryote, lives inside the host and is able to provide various functions which are unavailable to the host (Nowack & Melkonian, 2010). Because of that, endosymbiotic relationships in eukaryotes are often regarded as an acquisition of new biochemical capabilities in evolutionary history. Novel endosymbiotic associations and resulting abilities usually take place within protists as they do not have a germ line and genetic integration of the symbiont is therefore more readily established (Nowack & Melkonian, 2010). Metabolic symbiosis is the best understood type as it brought important biochemical functions throughout evolutionary history for the eukaryotic cell. It is believed that the origin of the eukaryotic cell started with the engulfment of an alpha proteobacteria capable of aerobic respiration for ATP synthesis. Other metabolic functions which were obtained in certain eukaryotic species include photosynthesis, nitrogen fixation, methanogenic respiration, sulphide oxidation and syntrophy (Husnik et al., 2021) (Nowack & Melkonian, 2010). Besides nutritional functions, endosymbionts can provide defence against predators, pathogens or to help eliminate competition. A solely unique and rare symbiotic relationship found in protists helps with moving the host such as chemotaxis or initiating movement of the flagella (Husnik et al., 2021).

The most well-known case of endosymbiosis and it's evolutionary consequence is that of the mitochondria and chloroplast, whose ancestors were alpha proteobacteria and cyanobacteria respectively which were taken up by a proto-eukaryotic cell (Archibald, 2015). The creation of organelles is the most extreme case of endosymbiosis, however there is no clear cut distinction between bacterial endosymbionts and a fully integrated organelle but multiple criteria have been set up. (I) Genetic integration by transferring the endosymbionts genes to the host's nucleus is arguably the most critical distinction. Due to the loss of genes the respective gene products are expressed by the host and targeted back to the symbiont by an established protein transport mechanism. (II) Cellular integration allows the host to control and sync the endosymbiotic division to the host cell cycle. (III) Metabolic integration where the metabolism of the two species complement each other (Keeling & Archibald, 2008).

Historically endosymbiosis is defined as a symbiotic, mutual interaction, where one of the participants receives something from their partner and gives something in return. Recently this relationship has been redefined as a spectrum of beneficial and exploitative interactions and it's even argued that endosymbiosis is in reality rarely ever mutualistic (Keeling & McCutcheon, 2017). In exploitative interactions the host exploits the functions of its endosymbiont and is able to reduce the symbiont into a non-self- sufficient organism (Husnik et al., 2021).

This can be achieved by reducing the genome of the endosymbiont, transferring genes to the host's nucleus and establishing a protein/metabolite transport mechanism between the two

species. When an endosymbiont's genome is dramatically reduced it can lead to the host discarding the endosymbiont and replacing it with a new, fresh one in multiple recurring cycles (Husnik & Keeling, 2019). This phenomenon is best described in Euplotes and their polynucleobacter endosymbionts for protists and symbiotic replacements were also observed in sap feeding insects (Husnik et al., 2021). With dramatic ecological shifts or capabilities (nutrients and energy) the endosymbiont has to remain in the host and it can turn into a dependent organelle such as the mitochondria or chloroplast (Keeling & McCutcheon, 2017). As the endosymbiotic relationship is dependent on the ecology of the organisms, endosymbionts can be lost over time as their function can become irrelevant to the host cell. In the case of plastids, a phototrophic lifestyle for the host can be energy costly, limiting to only a few environments and can result in reactive oxygen species. Due to more readily accessible carbon sources from other organisms or limited light some apicomplexan parasites lost their plastids as they switched to a heterotrophic lifestyle (Oborník, 2022).

On the other hand "professional symbionts" such as *Rickettsiales*, are usually bacteria with reduced but still self-sustaining genomes. They are able to switch symbiotic hosts and can even control the symbiosis by encoding effectors and secretory systems (Salje, 2021). It can be assumed that most endosymbionts are actually pathogens who invaded and are costly for the host. But with changing environmental contexts and potentially beneficial capabilities of the symbionts the role can shift and the dynamic ends up exploitative for the endosymbiont (Husnik & Kneeling, 2019).

Whether a symbiotic relationship is beneficial or exploitative, nutrients/metabolites/proteins are often exchanged between the two species. One way to overcome the different membrane barrier of cellular components, vesicles are employed for molecule transport (Cooper, 2000). Similar to the cell membranes, vesicles are made out of a lipid bilayer and transport molecules by budding off from their original component and fuse with the membrane of their target to release the cargo. Proper vesicle formation and transport to the desired target is facilitated by proteins coated around the exterior vesicle surfaces which are recognised by the transmembrane proteins of the target membrane (Cooper, 2000). Extracellular vesicles are secreted by most cells outside in order to communicate and transport bioactive molecules with other cells in a cooperative or antagonistic way. Pathogenic bacteria use extracellular vesicles to deliver toxins and avoid the immune system while a host cell uses its extracellular vesicles to defend against pathogens by neutralising toxins, activate immune responses and remove damaged cell parts (Zou et al., 2022).

1.1.1. Mitochondria

The mitochondria is an important organelle and most commonly known to provide the eukaryotic cell with energy in the form of ATP (Adenosine triphosphate). Energy is produced by a complex aerobic process where sugars are glycolysed to pyruvate which is then converted into Acetyl-CoA and enters the tricarboxylic acid cycle (TCA) (Osellame et al., 2012). In the cycle the acetyl group of Acetyl-CoA is transferred to oxaloacetate which forms a citrate. The citrate undergoes multiple enzymatic steps and intermediates to oxidise back to oxaloacetate. In the process the electrons are transferred to cofactors NADH and flavin and any excess carbon is released as carbon dioxide. The produced cofactors carry the electrons to the electron transport chain and are oxidised. The electrons are passed down the chain and the resulting energy pumps out protons from the matrix to the intermembrane

space and generates a transmembrane potential. At the end of the chain electrons are transferred to oxygen and the proton uptake produces water. Due to the transmembrane potential and the flow of protons back to the matrix is used to phosphorylate ADP into the final ATP product (Osellame et al., 2012). While oxidative ATP phosphorylation is the most prominent feature of mitochondria, the energy producing role has been lost in anaerobic eukaryotes and the organelle reduced to hydrogenosomes/mitosomes (Howe, 2008).

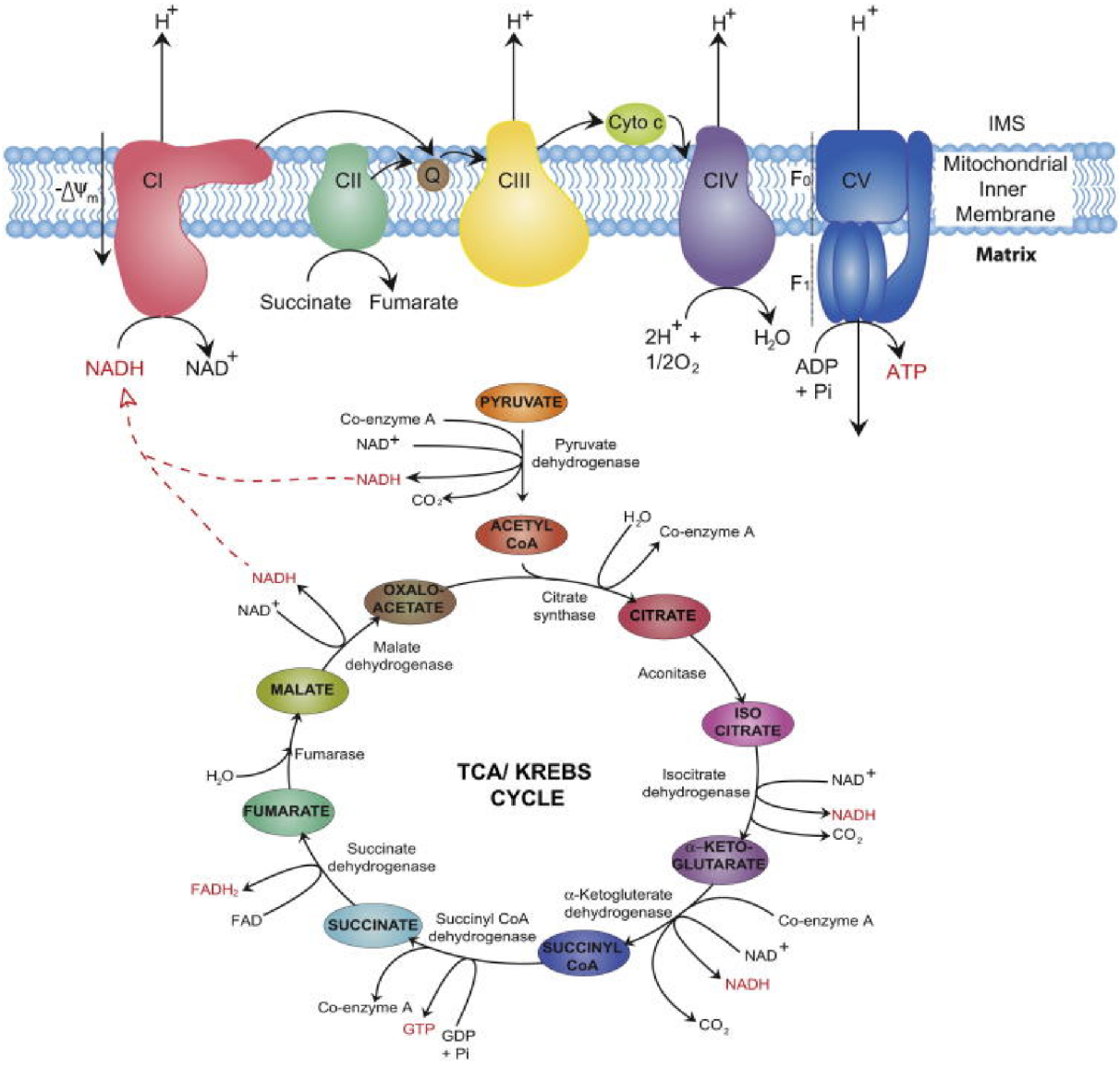

Fig.1: Schematic drawing of the Electron transport chain/TCA cycle to produce ATP and NADH (Osellame et al., 2012)

One function remains in all mitochondria and their derivatives and only disappears with the complete loss of the mitochondria: The Iron-Sulphur cluster biogenesis (Roger et al., 2017). Iron/Sulphur proteins are essential electron carriers and catalysts mediating cellular respiration, enzymatic conversions in anabolism/catabolism, radical chain reactions, DNA synthesis/repair, proteins translation and antiviral defence (Braymer et al., 2021). Because Iron and Sulphur are redox active elements which can be toxic to the cell in excess, a proper assembly and delivery to required apoprotein mechanism is required. The “Iron-Sulphur cluster assembly” (ISC) consists of 18 known mitochondrial proteins responsible for the proper assembly and trafficking of Fe/S clusters in the mitochondrion (Braymer & Lill,

2017). Additionally Eukaryotes have a "cytosolic Iron-Sulphur protein assembly" (CIA) with 11 known proteins in the machinery to support cytosolic and nuclear Iron/Sulphur cluster proteins but it requires precursors from the ISC system.

Other functions which are also attributed to the mitochondria are metabolism regulation, cell cycle control, protein signalling, cell growth and differentiation and cell death (McBride et al., 2006) (Osellame et al., 2012).

The Mitochondria is also one of the most prominent cases of Endosymbiosis as the acquisition of the organelle had a huge impact on eukaryotic cell evolution and aerobic development of life. In a single evolutionary event an alpha proteobacterium invaded a proto-eukaryotic cell (Dyall et al., 2004). The mitochondrion ancestor is believed to be a sister group of the *Rickettsiales* which are intracellular parasites and even the pro-mitochondria bacteria might have actually been an energy parasite (Wang & Wu, 2014). However, mitochondrial genes are highly divergent which makes it more difficult to accurately determine a possible ancestor. Despite the possible parasitic origins of the mitochondria it provided an essential function to the pro-eukaryotic host (efficient ATP generation, iron sulphur clusters) previously unavailable to them. This essential function caused the pro eukaryote to become dependent on the symbiont and in turn to start dominating the relationship to retain the symbiont function. This leads to extreme genome reduction of the endosymbiont and transfer to the host nucleus (Husnik & Kneeling, 2019). Over an evolutionary period, protein import system, targeting peptides and biochemical pathway between the host and the symbiont were established, which is considered a major distinction between organelle and endosymbiont (Dyall et al., 2004)(Wernegreen, 2012). Multiple evolutionary stages also led to the loss of independence of the endosymbiont as it lost major genes, the ability to control its own cycle and distribution and started to rely on the host for nutrients and proteins (Roger et al., 2017).

1.1.2. Endosymbionts associated with mitochondria

The *Candidatus Midichloria mitochondrii* is so far the only known endosymbiont to live inside of the mitochondria in ovaries of the female *Ixodes ricinus* ticks (Sassera et al., 2006). These endosymbionts occupy the periplasmic space between the two mitochondrial membranes and consume the inner mitochondria to reduce the mitochondrial matrix and multiply within there (Sassera et al., 2006). While the relationship and function of the ticks mitochondria and *Ca. M. mitochondrii* is still unknown, *Ixodes ricinus* larvae without the endosymbionts had a reduced blood feeding success rate than ticks harbouring the *Ca. M. mitochondrii* (Guizzo et al., 2023). The most prevalent theory is that the endosymbionts aid in cellular respirations during blood feeding which is believed to be a hypoxic state. The supply of metabolites or vitamins to the tick and regulation of mitochondrial reactive oxygen species are also potential functions of the symbiont (Stavru et al., 2020). A more parasitic reason for the residence inside the mitochondrial matrix may be for transmission purposes to the next generation of ticks by hijacking the maternally inherited mitochondrion (Stavru et al., 2020). However recent mathematical models do not support a predatory behaviour of the *Ca. M. mitochondrii* towards the mitochondrions of the ovarian tick cells due to the number of symbionts being too high for the mitochondrion to sustain before undergoing lysis.

Additionally dividing bacteria were rarely observed inside of the organelle (Comandatore et al., 2021).

1.2. Diplonemids

Diplonemids are described as flagellated heterotrophic protists, which are unicellular eukaryotes and classified as members of the Euglenozoa kingdom (Lara et al., 2009). Until recently they were discarded as a small eukaryotic group with only a handful of species described (Lukeš et al., 2015).
With the help of 18S ribosomal DNA primers and the Tara Ocean Expedition the hidden diversity of diplonemids could actually be investigated (Lara et al., 2009). The Tara Ocean Expedition (2009-2012) with its aim to determine eukaryotic diversity across the world from the photic zone revealed that Diplonemids are much higher in diversity than classic plankton groups (de Vargas et al., 2015). The extended Tara datasets, including samples from the photic and mesopelagic zone showed that Diplonemids might be the one of the most species rich marine eukaryotes and that their abundance increases with depth (Flegontova et al., 2016).
Nowadays Diplonemids are regarded as the fifth most abundant and most diverse eukaryotic group (Kostygov et al., 2021). Despite their species richness and abundance, most Diplonemid data consists only of environmental 18S rRNA genes but little morphological and characterising information is available (Gawrylluk et al., 2016).

As of now Diplonemids are sub-categorized into four clades: *Diplonemidae, Hemistasiidae, deep sea pelagic diplonemid (DSPD) I and II*. 97% of Diplonemid Species fall under the *DSPD I* clade while the others category account for 1% each of the species diversity (Kostygov et al., 2021). Deep sea dilponemids abundance increases with depth which excludes a phototrophic lifestyle while large reads from the SSU V9 region suggest parasitism and gene fragments from different prokaryotes/eukaryotes may indicate predatory behaviour (Gawrylluk et al., 2016). Additionally, different DSPD species were found within a single plankton sample, suggesting the coexistence of diplonemid phylotypes in a homogenous environment. This means that different species are not in competition with each other due to the requirement of different resources or a paradoxical situation where limited resources support diplonemid diversity (Lara et al., 2009). While the planktonic deep sea diplonemids inhabit nutrient poorer deep seas, the classic *Diplonemidae* were found in various benthic environments (coastal surfaces, cold anoxic seeps, hydrothermal vents, sea floor) but also in photic plankton communities, aquaria and fresh waters (Kostygov et al., 2021). *Hemistidae* clade was almost always detected from planktonic samples in the photic zone from cold to tropical regions (Kostygov et al., 2021).
Both *Diplonemidae* and *Hemistiidae* follow a large variety of heterotrophic lifestyles such as predation, scavenging, ectocommensalism and endoparastism (Kostygov et al., 2021). *Hemistasiidae* are categorised as omnivorous predators who feed on whole cells or consume larger organisms from within but are not reported to consume live bacteria. Different *Diplonemidae* species feed on living or dead bacteria, microalgae or consume the cytoplasm of large diatoms. Diplonemids were also found to associate with marine animals and plants, either commensally or pathogenetically (Tashyreva et al., 2022). So far phototrophy is the one lifestyle that can be definitely excluded for Diplonemids due to ecological and phylogenetic reasons. A possible parasitic lifestyle for the majority of Diplonemids is also

questionable as their numbers increase with oceanic depth while the possibilities of suitable hosts decrease (Lukeš et al., 2015). Cultured Diplonemids in nutrient rich environments were observed to use osmotrophy and some species are able to switch between osmotrophy to phagotrophy in order to ingest nutrients (Prokopchuk et al., 2022).

Diplonemids are generally sac shaped with their plasma membrane being suspended by interlinked microtubules. Beneath the microtubular corset or over the mitochondrial branches, sacs of rough and soft endoplasmic reticulum can be found. Tubular extrusomes can be found in several species and are located near the cell's feeding apparatus which suggests that they might be used for predation or penetration of prey/hosts. Cytosomes encircled by a rigid c shaped collar opens into the feeding apparatus which further extends as a long, tubular cytopharynx. A major characteristic of diplonemids is their asymmetric appearance of the anterior part caused by the apical papilla which connects the subapical flagellar pocket and the cytostome (Tashyreva et al., 2022). Two flagella emerge from the basal body and are joined by a fibrous connective. Generally the axonemes are arranged in "nine doublets and a central pair" of microtubules with the central axoneme penetrating through the distal plate. Paraflagellar rods are present in most diplonemid species but are only present in certain cell stages for other diplonemids (Tashyreva et al., 2022).

Even before the relative abundance and diversity of diplonemids was known, their unusual mitochondrial structure and gene editing already gathered attention (Lara et al., 2009). The classic model Diplonemid *Diplonema papillatum* has one giant mitochondrial and its DNA forms a network composed of thousands of monomeric circular mtDNA molecules (Marande et al., 2005). While the majority of eukaryotes have one mitochondrial chromosome, Diplonemids are estimated to possess 56 distinct chromosomes, making it one of the largest mitochondrial genomes (Marande et al., 2005). The total size of the mitochondrial DNA is estimated to be 260 Mbp which is dispersed through a large region of the mitochondria. Despite the large DNA content, the *D. papillatum* mitochondria only contains 18 fragmented genes and also no DNA splicing system (Lukeš et al., 2018). Instead the Coding regions are located on different chromosomes and transcribed separately from each other. The gene fragments are joined together by a "trans-splicing" mechanism (Marande et al., 2005). It is unknown if there are any benefits in having such a DNA rich mitochondria but a potential reason could be that the mitochondria is used to store energy in the form of purine/pyridine or serve as a "filler" organelle (Lukeš et al., 2018).

Diplonemids undergo different life stages with different characteristic morphological changes, depending on nutrient availability. The main stage is the trophic phase and exists under nutritious conditions where cells are larger and glide on surfaces. Under a lack of nutrients the cells grow smaller and their flagella elongates allowing the cells to enter the swimming stage and search for nutrients. Species from the *Diplonema* clade have the additional sessile stage which serves as a transition between the trophic and the starving swimming stage (Tashyreva et al., 2022).

1.2.1. Diplonemid endosymbionts

As of now endosymbionts were detected in three Diplonemid species: *Diplonema aggregatum*, *Diplonema Japonicum* and the more distantly related *Namystynia karyoxenos* (George et al., 2020).

Namystyina karyoxenos is the first known *Hemistasiidae* to contain endosymbionts, namely the rod shaped *Canidatus Sneabacter namystus* from the *Rickettsiaceae* family. They're found in abundance in the host's cytoplasm and especially under starving conditions they're located near the mitochondria or even seem to penetrate it. The alpha proteobacteria has also been found inside the nucleus surrounded by heterochromatin where they multiply within (Prokopchuk et al., 2019).

D. aggregatum from the *Diplonemidae* clade contains the endosymbiont *Cytomitobacter indipagum* which are short bacterial rods from the *Holosporaceae* family. Transmission Electron microscopy (TEM) shows that the endosymbionts reside within the cytoplasm and adjacent to the mitochondrial network. In extremely rare cases it even appears as if the endosymbionts are inside the organelle however TEM images only provide a 2D view and can not reliably prove this. When the diplonemid host is starved, only few endosymbionts can be seen and they arrange themselves in small clusters. Additionally their numbers can be reduced by incubating *D. aggregatum* with the antibiotic kanamycin (Tashyreva et al., 2018).

Meanwhile *D. japonicum*, also from the *Diplonemidae* clade, harbours two Holosporaceae endosymbiont species of different but related genera. One of the endosymbionts is *Cytomitobacter primus* is a rod shaped bacterium closely related and similar in size (1.0-1.4 µm long) to the endosymbiont species of *D. aggregatum*. The other endosymbiont *Nesciobacter abundans* is smaller in size (0.9-1.2 µm long) than its cohabitor but is more abundant in the *D. japonicum* cell throughout all life stages. Both endosymbionts are randomly distributed through the cell and their numbers decrease with a starving host. Similarly to the other diplonemid species both endosymbionts are found in the cytoplasm and near or even within the mitochondrial network of the cell (George et al., 2020) (Tashyreva et al., 2018).

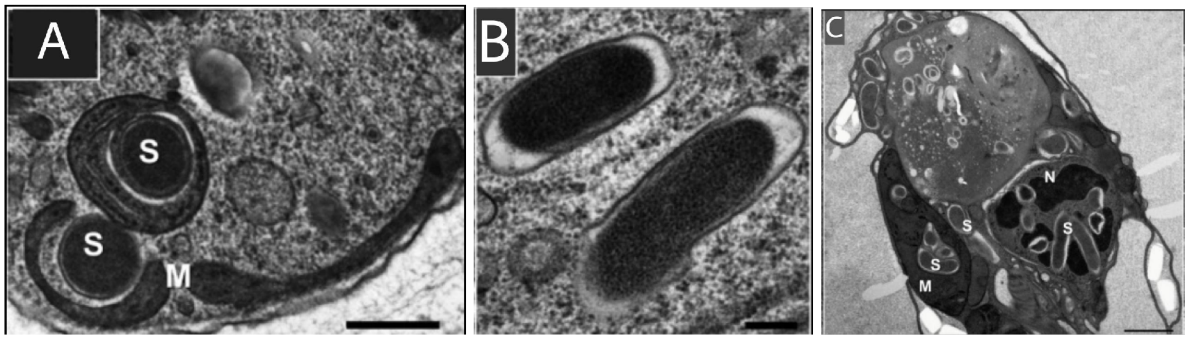

Fig.2: TEM images of *D. japonicum* endosymbiont being in close contact and surrounded by the mitochondria (A), rod shaped endosymbionts of *D. aggregatum* (B) and *Namystynia karyoxenos* endosymbionts who appear to be inside the mitochondria/nucleus (C). (S-Symbiont, M-mitochondria, N-Nucleus). Scale bars: 0.5 µm (A), 0.2µm (B) and 1 µm (C). Modified from Tashyreva et al., 2018 (A,B) and Prokopchuk et al., 2019 (C).

Genomes of the diplonemid endosymbionts are among the smallest in size for protist endosymbiont but are also very gene dense and contain proteins that could potentially facilitate intracellular life such as protein secretion, effectors and putative toxins (George et

al., 2020). Their genome is however not able to encode fully functioning metabolic pathways such as glycolysis, TCA cycle, oxidative phosphorylation complexes or the synthesis of essential metabolites (amino acids/vitamins) and suggests that the endosymbionts depend on their host for metabolites. In all endosymbionts ATP synthase was retained despite the lack of a respiratory chain. Additionally ADP/ATP translocase and metabolite transporters are encoded by the endosymbionts and hints at potential "energy parasitism" due to their closeness to the mitochondria. Despite being from different bacterial families the endosymbiont genome shows similarity in size and content which suggest evolutionary convergence within the Diplonemid host (George et al., 2020).

The exact purpose of the endosymbionts is still unknown and there have been no successful attempts at eliminating the endosymbionts of both *Diplonema* species and in the case of *D. aggregatum* their numbers could only be reduced. However the interaction between the organisms seems stable and harmless for the host (Tashyreva et al., 2018). The reason for the failure of removing the endosymbionts may lie in their antibiotic resistance or the inability of the drugs to permeate the host's membrane. In addition to the potential antibiotic resistance the endosymbionts of diplonemids encode toxins which hypothesise that they aid in defence against pathogens and bacterial infection (Kostygov et al., 2021).

The endosymbionts in all three diplonemid hosts belong to the alphaproteobacteria order and members of both families Rickettsiaceae and Holosporacea are considered "professional symbionts" or parasites as they are adapted to an intracellular lifestyle (Husnik et al., 2021). The number of endosymbionts is the highest in trophic cells for the more closely related *D. aggregatum* and *D. japnoicum* (Tashyreva et al., 2018). Additionally all four endosymbiont species are in close proximity to the mitochondria and some even appear to penetrate or reside within the organelle network (Tashyreva et al., 2018) (Prokopchuk et al., 2019).

1.2.2. Diplonema japonicum

Diplonema japonicum belongs to the classic *Diplonemidae* clade and was first isolated from the coastal surfaces and deep- sea waters around Japan (Tashyreva et al., 2018).

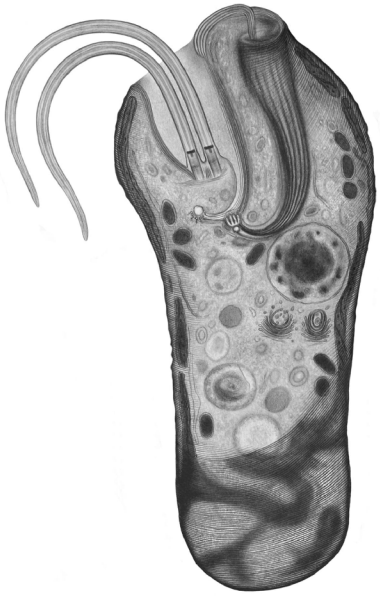

Fig.3: Illustration of a *Diplonema japonicum* cell depicting the general structure of a trophic *D. japonicum* cell (Tashyreva et al., 2018)

The cell cycle consists of three stages:

- Trophic stage in a nutrient rich environment: The cell has an elongated shape with round ends (19.9 ± 1.9 µm length and 5.8 ± 0.6 µm width) and two equally thin and short flagella (a third of the cell body in length) which are inserted subapically into a flagellar pocket. The cell glides and changes its shape due to metabolic contortion or attaches to surfaces with one flagella and rotates around their anterior. Apart from the anterior part numerous refractive vacuoles are found in the cytoplasm (Tashyreva et al., 2018).
- Sessile stage in nutrient poor environment: The cell body appears rounder and smaller (diameter from 6.5 to 8.5 µm) but is actually folded up and turns itself within to produce the longer flagella which wraps around the cell body. The Cell is enclosed in a translucent mucilaginous coat which allows the cell to stick to surfaces. Tubular extrosomes and paraflagellar rods are present (Tashyreva et al., 2018).
- Swimming stage after prolonged starvation: The cell is smaller in size (12.7 ± 1.0 µm length and 4.6 ± 0.4 µm width) but possesses two thick,unequally long flagella. The flagella are double the length of the cell body and are highly motile. Tubular extrosomes and paraflagellar rods are present but lacks refractive vacuoles. This stage lasts usually only for 12 hours and under continued starvation the cell shrinks further, its flagella disassembles and shortens until the cell undergoes lysis after several days. However when swimming cells are placed in a nutritious environment they return back to the trophic stage (Tashyreva et al., 2018).

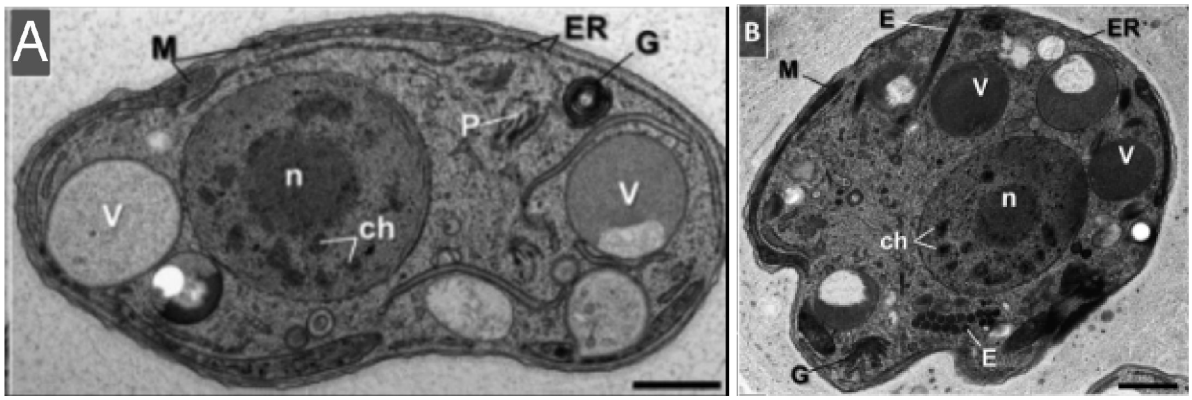

Fig.4: Transmission electron microscopy images showing the ultrastructure of a trophic (A) and a sessile (B) *D. japonicum* cell. (ch-chromatin, E-extrusomes, ER-endoplasmic reticulum, G-Golgi, M-Mitochondria, n-nucleus, V-Vacuole). Scale bars: 1 µm. Modified from Tashyreva et al., 2018.

Diplonema japonicum and *Diplonema aggregatum* were sourced from the same site, are closely related and share many characteristics with each other (Tashyreva et al., 2018). Both of them are the first recorded *Diplonema* to have a swimming stage, a lack of a paraflagellar rod in the trophic stage, major morphological differences in their cell stages and endosymbionts (Tashyreva et al., 2018). The feeding apparatus of both Diplonemids has additional supporting bundles (together with *Diplonema ambulator* and *hemistasiids*) and their extrusomes only develop in the sessile/swimming stage. The temporary swimming stage suggests extrusomes may be used for scavenging a steady nutrient supply (Tashyreva et al., 2022). Next to the J-shaped turn of the feeding apparatus the large, vesicular nucleus is

located which contains one nucleolus and condensed heterochromatin. The flat, single mitochondrial network extends at the outer edge of the cell and is made up of multiple electron dense DNA patches that are distributed uniformly and few but large lamellar cristae (Tashyreva et al., 2018).

Other than *D. aggregatum*, the *D. japonicum* cells have a longer swimming stage and contain two different endosymbiont families instead of just one. *Diplonema japonicum* has been found to feed on various nutrient sources such as heat killed bacteria and living/dead microalgae (Tashyreva et al., 2022).

1.3. Electron microscopy

Electron microscopy is a microscopy technique that can acquire high resolution images of cell cultures/ tissues, their ultrastructure and nano sized molecules. Compared to photons, electrons have a shorter wavelength and can be accelerated and focused into a beam as a illumination source to reveal smaller structures than light ever could (Merenich et al., 2022).

There are two major types of electron microscopy: Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM).

TEM utilises the electron beam by passing it through an ultrathin specimen section and allows the electrons to interact with the specimen from which then a projection image is generated (Jensen, 2012). The generated electrons are accelerated by an external voltage and are focused by condenser lenses into a beam and after passing through the sample the objective lens focuses the electrons to form a diffraction pattern and the first image. The diffraction pattern is magnified by a projector lens onto the detector which converts the electrons into digital pixels and creates the final image (Inkson, 2016). Due to the strong magnification power and high resolution of TEM (100 um to atomic level) the produced images show elemental and compound structures of the sample. Because TEM samples need to be prepared into ultrathin sections the procedure is time consuming, during sample preparation the specimen structure can be changed and TEM only allows a small field of view. Additionally the ultrathin sections can be destroyed by the electron beam during imaging. (Jensen, 2012).

In SEM the beam “scans” the surface of the sample by focusing the electron beam to interact with the atoms of the sample resulting in various signals. Similarly to TEM electrons are generated by an electron gun, accelerated with an external voltage and focused into a thin beam with condenser lenses. The specimen is scanned in a raster scan pattern by the electron beam and the detector combines the position of the beam with the various signal intensities of emitted electrons to form an image (Inkson, 2016). The resulting signals reveal information of the sample surface topography, composition and electrical conductivity. Large areas of the sample can be scanned with a tremendous depth of focus and brilliant image contrasting in addition to a simple sample preparation (Jensen, 2012).

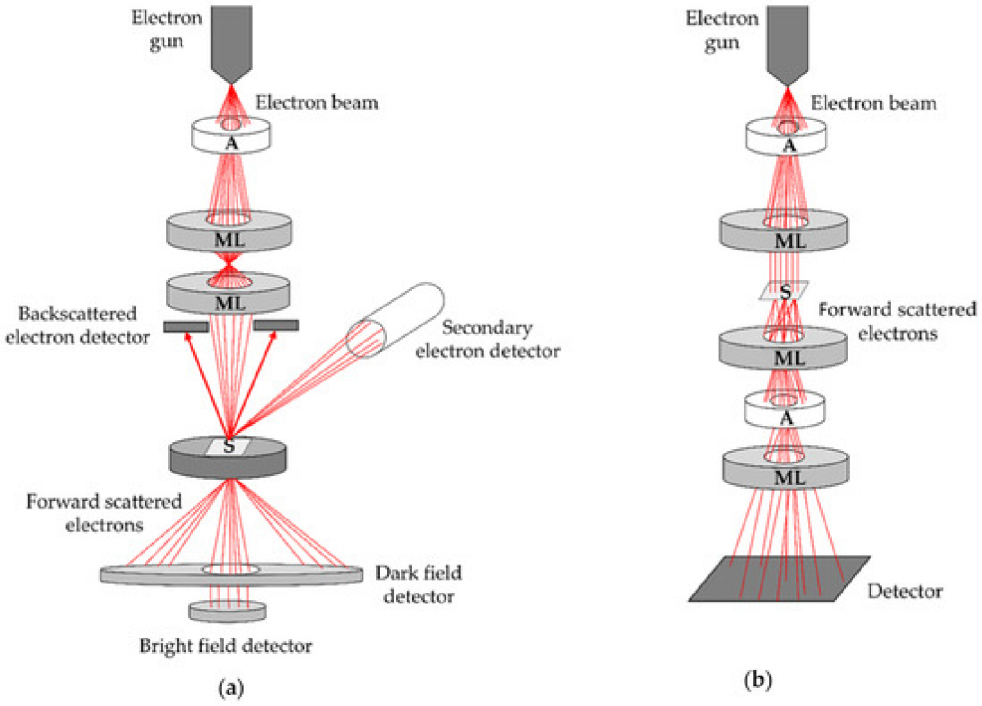

Fig.5: Schematic drawing of SEM (a) and TEM (b) apparatus and their imagination principle. (A-aperture/condenser, ML-magnifying lens, S-specimen/sample). (Malenica et al., 2021)

1.3.1. Serial Block Face Scanning Electron Microscopy

Serial Block Face Scanning Electron Microscopy (SBF-SEM) is a scanning electron microscopy technique which acquires high resolution 3D images. The fixed specimen is stained with heavy metals to improve the contrast in the images and is then embedded in resin to properly place the sample in the microscope (Titze & Genoud, 2016). The vacuum chamber is equipped with an ultramicrotome to place the sample and a diamond knife (Courson et al., 2021). The surface of the sample is scanned by the electron beam in a raster pattern and the backscattered electrons generate a digital image. After the first scan the diamond knife cuts away an ultrathin section from the sample and the ultramicrotome raises the sample upwards so the new layer can be imaged. This automated process is repeated until every layer of the sample has been scanned and the acquired images are arranged in a stack that represents the ultrastructure of the specimen (Courson et al., 2021).

In SBF-SEM the slice thickness is limited to 20 nm and reduces the ultimate resolution in the z-axis. Additionally secondary electrons, generated during the scanning process, can accumulate on the non conductive sample and cause “tissue charging” which produces an electric field that distorts the image or decreases the signal to noise ratio (Courson et al., 2021). Despite lower resolutions and the potential tissue charging, the entire cell or tissue ultrastructure can be quickly and reliably visualised with relative ease. The “cutting and raising the sample” process is fully automated and allows the sample to remain in the vacuum chamber for the entire duration. Compared to other 3D imaging methods, cutting the sample takes only a few seconds and makes the process much more time efficient (Smith & Starborg, 2019).

2. Aims

Verify if there are bacterial endosymbionts really residing fully inside of the *D. japonicum* mitochondria.

Determine the spacial relationship between the bacterial endosymbionts and *D. japonicum* and their interactions.

Check if the number of endosymbionts depends on the cell stage of the host.

Determine if there are differences in the two bacterial endosymbiont species and if we are able to distinguish the 2 bacterial species by their ultrastructural features.

Check if the mitochondrial network is fragmented.

Search for vesicles between the bacteria/mitochondria that might serve in communication between the species.

3. Methods and Materials

3.1. Sample Preparation

3.1.1. Fixation

In order to preserve and stabilise the cell culture in its native state the *Diplonema japonicum* cells were placed in a seawater based medium and then frozen by High pressure freezer (HPF). The fixed cell culture was removed afterwards from the Freeze Substitution (FS) temperature at 4°C.

3.1.2. Contrasting

To enhance the contrast and signal for the SBF-SEM images the fixed cell samples went under heavy metal staining. First the sample was incubated with 2% osmium tetroxide in 100% acetone for seven days at FS temperature. Afterwards the sample was washed three times with 100% acetone for 15 minutes each followed by the addition of 1% tannic acid and the sample was incubated for 30 minutes at room temperature. Once again the sample was washed three times with 100% acetone for 15 minutes each and 2% OsO4 acetone solution was added to the sample tube for 1.5 hours and washed with acetone afterwards. The sample was added in a 1% thiocarbohydrazide in acetone solution for 30 minutes and following the washing step with acetone the sample was added in 2% osmium tetroxide solution for one and half hours. The acetone washing step was repeated and the sample was then incubated overnight in 1% uranyl acetate solution at 4°C.

3.1.3. Resin

Following metal staining the sample was washed with acetone once again and then prepared for resin embedment by gradual infiltration of resin. The sample was incubated in a resin/acetone solution (1:2 ratio) for two hours at room temperature. This step was repeated with 1:1 and 2:1 resin/acetone mixtures. Afterwards the sample was put in pure resin overnight and was subsequently embedded into resin and left to polymerize for 48 hours.

The acquired resin blocks were cut and trimmed to form a trapezoid block face with the sample visible on the surface.

3.2. SBF-SEM Imaging

The resin block sample was mounted on a stub and placed in the microtome holder of the scanning electron microscope (Apreo ThermofisherScientific) and the pin was moved manually until the sample surface is at the same level to the knife's cutting edge. The SEM chamber was closed and evacuated to create vacuum conditions at 30 Pascal. On a connected computer were the control settings for the electron microscope where the imaging parameters are set.

An Electron beam which scans the sample was accelerated by an external voltage of 3.77 kV, probe current 50 pA, dwell time of 3 µs and pixel size 6 nm. First the surface of the sample was scanned to determine a region of interest with four tiles. The resin block was cut into thin slices of 75 nm by the diamond knife, raised to the focal point and imaged by the electron beam automatically. This process was repeated until each slice of the sample had been imaged and was saved digitally. The resulting stack contained 662 images composed of four tiles with an overlap of 10% and a resolution of 6144x6144 pixels for each tile.

3.3. Image Processing and observation

The obtained images of the cell culture were assembled into a 3D stack using the Microscopy Image Browser software (MIB) and saved as an amira file. MIB was also used for all further image processing, adjustments, observation and statistics. The stack of images was normalised to adjust their brightness and contrast which allows to view the individual organelles.

First the overall cell culture was observed to see the different types of interactions between the endosymbionts and the mitochondrial network. Cells which had close or unusual contact to the mitochondrion were documented in an excel file with their stack coordinates, slides and cell stage. To remember which cells had already been looked at they were marked with paint every 10 slides using the segmentation tool.

To get a reliable statistical count on how often close interactions between the bacterias and their mitochondria occur, 20 randomly selected cells from the image stack were used. The possible bacteria-mitochondria interactions were divided into “free in cytoplasm” “bacterium touching mitochondria” “half of bacteria surrounded by mitochondria” “bacteria almost surrounded” and “bacteria completely surrounded by mitochondria". For each selected cell the total bacterial count with their mitochondrial proximity was noted. Additionally the cell stage and possible mitochondrial fragmentation was noted. Calculations and the generation of diagrams was performed with Excel.

In order to verify potential interactions between the endosymbionts and the mitochondria, the existence of vesicles between them and possible fragmentation of the mitochondrial network were investigated.

3.4. Image segmentation and 3D modelling

One cell was found to harbour multiple endosymbionts enclosed by the mitochondrion and was cropped out from the stack. By using the MIB segmentation tool the endosymbionts, mitochondria and the entire cell cytoplasm were segmented and saved as a model.

The segmentation model was imported to the Amira software and converted to a 3D Model which allows the full view of the cell. With the constructed model the endosymbiont structure and the interactions with the mitochondrion could be better visualised and investigated. By comparing the constructed model with the SBF-SEM images the shape of the endosymbionts was determined.

The MIB program includes a statistics tool which is able to calculate the size of segmented objects which is used to determine the volume of the endosymbionts. With the help of the model and the MIB program the endosymbionts are compared based on their size/shape/ongoing bacterial division and their interaction with the mitochondria. Similarly to the previous section the diagrams were generated with excel.

Besides determining differences in the endosymbionts the 3D model is also used to investigate for mitochondrial fragmentation in the selected *Diplonema Japonicum* cell.

4. Results

4.1. Endosymbiont Mitochondria interaction

The obtained images from the Scanning electron microscope were aligned into stacks with each image representing one slice of the fixed cell culture in resin. This allows for the viewing of the internal ultrastructure of each Diplonemid cell in 3D.

The mitochondria look like long, dark grey tubes at the edge of the cell and appear at first discontinuous. However by looking at multiple slices it shows that all of the mitochondrion strings are actually connected and form one large network. The endosymbionts are black and circular/cylindrical spots which are scattered throughout the cell visible on several slices.

By being able to see the inside of the cells, the varying arrangements between endosymbionts and the Diplonemid mitochondria could be seen. Multiple cells in the stack were investigated and notable interactions between the endosymbionts and mitochondria were noted until a pattern could be observed and the interactions between the species could be categorised.

The obtained cell culture has mostly trophic cells with a few sessile ones and no swimming cells. Regardless of cell stage, all diplonemid cells contained multiple endosymbionts varying in their interactions with the mitochondria.

In the SBF-SEM image stack the bacteria placement in relation to the mitochondria could be categorised in five categories/groups:

- **Bacteria free in cytoplasm**: The endosymbiont resides in the cell cytoplasm and is not close to the mitochondria.

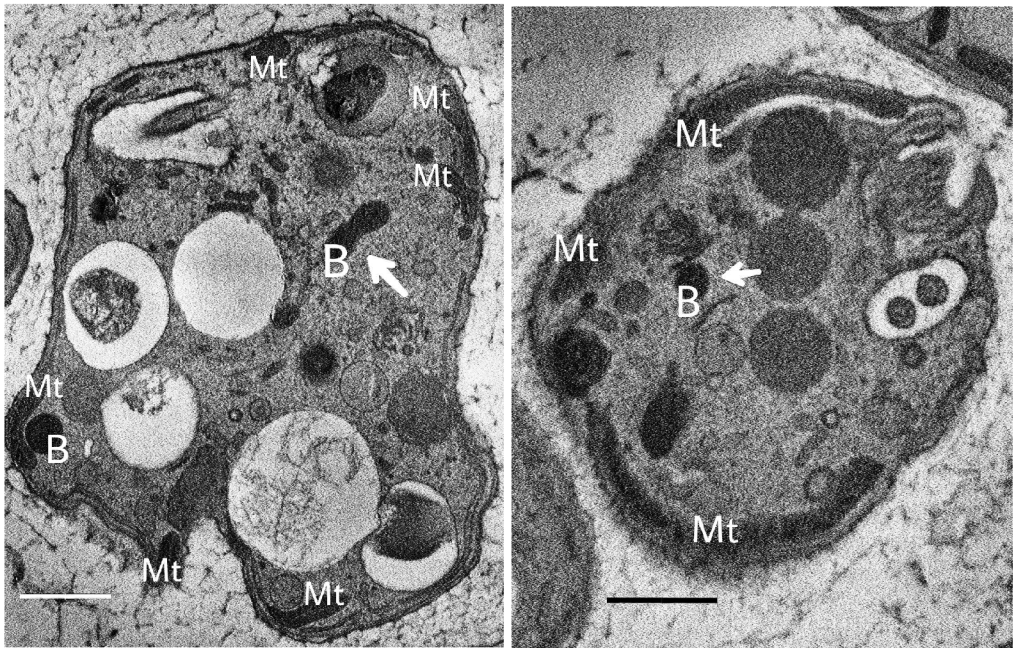

Fig.6: Trophic cells with endosymbionts in the cytoplasm (arrow) (B-bacteria, Mt-mitochondria). Scale bars: 2 µm

- **Bacteria touching mitochondria:** The endosymbiont is placed in close proximity to the mitochondrial network and appears to "touch" the organelle. However the bacteria is for the most part exposed to the cytoplasm.

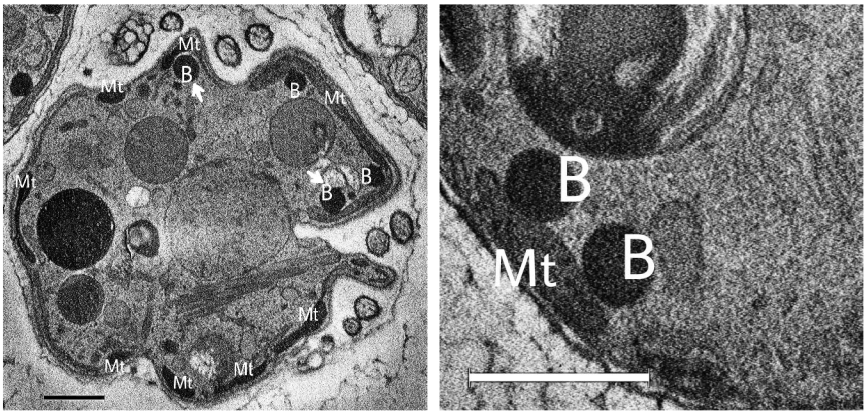

Fig.7: Sessile cells (left) with bacteria adjacent to mitochondria (arrow) and close up of two bacteria touching the mitochondria of a trophic cell (right). Scale bars: 2 µm

- **Half of the bacteria is surrounded by the mitochondria:** It appears as if the mitochondria is around the bacteria like a hook but half of the endosymbiont is still exposed to the cytoplasm.

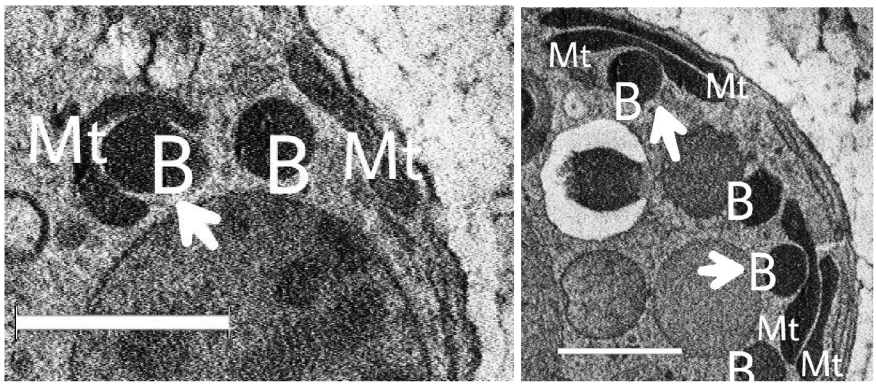

Fig.8: Close up of half surrounded bacteria (arrow) in two different trophic cells (B-bacteria, Mt-mitochondria). Scale bars: 2 µm

A subtype of this interaction is where the mitochondria wraps around multiple endosymbionts at once giving it a snake-like shape. While it can be found in both cell types, it is more common in sessile cells.

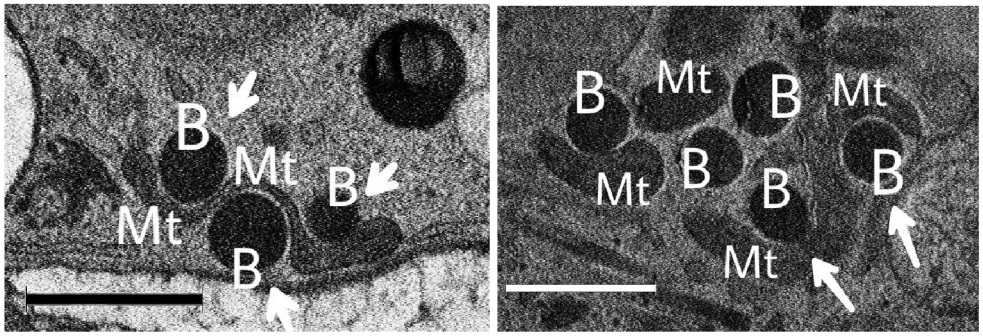

Fig.9: Multiple half surrounded bacteria by one mitochondrial string in different cells (arrow) (B-bacteria, Mt-mitochondria). Scale bars: 2 µm

- **The Bacteria is surrounded by the mitochondria with an opening:** The endosymbiont is surrounded by the mitochondrial network but there are still open channels and free communication with the cytoplasm.Here the 3D imaging provided by SBF-SEM proved to be useful as this type of interaction requires the use of multiple slides the most as the endosymbiont can look only half surrounded on one slice but fully surrounded at the next slice.

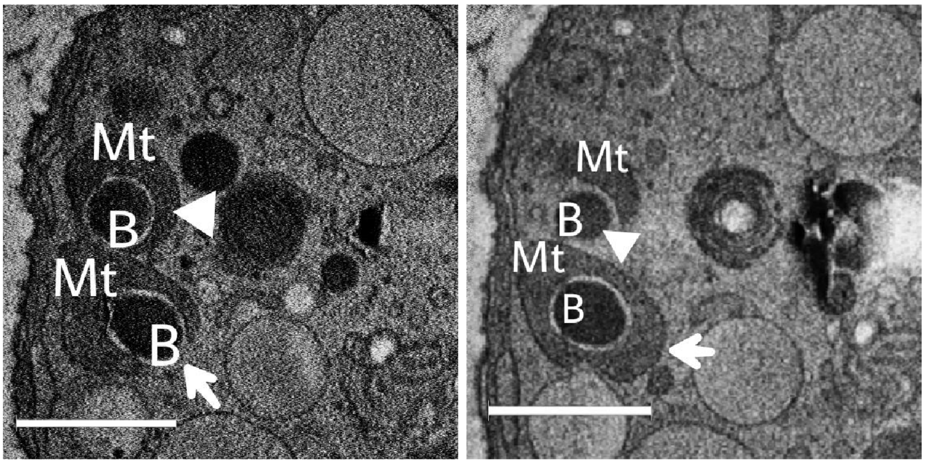

Fig.10: Different sections of the same bacteria (arrow) are exposed to the cytoplasm (left) and surrounded by the mitochondria (right). The adjacent bacteria (arrowhead) is surrounded by the mitochondria in the first section (left) and exposed to the cytoplasm in the following section (right). (B-bacteria, Mt-mitochondria). Scale bars: 2 µm.

- **Bacteria fully surrounded by mitochondria:** The endosymbiont is surrounded by the mitochondrial network without an opening to the cytoplasm.

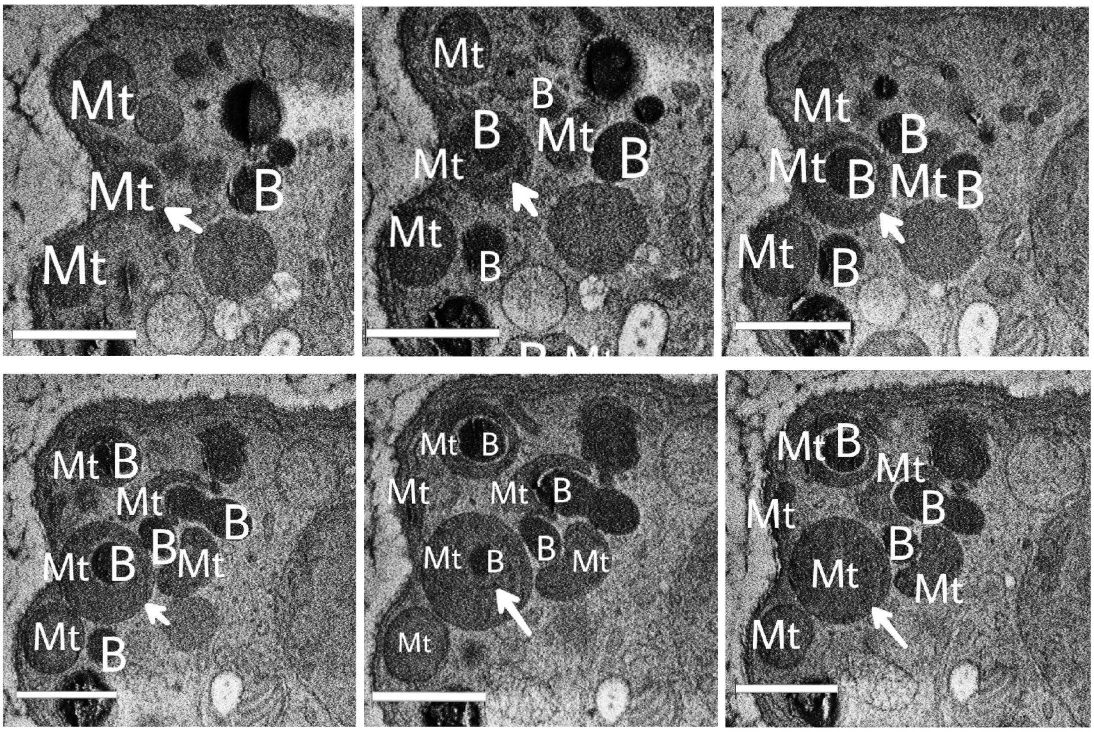

Fig.11: Stack of images showing bacteria fully surrounded by the mitochondria (arrow) for all sections in the cell.(B-bacteria, Mt- mitochondria) Scale bars: 2 µm

4.1.1. Bacterial count

From the image stack twenty cells were randomly selected and their endosymbionts and mitochondrial relationship was investigated to determine how common close interactions between the two species are. The majority of cells are in the trophic stage with a few sessile ones. No swimming stage cell could be found in the obtained stack of images.

Table 1: Number of endosymbionts and their interactions with the mitochondria in randomly selected trophic Diplonema Japonicum

Trophic cells	b free in cy	b touching mt	half of b surrounded by mt	b surrounded by mt with opening	b fully surrounded by mt	total b count
Cell 3	24	35	5	1	0	65
Cell 1	23	16	5	0	0	44
Cell 2	13	23	4	0	0	40
Cell 4	13	22	3	5	0	43
Cell 5	16	13	2	1	0	32
Cell 6	22	21	2	0	0	45
Cell 7	11	20	1	0	0	32
Cell 8	10	32	3	1	0	46
Cell 9	11	28	1	0	0	40
Cell 10	16	17	1	2	0	36
Cell 11	11	27	2	0	0	40
Cell 12	9	17	4	0	1	31
Cell 13	22	17	6	1	1	47
Cell 14	10	25	2	1	0	38
Cell 15	10	16	1	0	0	27

Table 2: Number of endosymbionts and their interactions with the mitochondria in randomly selected sessile Diplonema japonicum

Sessile cells	b free in cy	b touching mt	half of b surrounded by mt	b surrounded by mt with opening	b fully surrounded by mt	total b count
Cell 1	26	20	4	0	0	50
Cell 2	54	44	3	1	0	102
Cell 3	12	29	2	0	0	43
Cell 4	25	38	5	1	1	70
Cell 5	10	26	0	0	0	36

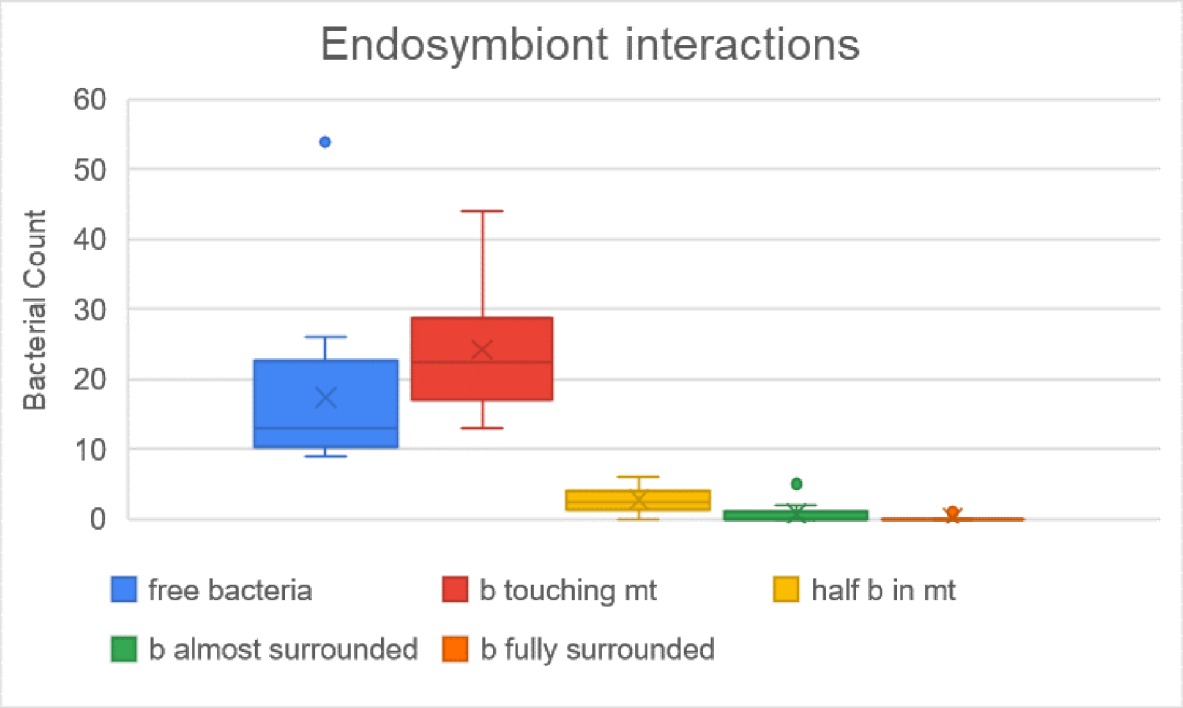

Fig.12: Boxplot generated by Excel of endosymbiont-mitochondria interactions per cell visualising how common the interaction types are. Boxes represent the number of bacteria in a cell with a certain interaction for 50% of the collected data with both quartiles containing 25% each. The middle line represents the median which is the middle count for the number of bacteria in a cell found. Horizontal lines depict 25% of the lower and higher values of the data each and the whiskers stand for the minimum/maximum value and its variability in comparison to the number of bacteria represented by the box. The dots represent outlier values while the cross inside the box stands for the mean value of bacteria with a certain interaction in a host cell.

For every cell an endosymbiont-mitochondria relationship was observed and in most cases more than half of the symbionts were in contact with the organelle. However, very close interactions where the bacterium is engulfed by the network (with or without openings) are rarely encountered and are for the most part restricted to only one bacterium in the entire cell (Table1 and Table2). Endosymbionts that are only half enveloped by the organelle are more common and are found in almost every cell. But even that type of closer interaction is only represented by less than 10% of the total bacterial count (Table 3).

Table 3: Average bacterial count and their interaction with the mitochondria in the randomly selected Diplonema japonicum cells

	B free in Cy	**B touching Mt**	**half of B surrounded by Mt**	**B surrounded by Mt with opening**	**B fully surrounded by Mt**	**total B count**
trophic	14.7 ±5.42	21.9 ± 6.4	2.8 ± 1.67	0.8 ± 1.32	0.13 ± 0.35	40.04 ± 9.09
sessile	25.4 ± 17.57	31.4 ± 9.58	2.8 ± 1.9	0.4 ± 0.55	0.2 ± 0.45	60.2 ± 26.6
total	17.4 ±10.44	24.3 ± 8.2	2.8 ± 1.67	0.7 ± 1.17	0.15 ± 0.37	45.35 ± 16.94

Disregarding the sessile outlier (Table2: Cell2) the number of endosymbionts and their closeness to the mitochondria appears relatively similar between both cell stages. The amount of endosymbionts in a cell also does not seem to correlate with their closeness to the

mitochondria since the highest (Table1: Cell3 and Table2: Cell2) and lowest (Table1: Cell 15 and Table2: Cell 5) count for both stages have no fully surrounded bacteria. However the trophic and sessile cell with the most endosymbionts both have one endosymbiont that is almost engulfed in the mitochondria and additionally they have more endosymbionts that are half surrounded by the mitochondria.

4.1.2. 3D Model

While investigating and categorising the endosymbiont-mitochondria interactions, a trophic cell with an unusually high amount of engulfed bacteria has been discovered. The cell which will be now referred to as "model cell" was cropped from the stack and was used for further analysis.

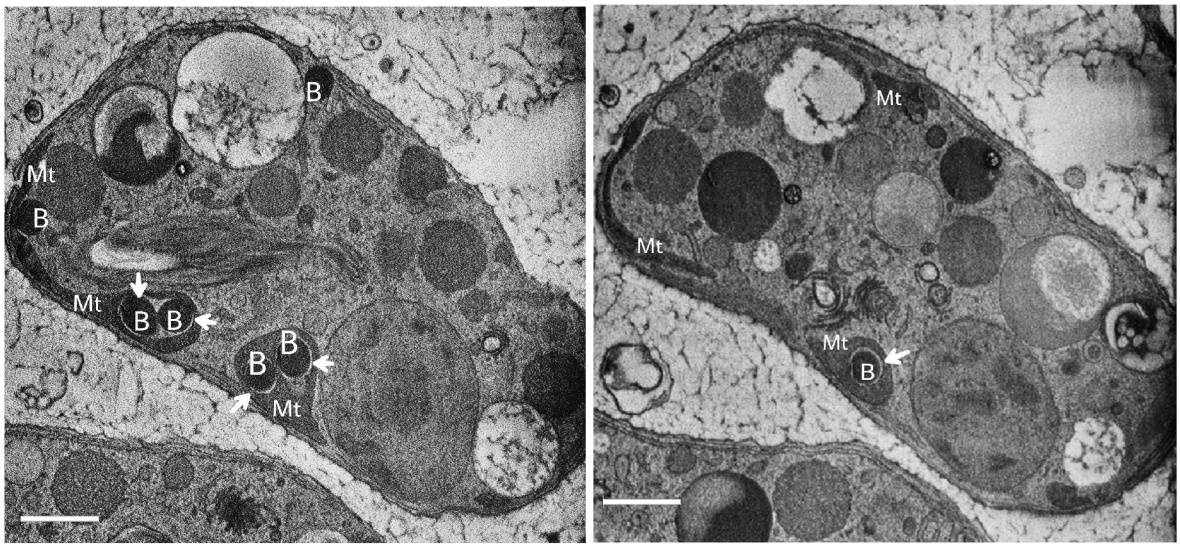

Fig.13: Subsequent section of the model cell with surrounded bacteria (arrow). (B-bacteria, Mt-mitochondria). Scale bars: *2 µm*

Similarly to the cells in Tab1 and Tab2 from the bacterial count, the types of endosymbiont-mitochondria interaction were counted and categorised for this model cell.

Table 4: Endosymbiont count and their relationship to the mitochondria

trophic cell	b free in cy	b touching mt	b half surrounded	b almost surrounded	b fully surrounded	total b count
	10	7	9	7	3	36

The resulting model in Amira shows the mitochondrial network of the model cell and its endosymbionts.

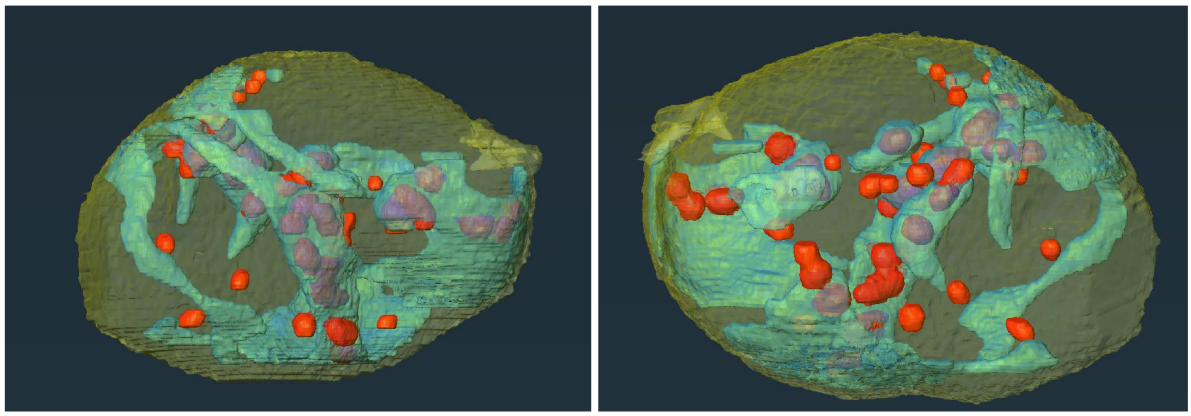

Fig.14: Constructed 3D model in Amira of the segmented model cell from different viewpoints (cytoplasm/cell-yellow, mitochondria-blue, bacteria-red)

The model shows how certain endosymbiont-mitochondria interactions look like when viewing the whole endosymbionts at once (Fig.15). In addition the model shows how a fully surrounded bacteria and the mitochondria look like as a whole (Fig.16)

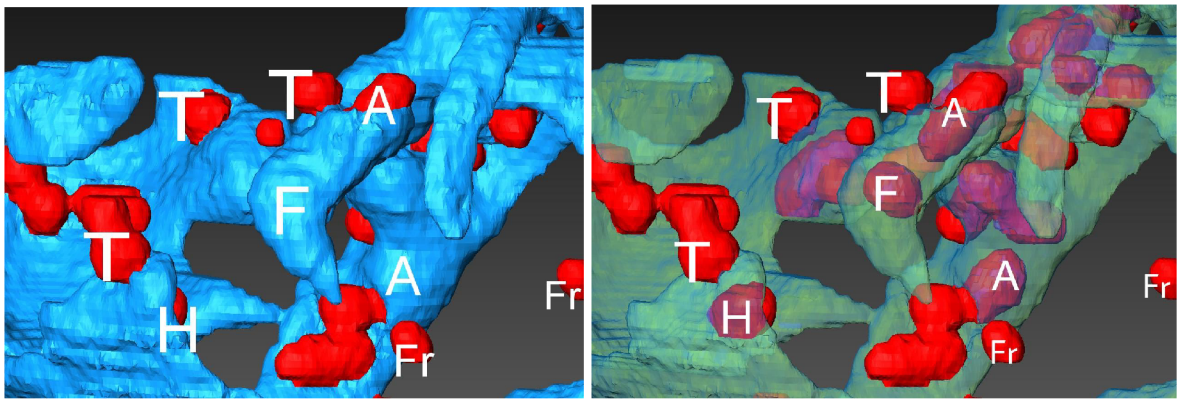

Fig.15: Multiple Endosymbiont-Mitochondria interactions in the Amira Model with non transparent mitochondria (left) and transparent mitochondria (right) to visualise bacteria fully/partially surrounded by the mitochondria. (mitochondria-blue, bacteria-red, F-Fully surrounded, A-Almost surrounded, H-Half surrounded, T-touching mitochondria, Fr-Free in cytoplasm)

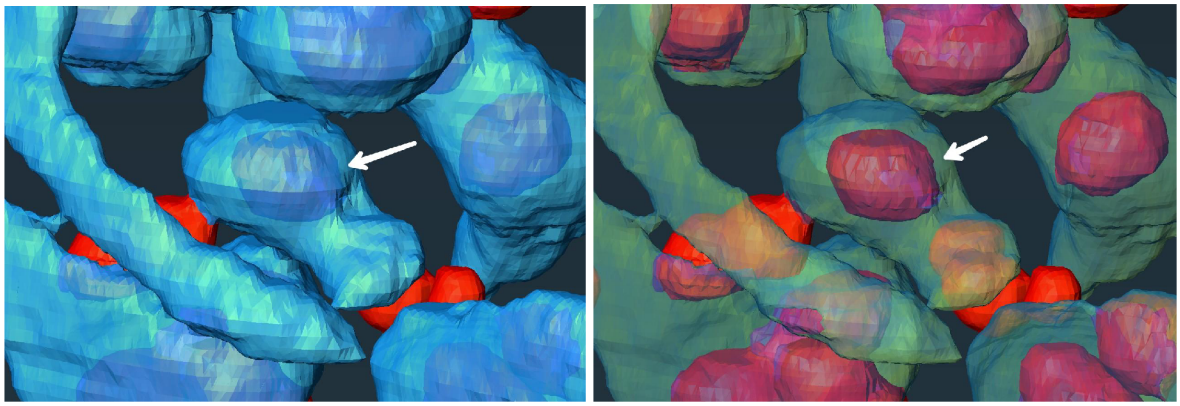

Fig.16: Fully surrounded bacteria (arrow) in Amira Model with partially transparent mitochondria (left) and more transparent mitochondria (right) of the same region. (mitochondria-blue, bacteria-red).

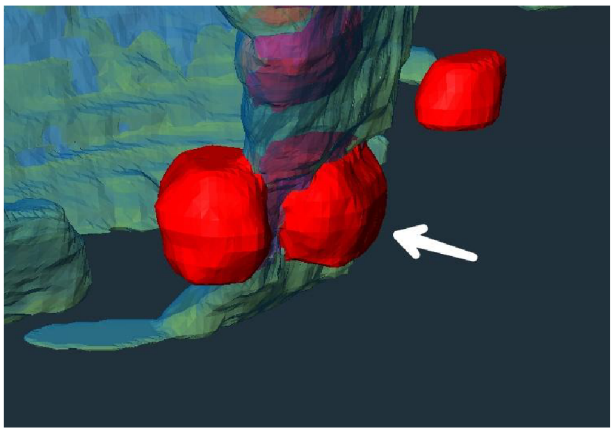

Fig.17: Half surrounded bacteria (arrow) in the 3d model. (mitochondria-blue, bacteria-red).

4.2. Endosymbiont size and species

D. japonicum harbours two different endosymbiotic bacterial species but in the SBF-SEM images the endosymbionts are only depicted as dark grey shapes. At most the endosymbionts can only be distinguished by their shape as some bacteria appear round and others more cylindrical (Fig.13). But even the shape distinction can be unreliable as cylindrical endosymbionts can be aligned with the z-axis and therefore make it appear rounder due to the top view of the stack images.

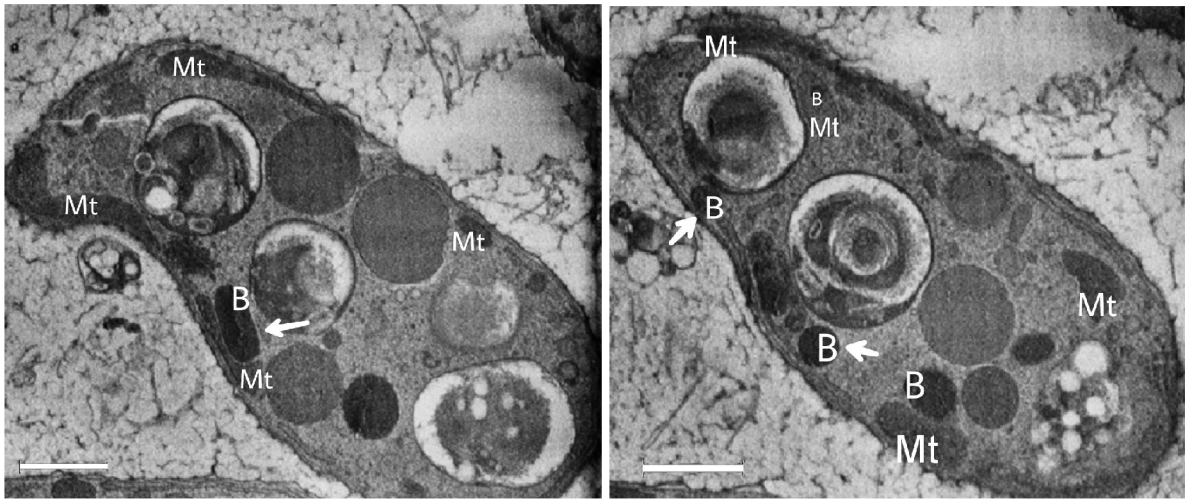

Fig.18: Oval endosymbiont (left) and round endosymbiont (right) inside the model cell shown by arrow (B-bacteria, Mt-mitochondria). Scale bars: 2µm

By using the "Statistics" tool in the MIB program the total number of pixels of a segmented endosymbiont could be determined and is then converted into cubic micrometres. The "Statistics" tool in MIB determines an object's volume based on the overlapping and connected segments. This can lead to two different endosymbionts who are spatially close to another to be considered as one object. To overcome this the segmentation of two close endosymbionts is removed to create more distance for the program to consider them as two individual objects. This compromise results in size values smaller than their true volume for some of the bacterial endosymbionts.

Each bacteria was categorised by their size, shape, mitochondria interaction and if they are undergoing bacterial division by comparing the SBF-SEM slices and the Amira model. Round endosymbionts undergoing division appear as two bacterias who are merged into one large object (Fig19). Due to the segmentation tool causing two closely neighbouring bacteria to appear as one object, the 3D model was more unreliable in distinguishing dividing endosymbionts. For oval bacterias no distinct feature could be found to determine if they are undergoing division or not.

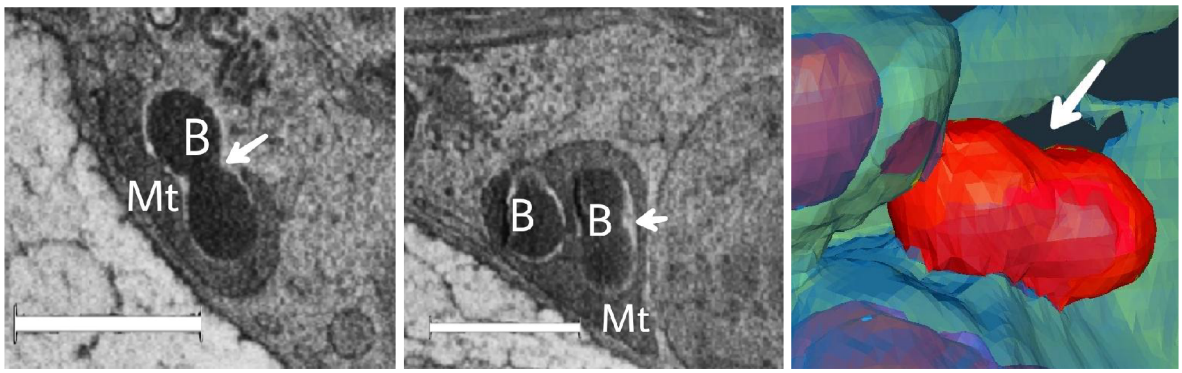

Fig.19: Dividing Bacteria (arrow) in the SBF-SEM images (left,middle) and 3D model (right). (mitochondria-blue, bacteria-red, B-bacteria, Mt-mitochondria). Scale bar: 2µm.

Table 5: Characteristics of the Endosymbionts inside the model cell from the MIB program and the Amira model

Size (μm^3)	**Shape**	**Bacterial division**	**Interaction**
1.272	round	dividing	fully
1.207	round	dividing	almost
1.111	round	dividing	almost
0.975	oval		almost
0.927	round	dividing	touching
0.907	oval		almost
0.869	round	dividing	touching
0.802	oval		half
0.769	oval		half
0.724	oval		half
0.673	oval		touching
0.634	round	dividing	half
0.600	round	dividing	almost
0.573	oval		touching/half
0.549	oval		half
0.510	round		almost
0.475	oval		half/touching
0.449	round		half
0.439	round		fully
0.417	round		free
0.405	round		half
0.390	round		free
0.380	round		half
0.365	round		fully
0.333	oval		free
0.327	round		free
0.321	round		almost
0.297	oval		free
0.272	round		free
0.251	round		free
0.178	round		free
0.148	round		free
0.127	round		touching
0.124	round		touching
0.108	round		free
0.101	round		touching

There is a higher count of round bacteria in the model cell over the oval ones but both types are in contact with the organelle.

Table 6: Endosymbiont-Mitochondria interactions between the two different bacterial species inside D.Japonicum

	b free in cy	b touching mt	b half surrounded	b almost surrounded	b fully surrounded	total b count
oval	2	2	5	3	0	12
round	8	5	4	4	3	24

Only round bacteria have been found to be fully enveloped by the mitochondrial network. However the oval shaped ones are more likely to be in closer contact to the mitochondria and only a small number of them are free in the cytoplasm.

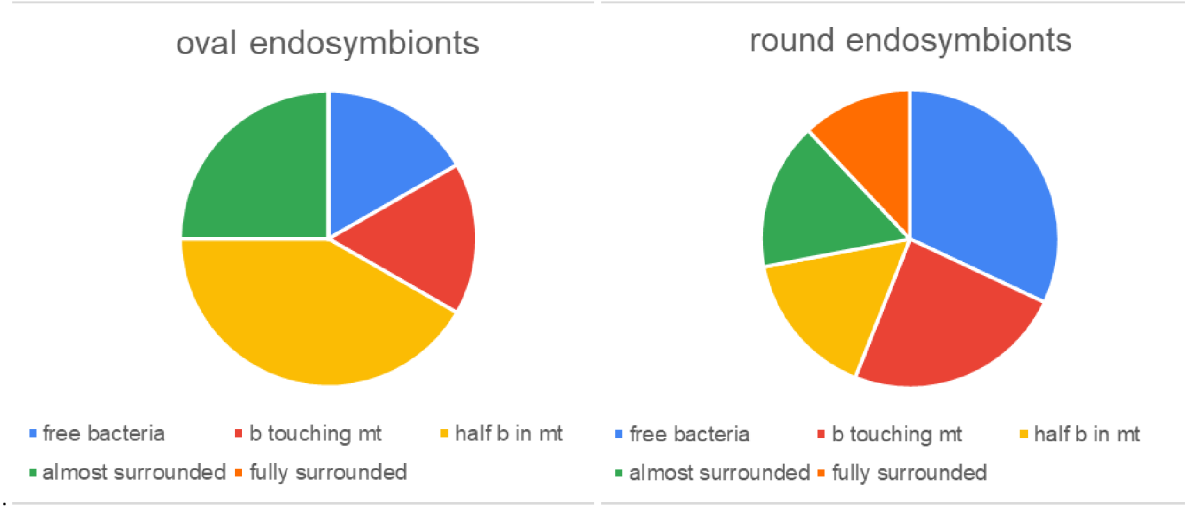

Fig.20: Pie chart showing the distribution of endosymbiont-mitochondria interaction of the two bacterial shapes inside the *D. japonicum* model cell

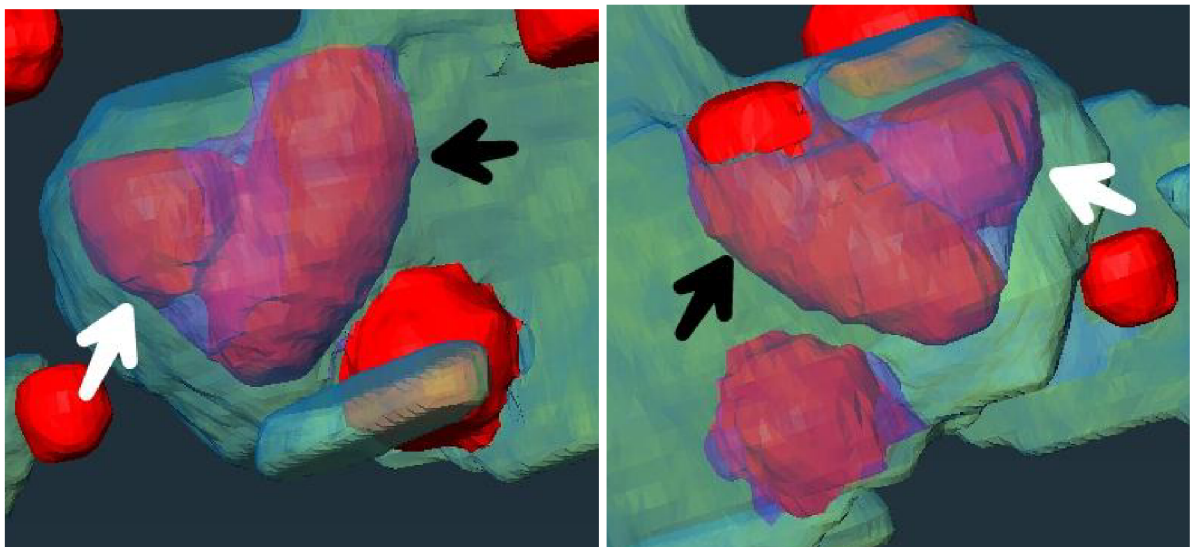

Fig.21: Fully encircled round bacterium (white arrow) and oval bacteria with gap to the cytoplasm (black arrow) next to each other from back (left) and front (right) view (mitochondria-blue, bacteria-red)

Bacterias with larger volume tended to be closer to the mitochondria than smaller ones which were more likely to be free in the cytoplasm (Table 5.). Some of the larger

endosymbionts were undergoing bacterial division and were all in contact with the mitochondria. The largest endosymbiont was undergoing bacterial division and was also fully surrounded by the network. Three of the seven dividing endosymbionts were surrounded by the mitochondria with openings to the cytoplasm. The remaining three dividing endosymbionts are either touching or half surrounded by the network.

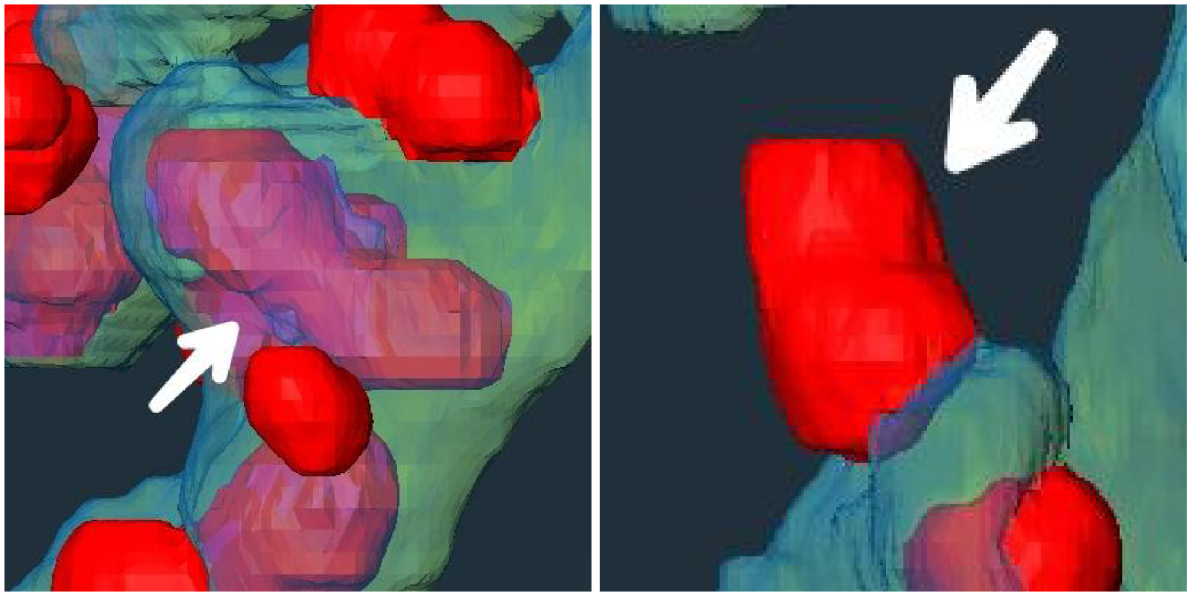

Fig.22: Dividing bacteria inside the mitochondria (left) and touching the mitochondria (right) shown by an arrow (mitochondria-blue, bacteria-red).

4.3. Mitochondrial fragmentation

For each cell their mitochondrial network was examined. For the majority of cells the network is fully connected, however 4 trophic cells and 1 sessile cell show possible fragmentation and one sessile cell appears to have a fractured network. However the cross section of the cells shows the mitochondria as multiple separated strings and only by following those strings over multiple slides is it apparent that the mitochondria is a connected network. Because of that it can be difficult determining mitochondrial fractions with 2D images as the connecting branch between mitochondrial strings can be extremely thin and not visible at first while actual fragments can be easily overlooked.

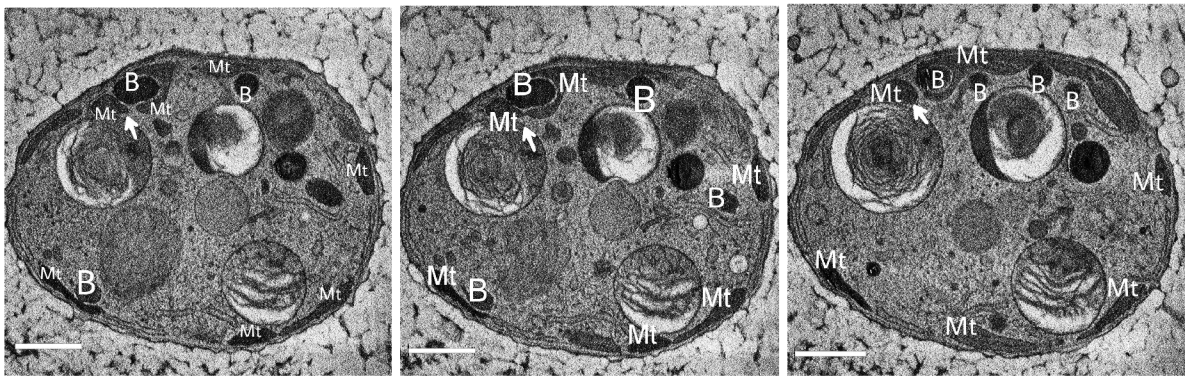

Fig.23: Stack of images showing a small potential mitochondrial fragment (arrow). However the middle image shows a small potential connection between the mitochondrial pieces (B-bacteria, Mt- mitochondria). Scale bars: 2µm

In the cell that was used to construct a 3D model for endosymbiont-mitochondria interaction no fragmentations were observed at first from the stack of SBF-SEM images. But in the model a mitochondrial fragment was found.

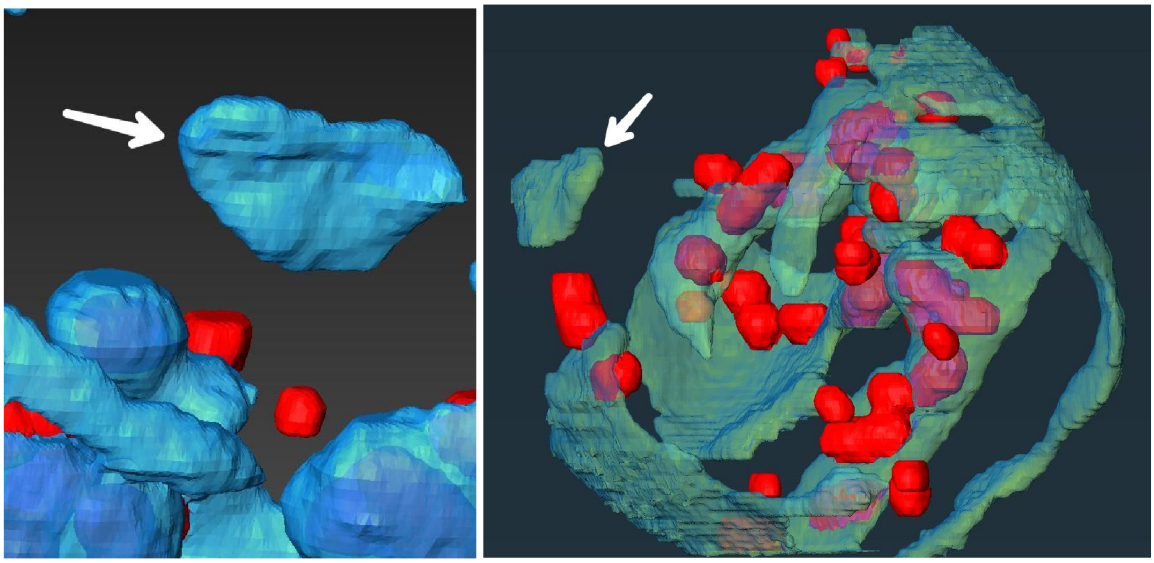

Fig.24: Potential mitochondrial fragment (arrow) in the 3D model, close up (left) and comparison to the total mitochondrial network (right). (mitochondria-blue, bacteria-red)

In the SBF-SEM images the fragment corresponds to a very dark grey mitochondrial string at the upper edge of the cell. From the slices (Fig. 35) the mitochondrial string doesn't seem to link with another large mitochondrial piece and therefore connect it to the larger network. However the fragment might actually be connected to the larger network but the string is not as visible due to poorer contrasting.

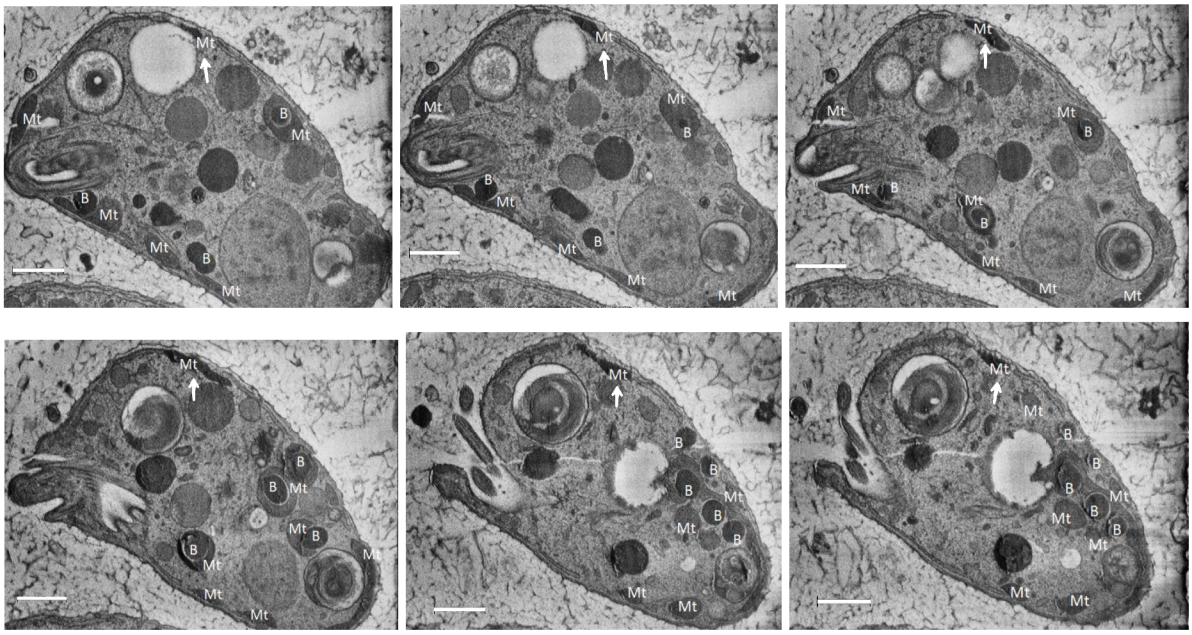

Fig.25: Stack of images showing Potential mitochondrial fragment (arrow) in the SBF-SEM images (B-bacteria, Mt-mitochondria). Scale bars: 2µm

At the top of the 3D model two small mitochondrial pieces were found where a bacterium appears to be in between and therefore disconnecting one piece from the larger network.

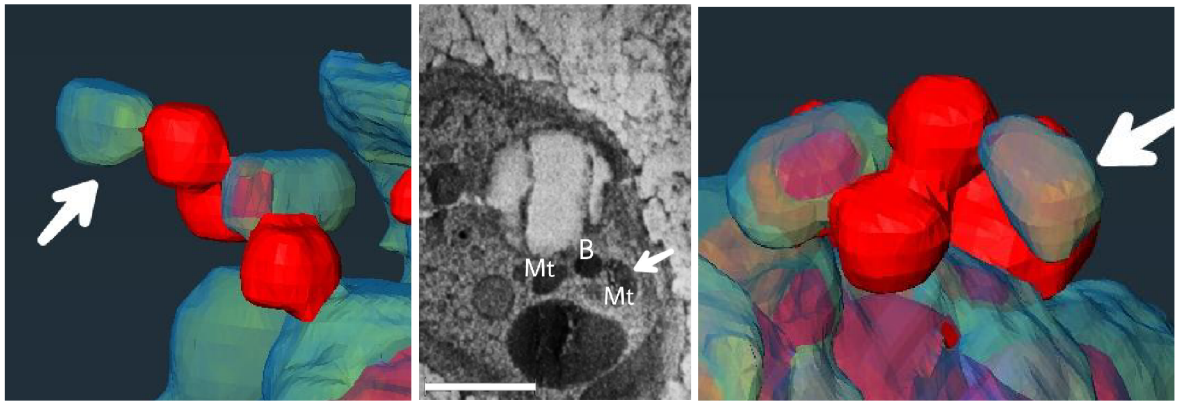

Fig.26: Visualisation of one of the mitochondrial fragment (arrow) in the 3D model (left and right) and the SBF-SEM image stack (middle). (mitochondria-blue, bacteria-red, B-bacteria, Mt-mitochondria) Scale bar: 2µm

In the SBF-SEM images the fragment's colour and placement near an endosymbiont makes it appear as a mitochondrial part. But because of the small size and shape of the fragment, it could have been a different cellular component or an artefact of the 3D model.

Mitochondrial parts which are fully surrounding bacteria were sometimes found to only have a thin and small connection to the total network. While this can not be considered fragmentation it could be argued that these mitochondrial parts are less strongly attached to the whole network.

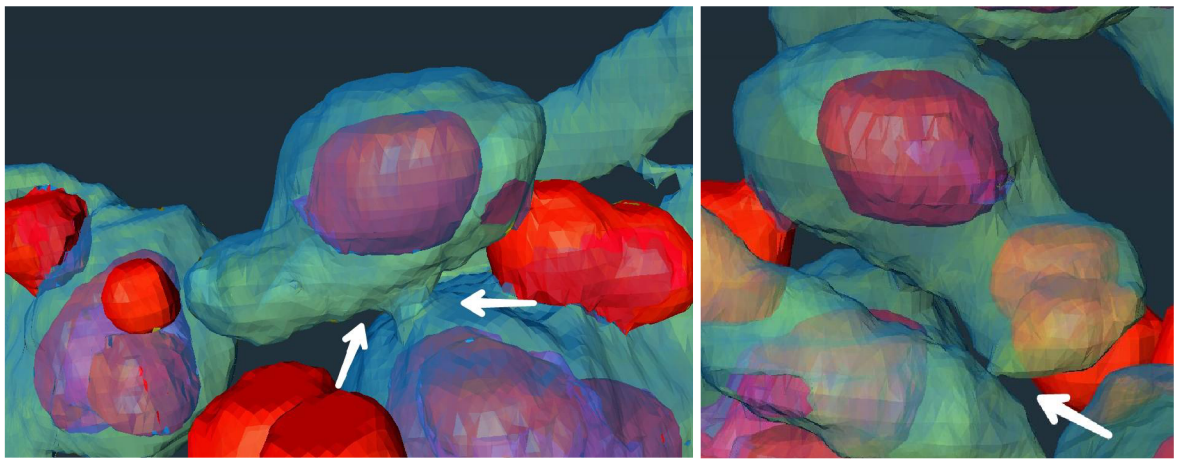

Fig.27: Bacteria and the mitochondrial parts fully surrounding them showing a thin connection to the mitochondrial network, arrow (mitochondria-blue, bacteria-red)

4.4. Vesicles

Most of the endosymbionts in a *D. japonicum* cell are adjacent to the mitochondria or even reside inside the network. However the SBF-SEM images of fully enclosed endosymbionts shows a gap between the mitochondria and the bacterium indicating that the bacteria isn't residing in the mitochondrial matrix. In the case that the two species exchange metabolites they would require carriers or adaptations to overcome the membrane barrier.

The image stacks were investigated for potential vesicles near the endosymbionts however nothing could be found due to the insufficient resolution of the SBF-SEM dataset.

5. Discussion

After observing multiple slices of SBF-SEM images it is now confirmed that some endosymbionts are fully enclosed by the mitochondria (Fig.11) and the constructed 3D model of a *D. japonicum* cell shows how some endosymbionts appear to penetrate the network (Fig.15). The 3D model in particular shows that dividing endosymbionts are close to the network and bacteria larger in volume size generally tend to be in close contact with the mitochondria (Table 5). This could suggest that the endosymbionts may use ATP directly from the mitochondria for bacterial replication and growth. Besides ATP, the endosymbionts of *D. japonicum* could take nucleotides from the organelle since the Diplonemid mitochondria contains a huge amount of non coding DNA (Lukeš et al., 2018). According to previous observation the sessile stage has a lower count of endosymbionts (Tashyreva et al., 2018) but in our experiment the number was equal to the trophic stage and one sessile outlier even exceeded the average count (Table 1 and Table 2). The endosymbionts in trophic cells show more "close interactions" with the mitochondria than their sessile counterpart but this might be due to the lower number of sessile cells in the sample. The trophic stage exists in a nutrient rich environment and is more likely to provide ATP to the endosymbionts which is why they might be closer to the mitochondria (Tashyreva et al., 2018).

In the SBF-SEM image stack the two different endosymbiont species could not be distinguished as no ultrastructural differences were seen. The only identification of the individual endosymbiont species would be based on their shape and size (George 2020) which unfortunately was unreliable with the SBF-SEM individual images as they showed only the cross section of each layer and can make oval shaped bacteria appear round. The 3D model is therefore more reliable in differentiating the endosymbionts by their shape. Rounder bacteria are more commonly found in the cell than oval ones and were additionally found to be fully surrounded by the mitochondria. However oval ones were more likely to be in contact with the mitochondria with only a small fraction residing freely in the cytoplasm. No definitive proof for the different endosymbiont species can be made based on the bacterial shape alone but previous research found that the *Nesciobacter abundans* is smaller and also more abundant in the cell meanwhile *Cytomitobacter primus* is more elongated (George et al., 2020). Based on that it may be assumed that the rounder endosymbionts are *Nesciobacter abundans* as they are more prominent in the host cell and the oval ones would be *Cytomitobacter primus*. However since some endosymbionts undergo bacterial division it is more difficult and unreliable to only distinguish them by their size and shape.

Recently George et. al. found vesicle-like structures in the outer membrane surface of the two *D. japonicum* symbionts but it's unknown if the vesicles are derived from the *D. japonicum* host or the endosymbionts (George et al., 2022). The discovery of vesicles indicates possible communication between the two species. In a cooperative case the vesicle suggests a transport mechanism of proteins but in a more antagonistic way the lipid structure could hint at the host defending itself or the bacteria weakening the host's immune system (Zou et al., 2022). Because of their small size, vesicles could not be detected in the obtained image stack of this study due to the poorer resolution of Scanning Electron Microscopes.

Both endosymbiont species of *D. japonicum* belong to the *Holosporaceae* family which include members of obligate endosymbionts or pathogens and could indicate that the bacteria

receive the benefits of the endosymbiotic relationship. Additionally the genome of all known diplonemid endosymbionts are strongly reduced with most energy metabolic pathways such as glycolysis, TCA cycle, oxidative phosphorylation complexes being absent or incomplete. The diplonemid endosymbionts however encode genes for ADP/ATP translocase which suggests that they take up ATP from their host (George et al., 2020). In another Diplonemid species *Namystynia karyoxeno*, the endosymbiont *Candidatus Sneabacter namystus* have been observed to replicate inside the Diplonemid nucleus (Prokopchuk et al., 2019). Although the endosymbionts of *D. japonicum* and *N. karyoxenos* belong to two different bacterial families and reside in different organelles, the endosymbionts might use their Diplonemid hosts for a similar reason.

The only other known case of an endosymbiont inside the mitochondria is that of the *Canidatus Midichloria mitochondrii* which resides in the mitochondria of ovarian cells of the female *Chlamydia psittaci* ticks. The *Ca. M. mitochondrii* was speculated to consume the mitochondria for bacterial replication due to the mitochondrial matrix appearing reduced (Sassera et al., 2006) which would mirror the theoretical case of the *D. japonicum* endosymbionts. Recent research and models however suggest a more functional behaviour for the tick host rather than a parasitic/predatory one. *Ixodes ricinus* larvae which had their endosymbiont removed have a lower success rate in blood feeding compared to the wildtype counterpart (Guizzo et al., 2023). During blood feeding the tick might enter a hypoxic state where the endosymbiont assists the host's cellular respiration by low oxygen tension phosphorylation (Stavru et al., 2020). Diplonemid diversity generally increases with deep sea waters which is an environment of lower oxygen and could benefit from a *Ca. M. mitochondrii* like function. A functional relationship for *D. japonicum* is more unlikely because the genome of diplonemid endosymbionts lacks or only has reduced essential metabolic pathways and instead contains mostly protein secretion systems (George et al., 2020). Most of the essential metabolic pathways are absent or reduced, ATP synthase and the non oxidative pentose phosphate pathway is still retained in diplonemid endosymbionts (George et al., 2020). The replication theory is also more relevant in the case of *D. japonicum* since all dividing bacteria in this study were found inside or in contact with the mitochondria while replicating *Ca. M. mitochondrii* were mostly found outside the mitochondria (Comandatore et al., 2021).

Another argument for a non predatory interaction for *Ca. M. mitochondrii* is the predator model of *Ca. Midichloria mitochondria*. According to that model the mitochondrion can only support up to 3 bacteria inside before undergoing lysis while in reality up to 10 symbionts can be found inside a single mitochondrion (Comandatore et al., 2021). On the other hand *Diplonema Japonicum* only has a singular mitochondrial network instead of individual mitochondrions and could theoretically sustain more bacteria before undergoing degradation. In this study fully surrounded bacteria are also more rare in *D. japonicum* compared to *Ixodes ricinus* and in the case of the model cell the 3 fully and 7 almost surrounded bacteria could have led to mitochondrial fragmentation.

For the most part the mitochondria consists of a single network but some cells were suspected to have mitochondrial pieces that are not connected to the larger network. While mitochondrial fragments were overlooked at first in the SBF-SEM images of the model cell, the 3D model shows fragments of the network. The largest fragment is not in contact with

any endosymbiont and is distant from the larger network but the shape in the stack slices and 3D model indicates it is a part of the mitochondria (Fig.24). However the fragmented piece could be attached to the larger network but the connecting piece might not be as clearly visible due to it not being well contrasted or the string being too thin. For the smaller fragment the model makes it appear as if an endosymbiont came between the fragment and split it off from the larger network (Fig.26). Both of the disconnected mitochondrial pieces are not reliable as the smaller piece could have been a different cellular component which just looks similar to the mitochondria in the SBF-SEM images. Mitochondrial fragmentation is often associated with oxidative stress and even cell death and could hint at a negative relationship between host and endosymbiont (Zorov et al., 2019). Endosymbionts could either try to "steal" the limited reserves of ATP or other metabolites and therefore consume the mitochondria. However the SBF-SEM image stack view limits further exploration and for the 3D model fragmentations may be damaged mitochondrial parts unrelated to the endosymbionts, particularly because the largest fragment is not in contact with any bacterium.

6. Conclusion

With the help of the Serial Block face scanning electron microscope, 662 sections of a *Diplonema japonicum* cell culture were imaged. This allowed us to view the internal structure of entire cells and to analyse the interaction between the endosymbionts and the mitochondria of multiple cells. In addition a 3D model based on the SBEM images of a cell was constructed to visualise the whole cell.

Most of the endosymbionts are placed adjacent to the mitochondria and in rare instances it is fully surrounded by the organelle which makes it the second known case so far. Across the two cell stages “trophic” and “sessile” the number of endosymbionts is similar and in this study specifically it is higher in sessile cells on average. Nevertheless almost and fully surrounded bacteria are more commonly found in trophic cells. *Diplonema japonicum* harbours two different endosymbiotic species but couldn’t be properly distinguished in the SBF-SEM images. Instead bacteria could only be differentiated based on their shape “round” or “oval”. Both shape types are in contact with the mitochondria but in this study only round bacteria were found to be fully surrounded while oval ones are more likely to be in closer contact with the organelle (half and almost surrounded). Endosymbionts which are in close contact to the mitochondria tend to be larger in size and/or undergo bacterial division. Because of that, the assumption in this study has been made that both endosymbiotic species directly take up ATP or other metabolites from the mitochondria for bacterial growth and replication. This interaction might be harmful for the cell since the 3D model showed a mitochondrial fragment. It is however difficult to confirm the existence of those fragments and even more so to prove that they are connected to the endosymbionts. Finally vesicles which may aid in the transfer of metabolites between the two species could not be found in this study due to the poor resolution of Scanning Electron Microscopes.

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