Chapter 2

Isolation and Cultivation of Diplonemids

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Abstract

Marine diplonemids (Diplonemidae and Hemistasiidae), once considered difficult to culture, can now be introduced into stable axenic cultures and grown to high densities. This chapter provides detailed protocols on establishing clonal cultures from environmental cells, their axenization and routine maintenance, as well as instructions on mid- and large-scale cultivation, estimation of cell density, and cryopreservation of selected species.

Key words Diplonemids, Cultivation, Isolation, Axenization, Cryopreservation, Large-scale cultivation

1 Introduction

Diplonemids are a group of predominantly marine flagellates belonging to the phylum Euglenozoa, which also encompasses free-living and parasitic kinetoplastids, photosynthetic and heterotrophic euglenids, and cryptic symbiontids [1]. Diplonemids (or class Diplonemea and order Diplonemida) include four major lineages: classical diplonemids (or family Diplonemidae), hemistasiids (or family Hemistasiidae), eupelagonemids (or family Eupelagonemidae; also known as DSPD I), and a so far unranked lineage termed DSPD II [1, 2].

Unlike euglenids and kinetoplastids that have been known for centuries, diplonemids have remained obscure until recently due to their inconspicuous morphology and difficulties in introducing them into axenic cultures. Indeed, only two Diplonemidae species, *Isonema nigricans* and *Paradiplonema papillatum* (also designated *Diplonema papillatum*), were available in private culture collections until late 1980s, when stable axenic cultures of several diplonemid

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species were deposited in the American Type Culture Collection (ATCC) [3]; stable culture of the first hemistasiid became available even later [4]. Thanks to focused efforts, the diversity of cultured Diplonemidae and Hemistasiidae has markedly expanded since then [5–7]. However, the other two diplonemid clades, DSPD II and eupelagonemids, currently remain uncultured [2].

The recent publication of the high-quality genome and transcriptome of the model species *P. papillatum* [8], together with a toolbox for its genetic manipulation [9], has made diplonemids an attractive model for studying various cellular and molecular processes. In this chapter, we summarize our experiences in culturing diplonemids and provide detailed protocols for establishing cultures, routine and large-scale cultivation, estimation of cell density, as well as cryopreservation of selected species.

2 Materials

2.1 Isolation from the Environment

1. For collecting samples: plankton nets with mesh size of 20 μm; cell scrapers, scalpels, and tweezers for samples of algal biofilms; 2 or 3 mL disposable plastic Pasteur pipettes to sample sediment; 50 mL Falcon tubes; 1 L glass bottles or jars for water samples; Parafilm; thermometer; salinity meter.
2. For concentrating cells from water samples: reusable Sterifil filtration systems (Merck); 0.8 μm polycarbonate filters (diameter 47 mm, Millipore, Merck); hand pump compatible with filtration system (alternatively: a centrifuge that accommodates 50 mL Falcon tubes).
3. For establishing cultures from single cells: disposable Petri dishes (diameter 50 mm); Parafilm; 1 mL aliquots of molecular grade ethanol in sterile microcentrifuge tubes; glass microscopy slides; 1 mL aliquots of filter-sterilized seawater in sterile microcentrifuge tubes; cleaning tissues for microscope lenses; fine-tip tweezers and scissors; Bunsen burner; 24-well plates; plastic Pasteur pipettes or 10–100 μL pipettors and compatible filter tips; glass Pasteur pipettes; flexible PVC plastic tube (outer diameter 7 mm), 1000 μL sterile pipette tips with filters; inverted microscope with 10×, 20×, and 40× objectives, preferably equipped with left-handed stage controller (if the isolator is right-handed and vice versa), a Petri dish holder, and Plast DIC or phase contrast; Hemi medium of variable salinity (refer to Subheading 2.2.1 for formulation) supplemented with penicillin, streptomycin, and neomycin antibiotic cocktail in 1:100 ratio (P4083, Merck); cell culture incubator with adjustable temperature (10–25 °C).

2.2 Cultivation

2.2.1 Culture Media

Three types of media are currently used for cultivating marine diplonemids: a universal Hemi medium that supports the growth of all currently cultivated diplonemids, and two additional media optimized for high-density growth of *P. papillatum* (referred to as OSS medium and *P. papillatum* medium).

1. Standard Hemi medium: Dissolve 36 g of anhydrous sea salts in 900 mL of deionized water, add 1 mL of Luria-Bertani Broth (LB) prepared according to the manufacturer's protocol and 10 mL of heat-inactivated horse serum (HS), mix well, and adjust the volume to 1 L in a graduated cylinder. To sterilize, filter through a 0.22-μm filter system (connected to an electric pump) into a heat-sterilized glass bottle. Close the bottle aseptically in a laminar flow chamber and store at 4 °C. Optionally, supplement with penicillin, streptomycin, and neomycin antibiotic cocktail at a 1:100 v/v ratio (P4083, Merck) (*see* **Note 1**).
2. *P. papillatum* medium: dissolve 36 g of anhydrous sea salts in 900 mL of deionized water, add 1 g of tryptone powder and 10 mL of heat-inactivated fetal bovine serum (FBS), stir until combined, and adjust the volume to 1 L in a graduated cylinder. Optionally, to prevent bacterial contamination, supplement with 2.5 mL of 40 mg/mL chloramphenicol stock to yield 100 μg/mL final concentration. Prepare the stock by dissolving 2 g of chloramphenicol in 50 mL of ethanol and store at −20 °C. Sterilize and store the medium as above (*see* **Note 1**).
3. OSS medium**:** dissolve 33 g of Instant Ocean sea salts in 1 L of deionized water and autoclave (or alternatively, use a sterile 0.22 μm filter). If any precipitate forms after autoclaving, decant into a sterile bottle/container before use to remove any insoluble content. Add 10 mL horse serum to 1 L of the autoclaved ocean salt (1× OS) solution just before use, thus creating 1× OSS. If stored for >24 h, keep at 4 °C. Optionally, supplement with ethanol-dissolved chloramphenicol to 40 mg/L to prevent bacterial contamination (*see* **Notes 1** and **2**).

2.2.2 Routine Maintenance of Cultures

Work aseptically in a laminar flow chamber using sterile cell scrapers, pipette tips with filters, and tubes. Disinfect gloves, all surfaces, and items by spraying with 70% ethanol solution. Cultures are maintained in plastic vented cell culture flasks with 25 cm^2 surface area or 12.5 or 25 mL volume. Optimal growth temperature differs among diplonemids species, therefore incubators with adjustable temperature (10–25 °C) may be necessary. An inverted microscope, with 10×, 20×, and 40× objectives, preferably equipped with phase contrast, is required for monitoring growth in flasks.

2.2.3 Mid-Scale Cultivation (0.5–1.5 L)

In addition to materials listed in Subheading 2.2.2, prepare the following: hemocytometer (e.g., Improved Neubauer chamber) with a coverslip, 95% ethanol, a reusable, large surface-area rectangular glass bakeware (ideally at least 15 × 25 cm; preferably Pyrex), fitted covers (use either a plastic lid that comes with the glass bakeware, or tightly cover the container with an aluminum foil), and a rotary platform or a large cultivation chamber with agitation (able to support the weight of the medium-filled container).

2.2.4 Large Scale Cultivation in a Carboy Setup with Active Aeration (>6 L)

1. Medium: the carboy setup has been tested with OSS medium described in Subheading 2.2.1 supplemented with 0.05% (w/v) yeast extract and 1% (v/v) horse serum for *P. papillatum*.
2. Aquarium air pump: aquarium air pumps with additional flow regulators (e.g., Fedour Aquarium Air Pump, Ultra Quiet Aquarium Bubbler for up to ~1100 L with two outlets, 5 W) are recommended to gently aerate the culture and lower the chances of damaging the cells (*see* **Note 3**). Depending on the air pump, the tube diameter of the lines provided by the manufacturer may be smaller than the tubing used in the carboy. In such cases, additional connectors may be needed.
3. Carboy: use of a carboy fitted with a spout is highly recommended to ease sample collection and ensure sterility during the process. Although glass containers can be used, polypropylene Nalgene carboys (e.g., Thermo Scientific Nalgene Polypropylene 23190020 Carboy; cat. no. 02-963-2A) may be safer in case gentle shaking is used to dislodge cells from the surface prior to sampling cultures. A multi-port carboy cap with a minimum of two ports, for inlet and outlet airflow, is required (e.g., ULAB Scientific Plastic Universal Carboy Venting Cap, TPE Gasket and Port Caps, Quick Filling, UCA1004). All parts must be autoclavable.
4. Flexible tubing: tubing that goes into the carboy (e.g., Masterflex® Transfer Tubing, C-Flex®, Clear, 6.35 mm ID × 9.525 mm OD; cat. no. MFLX06422-10) must be autoclavable and should fit snugly through the holes of the multi-port carboy cap. Additional non-autoclavable tubing can be used to connect the carboy to the air pump.
5. Unidirectional vent filters: these are recommended to filter the airflow and minimize contamination and should be autoclaved to ensure sterility (e.g., Cytiva Whatman™ HEPA-Vent Filter, 67235000).
6. Optional parts: autoclavable T-shaped connectors (e.g., 6.35 mm three-way hose barb fitting splicer for liquid/air three-way connector to match the inner diameter of the tubing) to form loops at bottom of the main air input line; flow regulators in the air pump to aerate the culture more gently.

These may come with the aquarium air pump; additional connectors to connect between the carboy and air pump system in case tubing with different sizes are used.

2.3 Cell Counting

A hemocytometer such as a Bürker-Türk or an Improved Neubauer counting chamber with a coverslip, microcentrifuge tubes, sterile 10 mL graduated glass pipettes, pipette gun, deionized water, inverted microscope with a 10× objective. Optionally: cell scrapers.

2.4 Cryopreservation

Sterile 1–2 mL cryovials (e.g., Simport, T311-1); DMSO; 50% (v/v aq.) glycerol solution sterilized by filtering through a 0.22 μm filter; Mr. Frosty cooling unit (Nalgene) or two to three nested styrofoam boxes (a "matryoshka" setup assembled by placing a styrofoam rack into a ~2 cm thick-walled styrofoam box, enclosed into another larger ~3 cm thick-walled styrofoam box).

3 Methods

3.1 Establishing Diplonemid Cultures from Environmental Samples

3.1.1 Collecting Samples

For samples of plankton taken from the ocean, lower the net to a depth of 1–5 m to draw approximately 10 L of seawater, slowly pull upwards, and let water pass through the mesh until the volume is reduced to ~1 L. Swirl the water inside the net and collect the 10× concentrated plankton sample in a 1-L glass bottle (*see* **Note 4**). If a plankton net is not available, refer to Subheading 3.1.2. Store at 15 °C with a cap/lid slightly open until use (typically up to a week), preferably in an incubator with 12:12 h light-dark cycle.

Collect samples of algal biofilms from rocks by pulling them with tweezers or scraping off with a scalpel and use a cell scraper to collect biofilms from aquarium walls. Before proceeding to a new sample, wipe dry with a tissue paper. Draw 2–3 mL of a sediment from coastal shallow water or an aquarium tank with a clean disposable plastic Pasteur pipette. Place the material into a 50 mL Falcon tube and fill it up to 15 mL with seawater from the site (*see* **Note 4**). Never fill to >20 mL to avoid anoxic conditions. For long-distance transportation, seal Falcon tube caps with Parafilm. Store in an incubator as described above with caps slightly open.

3.1.2 Concentrating Samples

In the absence of a plankton net or if a sample requires further concentration, pass the water through a 0.8 μm polycarbonate filter placed inside the Sterifil filtration system. Soft fragile diplonemids are best preserved when the water is allowed to pass by gravity, but the process can be sped up by the occasional use of a handpump. Collect cells concentrated into 20–50 mL with a disposable plastic Pasteur pipette and transfer to a Falcon tube or a small glass bottle. Alternatively, cells can be concentrated by centrifugation in 50 mL Falcon tubes. Diplonemid cultures are typically spun for 10 min at 3000 × *g* in a refrigerated centrifuge at 15 °C, but lower speed should be tried first for mixed environmental samples.

3.1.3 Establishing Cultures from Single Cells

1. Dispense 1 mL of Hemi medium supplemented with antibiotics (penicillin, streptomycin, and neomycin antibiotic cocktail at a 1:100 v/v ratio) into each well of 24-well microplates. Wash a glass microscopy slide thoroughly with distilled water and disinfect with a cleaning tissue for microscope lenses soaked in 100% ethanol. Regular tissue papers are not recommended as these leave fibers on glass slides.
2. Draw glass Pasteur pipettes manually into fine capillaries: hold the tip of a pipette with tweezers and heat the adjacent part in the flame of a Bunsen burner until the glass becomes soft. Withdraw the pipette from the flame and immediately draw by rapid pulling of the tip with tweezers. If necessary, cut the tip of the capillary with scissors. The inner diameter of the capillary for cell picking should be ~20 μm. Additionally, prepare a capillary with ~100 μm diameter for dispensing water droplets onto glass slides. Optionally: before drawing into microcapillary, bend the thin part of the pipette at a 45° angle (*see* Fig. 1).
3. Pour ~7 mL of sample into a disposable Petri dish, wrap the edges with Parafilm to prevent evaporation, and let it stand for 15 min to 1 h at 15 °C, allowing suspended cells to sink to the bottom of the dish (*see* **Note 5** for an optional enrichment step).
4. Assemble the cell-picking device as shown in Fig. 1: connect sterile 1 mL filter tips to both ends of a ~1 m long flexible PVC tube. Label one side of the tube as a mouthpiece and attach a glass capillary prepared in **step 2** to the other side. Disinfect the capillary by drawing in and pushing out a small amount of molecular grade ethanol.
5. Place the Petri dish in the dish holder attached to the microscope stage. Locate diplonemid cells in the Petri dish under the inverted microscope at 100× or 200× total magnification. For typical diplonemid size and morphology, refer to [1, 5, 10]. Most commonly, diplonemids are found either gliding at the bottom of a Petri dish, or freely swimming in the water column, occasionally stopping at the bottom for a few seconds. Dispense two 3 μL droplets of sterile sea water onto the disinfected glass slide. Draw a small volume (1–2 μL) of sterile seawater into the glass capillary from **step 4** through the mouthpiece and then pick a cell from the Petri dish. Place the slide on the microscope stage and gently push out the cell into one of the droplets, disinfect the capillary with ethanol and withdraw the cell from the droplet. If multiple cells are in the droplet, repeat the procedure by transferring the cell in the next droplet (*see* **Note 6**). Finally, place the cell into a well of 24-well plate containing Hemi medium supplemented with antibiotics (*see* **Note 7**).

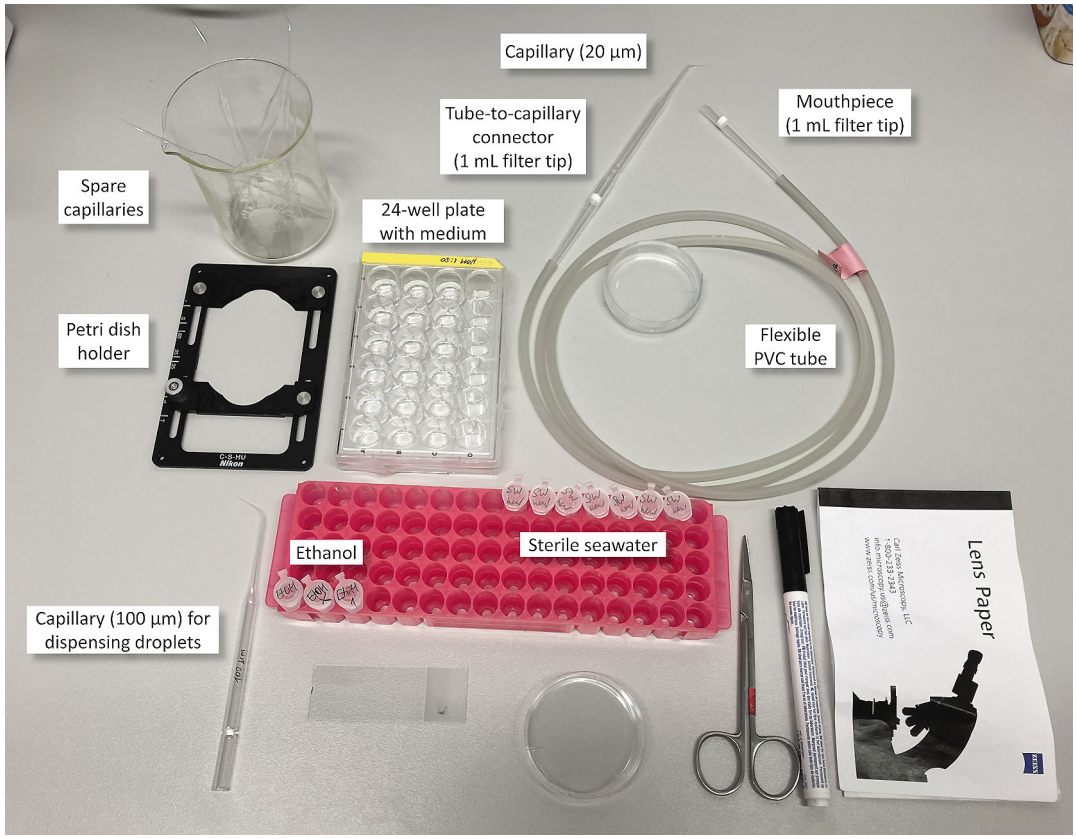

Fig. 1 A set of materials and tools required for isolating environmental diplonemid cells in culture by single-cell picking

6. Wrap the edges of the plate with parafilm to prevent evaporation and incubate at 15 °C in the dark for up to 2 weeks. If isolation is successful, diplonemid cells usually manifest in larger quantities after 1 week. The identification of the isolated organism as a diplonemid should be confirmed by PCR with EukA and EukB primers (5′- AACCTGGTTGATCCTGC CAGT-3′ and 5'-TGATCCTTCTGCAGGTTCACCTAC-3′; expected product length is ~2050 bp) targeting the 18S rRNA gene and sequencing.

3.2 Maintenance and Cultivation

3.2.1 Routine Maintenance

While some diplonemids (hemistasiids, some *Lacrimia* species, *P. papillatum*, *Sulcionema*) form homogenous suspensions or swim freely in the medium, most species glide at the bottom of culture flasks or adhere to surfaces (*Rhynchopus* and *Natarhynchopus* spp., *Diplonema* spp., *Flectonema*, some *Lacrimia* species). Therefore, before passaging, it is recommended to shake flasks vigorously or detach firmly adhered cells with a cell scraper. Transfer a small volume (50–100 µL) of dense culture into a new flask containing 10–15 mL of fresh Hemi medium, or 500 µL for species

that typically do not grow to high densities (hemistasiids). Although the optimum growth temperature differs among diplonemids, most will propagate at 13–15 °C (*see* **Note 8**). Keeping flasks in a horizontal position will increase cell density of adhering species and provide sufficient aeration. Regularly monitor cultures using an inverted microscope for signs of contamination (e.g., bacterial and fungal growth) and assessment of cell morphology and density. Depending on the species, subculture every 5–14 days (*see* **Note 9**).

3.2.2 *Mid-Scale Cultivation of* P. papillatum, F. neradi, and R. euleeides

Depending on the size, the medium capacity of a single container is restricted to ~0.5–1.5 L due to the limited height of the water column (<1.5 cm optimally), which is needed to obtain sufficiently large surface area to volume ratio for (mostly) passive aeration. For large-scale growth (>6 L), bench space and maintenance of multiple containers can become rapidly limiting. For higher yields, growth media can be modified (*see* **Note 10**). For larger volumes, employ a carboy system described in Subheading 3.2.3.

1. Prepare an inoculum. Estimate the cell density as described in Subheading 3.3. Passage cells from the late exponential or early stationary phase culture (1 to 5×10^6 cells/mL) by transferring ~5% of the original volume to a culture flask with the medium of choice (e.g., 500 μL to 10 mL) (also *see* **Note 11**). At 20 °C, the wild-type *P. papillatum* strain should enter its late exponential phase in ~72 h, while at 16 °C, it will take additional ~24 h.
2. Prepare the cultivation dish(es). Sterilize a rectangular glass bakeware in an oven at ≥140 °C for at least 2 h, preferably several hours in advance before the inoculation, so that the container has time to cool down to room temperature. To sterilize the container's lid (be it plastic or aluminum), wipe it thoroughly with a paper towel soaked to saturation with 95% ethanol, then immediately put the cover on the still hot bakeware as soon as taken from the oven and wait for ~10 min until ethanol evaporates (*see* **Note 12**). The same procedure can be used to sterilize the container, with any residual ethanol from containers removed by setting it on fire (e.g., with a Bunsen burner); this is usually very brief (1–2 s). While the flame is not necessary, it enhances the sterilization process.
3. Pour sufficient volume of medium into the container to create a layer of 1–1.5 cm, e.g., 500–750 mL to a 30 cm × 18 cm dish. This ensures sufficient aeration of the cells even without continuous shaking (*see* **Note 13**). Add the inoculum prepared in **step 1** to the culture medium at a 1:100 ratio. To reach the late exponential/early stationary phase (i.e., cell density ~ 2.5×10^6 /mL for *P. papillatum* in the regular OSS medium), incubate at

16 °C for ~5–7 days. Alternatively, dilute the inoculum at a 1:20 ratio to reach similar growth phase in 4–5 days to enter this phase.

4. Monitor the growth of the culture regularly (e.g., once a day) by taking a 10 μL aliquot (from a culture that has been agitated at 30–50 rpm for >10 min to homogenize its content) and screen for the presence of bacteria or fungi under a microscope. Estimate cell density as described in Subheading 3.3. The optimal growth phase to stop the cultivation at depends on the aim of the experiment, so should be determined beforehand in small-scale cultures.
5. Before harvesting the cultures, place the containers on a shaker (or a rotary platform) for 1–3 h (15–20 °C) to detach all surface-adherent cells with gentle, continuous agitation or rocking (20–50 rpm, depending on the container).
6. Harvest the cells by centrifugation (2000 × *g*, 4 °C, 5–10 min). Discard the medium and resuspend the cells in fresh sea salt solution without any carbon source. Spin as before and discard the supernatant. Continue with downstream sample processing as required.

3.2.3 Large Scale Cultivation in a Carboy Setup with Active Aeration (>6 L)

Mid-scale cultivation in dishes is gentler, providing a lower risk of damaging flagella, yet total cell yields may be insufficient for experiments requiring large cell quantities. The following protocol describes a simple set-up for culturing large quantities of *P. papillatum* using a carboy and a fish tank aeration pump (Fig. 2), similar to methods used previously for large-scale cultures of other planktonic protists and algae [11, 12]. Higher densities of cells were obtained by this carboy method than from the Pyrex dish method in the same amount of time (Fig. 3). Although there is still variability in the carboy-grown cultures (Fig. 4), higher total cell yields with significantly less space and labor can be obtained using this setup.

1. Preparation and initial assembly setup: fit the main tubing into the carboy cap, attach in-line vent filters to all lines connected to the carboy cap and connect to the air pump (*see* Fig. 2 for sample set up). Make small perforations in the region of the tubing that will rest near the bottom of the carboy in contact with the medium. Alternatively, perforations can be made on a separate, smaller piece of tubing. This tubing fragment can then be attached as a loop to the main air input tubing using a T-shaped connector (Fig. 2). Check that the vent line is functional and there are no unwanted leaks in the system: when the vent line is placed inside a beaker with water, bubbles formed from the vent line should be steady (1–2 bubbles per second), but not vigorous. Sterilize all parts exposed to the

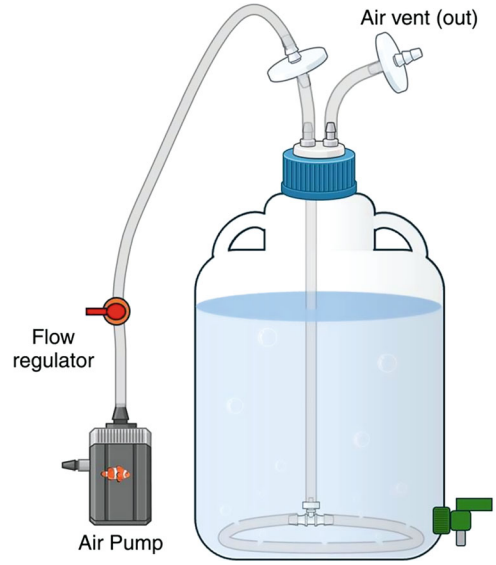

Fig. 2 A schematic of a carboy setup. Parts discussed in the methods are labelled with black text. The airflow of the system is shown in light blue

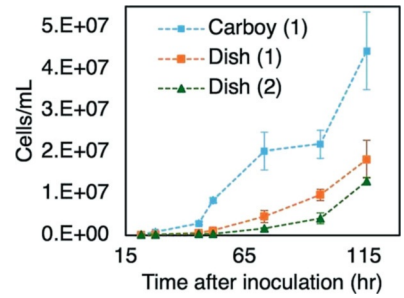

Fig. 3 Growth curves of *P. papillatum* grown in a carboy with 3 L of medium (blue squares) compared to two separate cultures grown in a Pyrex dish with 1 L of medium each (orange square and green triangle). Cells were grown in OS medium with 0.5 g/L yeast extract, 1% (v/v) horse serum, and 40 mg/L chloramphenicol. Cultures were inoculated with 10 mL of the starter culture per 1.5 L of growth medium. These cultures were grown in parallel, at room temperature (22 ± 2 °C). Cells were counted in triplicates using a hemocytometer, and error bars show the average deviation (except the last point in Dish (2), where the error bar is from two counts)

culture medium by autoclaving, except for the components used for the vent line (does not need to be autoclaved due to the positive pressure of the system).

2. Assemble the carboy for culture under a sterile environment (such as a laminar flow chamber) and seal the lines exposed to the outside environment with unidirectional vent filters.

   As foam will form during aeration, fill the carboy up to ~80% of the maximum capacity with cooled, autoclaved

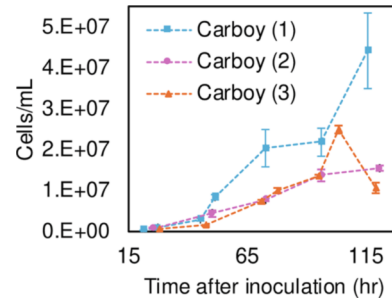

Fig. 4 Growth curves of *P. papillatum* grown in OS medium containing 0.5 g/L yeast extract, 1% (v/v) horse serum, and 40 mg/L chloramphenicol. Shown are growth curves from three independent cultures (3 L blue, 6 L mauve, 6 L orange). These cultures were grown at room temperature (22 ± 2 °C). Cells were counted in triplicates using a hemocytometer, and error bars show the average deviation

medium with desired additives (e.g., OSS medium with 1% (v/v) horse serum and 0.05% yeast extract) mixed with appropriate antibiotics (e.g., 40 mg/L chloramphenicol when culturing wild-type *P. papillatum*).

3. Inoculate the media with ~10–15 mL of starter culture grown to the late exponential phase per liter of media. Cap all exposed tubes by fitting unidirectional vent filters and make sure that the direction of the filters follows the direction of the airflow. This ensures that the medium is protected from potential contamination when the carboy is taken out of the sterile environment. Place the carboy in a cool location away from sunlight. Sufficient growth was observed with carboys placed on a lab bench inside a well-ventilated temperature-controlled room (22 ± 2 °C). Fit the air input lines to the air pump ensuring that the air pump is facing away from machines that output warm air (e.g., tabletop centrifuges). Establish gentle aeration by adjusting the airflow, preferably using the flow regulator. Check the flow rate by placing the vent line inside a clean beaker filled with water. Always maintain positive pressure in the system to prevent unfiltered air from getting inside the carboy.
4. Sampling during growth: dislodge cells from the carboy using a swirling motion. If the carboy is fitted with a spout, spray the spout with 70% ethanol solution before and after opening the spout to prevent contamination. Alternatively, the culture can be sampled by opening the carboy cap in a sterile environment, such as inside a laminar flow chamber. Check the cells under the microscope to ensure that cells swim freely in solution. For *P. papillatum*, fast, freely swimming cells were observed within 24 h post inoculation. Cells are harvested as described in Subheading 3.2.2.

3.3 Cell Counting

1. Scrape adherent cells off all surfaces of a culture flask and homogenize the culture by repeated passing up and down through a large-volume glass or plastic pipette (e.g., 10 mL) attached to a pipette gun. For non-adherent species, agitate the flask thoroughly to achieve a uniform cell distribution.
2. Transfer 180 μL of culture into a microcentrifuge tube containing 20 μL distilled water and immediately mix well to create a slightly hypotonic solution. This treatment will immobilize any fast-swimming cells. Directly proceed to **step 3** as long-term (>1 h) exposure to hypotonic solution may cause cell lysis in certain species.
3. Place the coverslip over the counting grid. Once positioned, the coverslip should remain stationary, as any movement during or after the sample loading may disrupt even distribution of cells and affect accuracy. Fill both chambers by pushing 10 μL of the cell suspension into the lateral capillary gap between the coverslip and the chamber base with a counting grid. Allow the capillary action to evenly distribute the sample under the coverslip.
4. Position the counting chamber under a microscope equipped with a 10× objective lens and focus on the central grid, which consists of nine large primary squares. Count cells within the four corner squares, following standard counting rules: include cells that intersect the top and left borders, but exclude those on the bottom and right borders. For improved accuracy, count cells from the same sample in three chambers resulting in 12 squares in total (*see* **Note 14**).
5. Calculate the concentration of cells per 1 mL as follows:

$$C(\text{cells/mL}) = -X/0.9 \times 10^4$$

Where C—concentration; X—arithmetic mean of cell counts per square; 10^4—multiplication factor converting a volume of 0.1 mm^3 (corresponding to one large square) into cm^3, or mL; 0.9—dilution factor introduced in **step 2**.

3.4 Cryopreservation

3.4.1 Cryopreservation of Paradiplonema papillatum

1. Grow *P. papillatum* in a vented flask to a density of ~5 × 10^6 (exponential phase) in 10–15 mL of medium of choice. Check the culture under the microscope for bacterial or fungal contamination and the condition of diplonemid cells. In a healthy culture, most cells swim and only few stick to the bottom.
2. Harvest cells by centrifugation (2000 × *g*, 4 °C, 5 min). Discard the medium.
3. Add 1.5 mL fresh medium supplemented with sterile DMSO to 8% (5–10% has been successfully tested). Resuspend the cells and distribute to three cryo-tubes (0.5 mL each).

4. Incubate the cryo-tubes at 4 °C or on ice for ~30 min, then transfer in a tube rack −80 °C for at least 3 h (*see* **Note 15** for an alternative protocol).
5. Store cryopreserved cultures at −80 °C for several months (up to a year, if absolutely necessary). Transfer the cryotubes into liquid nitrogen for long-term storage (up to ~15 years).

3.4.2 Cryopreservation of Diplonema ambulator, Flectonema neradi *and* Rhynchopus euleeides

1. Cultivate in 10–15 mL of medium of choice to the late exponential phase (cell growth curve depends on the species and should be determined beforehand). For the tested species, OSS with 2–3% horse serum appears optimal; use the same concentration of horse serum in all steps throughout the procedure described here.
2. Harvest by centrifugation (1000 × *g*, 4 °C, 5 min). Work on ice. If not possible to discard the supernatant without disturbing the cell pellet by decanting, remove as much as possible using a pipette, then resuspend the pellet in the remaining supernatant. Transfer to a 1.5 mL tube, re-spin in the same conditions, and remove the supernatant (*see* **Note 16**).
3. Resuspend in cold medium (1–4 °C) supplemented with cryoprotectants: (1) for *F. neradi*, use OSS with 15% glycerol; (2) for *R. euleeides*, use OSS with 8% DMSO or 5% glycerol; (3) for *D. ambulator*, use OSS with 10% DMSO or 15% glycerol. Keep on ice (or at 4 °C in the fridge) for ~30 min.
4. Transfer the tubes into a styrofoam rack, place it into the "matryoshka" styrofoam box setup, then transfer the whole nested assembly into the −80 °C freezer for >12 h to allow the gradual freezing of the samples before transferring the cryotubes into liquid nitrogen. Alternatively, use a dedicated freezing container (e.g., Mr. Frosty™) instead.

3.4.3 Starting a Diplonemid Culture from a Cryopreserved Stock

1. Place a frozen cryovial on ice and let it slowly thaw.
2. Once thawed, transfer the entire contents into a culture flask with 10–15 mL of medium pre-cooled down to 4–8 °C, but avoid using ice-cold medium as this can enhance cell autolysis.
3. Incubate at 20 °C (or 16–22 °C); recovered cells will start to swim.

4 Notes

1. The salinity of all media can be adjusted. Certain supplements (LB, yeast extract, antibiotics, horse serum or fetal bovine serum, tryptone) can be aliquoted and stored frozen at −20 °C. For OSS medium, it is recommended to prepare 10% stock solutions of tryptone and yeast extract. To prepare them,

dissolve 5 g of yeast extract/tryptone in 50 mL distilled water, filter-sterilize with a 0.22 μm filter or autoclave.

2. To enhance mitochondrial content in *P. papillatum* cultures, supplement with sterile yeast extract to 0.05%. To enhance cell number in *P. papillatum* cultures, supplement with tryptone to 0.05%. For more details see Chapter 15 in Vol. II.
3. Air stones should not be used due to potential difficulties with cleaning and sterilization.
4. Measure the temperature and salinity of samples immediately after collection.
5. Before single-cell picking, diplonemids can be enriched by incubating 100 μL of an environmental sample in 7–10 mL of Hemi medium with antibiotics for 3–4 days. Elimination of bacteria with antibiotics essentially suppresses the growth of bacterivorous protists, allowing only some free-living osmotrophic, parasitic, and certain predatory protists to propagate. Enrichment cultures should be monitored every day because diplonemids can be rapidly eliminated by predatory ciliates and amoebae, or overgrown by fungi.
6. Small droplets of seawater tend to evaporate fast, which dramatically increases the water salinity, and thus, transferring cells into the droplets should happen within 1–2 min after dispensing. If not possible, leave the picked cell inside the 20 μm capillary and dispense a new droplet onto the glass slide with a 100 μm capillary. Increasing the volume of droplets is not recommended because this will make locating diplonemid cells difficult.
7. In certain cases, penicillin-streptomycin-neomycin cocktail does not completely eliminate bacteria. For such samples, other antibiotics can be additionally supplemented, such as 50 μg/mL ampicillin, 50 μg/mL kanamycin, or 10 μg/mL gentamycin. Axenization of cultures is recommended because in many cases bacteria suppress diplonemid growth.
8. Most cultured diplonemids prefer 13–15 °C; hemistasiids isolated from warm waters prefer higher temperatures for growth (20–25 °C); the optimal temperature for *P. papillatum* is 16–22 °C.
9. Most diplonemids can survive extended periods at 4 °C. Low temperature slows down culture growth, which can be exploited for culture preservation (requires passaging once or twice a month). Old batch cultures of adhered cells can be rescued by replacing most of the culture fluid with fresh medium.
10. Maximum cell density can be increased by adjusting the content of horse serum: up to 2% for *P. papillatum*, but even up to

3% (v/v) for diplonemids that do not grow to high density in the regular OSS medium (e.g., *Flectonema neradi*, *Rhynchopus euleeides*). In the case of *P. papillatum*, it is beneficial to add yeast extract up to 0.05% (w/v) to enhance mitochondrial content, or tryptone up to 0.05% (w/v) to enhance cell number. However, adding more of these supplements or combining them is not recommended as cells will begin to swell up and become more sluggish, which indicates a non-physiological response. Not all diplonemids benefit from these supplements, so their influence needs to be tested prior to large-scale cultivation; e.g., the growth of *R. euleeides* is inhibited by the addition of yeast extract >0.01%.

11. While preparing the inoculum is not absolutely necessary, it is a recommended step to ensure that the cell culture has been pre-adapted to the medium and temperature of choice. *P. papillatum* propagates best in the range of 16–22 °C (*see* **Note 8**). In the upper range (22–27 °C), cells grow and divide but are heat-stressed (>28 °C will lead to growth arrest and cell death). In the lower range (4–16 °C), cells divide more slowly than at higher temperatures. Growth temperature and the composition of the medium will ultimately result in physiological changes, e.g., accumulation of storage lipids, changes in composition of the membranes, rate of the mitochondrial metabolism, or the relative and absolute content of most proteins. Therefore, the optimal growth conditions for a particular experiment should be determined beforehand in a small-scale setup.
12. Flat glass containers with plastic covers are more practical and better protect against evaporation, but in the absence of fitted covers, a large piece of aluminum foil can be used to shield the contents. If sterilizing containers using ethanol, add plastic covers onto the containers only once the flame has extinguished. If the bakeware cannot be taken out of the oven while still hot, sterilize it covered with a temporary aluminum foil to ensure the container's sterility. If using just an aluminum foil to cover the container, the whole assembly can be sterilized in the oven without ethanol wiping.
13. Occasional (i.e., once or twice a day) gentle waving motions at 30–50 rpm improve culture aeration and are strongly recommended. Avoid extensive stirring or shaking, which can lead to flagella loss. If an incubator shaker is not available, occasional shaking can be done by hand or on a rotary platform.
14. The optimum cell concentration range is 2.5×10^5 to 1×10^6 cells/ml that corresponds to 25 to 100 cell per square. It is recommended to dilute denser cultures.

15. Alternative for **steps 3–4**: Mix 200 µL of sterile 50% glycerol solution and 800 µL *P. papillatum* cell culture in labelled 1.2 mL cryovials and mix gently using the pipette. Place the cryovials in a dedicated freezing container (e.g., Mr. Frosty^{TM}) and let it freeze at −80 °C.
16. The low-speed spins and slow cooling are particularly critical to ensure cell viability after thawing.

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