Isolation of mitochondria from Diplonema papillatum

Version 2

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PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

- Culture conditions may require optimization. Variables, known to influence the yield of cells and of mitochondria, include the cultivation temperature, the sea salt batch/manufacturer, and the presence of tryptone instead or in addition to yeast extract.

- Avoid salt precipitates in the OS solution, as these tend to inhibit *Diplonema*'s growth. If present after autoclaving, let the precipitates sediment and decant the medium into a new sterile bottle.
- In order to sufficiently aerate the culture, the ratio Air:Medium in the flask/container should be at least 3:1. The optimal cell culture conditions are achieved when the height of the medium in the flask/container is 1-2 cm. Cultivation in tall flasks with small surface is not recommended, because it requires vigorous shaking to sufficiently aerate the culture, which in turn leads to higher amount of damaged cells and hampers isolation of intact sub-cellular fractions. Oven-sterilized large-surface glass bakeware works well. (Note that cells can stick to the surface of some glass types; *Pyrex* glass is among the least sticky with *Diplonema*.)
- When washing cells, solubilization by gentle vortexing or hand-swirling is recommended instead of pipetting to avoid breaking cells' flagella. Resuspending only a single aliquot (to be opened in the cavitation instrument) at a time can prevent cell autolysis during extended incubation in ice-cold SoHE.

- Optimize the stirring conditions (size of the stirring bar, speed, position on the magnetic stirrer) before the isolation to ensure that the solution in the nitrogen cavitation pressure chamber is properly mixed when the chamber is closed.
- To avoid repeated thawing/freezing cycles of the samples, either perform the final spin in 1.5-mL tubes, or distribute the final mitochondrial pellet into several 1.5-mL tubes.
- Keep small aliquots (at least 200 µL in case of a large-scale prep) of all isolation fractions, i.e., cells before breakage, cells after breakage, supernatant after ultracentrifugation, supernatant/36% sucrose band, 36%/60% sucrose band, supernatant after the wash of the 36%/60% sucrose band, and final mitochondrial pellet. These are used to verify the efficacy of the procedure and the enrichment of mitochondria with a method of choice (e.g., extraction of mtDNA, extraction of total RNA, measurement of enzymatic activities, Western blotting, etc.). We routinely perform nucleic acids extraction, RT-PCR, and citrate synthase assay.

MATERIALS

NAME	CATALOG #	VENDOR
Chloramphenicol	View	P212121 P212121
Sucrose	View	P212121 P212121
Sorbitol	View	P212121 P212121
Ethylenediaminetetraacetic acid disodium salt dihydrate	E4884	Sigma Aldrich
Yeast Extract	Y1625	
HEPES	BP310	Fisher Scientific
Instant Ocean aquarium sea salt mixture	SS15-10	
Horse serum	16050122	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
cOmplete™, EDTA-free Protease Inhibitor Cocktail	05056489001	Sigma Aldrich
Tube, Thinwall, Ultra-Clear™, 38.5 mL, 25 x 89 mm	344058	Beckman Coulter
Tube, Thinwall, Polypropylene, 13.2 mL, 14 x 89 mm	331372	Beckman Coulter

BEFORE STARTING

- Prepare OSS growth medium: 33 g/L *Instant Ocean* sea salt (sterilize by autoclaving or filtration), 1% horse serum (add just before use); store at 4 °C.
- Prepare OS solution: sterile 33 g/L *Instant Ocean* sea salt (without serum).
- Prepare SoHE buffer: 1.2 M sorbitol, 20 mM HEPES pH 7.5, 2.5 mM EDTA pH 8.0.
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- Prepare 36% (~1.2M) and 60% (~2.2M) sucrose solutions buffered with 20 mM HEPES (KOH), pH 7.5, and 2 mM EDTA.
- Ensure the availability of the following equipment:

- Plastic or glass flasks. (Use of large-surface glass bakeware, which can be heat-sterilized in an oven and reused, is recommended.)
- Cultivation room/box/incubator. (For temperatures 15-20 °C; no fungi and/or bacteria grown in proximity, if possible to avoid contaminations.)
- Shaker, rotary platform, or large cultivation chamber with agitation. (For practical reasons, for a single machine, at least 20 kg weight limit is recommended.)
- Nitrogen cavitation chamber. (Parr Instrument Company)
- Magnetic stirrer and a sterile stir bar.
- Syringe (10 mL) and hypodermic needle (gauge 18G).
- Refrigerated centrifuge (at least 20,000×g).
- Ultracentrifuge with a swinging bucket rotor (at least 150,000×g) and corresponding tubes (e.g., Beckman SW28 rotor and 25×89 mm tubes; for smaller preparations, e.g., Thermo Scientific TH-641 or Beckman SW41 Ti rotor and 14×89 mm tubes).
- Ice dispenser.
- Liquid nitrogen (or dry ice) supply.
- Cold room (for temperatures 4-10 °C).

1 Inoculate 50 mL of OSS growth medium supplemented with yeast extract (0.05%) and chloramphenicol (40 mg/L) with an axenic stock of *Diplonema* cells. Cultivate at 16 °C with occasional shaking (e.g. 4× per day) to cell density approx. 5×10^6 – 10^7 per mL. This corresponds approximately to the late exponential phase and usually takes 3-6 days, if starting from a frozen stock of cells.

- *Cultivation at 16 °C seems optimal, but any temperature in the range from 15 °C to 20 °C works.*
- *Note that* Diplonema papillatum *will grow well in the range from 4 °C to 26 °C; however, the cultivation times will vary (e.g., slower growth at low temperatures) and the N_2 pressure required for optimal lysis (see step #9) will need to be optimized (e.g., we noticed that cultivation in the cold generally results in higher pressures needed to disrupt the cells, which nevertheless makes avoiding mitochondrial leakage trickier).*

2 Prepare 4 L of fresh OSS (or OSSYE) medium by adding horse serum (1% final) and, optionally, yeast extract (0.05% final) to the sterile artificial see water. Transfer the inoculum into large volume of the fresh medium (10 mL per 1 L, i.e., 100× dilution) and distribute the fresh culture (4 L) into large sterile flasks/containers with high Surface:Height ratio (see the guidelines).

- *Adding yeast extract (0.05% final concentration) improves the yield of mitochondria (inferred from higher citrate synthase activity and larger amount of mitoribosomes) compared to the OSS medium, though only slightly (by ~10-20%).*

3 Cultivate at 15–20 °C for 3–4 days to the late exponential phase. Occasionally slowly shake for 15-30 min (e.g., twice daily; max. at 30 rpm).

4 Place the cell cultures on a shaker (or a rotary platform) for 1–3 hours (15–20 °C) prior to isolation to detach all surface-adherent cells with gentle, continuous agitation or rocking (10–30 rpm, depending on the container).

- *Synchronizing the cells prior to isolation can improve the yield, though it is not required. After the 3-day cultivation, transfer the cultures into a cold room (4–10 °C) for 12–36 hours, then place them on the shaker (i.e., resume at the step #4).*

5 Collect the cells at 2,000×g, 4 °C, for 5 min. Solubilize the pellets in 80 mL of OS at ambient temperature to wash away the remnants of serum, distribute into two 50-mL tubes, then spin (3,000×g, 4 °C, 5 min).

6 Solubilize each pellet in 30 mL of ice-cold SoHE and spin (8,000×g, 4 °C, 10 min).

- *Longer, low-speed spin (3,500×g, 4 °C, 20 min) works, too. Note, however, that material loss may occur, as cells will not completely adhere to the bottom of the tube.*

7 Weigh the pellets (1-2 g/L of the culture are expected). Solubilize the pellets in ice-cold SoHE to 1 g (wet-weight) per 10 mL. From this step on, work on ice (unless otherwise stated).

8 While harvesting and washing the cells, prepare a two-step sucrose gradient. Into a 25×89 ultracentrifuge tube, add 6 mL of a HEPES-buffered 60% sucrose solution, then carefully overlay with 14 mL of HEPES-buffered 36% sucrose solution. Store in ice until use.

- *For small-scale preparations, use 14×89 ultracentrifuge tubes and add 2 mL of 60% and 4.5 mL of 36% sucrose.*
- *Note that 36% sucrose corresponds to 1.2M, thus preserves the osmolarity of the SoHE buffer, which seems critical to avoid break-up of Diplonema's mitochondria.*
- *Adding EDTA to 2 mM into the HEPES-buffered sucrose solutions slightly decreases the contamination by cytosolic ribosomes.*

9 Prepare 10–20 mL aliquots of cell suspension. Add protease inhibitor cocktail (20× in SoHE to 1× final) before transferring the cell suspension into a pre-cooled N_2-cavitation chamber (on ice). Add a sterile stir bar. Apply 30 bar (435 psi/29.6 atm) N_2 pressure for 3 min, while placing the chamber on a magnetic stirrer to ensure thorough mixing during the incubation.

- *The volume of aliquots is imposed by the volume of the N_2-cavitation chamber. Adjust according to the pressure chamber used.*
- *At 30 bar, which is the minimum pressure to open >80% cells, using SoHE at the concentration of 1.2 M sorbitol is necessary to avoid breaking up mitochondria and losing the contents of mitochondrial matrix into the solution. If one is only interested in mitochondrial membranes, it is possible to use 60 bar pressure in 0.65 M sorbitol to break virtually all cells.*
- *Prior to adding the cell suspension, verify the speed and the position of the chamber on the stirrer at which the bar rotates optimally.*

10 Release the suspension from the chamber into a pre-cooled 50-mL tube. Put in ice and wait for the phase separation of the foam and suspension of broken cells. Repeat the steps #9 and #10 with the remaining aliquots.

- *When using the cavitation chamber repeatedly, rinse it after each aliquot lysis with ice-cold SoHE to wash away remnants of lysed cells.*

11 Combine the suspensions from all aliquots (avoiding the foam) and overlay on the top of the sucrose gradients, distributing evenly the suspension. When using 25×89 ultracentrifuge tubes, spin using SW28 rotor (or equivalent) at 134,000×g (27,300 rpm), 4 °C, for 30–60 min.

- *When working with 14×89 ultracentrifuge tubes use TH-641 rotor (or equivalent).*
- *For higher purity of mitochondria, two successive centrifugations (2,000×g, 4 °C, 10 min) can be performed to remove unlysed cells and nuclei prior to loading the mitochondria-containing supernatants on the sucrose gradient. (Note that the final yield will be >5 times lower.)*

12 Collect the mitochondria-enriched band from the interface of 36%/60% sucrose using the syringe with an 18G needle.

- *The relatively large bore of the 18G needle prevents damage (because of the shearing forces) to the mitochondria.*

13 To remove the sucrose (as well as cellular contaminants), dilute the mitochondrial fraction by adding with 3.5 volumes of SoH, gently, but thoroughly swirling the solution and then resuspending once or twice with a 18G-needle syringe stroke.

- *To resuspend the collected, sucrose-containing mitochondrial fraction in SoH, one syringe stroke is usually enough. Being quick (and gentle) is essential, as an extended incubation with the buffer seems to favor the lysis of mitochondria and loss of the matrix into the supernatant after the subsequent centrifugation.*
- *If isolating just crude mitochondria and if sucrose content is irrelevant for downstream steps, flash-freeze the collected mitochondrial fraction without further manipulations.*

14 Pellet the mitochondrial fraction (20,000×g, 4 °C, 10 min). Remove the supernatant and flash-freeze the final mitochondrial pellet in liquid nitrogen (or alternatively in dry ice).

- ***Pause point:** Mitochondria can be stored at -80 °C for at least three months without any apparent quality loss.*