Chapter 15

Biochemistry of Diplonemids

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Abstract

Diplonemids are heterotrophic marine flagellates that have attracted attention and interest due to their eccentric mitochondrial genome and gene expression. This has led to the development and optimization of experimental procedures aimed at better understanding of the unusual mitochondrial processes that occur in this group. Here we present a collection of protocols established in the diplonemid type species *Paradiplonema papillatum*, which range from DNA preparation for direct long-read sequencing of both mitochondrial and nuclear DNA, to isolation of mitochondria and separation of mitochondrial ribosomes, to assays for assessing metabolic functions through measurement of respiration, mitochondrial membrane potential, and peroxide-scavenging activities, as well as tracking metabolic routes via stable-isotope labeling of metabolic intermediates.

Key words Diplonemid, *Diplonema*, Respiration, Bioenergetics, Subcellular fractionation, Mitochondria, Mitoribosome, Stable-isotope labeling, Reactive oxygen species, Nanopore sequencing

1 Introduction

Diplonemids are euglenozoan protists and the sister lineage of the well-known kinetoplastids, whose representatives, *Trypanosoma* and *Leishmania*, have been intensively studied not only for their pathogenicity, but also for their unconventional mitochondrial metabolism [1], genome structure and inheritance [2], and guide-RNA directed uridine insertion and deletion RNA editing [3]. Diplonemids display a large array of similarly unorthodox features, but of their own making [4, 5]. Notably, diplonemid hallmarks include a mitochondrial genome consisting of numerous small circular DNA molecules that mostly contain non-coding regions in addition to small gene fragments that are separately transcribed. Many gene-fragment transcripts undergo RNA editing of three to five biochemically distinct kinds, and are then assembled into functional rRNAs and mRNAs by an as-yet-unknown mechanism [6–9].

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In contrast to the intricate mechanism of mitochondrial DNA (mtDNA) transmission in kinetoplastids, diplonemids appear to ensure that all gene fragments are passed on to the next generation by simple hyper-amplification of their mitochondrial chromosomes—to the extent that in the type species *Paradiplonema (Diplonema) papillatum*, mtDNA represents >50% of total cellular DNA [8, 10]. This amplification, together with abundant repeats and transposable elements in the nuclear genomes, makes it challenging to sequence the two diplonemid genomes with traditional approaches that rely on physical DNA fragmentation and in silico reassembly of the sequenced fragments [11].

For the above reasons, we adapted previously published protocols [12–14] to generate high-molecular weight (HMW) diplonemid DNA for direct sequencing using Oxford Nanopore Technologies (ONT) platforms. For instance, we used the HMW DNA extracted from *P. papillatum* as described below, to prepare several Nanopore sequencing libraries using standard ligation kits and generated reads of high quality and depth (e.g., at 14 Gbp throughput: read N50 ~40 kbp, ~2000 reads >100 kbp long, 10 reads >200 kbp long), ensuring contiguous assembly of many nuclear chromosomes, as well as comprehensive coverage of the heterogeneous mitochondrial chromosome population.

So far, studies of mitochondrial proteins and RNAs have been a staple of diplonemid research. Protocols developed to isolate mitochondria, primarily from *P. papillatum*, have allowed characterization of a divergent respiratory chain Complex I [15] and an extremely protein-rich mitochondrial ribosome (mitoribosome) with diminutive ribosomal RNAs [16]. The protocols involve disrupting the outer cell membrane and then collecting the released membrane-bound structures through differential centrifugation and sucrose gradient-based fractionation. We describe procedures where cell disruption is achieved by nitrogen cavitation, hypotonic lysis, sonication or digitonin treatment. The appropriate isolation method is selected based on the intended use of the mitochondria or the quantity of the starting material.

Originally developed for *Trypanosoma brucei* (e.g., [17]), the nitrogen cavitation method generally yields the highest quality mitochondria-enriched material in the diplonemid type species and is best suited for large-scale preparations. The other three methods are simpler, quicker, and more appropriate for screening a larger number of samples or for specific assays. Hypotonic lysis has minimal requirements for equipment, its principle being the sequential incubation of cells in two solutions with different osmolarity, which causes the cells to swell and ultimately burst when passed through a needle. Sonication is a cost-effective method of cell lysis under defined conditions; its advantage compared to hypotonic lysis is its reproducibility. Digitonin treatment is specifically recommended for cellular fractionation from a low number of cells [18]. Note, however, that the amount of digitonin required to

dissolve the membranes of individual cell compartments strongly depends on the cultivation conditions because the composition of the growth medium influences the sterol constitution of the membranes, on which digitonin acts. Nevertheless, this approach is particularly suitable for preparing permeabilized cells via digitonin titration inside the O2k chamber (*see* the protocols on respirometry and membrane potential measurement), allowing real-time monitoring of digitonin's permeabilizing effect on cellular membranes [19].

Healthy mitochondria preserve membrane potential that ensures, among other intracellular activities, the import of nucleus-encoded proteins synthesized on the cytosolic ribosomes which in diplonemids, as in other eukaryotes, constitute the bulk of the mitochondrial proteome. We present a protocol in which the mitochondrial membrane potential (mtMP) is easily assessed by preparing permeabilized cells via digitonin titration within the O2k chamber using a high-resolution respirometry system with blue light-range excitation and corresponding emission detection, because the mtMP drives safranin accumulation inside the mitochondrial matrix. The degree of safranin self-quenching serves as an indicator of the mitochondrial metabolic status, reflecting the magnitude of the mtMP.

Reactive oxygen species (ROS) are a natural by-product of metabolism, particularly in mitochondria, which serve as the main hub for energy conversion. ROS are essential for some cellular functions but harmful at excessive levels. Catalase and ascorbate peroxidase maintain the hydrogen peroxide (H_2O_2) concentration at a physiological level by degrading it in various cellular compartments. We describe a simple procedure, optimized for diplonemids, that quantifies these enzymatic activities by spectrophotometric monitoring of H_2O_2 decomposition or ascorbate reduction [20].

The complexity of mitochondrial metabolism and its changes triggered by external conditions can also be monitored using non-radioactive labeling of metabolites and subsequent identification and quantification thereof by liquid chromatography-mass spectrometry (LC-MS). As we detail in the last protocol of this chapter, the high content of proline and its metabolic utility in *P. papillatum* make this amino acid a particularly suited substrate for such analyses [21].

2 Materials

2.1 DNA Preparation for Long-Read Sequencing

1. Refrigerator (4 °C).
2. Centrifuge, 4–22 °C, up to 20,000× *g*.
3. Ice and styrofoam icebox.
4. 1.5 mL and 50 mL centrifugation tubes, sterile.
5. (Dry) bath or thermoblock.

6. Glass hook or capillary.
7. Large-bore pipette tips. If not available, cut off the very end of regular tips with scissors to obtain an opening of 1.5–2 mm in diameter.
8. 0.5× SoHE buffer: 0.6 M sorbitol, 10 mM HEPES, pH 7.5, 1.25 mM EDTA, pH 8.0.
9. Cell lysis buffer: 100 mM Tris–HCl, pH 8.0, 100 mM EDTA, pH 8.0, 1.15% polyvinylpyrrolidone (PVP), 5 mM spermidine. Preferably prepare it fresh before use.
10. Phenol (saturated, pH 6.6/7.9 Liq.): chloroform:isoamylalcohol mixture (25:24:1).
11. 10% (w/v) N-lauryl sarcosine (sarcosyl) (or sodium dodecylsulfate (SDS)).
12. Precipitation solution: 95–100% ethanol, 0.5 M ammonium acetate.
13. 80% ethanol.
14. 5 mg/mL RNase A or RNase I.
15. 10 mg/mL proteinase K.
16. 10 mM Tris-HCl, pH 8.5.

2.2 Isolation of Mitochondria

1. Refrigerator (4 °C).
2. Freezer (−20 °C and −80 °C).

2.2.1 Nitrogen Cavitation

3. Ice and styrofoam icebox.
4. Liquid nitrogen (or dry ice) supply and appropriate equipment for its manipulation (e.g., gloves, ladle).
5. Cold room, 4–10 °C.
6. Plastic or glass flasks. We recommend large-surface glass bakeware, which can be heat-sterilized in an oven and reused.
7. Cultivation room/box/incubator, for temperatures 15–20 °C. To avoid contaminations, do not grow fungi or bacteria in proximity.
8. Shaker, rotary platform, or large cultivation chamber with agitation that can support at least 20 kg.
9. Nitrogen cavitation chamber (Parr Instrument Company).
10. Magnetic stirrer and a sterile stir bar.
11. Syringe (10 mL) and hypodermic needle (gauge 18G).
12. Centrifuge at 4–22 °C, up to 20,000× *g*.
13. 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).

14. 1.5-mL and 50-mL Centrifugation tubes, sterile.
15. OSS medium (1% horse serum for the inoculum, 1.5% horse serum for the large-scale cultivation).
16. 10% Yeast extract, sterile.
17. Chloramphenicol 20 g/L (in ethanol).
18. OS medium (i.e., sea salt solution).
19. 1× SoHE buffer: 1.2 M sorbitol, 20 mM HEPES, pH 7.5, 2.5 mM EDTA, pH 8.0.
20. 1× SoH buffer: 1.2 M sorbitol, 20 mM HEPES, pH 7.5.
21. 36% (~1.2 M) and 60% (~2.2 M) sucrose solutions buffered with 20 mM HEPES-KOH, pH 7.5, and 2 mM EDTA.
22. cOmplete™, EDTA-free Protease Inhibitor Cocktail; 100× solution corresponds to 1 tablet dissolved in 500 µL of water.
23. 25 × 89 mm or 14 × 89 mm ultracentrifugation tubes (e.g., Beckman Coulter, Catalog #344058 or #331372).

2.2.2 Hypotonic Lysis

1. Ice.
2. Syringe (10 mL) and hypodermic needle (gauge 25G).
3. NET solution: 0.15 M NaCl, 0.1 M EDTA; 10 mM Tris–HCl, pH 8.0.
4. DTE: 1 mM Tris–HCl, pH 7.9, 1 mM EDTA.
5. 60% (w/v) sucrose.
6. STM: 0.25 M sucrose, 20 mM Tris–HCl, pH 7.9, 2 mM $MgCl_2$, 1 tablet of cOmplete™ Mini, EDTA-free Protease Inhibitor Cocktail per 10 mL of solution.
7. STE: 0.25 M sucrose, 20 mM Tris–HCl, pH 7.9, 2 mM EDTA, 1 tablet of cOmplete™ Mini, EDTA-free Protease Inhibitor Cocktail per 10 mL of solution.
8. Centrifuge, 4–22 °C, up to 21,000× *g*.
9. DNAse I solution: 2 mg of DNAse I in 1 mL of 10 mM $CaCl_2$, 50% (w/v) glycerol, 10 mM Tris–HCl, pH 7.4.
10. 1 M $MgCl_2$.

2.2.3 Sonication

1. Same materials as for Subheading 2.2.2.
2. Bioruptor Plus Sonication System (Diagenode SA).

2.2.4 Digitonin Treatment

1. Digitonin (10 mg/mL in deionized water, solubilized at 86 °C for 15 min).
2. 0.5 M aminocaproic acid (ACA).
3. Centrifuge, 4–22 °C, up to 21,000× *g*.
4. Ice.

2.3 Separation of Mitochondrial Ribosomes on Sucrose Gradients

1. Refrigerator (4 °C).
2. Freezer (−80 °C).
3. Ice and styrofoam icebox.
4. Liquid nitrogen (or dry ice) supply and appropriate equipment for its manipulation (e.g., gloves, ladle).
5. Ultracentrifuge with a swinging bucket rotor (up to 250,000× *g*) and corresponding tubes (e.g., Thermo Scientific AH-650 rotor or Beckman SW55 Ti and 13 × 51 mm tubes; for larger preparations, e.g., Thermo Scientific TH-641 or Beckman SW41 Ti rotor and 14 × 89 mm tubes).
6. 13 × 51 mm or 14 × 89 mm ultracentrifugation tubes (Beckman Coulter, Catalog #367280 or #331372).
7. TissueLyserII (or equivalent) and 25-mL steel grinding jar with a 10-mm steel ball.
8. 1.5-mL and 50-mL Centrifugation tubes, sterile.
9. Centrifuge, 4–22 °C, up to 20,000× *g*.
10. Gradient maker and peristaltic pump with appropriate tubing.
11. 100× cOmplete EDTA-free protease inhibitor cocktail (1 tablet dissolved in 500 μL of water).
12. Pre-lysis buffer: 30 mM Tris–HCl, pH 7.6, 30 mM KCl, 30 mM $MgCl_2$, 3 mM DTT, 7.5× cOmplete, EDTA-free.
13. Buffered solutions of 10% and 30% sucrose: 20 mM Tris–HCl, pH 7.6, 20–100 mM KCl, 20 mM $MgCl_2$.
14. 1 M DTT.
15. 10% mild detergent (e.g., Triton X-100, dodecylmaltoside (DDM)).
16. *Optional*: Ultrafiltration device (exclusion limit ≤300 kDa) (e.g., Amicon Ultra-0.5, Sartorius Vivaspin 500).

2.4 High-Resolution Respirometry

1. OROBOROS O2k-FluoRespirometer (OROBOROS Instruments GmbH, Innsbruck) (*see* **Note 1**).
2. Centrifuge, up to 4500× *g*.
3. Hamilton syringes (separate syringes of specific volume for cells (100 μL), digitonin (10 μL), substrates (25 μL), and inhibitors (10 μL)).
4. MiR05 solution: 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM D-sucrose, 1 g/L fatty acid-free bovine serum albumin, pH 7.1.
5. Digitonin (10 mg/mL in deionized water, solubilized at 86 °C for 15 min).
6. 1 M pyruvate (in water).

7. 1 M malate (in water).
8. 1 M NADH (in water).
9. 1 M succinate (in water).
10. 1 M KCN (in ethanol).
11. 1 M malonate (in water).
12. 1 M SHAM (in water).
13. 0.5 M ADP (in water).
14. 5 mM oligomycin (in ethanol).

2.5 Membrane Potential Measurement

1. OROBOROS O2k-FluoRespirometer (*see* **Note 1**).
2. Blue filter (excitation 495 nm and emission at 587 nm).
3. Centrifuge, up to 4500× *g*.
4. Hamilton syringes (separate syringes of specific volume for cells (100 μL), safranin (10 μL), digitonin (10 μL), substrates (25 μL), and inhibitors (10 μL)).
5. MiR05 solution: 0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6\ H_2O$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM D-sucrose, 1 g/L fatty acid-free bovine serum albumin, pH 7.1.
6. Digitonin (10 mg/mL in deionized water, solubilized at 86 °C for 15 min).
7. 200 μM safranin (in water).
8. 0.5 M ADP (in water).
9. 1 M pyruvate (in water).
10. 1 M malate (in water).
11. 1 M succinate (in water).
12. Valinomycin (10 mg/mL in ethanol).
13. 4.8 mM KCl (in water).
14. 1 mM FCCP (in ethanol).

2.6 Activity Measurement of Catalases and Peroxidases

1. UV-VIS spectrophotometer.
2. Centrifuge, up to 21,000× *g*.
3. Ice.
4. 1.5-mL Cuvettes for UV-VIS (Varian-Agilent Quartz Semi-Micro Cuvette Cell, Agilent).
5. 0.5 M potassium phosphate buffer, pH 7.2.
6. 35% hydrogen peroxide solution.
7. 1 M ascorbate (freshly dissolved in water).
8. 0.5 M aminocaproic acid (ACA).
9. 10% (w/v) dodecylmaltoside (DDM).

2.7 Metabolic Labeling by ^{13}C

1. Centrifuge, up to 4500× *g*.
2. Incubator, 17 °C.
3. Nitrogen gas cylinder.
4. Glass vials with caps.
5. 0.22 μm ultrafiltration units for centrifugation.
6. 3.6% (w/v) sea salt solution.
7. 270 mL polyethylene cultivation flask with a screw cap.
8. 10 μL of 1 M ^{13}C-proline ($^{13}C_5H_9NO_2$).
9. 10 μL of 1 M ^{12}C-proline ($^{12}C_5H_9NO_2$).
10. Extraction solution: methanol:acetonitrile:water at a 2:2:1 ratio.
11. 1 M 2,2-diphenylglycine.

3 Methods

3.1 DNA Preparation for Single-Molecule Long-Read Sequencing

1. Harvest ~150 mg wet-weight cells (~3 × 10^9 *P. papillatum* cells). Resuspend the pellet in 1 mL of 0.5× SoHE buffer and centrifuge (2000× *g*, 3 min, 20 °C). Remove the supernatant.
2. Homogenize the cells in 1.5 mL (10× volume of the pellet) of the cell lysis buffer (*see* **Note 2**).
3. Add N-lauryl sarcosine (sarcosyl) to a final concentration of 0.5% and mix thoroughly, but gently. The solution should become very viscous (*see* **Note 3**).
4. Add 40 μL of RNase and mix thoroughly, but gently (by inverting the tube or up-and-down pipetting using a wide-bore tip). Incubate for 15–30 min at 37 °C with occasional gentle mixing by inverting the tube.
5. Add 100 μL of proteinase K and mix thoroughly but gently (by inverting the tube or up-and-down pipetting using a wide-bore tip). Incubate for 30–60 min at 50 °C with occasional gentle mixing by inverting the tube.
6. Add a phenol:chloroform:isoamylalcohol (25:24:1) mixture at a volume equal to that of the reaction mix. Mix well by inverting the tube until the suspension becomes white and less viscous than originally. Centrifuge (4000× *g*, 10 min, 4 °C) to extract the DNA into the aqueous phase (*see* **Note 4**).
7. Transfer the aqueous phase into a new tube using a large-bore tip. Add an equal volume of the phenol:chloroform:isoamylalcohol (25:24:1) mixture. Mix well by inverting the tube several times and centrifuge (4000× *g*, 10 min, 4 °C) to re-extract the DNA into the aqueous phase (*see* **Note 5**).

8. Transfer the aqueous phase into a new pre-chilled tube. Add 2 volumes of an ice-cold precipitation solution. Mix thoroughly, but gently by inverting the tube. If the HMW DNA concentration is sufficient, a white filamentous precipitate will quickly start to form (*see* **Note 6**).
9. Prepare a new tube filled with 0.5–1 mL of pre-chilled 80% ethanol (0–4 °C). Use a glass hook/capillary to take out the filamentous precipitate and transfer it to the new tube (*see* **Note 7**).
10. Mix by gently inverting the tube several times and let the precipitate sink to the bottom. Carefully remove as much liquid as possible using a fine tip and without touching the precipitate.
11. Add 0.5–1 mL of pre-chilled 80% ethanol (0–4 °C), mix by gently inverting the tube several times. Once the precipitate has dropped to the bottom, again carefully remove as much liquid as possible using a fine tip and without touching the precipitate. Leave the tube open at room temperature until all the remaining liquid has evaporated.
12. Add 30–40 μL of 10 mM Tris–HCl, pH 8.5 (or Qiagen Elution buffer or equivalent) and let the precipitate slowly hydrate and solubilize overnight at 4 °C.
13. Resuspend the DNA by gently pipetting up and down the viscous solution several times using a large-bore tip. To spectroscopically measure the concentration and purity of the HMW DNA, dilute a small aliquot (e.g., 2 μL) 10× to 20× in the same resuspension buffer (*see* **Note 8**).

3.2 Isolation of Mitochondria

3.2.1 Nitrogen Cavitation

1. Inoculate 50 mL of OSS growth medium supplemented with chloramphenicol (40 mg/L) with an axenic stock of *P. papillatum* cells. Cultivate at 16–20 °C under occasional shaking (e.g., four times per day) to a cell density ~1–2 × 10^6 per mL (*see* **Note 9**). This corresponds approximately to the late exponential phase and usually takes 3–6 days when starting from a frozen stock of cells or 2–3 days when starting from a pre-cultured inoculum.
2. Prepare 4 L of fresh cultivation medium by adding horse serum (1.5% final) and, optionally, yeast extract (0.05% final) to the sterile artificial sea water. Inoculate the medium with the pre-culture at the 1:100 ratio (e.g., 40 mL into 4 L) and distribute it into large sterile flasks/containers ensuring that the liquid column does not exceed 1.5 cm (*see* **Note 10**).
3. Incubate the cultures at 16–20 °C for 5–6 days to the late exponential phase (i.e., cell density ~ 2.5 × 10^6/mL). Occasionally, slowly shake for 15–30 min (e.g., twice daily, at maximum of 50 rpm; *see* **Note 11**).

4. To detach all surface-adherent cells, shake the cultures gently on a rocking or orbital platform for 30–120 min at 20–50 rpm (depending on the container), ideally at the same temperature at which the cultivation was done.
5. Collect the cells by centrifugation (2000× *g*, 5 min, 8 °C). Solubilize the pellets in 80 mL of OS at ambient temperature to wash away the remaining serum (*see* **Note 12**). Distribute the cell suspension into two 50 mL tubes, then spin (2000× *g*, 5 min, 8 °C).
6. Remove the supernatant and solubilize each pellet in 30 mL of ice-cold SoHE and spin (6000× *g*, 4 °C, 10 min). Alternatively, resuspend the pellet in 0.5× SoHE and perform a low-speed spin (2500× *g*, 10 min, 4 °C).
7. Weigh the pellets (1–2 g per 1 L of cell culture is expected). Solubilize the pellets in ice-cold SoHE to 1 g wet weight per 10 mL. From this step onward, work on ice (unless otherwise stated).
8. While harvesting and washing the cells, prepare a two-step sucrose gradient. Into a 25 × 89 mm ultracentrifuge tube, add 6 mL of a HEPES-buffered 60% sucrose solution, then carefully overlay with 14 mL of HEPES-buffered 36% sucrose solution (*see* **Note 13**). Store on ice until use.
9. Prepare 10–20 mL aliquots of cell suspension (*see* **Note 14**). Add the protease inhibitor cocktail (20× in SoHE to 1× final) before transferring the cell suspension into an ice-cold N_2-cavitation chamber. Add a magnetic stir bar. Apply an N_2 pressure of 30 bar (435 psi/29.6 atm) for 3 min, while placing the chamber on a magnetic stirrer to ensure thorough mixing during the procedure.
10. Release the suspension from the chamber into a pre-cooled 50 mL tube (ideally at a rate of <1 mL per second). Put the tube on ice and wait for the phase separation of the foam and the broken cell suspension. Repeat the **steps 9** and **10** with the remaining aliquots. When using the cavitation chamber repeatedly to process larger volumes, rinse it after each aliquot lysis with ice-cold SoHE to wash away remnants of lysed cells.
11. Combine the disrupted cells from all aliquots (avoiding the foam) and layer the suspension onto the sucrose gradients by uniformly distributing it across multiple tubes (*see* **Note 15**). When using 25 × 89 mm ultracentrifuge tubes, spin using SW28 rotor (or equivalent) (134,000× *g* (27,300 rpm), 4 °C, 30–60 min). When working with 14 × 89 mm ultracentrifuge tubes, use TH-641 rotor (or equivalent).
12. Collect the mitochondria-enriched band from the interface between 36% and 60% sucrose using the syringe with an 18G needle (*see* **Note 16**).

13. To remove the sucrose (as well as cellular contaminants), dilute the mitochondrial fraction by adding 3.5 volumes of SoH, followed by a gentle, but thorough swirling motion, and then resuspend once or twice using a 18G needle syringe stroke (*see* **Note 17**).
14. Pellet the mitochondrial fraction (≥20,000× *g*, 10 min, 4 °C). Remove the supernatant and flash-freeze the final mitochondrial pellet in liquid nitrogen (or alternatively in dry ice). Mitochondria can be stored at −80 °C for at least 3 months without any apparent quality loss (*see* **Note 18**).

3.2.2 Hypotonic Lysis

1. Resuspend a pellet of 5×10^8 cells in 1.5 mL of hypertonic NET solution and incubate for 1 min to allow cells to shrink.
2. Centrifuge the suspension (2000× *g*, 10 min, 4 °C), discard the supernatant and keep the pellet.
3. Resuspend the pellet in 1.5 mL of hypotonic DTE and allow cells to swell.
4. Load the swollen cell suspension into a syringe and push the suspension through the 25G needle. Check for effective cell rupture under a microscope by screening for the presence of "cell ghosts," i.e., distorted cell remnants with apparently thin cell membranes and lacking the usual *P. papillatum* morphology; ideally a "cell ghost"-to-"typical cell" ratio of 10:1–20:1 should be attained.
5. Add 180 μL of 60% (w/v) sucrose to the ruptured cell suspension.
6. Centrifuge the suspension (21,000× *g*, 30 min, 4 °C) and collect the pellet containing membranes.
7. Resuspend the membrane pellet in 500 μL of STM. Add DNase I to a final concentration of 10 μg/mL and incubate on ice for 30 min.
8. Stop the DNase treatment by adding 500 μL of STE. Centrifuge the suspension (21,000× *g*, 30 min, 4 °C).
9. Wash the pellet twice by adding 500 μL of STE and spinning (21,000× *g*, 30 min, 4 °C).
10. Store the final pellet of mitochondrial membranes at −80 °C.

3.2.3 Sonication

1. Resuspend a pellet of 5×10^8 cells in 2 mL of STE.
2. Put the tubes on ice. Sonicate the cell suspension using, e.g., a Bioruptor Plus Sonication System (Diagenode SA). Apply six sonication cycles, each consisting of 20 s "ON" followed by 20 s "OFF."
3. Centrifuge the sonicated lysate (1800× *g*, 5 min, 4 °C).

4. Carefully collect the supernatant into a new tube. Discard the pellet containing intact cells and large debris.
5. Centrifuge the supernatant (21,000× *g*, 30 min, 4 °C).
6. Carefully collect the mitochondria-enriched pellet. Resuspend in 500 μL of STM. Continue with the DNase treatment and wash steps as described in Subheading 3.2.2.

3.2.4 Digitonin Treatment

1. Determine the number of cells corresponding to 1 mg of total protein.
2. Resuspend the cell pellets corresponding to 1 mg of protein in 0.5 M ACA.
3. Add 1 mg digitonin per 1 mg of total cell protein. Incubate the cell suspension at room temperature for 3 min.
4. Centrifuge the cell suspension (21,000× g, 10 min, 4 °C).
5. Carefully collect the pellet and discard the supernatant, which contains proteins from cellular compartments other than mitochondria. Continue with the DNase treatment and wash steps as described in Subheading 3.2.2.

3.3 Separation of Mitochondrial Ribosomes on Sucrose Gradients

3.3.1 Cryo-Milling of Mitochondria or Whole Cells

1. For higher lysis efficiency, especially when using frozen cell pellets or mitochondria or when cells have not been processed right after their harvest, cryo-mill the sample using a tissue grinder (e.g., TissueLyserII).
2. Fill a 50 mL tube with ~20–25 mL liquid nitrogen and let it cool down.
3. Prepare cryo-beads from the material to be cryo-pulverized. When using an enriched mitochondrial fraction (e.g., from the 36%/60% sucrose gradient interface), add 2 mL of pre-lysis buffer without detergent to 1 mL (or ~1 g) material (*see* **Note 19**). Mix well quickly by pipetting up and down, then let the suspension plunge in droplets of 40–80 μL into the tube filled with (>15 mL) liquid nitrogen. Cryo-beads created this way are easier to manipulate in subsequent steps (*see* **Note 20**).
4. Pre-cool a 25-mL grinding jar with a 10-mm ball in liquid nitrogen (*see* **Note 21**).
5. Aliquot ~1.5 g (1–2 g) of cryo-beads into the jar, add the ball, and close the lid. Perform cryo-pulverization 2–4 × 30 s at 30 Hz with intermittent cooling in liquid nitrogen (*see* **Note 22**).
6. Cool the jar after the last cycle before opening it. Scoop the powder with a pre-cooled spatula and transfer it into a pre-cooled 50 mL tube. The frozen powder can be stored at −80 °C for at least 1 year.

3.3.2 Preparation of a Sucrose Gradient

1. Prepare the sucrose gradients just before starting the sample processing (or during lysate incubation and centrifugation) to reduce diffusion.
2. To the buffered solutions of 10% and 30% sucrose prepared beforehand, add, just before use, the following components to the specified final concentrations: DTT, 1 mM; cOmplete EDTA-free PIC, 0.2×; and detergent at 1/100 concentration used for sample solubilization (e.g., 0.015% Triton X-100 if using 1.5% solution for the initial solubilization of mitochondria).
3. Set up the gradient maker, pump, and stirrer (*see* **Note 23**). To generate a 5 mL gradient in a 13 × 51 mm tube, pour 2.5 mL of 30% sucrose into the proximal chamber (with a magnetic stirrer) and 2 mL of 10% sucrose in the distal chamber. While using the BioRad Econo Pump EP-1 and tubing of 3.2 mm internal diameter, the optimal flow rate is 20%. (To generate a 11 mL gradient in a 14 × 89 mm tube, use 5.5 mL of 30% sucrose and 5 mL of 10% sucrose solutions.)
4. Open the 30% sucrose valve, start the pump at 20% of the maximum rate. Let the solution flow until 0.5 mL of sucrose has left the chamber, then open the 10% sucrose valve to allow mixing. (Stirring the liquid in the proximal chamber with a magnetic bar enhances the mixing of the two solutions.) Monitor closely that no bubble enters the connection between the two chambers, which can occur especially in the presence of detergents, and inhibits mixing.
5. After the ultracentrifuge tube is filled, put it immediately on ice to reduce sucrose diffusion.

3.3.3 Velocity Separation of Mitoribosomes

1. Weigh the required amount of cryo-powder: 0.2–0.5 g per ultracentrifuge tube when using mitochondria or ~0.5–1 g when using whole cells (*see* **Note 24**). Let the powder thaw on ice.
2. Once thawed and just before adding a mild detergent (e.g., Triton X-100 or dodecylmaltoside), add the necessary amount of buffer components (e.g., Tris, KCl, $MgCl_2$, DTT, protease inhibitors) to compensate for the subsequent detergent addition (*see* **Note 25**).
3. Add the detergent (1.5% final), mix well by pipetting up and down, and incubate on ice for 10 min.
4. Spin the lysate (≥20,000× *g*, 10 min, 4 °C) and transfer the supernatant into an ice-cold tube (*see* **Note 26**).
5. *Optional:* The smaller the volume of the loaded sample, the better the resolution after migration in the gradient. The loaded volume of the soluble lysate should be less than 10%

of the total gradient volume (i.e., <0.5 mL for a 5 mL ultracentrifuge tube), ideally ≤5%. If the volume is larger than required, the sample can be concentrated using an ultrafiltration device (*see* **Note 27**). For example, centrifugation in Amicon Ultra-0.5 (or equivalent) with a 100–300 kDa filter spin column (13,800× *g*, 10 min, 4 °C) can usually halve the sample volume in one (300-kDa filter) or about three (100 kDa filter) centrifugation rounds with intermittent resuspension of the concentrate.

6. Carefully load the lysate on top of the gradient (*see* **Note 28**). Set aside 2–5% of the lysate as the input control for RNA extraction and a similar amount for SDS-PAGE and follow-up western blot.
7. Perform ultracentrifugation. For a 5-mL gradient (13 × 51 mm tube in the rotor AH-650), use the settings 45,700 rpm (~247,500× *g*), 2 h 10 min, 4 °C. For an 11 mL gradient (14 × 8 mm tube in the rotor TH-641), use 38,000 rpm (~247,300× *g*), 4 h 30 min, 4 °C (*see* **Note 29**).
8. Collect 210-μL fractions from the top using a pipette. From a 5-mL gradient (13 × 51 mm tube), collect 24 fractions, and from an 11-mL gradient (14 × 89 mm tube), collect 48 fractions.
9. The simplest test to verify proper separation in the gradient is to extract RNA from 150 μL for every second (5 mL gradient) or every third (11 mL gradient) fraction (*see* the *Single-step DNA and RNA isolation* protocol in Chap. 1 of Volume 2: "Genetic Manipulation of *Paradiplonema papillatum*"), and determine the distribution of mitochondrial rRNAs across the fractions by agarose gel electrophoresis (*see* **Note 30**). Based on the estimated quantity of ribosomes, load 25–100% of the extracted RNA on the gel; 25–50% is usually enough for mitochondrial lysates, but up to 100% may be necessary when starting from whole cells.

3.4 High-Resolution Respirometry

1. Calibrate the OROBOROS O2k-FluoRespirometer with air using MiR05 medium. Ensure that the device is stable and calibrated prior to introducing the sample (*see* **Note 31**). Set the O2k chamber temperature to 25 °C.
2. Collect 5 × 10^6 cells from the medium by centrifugation (4500 × *g*, 10 min, 20 °C).
3. Wash the cell pellet with 500 μL of MiR05 medium and spin (4500 × *g*, 10 min, 20 °C).
4. Using a Hamilton syringe, introduce the washed cell suspension into the O2K chamber containing 2 mL of MiR05 medium.

5. Record the basal oxygen consumption of the intact cells. Allow the signal to stabilize.
6. Add gradually digitonin (2.5 μg/mL) into the chamber to permeabilize the plasma membrane and monitor the real-time effect (digitonin is added until oxygen consumption in the chamber stops). Keep the time intervals between individual additions for at least 15 min so that the oxygen consumption in the chamber stabilizes.
7. Add the following substrates to assess the electron flow and the contribution of respiratory complexes to respiration: pyruvate (final concentration 5 mM) and malate (final concentration 2 mM), or NADH (final concentration 10 mM), or succinate (final concentration 10 mM). Record the oxygen consumption rate.
8. Add ADP to a saturating concentration (final concentration 2.5 mM) to the chamber to assess the coupled state of respiration to ATP synthesis via OXPHOS. Record the oxygen consumption rate.
9. Add the following inhibitors to assess electron routes: KCN (final concentration 1 mM; complex IV inhibitor), malonate (final concentration 5 mM; complex II inhibitor), salicylhydroxamic acid (final concentration 250 μM; alternative oxidase inhibitor). Record the oxygen consumption rate after each inhibitor addition.
10. Add oligomycin to block the flow of protons back to the mitochondrial matrix. By comparing the oxygen consumption rate in the presence of ADP with the rate in the presence of oligomycin, the coupling efficiency of the mitochondria is assessed.
11. Analyze the oxygen consumption data, expressing respiration as oxygen flux per volume or per number of cells.

3.5 Membrane Potential Measurements

1. Calibrate the OROBOROS O2k-FluoRespirometer with air using MiR05 medium. Ensure that the device is stable and calibrated prior to introducing the sample (*see* **Note 31**). Set the O2k chamber temperature to 25 °C.
2. Configure the O2k-FluoRespirometer with the blue filter: excitation at 495 nm and emission at 587 nm.
3. Calibrate the MiR05 medium in the O2K chamber with safranin in 0.5 μM increments at 15 min intervals, until a final concentration of 2 μM is reached.
4. Collect 5×10^6 cells from the medium by centrifugation (4500× *g*, 10 min, 20 °C).
5. Wash the cell pellet with 500 μL of MiR05 medium and spin (4500× *g*, 10 min, 20 °C).

6. Using a Hamilton syringe, introduce the washed cell suspension into the O2K chamber containing 2 mL of MiR05 medium with safranin.
7. Record the initial safranin fluorescence under basal conditions. Allow the signal to stabilize. Add digitonin to a final concentration of 30 μg/mL to permeabilize the plasma membrane. Monitor the fluorescence signal.
8. Add substrates to build up the mitochondrial membrane potential in the permeabilized cells, using pyruvate and malate or NADH at the same concentration as for the protocol *3.4 High-resolution respirometry* (*see* **Note 32**). Record the fluorescence. A decrease in fluorescence indicates an increase in membrane potential.
9. Record the final fluorescence signal and determine changes in membrane potential (*see* **Note 33**).
10. Add valinomycin (5 nM) to collapse a pH gradient across the inner mitochondrial membrane.
11. Add gradually KCl to calibrate fluorescence changes (*see* **Note 34**).
12. Add FCCP (1.5 μM) to uncouple the membrane potential.
13. Calculate K^+ equilibrium potential in the presence of valinomycin from Nernst equation at 25 °C (considering $[K_{in}^+]$ as 120 mM and $[K_{out}^+]$ as a concentration in individual step): $\Delta\Psi = 58.9 \times \log (K_{in}^+/K_{out}^+)$ [mV].
14. Plot a calibration curve as ΔF/F as a function of the calculated values ΔΨ, where "F" is the fluorescence after FCCP addition and "ΔF" is "F" minus steady state fluorescence after KCl addition.
15. Use the calibration curve to evaluate the fluorescence from the measurement.

3.6 Measurement of Catalase and Peroxidase Activity

1. Prepare the cell lysate by incubating 5×10^8 cells in 0.5 M ACA with 2% (w/v) DDM for 1 h on ice.
2. Centrifuge the cell lysate (21,000× *g*, 30 min, 4 °C). Collect the supernatant to determine the enzyme activity.
3. Prepare 1.5 mL of the assay solution by diluting potassium phosphate buffer to 50 mM and supplement it with: (a) H_2O_2 to a final concentration of 0.005% (v/v) for catalase activity measurement, or (b) ascorbate to a final concentration of 15 μM for ascorbate peroxidase activity measurement.
4. Set the spectrophotometer to monitor absorbance at (a) 240 nm (catalase) or (b) 274 nm (ascorbate peroxidase).
5. Start the reaction by adding 20 μg of cell lysate proteins to the assay solution in the cuvette.

6. Start recording the decrease in absorbance for 2–4 min.
7. To calculate the activity, determine the difference in absorbance in the linear part of the reaction progress and use this value to calculate enzyme activity U (the change in molar concentration Δn [mol] per min) of the monitored molecule based on the Lambert Beer law: $U = \Delta A \times V_r/d \times \varepsilon \times V_s$. The individual abbreviations represent: ΔA—change in absorbance per min; V_r—reaction volume [1.5×10^{-3} L]; V_s—sample volume [corresponds to 20 μg of proteins in L]; d—the length of the cuvette [cm]; ε—molar extinction coefficient of the monitored molecule [H_2O_2: $\varepsilon_{240} = 14.900\ M^{-1}\ cm^{-1}$; ascorbic acid: $\varepsilon_{274} = 14.900\ M^{-1}\ cm^{-1}$]. The enzyme unit U is defined as the activity of the enzyme which catalyzes the conversion of 1 mol of H_2O_2 (catalase) or ascorbate (ascorbate peroxidase) per min. The specific activity is calculated as the number of enzyme units U per mg of cell protein [20].

3.7 Metabolic Labeling via ^{13}C

1. Harvest 10^8 cells (*see* **Note 35**) in the exponential growth phase from the culture by centrifugation (2000× *g*, 5 min, 17 °C).
2. Wash the harvested cells in 3.6% (w/v) sea salt solution (i.e., medium without serum and other supplements), spin (2000× *g*, 5 min, 17 °C), and remove the supernatant.
3. Incubate the washed cells in 50 mL of sea salt solution for 18 h at 17 °C to deplete intracellular carbon reserves.
4. Add 10 μL of 1 M ^{13}C-proline to the cell suspension (*see* **Note 36**). Incubate motionless for 2 h at 17 °C.
5. Prepare a parallel sample and add 10 μL of 1 M ^{12}C-proline as a negative control. Incubate motionless for 2 h at 17 °C.
6. Harvest both the ^{13}C-proline-labeled and ^{12}C-proline-labeled cells by centrifugation (2000× *g*, 5 min, 4 °C).
7. Wash the harvested cells with 3.6% (w/v) sea salt solution and spin (2000× *g*, 5 min, 4 °C).
8. Resuspend the washed cells in 100 μL of ice-cold extraction solution. Homogenize the cell suspension in an ultrasound water bath for 5 min. Use 30 s sonication intervals followed by 30 s cooling intervals on ice.
9. Remove residual cell debris by centrifugation (2000× *g*, 10 min, 4 °C). Collect the supernatant.
10. Filter the supernatant through a 0.22 μm ultrafiltration unit by centrifugation (5000× *g*, 5 min, 4 °C).
11. Dry the purified extracts under a stream of nitrogen gas.

12. Analyze the dried extracts using high-resolution liquid chromatography mass-spectrometry (LC-MS). Use 10 mM 2,2-diphenylglycine as an internal control for calibration.

4 Notes

1. Other instruments are available, such as Seahorse XF Analyzer (Agilent) or a standard Clark electrode, but the presented protocol was optimized for the use of the OROBOROS O2k-FluoRespirometer.
2. It is preferable to pipet the suspension up and down using a large-bore tip. Do not vortex to avoid damaging/breaking the DNA. Being a marine organism, *P. papillatum* is sensitive to low salt conditions, so, its cells start then, to lyse even before the addition of the detergent in the following step, and thus, the DNA is not protected in the cells anymore. If working with a lower cell quantity, it is preferable to downscale the protocol to maintain the cells: buffer ratio at 1:10 (high DNA concentration facilitates DNA precipitation).
3. SDS can be used instead of sarcosyl, but RNase digestion may not be as efficient.
4. Although the phenol/chloroform extraction also works without isoamylalcohol, its addition enhances DNA purity.
5. If a high quantity of HMW DNA has been extracted, the aqueous phase should be quite viscous. Re-extraction improves the purity of the HMW DNA. From this point on, use only large-bore tips.
6. Alternatively, add a sodium chloride solution at a final concentration of 0.4 M, mix well, and then add an equal volume of isopropanol. Mix well by inverting the tube to initiate DNA precipitation.
7. It is strongly recommended to use a glass tool, because after addition of 80% ethanol, the precipitate detaches more easily from this material compared to plastic or metal.
8. The DNA concentration of the diluted sample should be 0.5–1 μg/μL, A260/A280 ~1.8–1.9, and A260/A230 ~2.0–2.2. For subsequent nanopore sequencing, we recommend a ligation-based sequencing kit to preserve DNA modifications (especially for nuclear DNA). Use long fragment-enrichment buffers when the aim is to assemble the nuclear genome to the exclusion of the overabundant mitochondrial DNA.
9. Note that *P. papillatum* will grow well in the range from 4 to 26 °C; however, cultivation times will be slower at lower

temperatures and the nitrogen pressure required for optimal lysis (see below) will need to be optimized. For example, cultures grown in the cold require a higher pressure to disrupt the cells, which in turn increases the risk of leakage from the mitochondrial matrix.

10. Addition of yeast extract (0.05% final concentration) improves the yield of mitochondria (inferred from higher citrate synthase activity and larger amount of mitoribosomes) by up to 20% compared to the regular OSS medium. To ensure a healthy respiration, the culture must be well aerated. 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 1–1.5 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-grade glass is among the least sticky with *P. papillatum*.
11. Synchronizing the cells prior to isolation may improve the yield but is not required. After day 5 of cultivation, place the cultures in a cold room (4–10 °C) for 12–36 h.
12. While washing cells, it is recommended to suspend cells by gentle vortexing or hand-swirling instead of pipetting, which breaks the flagella. Resuspend only a single aliquot at a time (to be disrupted in the cavitation instrument) to prevent cell autolysis during extended incubation in ice-cold SoHE.
13. For small-scale preparations, use 14 × 89 mm ultracentrifuge tubes and add 2 mL of 60% and 4.5 mL of 36% sucrose. Note that 36% sucrose corresponds to 1.2 M, thus preserves the osmolarity of the SoHE buffer, which seems critical to avoid disintegration of *P. papillatum* mitochondria. Addition of EDTA at a final concentration of 2 mM to the HEPES-buffered sucrose solutions slightly decreases the contamination of the fraction by cytosolic ribosomes.
14. The volume of the aliquots is imposed by the volume of the N_2-cavitation chamber and needs to be adjusted according to the pressure chamber used. At 30 bar, which is the minimum pressure to open >80% cells, it is necessary to use SoHE at the concentration of 1.2 M sorbitol to avoid breaking up of the 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 (which is approximately isotonic with the sea salt medium) to break virtually all cells. Prior to adding the cell

suspension, optimize the stirring conditions (size of the stirring bar, speed, and position on the magnetic stirrer) to ensure that the solution in the nitrogen cavitation pressure chamber is properly mixed when the chamber is closed.

15. For higher purity of mitochondria, unlysed cells and nuclei can be removed by two successive centrifugations (2000× *g*, 4 °C, 10 min) prior to loading the mitochondria-containing supernatants on the sucrose gradient. (Note that the final yield will be >5 times lower).
16. The relatively large bore of the 18G needle prevents shearing-caused damage to the mitochondria.
17. One syringe stroke is usually enough to resuspend the collected sucrose-containing mitochondrial fraction in SoH. Being quick (and gentle) is essential, as an extended incubation with the buffer seems to increase the lysis of mitochondria and loss of the matrix into the supernatant after the subsequent centrifugation. When isolating just crude mitochondria and when sucrose content is irrelevant for downstream steps, flash-freeze the collected mitochondrial fraction without further manipulations.
18. 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 steps, i.e., cells before and after breakage, supernatant after ultracentrifugation, material at the supernatant/36% sucrose and 36%/60% sucrose boundaries, supernatant after the wash of the 36%/60% sucrose boundary material, and the final mitochondrial pellet. These aliquots are used to assess 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, SDS-PAGE and western blotting, etc.). We routinely perform nucleic acids extraction, RT-PCR, and citrate synthase assay on these aliquots.
19. The pre-lysis buffer contains all components to obtain a 1× final concentration. For mitoribosomes, 20 mM $MgCl_2$ is usually recommended, but Mg^{2+} concentrations should be adjusted based on the planned experiment. Similarly, KCl concentrations can be varied as well, but caution is required; while the default 20 mM KCl appears to ensure that all mitochondrial ribosomal proteins strongly associate with mitoribosomes, certain proteins may dissociate at concentrations >100 mM. Instead of Tris-HCl, pH 7.6, the same concentration of HEPES-KOH, pH 7.6 can be used throughout. Protease inhibitors are added at 5× higher concentration than

recommended because the protease activity of *P. papillatum*—and in particular its mitochondria—appears to be high, with some proteins being completely degraded after <30 min at 4 °C if only 1× inhibitors are used. Reducing the Mg^{2+} concentration decreases this protease activity; however, the reduced magnesium ion content destabilizes mitoribosomes and may lead to their progressive disassembly during the procedure. Note also that because later detergent addition will change the concentrations of the initially added components, it is always important to adjust the final concentrations, which should be planned out well in advance to avoid delays.

20. This step is important when working with mitochondria because of their high DNA content compared to the whole cells and because mitochondrial preparations generally contain more sorbitol and leftover sucrose, which makes the undiluted material distinctly viscous and sticky even during cryo-milling. When using whole cells, cryo-beads are best prepared right after harvesting the cells and without the addition of the pre-lysis buffer.
21. For manipulations with liquid nitrogen, use containers that withstand extremely low temperatures. If lacking in specialized equipment, a kitchen ladle can be used to aliquot and a styrofoam box to hold liquid nitrogen for the duration of the required manipulations described here.
22. Two cycles are sufficient for whole cells, whereas at least three cycles are necessary when pulverizing mitochondrial material due to its higher relative density of DNA.
23. If a gradient maker is not available, sucrose gradients can also be set up manually by layering solutions of decreasing concentration in increments of 5% on top of each other (e.g., 0.75 mL 30%, 1 mL 25%, 1 mL 20%, 1 mL 15%, 0.75 mL 10% sucrose). Although such gradients usually have poorer resolution, if the aim is to enrich mitoribosomes, a manual gradient can be exploited for preparative purposes because in the default conditions specified in this protocol, mitoribosomes migrate to ~20% sucrose.
24. If using freshly harvested *P. papillatum* cells, resuspend them directly in the lysis buffer of the desired composition, mix well by pipetting up and down, then add the detergent and mix by pipetting again.
25. For example, for 0.5 g, the initial volume will be ~0.5 mL. For a final volume of 0.6 mL and to obtain 1.5% Triton X-100, add 90 μL of the detergent together with 2 μL 1 M Tris, pH 7.6, 0.5 μL 4 M KCl, 2 μL 1 M $MgCl_2$, 0.2 μL 1 M DTT, 5 μL 100× cOmplete EDTA-free.

26. If the powder was properly cryo-pulverized and if lysate volume was 0.6 mL, the expected volume of the supernatant is at least ~0.3 mL for mitochondria and ~0.5 mL or more for whole cells (mitochondria being "fluffier" complicates the separation of the supernatant from the pellet).
27. At concentrations usually used to solubilize cells or organelles, detergents form micelles of sizes above exclusion limits of certain ultrafiltration devices. To avoid increasing the relative concentration of the detergent in the concentrate, which can destabilize RNP complexes, always verify the micelle size of the selected detergent (e.g., [22]) and perform optimizations. Filters with exclusion limits ≥100 kDa are considered safe to use with Triton X-100 and dodecylmaltoside.
28. Before loading the soluble lysate on the gradient, verify that it is less dense than the top of the gradient (10% sucrose). This is especially critical when working with mitochondrial lysates or if the sample was concentrated using an ultrafiltration device.
29. At the same relative velocity, the run time is approximately twice as long in the TH-641 rotor compared to the AH-670 rotor because their K-factor ratio is ~2 ($K_{TH\text{-}641} = 114$, $K_{AH\text{-}650} = 53$).
30. Under the conditions described here, the majority of *P. papillatum* mitochondrial rRNAs—being components of separate mitoribosomal subunits—migrates at 40–70% distance from the top of the gradient (i.e., in fractions 9–17 of a 5 mL gradient comprising 24 fractions).
31. Follow the OROBOROS O2k-FluoRespirometer manufacturer's instructions for operation and maintenance.
32. Succinate is not recommended for membrane potential measurements due to its interference with safranin fluorescence.
33. To compare the change in membrane potential on the mitochondrial membrane between two conditions (e.g., different cultivation conditions), the difference in safranin fluorescence between the two samples in units (AU) may be sufficient. To quantify the membrane potential, it is necessary to prepare a calibration curve of K^+ ions and calculate the membrane potential for individual points of the calibration curve using the Nernst equation (continuation from **step 10**) [23].
34. Concentration points [K^+] for calibration curve should range from 0.5 to 6 mM.
35. The indicated quantity provides sufficient material for triplicate high-resolution liquid chromatography-mass spectrometry (LC-MS) analysis, while ensuring that the cells are in the exponential growth phase.

36. Isotopically labeled compounds are selected to target specific metabolites or pathways. Given that *P. papillatum* poorly takes up glucose [24], amino acids like proline and alanine are the preferred labeled carbon sources.

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