bio-protocol

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Purification of Mitochondrial Ribosomal Complexes from *Trypanosoma cruzi* and *Leishmania tarentolae* for Cryo-EM Analysis

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Abstract

Kinetoplastids are unicellular eukaryotic parasites responsible for human pathologies such as Chagas disease, sleeping sickness or Leishmaniasis, caused by *Trypanosoma cruzi*, *Trypanosoma brucei*, and various *Leishmania spp*., respectively. They harbor a single large mitochondrion that is essential for the survival of the parasite. Interestingly, most of the mitochondrial gene expression machineries and processes present significant differences from their nuclear and cytosolic counterparts. A striking example concerns their mitochondrial ribosomes, in charge of translating the few essential mRNAs encoded by mitochondrial genomes. Here, we present a detailed protocol including the specific procedures to isolate mitochondria from two species of kinetoplastids, *T. cruzi* and *L. tarentolae*, by differential centrifugations. Then, we detail the protocol to purify mitochondrial ribosomal complexes from these two species of parasites (including ribosomal maturating complexes) by a sucrose gradient approach. Finally, we describe how to prepare cryo-electron microscopy (cryo-EM) grids from these two sorts of samples. This protocol will be useful for further studies aiming at analyzing mitochondrial translation regulation.

Keywords: Kinetoplastids mitochondria, Mitoribosome, Sucrose density gradient, Cryo-EM, Mitoribosome biogenesis, *Leishmania tarentolae*, *Trypanosoma cruzi*

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Background

Kinetoplastids are unicellular eukaryotic parasites, causative agents of several human and livestock pathologies (Stuart *et al.*, 2008). They are potentially lethal, affecting tens of millions of people worldwide (Stuart *et al.*, 2008). They have strongly diverged from other eukaryotic model species, in part because of their obligatory parasitic nature. Kinetoplastids evolved to live in and infect a large variety of eukaryotic organisms in extremely different molecular environments. Thus, beyond the general similarities, kinetoplastid species have diverged evolutionarily from each other and their protein sequence identity can be relatively low (Jackson *et al.*, 2016). They possess a single large mitochondrion, where gene expression machineries, namely the mitochondrial ribosomes (mitoribosomes), have also largely diverged (Amunts *et al.*, 2015; Desai *et al.*, 2017; Bieri *et al.*, 2018; Ramrath *et al.*, 2018; Waltz and Giege, 2020; Waltz *et al.*, 2020). The mitoribosomes are RNA-proteins complexes that translate the few mRNAs still encoded by mitochondrial genomes. Their composition and structure greatly diverged from that of the bacterial ancestor, with the most extreme case described to date being in fact the kinetoplastid mitoribosomes. With highly reduced rRNAs and more than 80 additional ribosomal proteins (r-proteins) compared to bacteria, the overall ribosome structure is significantly reshaped. Recent structural studies performed on *Trypanosoma brucei* have highlighted the particularities of this mitoribosome structure and composition as well as the assembly processes of the small subunit (SSU) (Ramrath *et al.*, 2018; Saurer *et al.*, 2019).

The comprehensive structural characterization of the full *T. brucei* mitoribosomal complexes required a sophisticated purification protocol based on affinity purification of several tagged potential ribosomal proteins/factors, not mentioning the expensive special growth medium required for the culture of *T. brucei* parasite (Ramrath *et al.*, 2018; Saurer *et al.*, 2019). Our study was based on *T. cruzi* and *L. tarentolae* species, which are more convenient and cheaper to grow *in vitro*. In addition, our sucrose gradient purification protocols are significantly simpler, as they do not require the tagging of any mitoribosomal proteins/factors, nor do they require affinity purification. Finally, our purification protocols can be adapted to enrich on other mitochondrial complexes, such as ATP synthase and different subunits and molecules from the respiratory chain complexes.

It is worth mentioning that around the same time of the publication of our study (Soufari et *al.*, 2020), two partially overlapping studies were published (Jaskolowski *et al.*, 2020; Tobiasson *et al.*, 2021).

Materials and Reagents

Cells

1. *Leishmania tarentolae UC strain* (ATTC®, catalog number: PRA-229™)
2. *Trypanosoma cruzi Y strain,* provided by Marcelo Sousa Silva (Universidae Nova de Lisbo, Lisboa, Portugal) and João Aristeu da Rosa (Department of Biological science, Araraquara, Brazil) (Rimoldi Ribeiro *et al.*, 2018)

Growth of cells

1. Difco™ Liver infusion broth (BD Bioscience, catalog number: 226920)
2. Bacto Tryptose (BD Bioscience, catalog number: 211713)
3. Sodium chloride (Sigma-Aldrich, catalog number: S5886)
4. Sodium phosphate dibasic (Sigma-Aldrich, catalog number: 53264)
5. Potassium chloride (Sigma-Aldrich, catalog number: P9333)
6. Glucose (Sigma-Aldrich, catalog number: G8270)
7. Hemin (Sigma-Aldrich, catalog number: H9039)
8. Fetal Bovine Serum heat inactivated (Dutscher Dominique, catalog number: 702780)
9. Brain Heart Infusion Broth (Sigma-Aldrich, catalog number: 53286)
10. Penicillin/Streptomycin 10,000 U/mL (Gibco™, catalog number: 15140-122)
11. LIT medium (see Recipes)
12. 1.25 mg/mL Hemin solution (see Recipes)
13. BHI medium (see Recipes)

Mitochondria purification

1. 38-mL Ultra clear tubes (Beckman Coulter, model: 344058 or equivalent)
2. 50-mL Falcon™ tubes
3. 1.5-mL Eppendorf Safe-Lock tubes
4. Sorbitol (Sigma-Aldrich, catalog number: S1876)
5. Sucrose (Sigma-Aldrich, catalog number: 16104-2)
6. Glucose (Sigma-Aldrich, catalog number: G8270)
7. Sodium Chloride (Sigma-Aldrich, catalog number: S5886)
8. Tris (Sigma-Aldrich, catalog number: 10708976001)
9. Hydrochloric Acid (Sigma-Aldrich, catalog number: 320331)
10. Magnesium Chloride ($MgCl_2 \cdot 6H_2O$) (Sigma-Aldrich, catalog number: M2670)
11. Histodenz™ (Sigma-Aldrich, catalog number: D2158)
12. EDTA (Sigma-Aldrich, catalog number: EDS)
13. DNase I, grade II from bovine pancreas (Roche, catalog number: 10104159001)
14. Sodium phosphate monobasic monohydrate (Sigma-Aldrich, catalog number: S9638)
15. Sodium phosphate dibasic heptahydrate (Sigma-Aldrich, catalog number: S9390)
16. Tris-HCl pH 7.5 (Sigma-Aldrich, catalog number: T2319)
17. Tris-HCl pH 8 (Sigma-Aldrich, catalog number: T2694)
18. Nitrogen cylinder
19. Phosphate buffer pH 7.9 (see Recipes)
20. Wash buffer (see Recipes)
21. SoTE buffer (see Recipes)
22. SoTE-EDTA buffer (see Recipes)
23. SoTM buffer (see Recipes)
24. Discontinous Histodenz™ gradient for *L. tarentolae* (see Recipes)
25. Discontinous Histodenz™ gradient for *T. cruzi* (see Recipes)

Mitoribosome purification

1. HEPES-KOH (Euromedex, catalog number: 10-110-C)
2. Triton X-100 (Sigma-Aldrich, catalog number: T9284)
3. Magnesium chloride hexahydrate (Sigma-Aldrich, catalog number: M2670)
4. Potassium chloride (Sigma-Aldrich, catalog number: P9333)
5. n-Dodecyl β-D-maltoside: DDM (Sigma-Aldrich, catalog number: D4641)
6. Complete EDTA-free protease inhibitor cocktail (Sigma-Aldrich, catalog number: 11873580001)
7. DL-Dithiothreitol (Sigma-Aldrich, catalog number: D0632)
8. Sucrose (Sigma-Aldrich, catalog number: S7903)
9. HEPES-KOH pH 7.6 (see Recipes)
10. Mitochondria Lysis buffer (see Recipes)
11. Monosome buffer (see Recipes)
12. 40% Sucrose cushion (see Recipes)
13. 10–30% Sucrose gradient (see Recipes)

Equipment

Growth of cells

1. PSM HeraSafe KS12 (Thermo scientific™, catalog number: 15227966)
2. Innova®-42 Incubator Shaker (Eppendorf, New Brunswick™, catalog number: M13350012)
3. Sterile 5-L Erlenmeyer (DURAN®, catalog number: 212167307) with silicone sponge closure (Sigma-Aldrich,

catalog number: C1046)
4. Malassez counting cell (Sigma-Aldrich, catalog number: BR719005)

Mitochondria purification

1. 45-mL Cell Disruption Bombs (Parr Instrument Co., catalog number: 16-6950)
2. Centrifuge (Beckman-coulter, model: Avanti j-26XP or equivalent) able to take fixed-angle rotor 4 × 1 L.
3. Fixed-angle rotor 4 × 1 L (Beckman-coulter, model: JLA9.1 or equivalent)
4. 4 × 1-L centrifuge bottles (Beckman-coulter, model: 1L Bottle Assy, J-Lite, PP, 95 × 191 mm, part number: A98813)
5. Fixed-angle rotor 12 × 39-mL (Beckman Coulter, model: 50.2 Ti or equivalent)
6. 26.3-mL centrifuge bottle (Beckman Coulter, model: 355654 or equivalent)
7. KIMBLE® Dounce tissue grinders, 15 mL (Sigma-Aldrich, catalog number: D9938)
8. 50-mL centrifuge (Sigma, model: 3-18KS or equivalent)
9. 10-mL Syringe (Terumo™, catalog number: 8SS10S21381)
10. Hypodermic needle N°25 (Terumo™, catalog number: 8AN1925R1)
11. Ultracentrifuge (Beckman Coulter, model: OPTIMA L80XP Or equivalent), able to take SW28 rotor
12. Swinging rotor 6 × 38-mL (Beckman Coulter, model: SW28 or equivalent)
13. Microcentrifuge for 1.5-mL tubes (Eppendorf: 5425R, catalog number: 5405000158)

Mitoribosome purification

1. Gradient master 108 (BioComp Instruments, catalog number: 108)
2. Piston Gradient Fractionator (BioComp Instruments, catalog number: 152)
3. Ultracentrifuge (ThermoFisher Scientific, model: Sorvall™ WX, or equivalent, catalog number: 15362177), able to take TH-641 tubes
4. Swinging rotor 6 × 13-mL (ThermoFisher Scientific, model: TH-641 or equivalent, catalog number: 12161690)
5. TH-641 tubes: 13.2-mL, Open-top polyclear™ centrifuge tube (Seton Scientific, catalog number: 7030)
6. Ultracentrifuge (Beckman Coulter, model: OPTIMA MAX XP or equivalent), able to take TLA 110 rotor
7. Fixed-angle rotor 6 × 4-mL (Beckman Coulter, model: TLA110.3 or equivalent)
8. TLA110.3 tubes: 3.5-mL, Open-top Thickwall Polypropylene tube (Beckman Coulter, catalog number: 349623)
9. A rotating wheel for 1.5-mL tubes (IKA™, catalog number: 0004016000)
10. KIMBLE® Dounce tissue grinders, 2 mL (Sigma-Aldrich, catalog number: D8938)

Mitoribosome cryo-grid preparation and data collection

1. Quantifoils R2/2 EM grids (Electron Microscopy Sciences, catalog number: Q210CR2)
2. Cryo-grid TEM grid boxes (Agar Scientific, catalog number: AGG3727)
3. Safematic CCU-010 carbon coater or equivalent (Labtech-EM, catalog number: 100001)
4. Glow discharge system from ELMO or equivalent (Agar Scientific, catalog number: AGB8958)
5. Thermo Fisher Vitrobot Mark IV or equivalent

Procedure

A. Growth of cells

The needed critical mass of cells is obtained by growing the parasites under agitation and by collecting the parasites at the late log phase*.

*The pre-culture of parasites for inoculation is always maintained in logarithmic phase, where cell density increases exponentially.

1. Epimastigote *Trypanosoma cruzi*

   Fill each of the four 5-L Erlen Meyer with 1 L of **LIT medium** inoculated with 8 × 10^5 parasites/mL (~16

mL of preculture at 5 × 10^7 parasites/mL). Incubate at 28°C under agitation (90 rpm) during 7 days. The final expected concentration is around 3 × 10^7 parasites/mL*.
*The final concentration of the culture is counted using a Malassez counting cell.

2. Promastigote *Leishmania tarentolae*
Fill each of the four 5-L Erlen Meyer with 1 L of **BHI medium** inoculated 1.3 × 10^6 parasites/mL (~26 mL of preculture at 5 × 10^7parasites/mL). Incubate at 26°C under agitation (140 rpm) for 2 days. The final expected concentration is around 6 × 10^7 parasites/mL.

B. Extraction and purification of *T. cruzi* and *L. tarentolae* mitochondria

This procedure is based on the disruption of the cell by nitrogen decompression, differential centrifugation and purification of mitochondria with Histodenz™ gradient*. The whole procedure should take in total one day and is summarized in Figure 1. All the buffers are prepared the day before the extraction of mitochondria and are stored at 4°C. All the instruments are pre-cooled at 4°C.
*Nitrogen decompression allows the complete and rapid disruption of cell membranes without altering the organelles. Differential centrifugation is performed to separate organelles from cellular debris. The use of the Histodenz™ anion gradient is the best way to obtain a good yield of intact mitochondria. The experimental steps are the same for *T. cruzi* and *L. tarentolae*, except the fact th at the fractions recovered from the Histodenz™ gradient are different (see Figure 1).

1. Harvest cells in four 1-L centrifuge bottles by centrifugation for 10min at 11,000 × *g* in a fixed-angle rotor at 4°C.
2. Wash the pellets twice with 1 L of wash buffer. Each time, the pellets are recovered by centrifugation for 10 min at 11,000 × *g* at 4°C and removal of the supernatants by gently inverting of the bottles.
3. Resuspend the four pellets with a volume of **SoTE buffer** calculated to reach the concentration of 2.5 × 10^9 cells/mL*.
*48 mL SoTE buffer for *T. cruzi*, 96ml SoTE buffer for *L. tarentolae*.
4. Transfer all the resuspended pellets into the adequate number of 15-mL glass dounce potters and homogenize them with 5 strokes*.
*The step prior the nitrogen cavitation using the Douncers is aimed at homogenizing the distribution of the parasite in the sample. This is to avoid aggregates and to optimize the contact with the nitrogen. Due to the volume of SoTE needed to resuspend the parasites, several Douncers are needed to perform the homogenisation.
5. Proceed to the cell disruption by nitrogen cavitation. Place the cells in the pressure vessel with a magnetic bar*. Close it and place on ice with a magnetic stirrer. Connect the nitrogen cylinder and inject the gas to obtain a pressure of 80 bar. Let the cells under this condition with agitation for 1 h. Then open the valve and recover the disrupted sample on a 50-mL Falcon tube.
*The maximum volume that is possible to load on the cell disruption bombs is 45 mL. It is thus necessary to repeat the cavitation step several times until the entire volume of parasites has been treated. During this time, the disturbed parasites can remain at 4°C.
6. Leave this crude lysate on ice for at least 10 min until the cellular debris settle. Collect with a serological pipette as much liquid (that contains the mitochondria) as possible and transfer it into two 26.3-mL centrifuge bottles. Wash again the sedimented pellet of cellular debris with 3 mL of **SoTE buffer** and collect again the liquid. Repeat this step until the foam color turns from brown to white*.
*Foam contains a lot of mitochondria, washing it allows to strongly increase the yield of mitochondria recovery.
7. Centrifuge for 10 min at 31,000 × *g* at 4°C on fixed angle 50.2 Ti rotor. Gently discard the supernatant.
8. Recover the pellet containing the mitochondria embedded in the genomic DNA with 1 volume of **SoTM buffer** supplemented with 100 µg/mL of DNaseI (final concentration).
9. Break genomic DNA further by pushing the extract through a hypodermic needle (No.25) by using a syringe.
10. Incubate for 30 min at 4°C. During incubation repeat step B9.
11. Stop the DNase treatment by adding an equal volume of **SoTE-EDTA Buffer.**
12. Transfer the extract into a 50-mL Falcon tube and complete to 50 mL with **SoTE Buffer**.

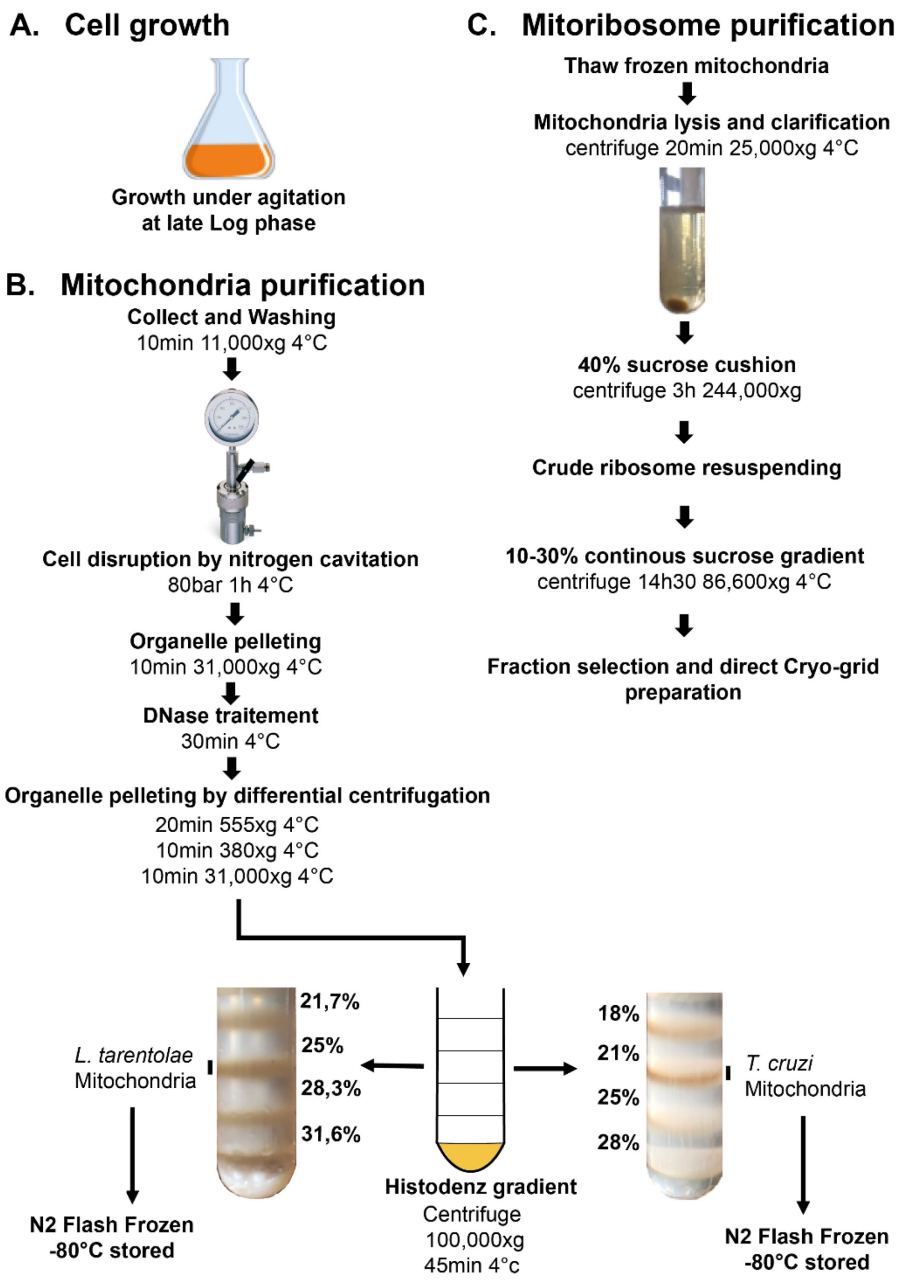

Figure 1. Workflow for mitochondria and mitoribosome purification from *T. cruzi* and *L. tarentolae* cultures.
(A) Cell culture; (B) Mitochondria purification: discontinuous Histodenz™ gradient for *L. tarentolae* is composed of 21.7%/25%28.3%31.6% phases and for *T. cruzi* is composed of 18%/21%/25%/28% phases. Mitochondria should appear at the 21%/25% Histodenz™ interphase for *T. cruzi* (right) and at the 25%/28% Histodenz™ interphase for *L. tarentolae* (left); (C) Mitoribosome purification.

13. Centrifuge at 555 × *g* and 4°C for 20 min on 3-18KS centrifuge.
14. Collect the supernatant and keep it on ice.
15. Resuspend the pellet with 15 mL of **SoTE Buffer** and homogenize using the 15-mL Dounce potter.
16. Transfer into a 50-mL Falcon tube and complete to 50-mL with **SoTE Buffer**.
17. Centrifuge for 10 min at 380 × *g* at 4°C on 3-18KS centrifuge.
18. Collect the supernatant. Mix with the supernatant from the step B14.

19. Distribute on four 26.3-mL bottles.
20. Centrifuge 10 min at 31,000 × *g* at 4°C in a fixed angle rotor 50.2Ti. The pellets contain the organelles.
21. Resuspend the organelles pellet with a total volume of 6 mL of **SoTE buffer** containing 50% Histodenz™.
22. Homogenize the resuspended pellet with 5 strokes using the 15-mL Dounce potter,
23. With a syringe load with a syringe 3 mL (that correspond to ~6 to 12 × 10^{10} cell-equivalents) of the homogenized solution onto the bottom of two discontinuous Histodenz™ gradient (21.7%/25%28.3%31.6% or 18%/21%/25%/28% for extracts from *L. tarentolae* or from *T. cruzi*, respectively) in 38-mL Ultra Clear tubes.
24. Centrifuge the gradients at 100,000 × *g* for 45 min at 4°C on swinging bucket rotor SW28.
25. After this stage the gradient should show four bands*, **.
    *For *T. cruzi*, Mitochondria should appear at the 21%/25% Histodenz™ interphase and for *L. tarentolae*, mitochondria should appear at the 25%/28% Histodenz™ interphase.
    **Bands visible by eyes appeared at the interfaces of the different Histodenz™ phases (highlighted in Figure 1B).
26. By pipetting, eliminate the upper part of the gradients until reaching the band of interest. Then, collect 5 mL of this band from each gradient.
27. Wash the mitochondria from excess of Histodentz™ by adding 4 volumes of **SoTE buffer**. Mix by inversion and centrifuge at 33,000 × *g* and 4°C for 15 min on two 26.3-mL bottles.
28. Recover each mitochondria pellet with 1 mL of **SoTE buffer**.
29. Transfer on two 1.5-mL Eppendorf tubes, Centrifuge at 10,000 × *g* and 4°C for 10 min.
30. Eliminate the supernatant, and weight the wet mitochondria pellet. The total expected amount is ~700 mg.
31. Snap freeze the dried pellets in liquid nitrogen and store them at -80°C.

C. Purification of mitoribosomes

In this part of the protocol, purification of the mitoribosome involves lysing the frozen mitochondria, making a sucrose cushion to recover the mitochondrial complexes, and then a sucrose gradient to separate the different high-molecular weight complexes (including different forms of the ribosome). Prepare stock buffer one day before, filter (0.2-µm), and store at 4°C; these can be kept for months. The buffers are finalized extemporaneously, by adding DTT and/or protease inhibitors, when relevant. Every step is done at 4°C.

1. Take 3 g of frozen mitochondria obtained from procedure B*. Fill each Eppendorf tube with 700 µL **Lysis buffer** and allow the frozen pellets to thaw on ice. Resuspend the pellets softly using a 1-mL automatic pipette until all aggregates are dissolved.
   *In procedure B, we have seen that it is possible to purify ~700 mg of mitochondria from 4 L of late log phase culture of parasites. For the purification of Mitoribosome, 3 g of dry weight mitochondria are required. So, before the mitoribosome purification, procedure B must be repeated several times to obtain the sufficient amount of mitochondria.
2. Pool the resuspended mitochondria solution into one 50-mL Falcon tube. Add 27 mL of **Lysis buffer**.
3. Transfer the resuspended mitochondria solution on the adequate number of 15-mL glass Dounce potters. Lyse the mitochondria using pestle A (which offers the less resistance to break the organelles). At least 10 strokes are necessary.
4. Incubate 15 min at 4°C.
5. Transfer to JA-25.50 tubes and centrifuge at 25,000 × *g* and 4°C for 20 min to clarify the lysate. Keep the supernatant (around 27 mL).
6. Gently load the mitochondrial lysate (supernatant of the previous step) on the top of three 40% sucrose cushions in 50.2Ti tubes. Each tube should contain 3 mL sucrose solution and 9 mL mitochondrial lysate corresponding to ~4 × 10^{10}–8 × 10^{10} cell-equivalents. To facilitate the observation of the pellet, the side of the tube that will be close to the outer part of the rotor should be identified.
7. Centrifuge the sucrose cushions loaded with the sample for 3 h at 244,000 × *g* at 4°C in an ultracentrifuge with a 50.2Ti rotor.
8. Carefully remove the supernatant. Start with a 10-mL serological pipette and end with a 200 µL pipette and suitable tips until the supernatant is completely eliminated. The pellets, which contain high-molecular weight mitochondrial complexes, should be clearly visible after removal of the supernatant.

9. Wash the pellets to eliminate the sucrose. For that, add 50 µL of **Monosome buffer** and let the liquid gently cover the pellet without resuspending it, then remove the buffer by pipetting.
10. Recover the pellets by adding 200 µL of **Monosome buffer** using a 200 µL automatic pipette*.
    *This step is delicate, one should not take the pellet directly but pipette softly the liquid up and down over the pellet without generating any bubbles.
11. Pool the resuspended pellets into a 1.5-mL Eppendorf tube; wash each 50.2Ti tube with 50 µL of **Monosome buffer** and add to the Eppendorf tube. Again with 200 µL automatic pipette, continue the resuspension by softly pipetting the solution up and down. Let the Eppendorf tube on a rotating wheel at 4°C at medium speed for 60 min to complete the resuspension of the complexes and until the continuous sucrose gradient is ready (see Recipes).
12. These pellets contain high-molecular weight mitochondrial complexes: ribosomes, dissociated ribosomal subunits, but also respiratory complexes, *etc*.
13. Quantify the mitochondrial complexes using a NanoDrop™ device*.
    *The ribosome is a ribonucleoprotein complex that absorbs at 260 nm by its RNA and at 280 nm by its proteins. So, by using BSA equivalent measurement, 2 peaks are visible, which confirms that the sample contains some mitoribosomes. The total protein quantity that can be expected is around 2 mg.
14. Prepare continuous 10–30% sucrose gradients (long gradients, short caps) in TH-641 tubes using the BioComp gradient master 108.
15. Gently layer the mitochondrial complexes solution (100 µL per gradient) onto two 10–30% sucrose gradients.
16. Centrifuge the gradients at 86,600 × *g* and 4°C for 14h 30min on swinging bucket rotor TH641.
17. Fractionate the gradients using the BioComp piston gradient fractionator device; 30 fractions of 350µl should be collected. Monitor the absorbance at 260 nm and 280 nm to select the ribosome-containing fractions. Usually, 3 major peaks are observed, corresponding (from lighter to heavier) to large subunits, small subunits, and monosomes (Figure 2). To confirm that the peaks contain mitoribosomal complexes, mass spectrometry analysis was performed. If several gradients were prepared, we pool all fractions corresponding to the same peaks. It is worth mentioning that in contrast to other known mitoribosome structures, the small ribosomal subunit in kinetoplastids is astonishingly larger, even larger than the "large ribosomal subunit", which explains why it is found at a slightly higher molecular weight in the sucrose gradient.

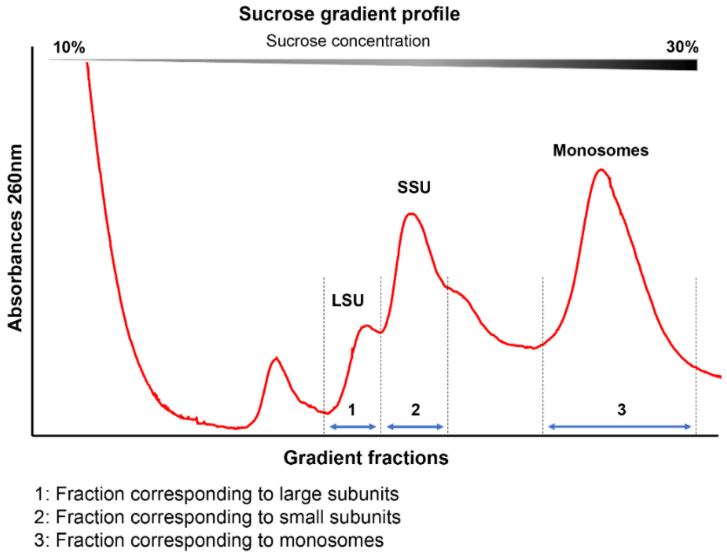

Figure 2. Sucrose gradient profile after mitochondrial complexes separation.
Mitoribosome complex profile on 10–30% sucrose gradient, showing 3 peaks. The lightest fraction contains the large subunits (LSU) (1), the heaviest fraction contains the monosomes (3), and between them are the small subunits (SSU) (2).

18. Pellet the ribosomes by centrifugation at 490,000 × *g* and 4°C for 2h 30min on a fixed angle rotor TLA110.3. Mark the tubes to remember their orientation.
19. Discard the supernatants; clear yellowish pellets should be visible at the bottom of the tubes. For Cryo-EM analysis, make sure to remove the totality of the supernatant, as sucrose will hamper the quality of the micrographs (this step is less crucial for proteomics or RNA analyses).
20. Resuspend the ribosome pellets in 30 µL of **Monosome buffer** and transfer to 1.5-mL Eppendorf tubes on ice. The pellets should be easy to resuspend. Again, it is crucial to avoid bubbles and minimize air/water interfaces.
21. Quantify the ribosomes using a NanoDrop™ device. The ideal concentration for cryo-EM is 1–2 µg/µL protein (using BSA equivalent measurement). The ideal 260/280 ratio is ~1.9, with a strong peak at 260 nm and a peak of half the height at 280 nm in the absorbance profile.
22. At this stage, cryo-EM grids should be directly prepared. Ribosomes at the desired concentration can also be aliquoted and flash-frozen in liquid nitrogen for further analysis (proteomics, RNA analysis, *etc.*). You can prepare cryo-EM grids from thawed ribosomes previously flash-frozen in nitrogen, but this will hamper the quality of your complexes.

D. Preparation of cryo-grids

1. At this stage, Quantifoil R2/2 300-mesh holey carbon grid, coated with thin home-made continuous carbon (thanks to vaporizing a thin layer of carbon of 2nm using the Safematic CCU-010 Carbon coater). Indeed, using this apparatus, carbon is evaporated on a mica sheet that is then plunged at 30° angle into a small box filled with demineralized water. The vaporized layer will separate from the mica sheet and float over the water surface. The Quantifoil grids are mounted below the surface that is then lowered by draining water from the box and the carbon layer will land on the top of plastic-free Quantifoil R2/2 grids.
2. Glow-discharge the grids using a glow discharged device at 3 mA for 20 s.
3. Prepare cryo-grids using a Vitrobot Mark IV device. The chamber parameters are: temperature 4°C and humidity 100%.
4. Use 4 µL of ribosome solution at 1 to 2 µg/µL concentration. Incubate the samples on the grid for 30 s and then automatically blot with a filter paper for 2 s at blot force 5, flash freeze in liquid ethane. Each sample should be at least prepared in duplicates.
5. Store grids in liquid nitrogen until data acquisition.

Recipes

Cells culture

1. LIT medium

3 g Liver infusion broth
5 g Bacto tryptose
4 g NaCl
8 g Na_2HPO_4
0.4 g KCl
1 g Glucose
Adjust at pH 7.2 with HCl
Adjust to 1 L with distilled H_2O
Before use add 10 mL Hemin solution at 1.25 mg/mL, 100 mL inactivated Fetal bovine serum and 10 mL Penicillin/streptomycin10,000 U/mL.

2. 1.25 mg/mL Hemin solution

Add 125 mg of Hemin into 100 mL of 20 mM NaOH, sterilize by autoclaving, and filter with 0.2 µm filter to remove aggregates.

3. BHI medium

Add 37 g of BHI powder into 1 L distilled H_2O and adjust at pH 7.4 with NaOH. Sterilize by autoclaving. Before use, mix 10 mL of Hemin solution at 1.25 mg/mL, 100 mL inactivated Fetal bovine serum and 5 mL Penicillin/streptomycin 10,000 U/mL.

Mitochondria purification

1. Phosphate buffer pH 7.9 0.2 M

Mix 93 mL of $Na_2HPO_4 \cdot 7H_2O$ 0.2 M with 7 mL of NaH_2PO_4 0.2 M.
Add distilled water until volume 200 mL.

2. Wash buffer

20 mM phosphate buffer pH 7.9
20 mM glucose
150 mM NaCl

3. SoTE buffer

600 mM sorbitol
20 mM Tris-HCl pH 7.5
1 mM EDTA

4. SoTE-EDTA Buffer

600 mM sorbitol
20 mM Tris-HCl pH 7.5
20 mM EDTA

5. SoTM buffer

600 mM sorbitol
20 mM Tris-HCl pH 8.0
5 mM $MgCl_2$

6. Discontinous Histodenz™ gradient for *L. Tarentolae*

Prepare 50% (w/v) Histodenz™ solution with SoTE buffer. Then dilute to have 10 mL of 21.7%; 25%; 28.3%; 31.6% Histodenz™ with SoTE buffer. Load 8 mL of each solution on 38-mL Ultra clear tubes.

7. Discontinous Histodenz™ gradient for *T. Cruzi*

Prepare 50% (w/v) Histodenz™ solution with SoTE buffer. Then dilute to have 10 mL of 28%; 25%; 21%; 18% Histodenz™ with SoTE buffer. Load 8 mL of each solution on 38-mL Ultra clear tubes.

Mitoribosome purification

1. HEPES-KOH pH 7.6 1 M

Prepare 150 mL of distilled H_2O in a suitable container.
Add 47.7 g of HEPES
Adjust solution to final desired pH using KOH.
Add distilled H_2O to reach a final volume of 200 mL.

2. Mitochondria Lysis buffer

20 mM HEPES-KOH Ph 7.6
100 mM KCl
20 mM $MgCl_2$
1 mM DTT

1% Triton X-100
2% W/V DDM
Complete EDTA-free protease inhibitor cocktail

3. Monosome Buffer

20 mM HEPES-KOH pH 7.6
100 mM KCl
20 mM $MgCl_2$
1 mM DTT
0.02% W/V DDM
Complete EDTA-free protease inhibitor cocktail

4. 40% Sucrose cushion

40% (w/v) sucrose
20 mM HEPES-KOH pH 7.6
100 mM KCl
20 mM $MgCl_2$
1 mM DTT
0.02% W/V DDM
Complete EDTA-free protease inhibitor cocktail

5. 10–30% Sucrose gradient

Prepare 10% sucrose solution
10% (w/v) sucrose
20 mM HEPES-KOH pH 7.6
100 mM KCl
20 mM $MgCl_2$
1 mM DTT
0.02% W/V DDM
Complete EDTA-free protease inhibitor cocktail

Prepare 30% sucrose solution
30% (w/v) sucrose
20 mM HEPES-KOH pH 7.6
100 mM KCl
20 mM $MgCl_2$
1 mM DTT
0.02% W/V DDM
Complete EDTA-free protease inhibitor cocktail
Mix using the Biocomp gradient master 108.

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Competing interests

The authors declare no competing interests.

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