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Purification and Cryo-electron Microscopy Analysis of Plant Mitochondrial Ribosomes

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[Abstract] Plants make up by far the largest part of biomass on Earth. They are the primary source of food and the basis of most drugs used for medicinal purposes. Similarly to all eukaryotes, plant cells also use mitochondria for energy production. Among mitochondrial gene expression processes, translation is the least understood; although, recent advances have revealed the specificities of its main component, the mitochondrial ribosome (mitoribosome). Here, we present a detailed protocol to extract highly pure cauliflower mitochondria by differential centrifugation for the purification of mitochondrial ribosomes using a sucrose gradient and the preparation of cryo-electron microscopy (cryo-EM) grids. Finally, the specific bioinformatics pipeline used for image acquisition, the processing steps, and the data analysis used for cryo-EM of the plant mitoribosome are described. This protocol will be used for further analysis of the critical steps of mitochondrial translation, such as its initiation and regulation.
Keywords: Plant mitochondria, Mitoribosome, Sucrose density gradient, Cryo-EM, Electron microscopy image processing

[Background] Mitochondria are essential organelles that act as both energy conversion powerhouses and metabolic hubs in eukaryotic cells. Mitochondria contain a genome and fully functional gene expression machinery. These gene expression complexes combine traits inherited from prokaryotic ancestors and specific features acquired during eukaryotic evolution (Gray, 2015; Waltz and Giegé, 2020). Mitochondrial research has huge societal implications given that numerous diseases are linked to mitochondrial dysfunctions in humans and many other eukaryotes (Nunnari and Suomalainen, 2012; De Silva *et al.*, 2015). For instance, in plants, male sterility resulting from recombined mitochondrial DNA is a trait widely used in agronomy (Chen and Liu, 2014). Among mitochondrial gene expression processes, translation is considered the most important in terms of regulation since it is clear that transcriptional processes are not limiting for mitochondrial gene expression. Still, the mechanistic details of translational regulation, and even the basic composition of the translational machinery, remain poorly understood for many eukaryotes. Recent functional, biochemical, and structural data have revealed an unexpected diversity of mitochondrial translation systems, in particular of their key players, the mitochondrial ribosomes (mitoribosomes) in diverse eukaryotes (Waltz and Giegé, 2020). In particular, the mitoribosomes of the land plant *Arabidopsis thaliana* and cauliflower were recently characterized at the biochemical and structural levels, revealing their unique and unexpected features such as the occurrence of novel domains and additional plant-specific ribosomal proteins (Waltz *et al.*, 2019, 2020a

and 2020b). This represents the first examples of mitoribosome characterization in photosynthetic organisms. These major advances have only been possible as a result of the recent advances in cryo-electron microscopy (cryo-EM) techniques, acknowledged by the Nobel Prize in Chemistry to J. Frank, J. Dubochet, and R. Henderson in 2017 (Cressey and Callaway, 2017). Cryo-EM is an electron microscopy technique that is used to investigate the structure of various types of biomolecules from single proteins to large complexes such as ribosomes. The technique relies on imaging isolated macromolecules and complexes that are embedded in vitreous water on a microscopy grid. To do so, the liquid sample containing the biomolecules is flash-frozen to cryogenic temperatures in liquid ethane. Thanks to this process, the complexes can conserve their native organization and structure. Near atomic resolution structures, and even atomic structures (Nakane *et al.*, 2020), of biological complexes, can be obtained through computational processing and averaging of multiple images, which ultimately allow 3D reconstruction of the complexes.

While ribosome purification and analysis by cryo-EM have been described elsewhere (Aibara *et al.*, 2018), the protocol described herein details the specific procedures required to prepare the individual plant mitoribosome large subunit (LSU) and small subunit (SSU) and to single out the intact 78S plant mitoribosome monosomes. In addition to the specific steps for the biochemical preparation of plant mitoribosome samples, the protocol also describes the specific bioinformatics pipeline used for the cryo-EM analysis of plant mitoribosome samples. Although significant progress has recently been made in this field, numerous questions remain completely unexplored, such as how the mitoribosome interacts with the mitochondrial membrane insertase systems, how translation initiation functions, and how translation is regulated. The protocol described herein will be instrumental in helping to solve these key questions regarding plant mitochondrial translation.

Materials and Reagents

Mitochondria purification

1. D-Mannitol (Sigma-Aldrich, catalog number: M4125)
2. Sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$) (Sigma-Aldrich, catalog number: 71515-250G)
3. BSA: Bovine serum albumin (EUROMEDEX, catalog number: 04-100-812-C)
4. Polyvinylpyrrolidone-25 (Sigma-Aldrich, catalog number: 81400)
5. β-Mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
6. EDTA (Sigma-Aldrich, catalog number: EDS)
7. L-Cysteine (Sigma-Aldrich, catalog number: W326305)
8. Phosphate buffer 1 M, pH 7.5
9. Miracloth (Milipore, catalog number: 475855)
10. Percoll® (Sigma-Aldrich, catalog number: P1644)
11. Pierce™ Coomassie (Bradford) Protein Assay Kit (Sigma-Aldrich, catalog number: 23200)
12. 3× Extraction buffer (see Recipes)
13. Wash buffer (see Recipes)

14. 28% Percoll® gradient (see Recipes)

Mitoribosome purification

1. 1.5-ml Eppendorf tubes
2. HEPES-KOH (EUROMEDEX, catalog number: 10-110-C)
3. KCl (Sigma-Aldrich, catalog number: P9541)
4. $MgCl_2$ (Sigma-Aldrich, catalog number: M8266)
5. DTT: DL-Dithiothreitol (Sigma-Aldrich, catalog number: D0632)
6. Triton X-100 (Sigma-Aldrich, catalog number: X100)
7. n-DDM: n-Dodecyl β-D-maltoside (Sigma-Aldrich, catalog number: D4641)
8. Sucrose (Sigma-Aldrich, catalog number: S7903)
9. Complete EDTA-free protease inhibitor cocktail (Sigma-Aldrich, catalog number: 11873580001)
10. Liquid ethane
11. Liquid nitrogen (N_2)
12. Lysis buffer (see Recipes)
13. 40% Sucrose cushion (see Recipes)
14. Resuspension buffer (see Recipes)
15. 10-30% Sucrose gradient (see Recipes)
16. Final resuspension buffer (see Recipes)
17. 10% n-DDM (see Recipes)
18. 50% Triton X-100 (see Recipes)

Equipment

Mitochondria purification

1. KIMBLE® Dounce tissue grinders, 15-ml and 2-ml (Sigma-Aldrich, catalog number: D8938)
2. Juice extractor, Moulinex Type 140.2.03 or equivalent
3. Centrifuge (Beckman Coulter, model: AVANTI-JE or equivalent), able to take JA-10 and JA-25.5 rotors
4. JA-25.5 fixed-angle rotor 8 × 50-ml Beckman Coulter or equivalent
5. JA-25.5 tubes: Round centrifuge tube polycarbonate, 50-ml (Nalgene, catalog number: 3117-0500)
6. JA-10 fixed-angle rotor 6 × 500-ml Beckman Coulter or equivalent
7. F500 bottles: Centrifuge bottle with sealing cap, PPCO, 500-ml (Nalgene, catalog number: 3141-0500)
8. Benchtop centrifuge (*e.g.*, Eppendorf, model: 5427R)
9. A round-type paintbrush (*e.g.*, Windsor & Newton watercolor sable n°10)

Mitoribosome purification

1. BioComp gradient master 108
2. BioComp piston gradient fractionator
3. Ultracentrifuge (Beckman Coulter, model: OPTIMA XE90, or equivalent), able to take 60Ti and SW41 rotors
4. Fixed-angle rotor 8 × 38.5-ml (Beckman Coulter, model: 60 Ti or equivalent)
5. 60 Ti tubes: 26.3-ml Polycarbonate bottle with cap assembly (Beckman Coulter, catalog number: 355618)
6. Swinging rotor 6 × 13-ml (Beckman Coulter, model: SW41 Ti or equivalent)
7. SW41 Ti tubes: 13.2-ml, Open-top thinwall ultra-clear tube (Beckman Coulter, catalog number: 344059)
8. Ultracentrifuge (Beckman Coulter, model: OPTIMA MAX XP or equivalent), able to take a TLA110 rotor
9. Fixed-angle rotor 8 × 5.1-ml (Beckman Coulter, model: TLA110 or equivalent)
10. A rotating wheel for 1.5-ml tubes

Mitoribosome cryo-grid preparation and data collection

1. Quantifoils R2/2 EM grids (Electron Microscopy Sciences, catalog number: Q210CR2)
2. Cryo-grid box storage
3. Safematic CCU-010 carbon coater or equivalent (Labtech-em, catalog number: 100001)
4. Glow discharge system from ELMO or equivalent
5. Thermo Fisher Vitrobot Mark IV or equivalent
6. Thermo Fisher Talos Artica 200kV or Titan Krios 300kV cryo-EM microscope or equivalent
7. Falcon III direct detector device or equivalent/superior (*e.g.*, Gatan Summit K2, K3, Falcon IV)

Software

1. RELION (MRC laboratory, https://www3.mrc-lmb.cam.ac.uk/relion/index.php/Main_Page)
2. CryoSPARC (Structura Biotechnology Inc, https://cryosparc.com/)
3. MotionCor2 (Shawn Zheng) (https://emcore.ucsf.edu/ucsf-software)
4. Gctf (Zhang, 2016; https://www2.mrc-lmb.cam.ac.uk/research/locally-developed-software/zhang-software/)
5. Resmap (http://resmap.sourceforge.net/)
6. Chimera (UCSF, https://www.cgl.ucsf.edu/chimera/)

Procedure

A. Extraction and purification of cauliflower mitochondria

Buffers are prepared the day before mitochondria purification and stored at 4°C. Percoll®-containing solutions must be inspected to ensure that no contamination has occurred. For solutions containing

BSA, L-cysteine, and β-mercaptoethanol, these components must be added on the day of mitochondria purification. Cauliflowers are bought fresh from the market and used within two days. Mitochondria purification should take half a day in total. The whole procedure is summarized in Figure 1A.

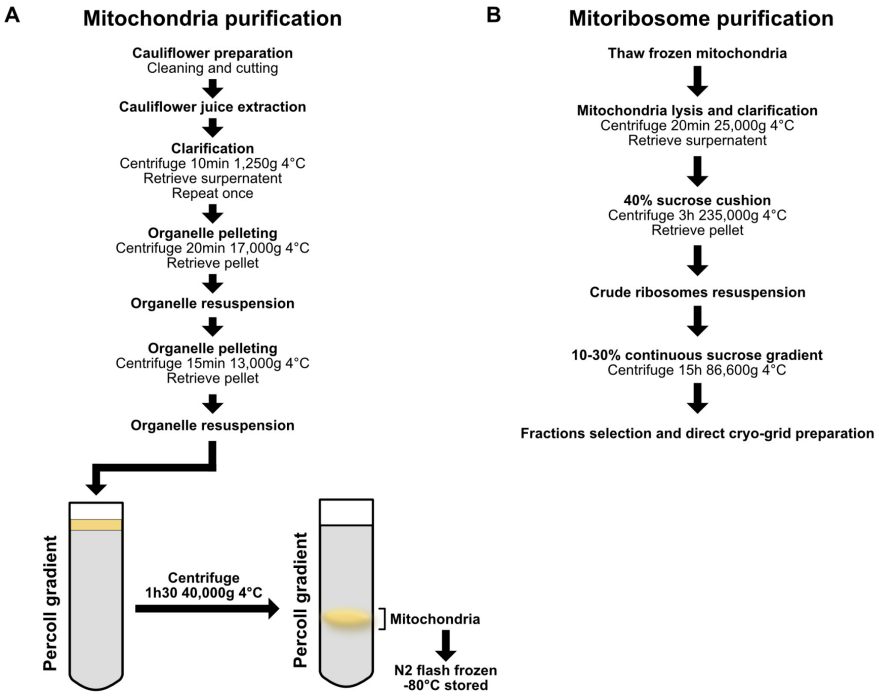

Figure 1. Overall workflow for mitochondria and mitoribosome purification. The overall workflow for mitochondria (A) and mitoribosome (B) purification is presented.

1. Take a 1-1.5 kg cauliflower. Using a knife, discard the leaves and the moldy spots (if any) to keep only the inflorescence.
2. Cut the cauliflower inflorescence into rough pieces of about 4 × 4 cm. The hard center piece of the cauliflower inflorescence can be discarded. The total remaining pieces should weigh 0.8-1 kg.
3. Prepare a 1-L beaker containing 300 ml 3× extraction buffer and place on a magnetic stirrer under slow rotation.
4. Place the pieces of cauliflower, one by one, in the juice extractor and retrieve the juice directly in the beaker containing 3× extraction buffer. The final total volume after complete extraction should be about 900 ml. If the total volume is below 900 ml, add distilled water until the desired volume is reached.
5. Make sure that the cauliflower juice is well homogenized with the rest of the extraction buffer.
6. Filter the solution through two layers of miracloth placed in a large funnel over another 1-L beaker used to retrieve the filtered solution.
7. Divide the solution into two F500 bottles and centrifuge at 1,250 × *g* for 10 min at 4°C.

8. Retain the supernatants and the repeat previous step. During these two steps, cell debris is removed.
9. Centrifuge the retained supernatants at 17,000 × *g* for 20 min at 4°C. During this step, the organelles are pelleted.
10. Discard the supernatants by inverting the bottles; the pellets will stick to the bottom of the containers.
11. Each bottle should contain a 4 × 2 cm slimy yellow/orange pellet.
12. Add 5 ml wash buffer to the bottles containing the organelle pellets. Do not pour wash buffer directly onto the pellets to avoid resuspending large chunks.
13. Gently resuspend the pellets using a round-type paintbrush. To do so, gently brush the pellets with the tip of the paintbrush while submerged in wash buffer, from the outer part of the pellet to the center to avoid resuspending large chunks. Once fully resuspended, wash the paintbrush with wash buffer to retrieve the remaining organelles.
14. Transfer the resuspended pellet to a 15-ml glass dounce potter. Rinse the bottle with 5 ml wash buffer and transfer to the Dounce potter. This step is performed individually for each pellet.
15. Using the Dounce potter and pestle A (which offers the least resistance to avoid disrupting the organelles), homogenize the resuspended pellet solution using 5 strokes.
16. Pool the resuspended and homogenized organelle pellet solutions and bring the total volume to 100 ml with wash buffer.
17. Separate the organelle solution into four JA-25.5 tubes and centrifuge at 13,000 × *g* for 15 min at 4°C (during the centrifugation, prepare for Step A22).
18. Carefully discard the supernatants by pipetting out.
19. Gently resuspend each pellet using a round-type paint brush in 1.5-2 ml wash buffer as in Step A13.
20. Transfer each pellet solution to a 2-ml glass Dounce potter by pipetting using a 1-ml pipette with the 1-ml tip cut at the end to give a larger opening. Homogenize the pellet solutions with 10 strokes using pestle A.
21. Rinse all JA-25.5 tubes with a total of 1 ml wash buffer, and pool the homogenized pellets together with the wash buffer used to rinse the tubes. The total volume should be about 18 ml.
22. Layer 2-3 ml homogenized organelle solution gently onto 30 ml 28% Percoll® gradient in JA-25.5 tubes. A total of 6-8 tubes are necessary depending on the final volume of resuspended organelle pellet obtained in Step A21.
23. Centrifuge the gradients at 40,000 × *g* for 1 h 30 min at 4°C without the brake to avoid gradient disturbance.
24. After this stage, mitochondria should appear in the gradients as a foggy yellow/white band at about 1/3 from the bottom of the tube (Figure 1A).
25. Discard the gradient solution on top of the mitochondria band by pipetting out until 0.5 cm gradient solution remains above the band.

26. Retrieve the mitochondria band using a 1-ml pipette with the 1-ml tip cut at the end to give a larger opening. A 5-ml pipette can also be used.
27. Pool all mitochondria together and dilute at least 6× with wash buffer.
28. Split the diluted mitochondria into 4-6 JA-25.5 tubes and centrifuge for 15 min at 18,000 × *g* at 4°C.
29. Carefully discard the supernatant, resuspend the mitochondria in the same volume as previously stated, and repeat the centrifugation step. The aim of this step is to remove most of the Percoll® from the mitochondria suspension.
30. Carefully discard the supernatant and resuspend each mitochondria pellet in 1 ml wash buffer. This constitutes purified mitochondria.
31. The final quantity of mitochondria can be evaluated by performing a Bradford assay. Usually, the final mitochondria solution obtained in Step A30 must be diluted at least 10× to perform the Bradford assay; otherwise, measurement saturation may occur, leading to an underappreciation of the actual quantity of purified mitochondria. A yield of 50 mg mitochondria can be expected from a 1 kg cauliflower.
32. Aliquot and pellet (16,000 × *g*, 5 min, 4°C) the mitochondria into 2-ml Eppendorf tubes, discard the supernatants, flash-freeze the mitochondria pellets in liquid nitrogen, and store at -80°C.

B. Mitoribosome purification

Prepare stock buffers the day before mitoribosome purification, filter (0.2-µm) and store at 4°C; these can be kept for months. The solutions that do not involve sucrose can be prepared the day before purification. DTT and protease inhibitors should be added immediately before use. For this procedure, at least 50 ml lysis buffer, 20 ml 40% sucrose cushion, and 25 ml 10% and 30% sucrose gradient solutions should be prepared. The overall mitoribosome purification procedure is similar to the classical ribosome purification and should be applicable to any type of mitochondria, although detergent and buffer composition will have to be optimized for the respective mitochondria samples. At all steps, be sure to work on ice.

1. From the end of Procedure A, mitochondria are dry pellets in 2-ml Eppendorf tubes. Take 40-50 mg frozen mitochondria. Fill the Eppendorf tubes with lysis buffer and allow the frozen pellets to thaw on ice. Every few minutes, resuspend the pellets using a 1-ml pipette with the 1-ml tip.
2. Once all the pellets are thawed and reasonably resuspended, pool the resuspended mitochondria solutions into one 50-ml Falcon tube. Dilute to a final concentration of 1-1.2 mg/ml; you should end up with 40-50 ml in total.
3. Transfer the resuspended mitochondria solution to a 15-ml (several steps are necessary) glass Dounce potter. Lyse the mitochondria using pestle B (which offers the most resistance in order to break the organelles). At least 10 strokes are necessary.
4. Transfer to JA-25.5 tubes and centrifuge for 20 min at 25,000 × *g* at 4°C. Retain the supernatant.
5. Gently layer the lysate onto 40% sucrose cushions in 60Ti tubes. Each tube should contain 3.5

ml sucrose solution and 9-11 ml mitochondria lysate. Mark the outside of the tubes, where the pellets should be after centrifugation.

6. Centrifuge the sucrose cushions for 3 h at 235,000 × *g* at 4°C in an ultracentrifuge with a 60Ti rotor or equivalent.
7. Carefully take the tubes out of the centrifuge. At this stage, clear round pellets could be observable at the bottom of the tubes. If not, they will be visible after the supernatant has been removed. These pellets contain high molecular weight mitochondrial complexes: ribosomes, dissociated subunits, but also respiratory complexes, *etc*.
8. Remove the supernatant from each tube using an autopipette. Remove the last few milliliters carefully using a 1-ml pipette with the 1-ml tip. Repeat using a 200-µl pipette with the 200-µl tip to ensure that all the supernatant has been removed.
9. Wash the pellets by adding 50 µl resuspension buffer and gently rolling the tubes to allow the liquid to wash over the pellets without actually resuspending them. The aim here is to remove the maximum remaining sucrose. Discard the buffer.
10. Resuspend the pellets by adding 100 µl resuspension buffer. Using a 200-µl or 100-µl pipette and the appropriate tip, resuspend the pellets by pipetting the liquid up and down. The pellets will be difficult to resuspend; try to avoid scratching the pellets with the tip of your pipette since the pellet will stick to the tip without resuspending. Moreover, try to avoid generating bubbles because the more the complexes are exposed to air, the more they tend to degrade, which will ultimately hamper the quality of the data.
11. Pool the resuspended pellets into a 1.5-ml Eppendorf tube; clean each 60Ti tube with 50 µl resuspension buffer and add to the Eppendorf tube. Using a 200-µl or 100-µl pipette and the appropriate tip, pipette the solution up and down to maximize resuspension. Place the Eppendorf tube on a rotating wheel at 4°C at medium speed for 30-60 min to allow for further resuspension of the complexes. During this step, prepare the continuous sucrose gradient for Step B13.
12. At this stage, you can perform a rough quantitation of the crude mitochondrial ribosomes using a NanoDrop™ device by measuring either the RNA or protein concentration (using BSA equivalent measurement). The absorbance profile should have two peaks, at 260 nm and 280 nm, indicating that the fraction indeed contains ribosomes. At this stage, you should have a total of 6,000-9,000 µg protein.
13. Prepare continuous 10-30% sucrose gradients (long gradients, short caps) in SW41 tubes using the BioComp gradient master 108.
14. Gently layer 3,000-6,000 µg crude ribosome solution (in 200-300 µl) onto the 10-30% sucrose gradients. Two gradients are usually required. If you have less than 4,000 µg crude ribosomes, do not split the solution into two; otherwise, this will lead to an excessive dilution of your complexes; try to load everything onto one gradient.
15. Centrifuge the gradients at 86,600 × *g* for 15 h at 4°C (with maximum acceleration and deceleration) overnight.

16. The next day, fractionate the gradients using the BioComp piston gradient fractionator device; 30 fractions of 350 µl 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 small subunits, large subunits, and monosomes (Figure 2). If several gradients were prepared, pool all fractions corresponding to the same peaks.

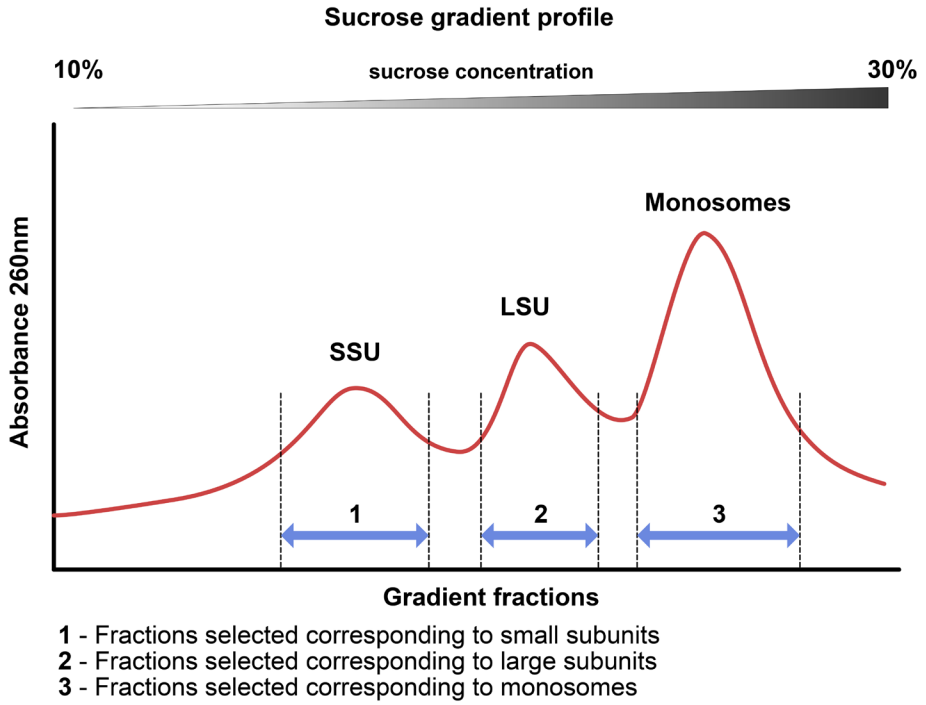

Figure 2. Representative sucrose gradient profile after crude mitoribosome separation. A representative profile resulting from mitoribosome separation on a 10-30% sucrose gradient is shown; absorbance at 260 nm is displayed. The composition of these fractions can be assessed by mass spectrometry analysis or direct cryo-EM inspection. SSUs are found in the lightest fraction and monosomes in the heaviest fraction. Contamination with other mitochondrial complexes can still occur, although usually limited to the lightest fractions, and cytosolic ribosomes (in the same fractions as monosomes) can also be found depending on mitochondria purity.

17. Pellet the ribosomes by centrifugation at 624,000 × *g* for 2 h at 4°C in an ultracentrifuge with a rotor such as TLA110. Mark the tubes to remember their orientation, as the pellets can be hard to see.
18. Discard the supernatants; clear yellowish pellets should be visible at the bottom of the tubes. Make sure to remove the totality of the supernatant, as sucrose will hamper the quality of the micrographs during cryo-EM analysis. However, for proteomics or RNA analysis, this is not crucial.

19. Resuspend the ribosome pellets in 30 µl final resuspension buffer and transfer to 1.5-ml Eppendorf tubes on ice. The pellets should be easy to resuspend. At first, the pellets should be resuspended in a small volume to avoid over-dilution of the ribosomes. Again, it is crucial to avoid bubbles and minimize air/water interfaces.
20. Quantitate the ribosomes as in Step B12 using a NanoDrop™ device. The ideal concentration for cryo-EM is 1-2 µg/µl protein (using BSA equivalent measurement). Here, the absorbance profile should be a strong peak at 260 nm and a peak of half the height at 280 nm. The 260/280 ratio should be about 1.9.
21. At this stage, cryo-EM grids should be directly prepared. You can also aliquot ribosomes at the desired concentration and flash-freeze them 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.

C. Cryo-grid preparation
1. At this stage, plastic-free Quantifoil R2/2 300-mesh holey carbon grids coated with thin homemade continuous carbon film (by vaporizing a thin layer of carbon (2 nm) using the Safematic CCU-010 carbon coater and floating it on top of the grids) are used.
2. Glow-discharge the grids using a glow discharge device at 3 mA for 20 s.
3. Liquify gaseous ethane in the Vitrobot Mark IV device cryo-receptacle using liquid nitrogen.
4. Prepare the cryo-grids using a Vitrobot Mark IV device. The chamber parameters are as follows: temperature 4°C and humidity 100%.
5. Apply 4 µl ribosome solution to the grids at a concentration of 1-2 µg/µl. Incubate the samples on the grid for 30 s, automatically blot with filter paper for 2 s at a blot force of 5, and flash-freeze in liquid ethane. Each sample should be prepared in at least duplicate.
6. Transfer the grids to liquid nitrogen and store until data acquisition.

Data analysis

Cryo-EM data processing will be different depending on the sample and the microscope used. An overview of the procedure is presented here, and a visual summary is also provided in Figure 3. Data collection is performed on a 200 or 300 kV cryo-EM instrument, such as a Talos Arctica or a Titan Krios instrument (Thermo Fisher). Depending on the sample concentration and intrinsic variability of the molecular complexes, a higher or lower number of micrographs should be collected. We recommend at least 4,000 micrographs at the magnification used here. Data are collected at a nominal underfocus of -0.5 to -2.7 µm with a 0.2 step. In our case, we collected at a magnification of 120,000×, yielding a pixel size of 1.21 Å, using a Falcon III direct electron detector (Thermo Fisher), but this will change depending on the microscope and camera type. Micrographs are recorded as movie stacks, and each movie stack is fractionated into 20 frames for a total exposure of 1 s corresponding to an electron dose of 60 ē/Å2. The general quality of the sample can be

assessed using on-the-fly processing provided by CryoSPARC Live (Punjani *et al.*, 2017).

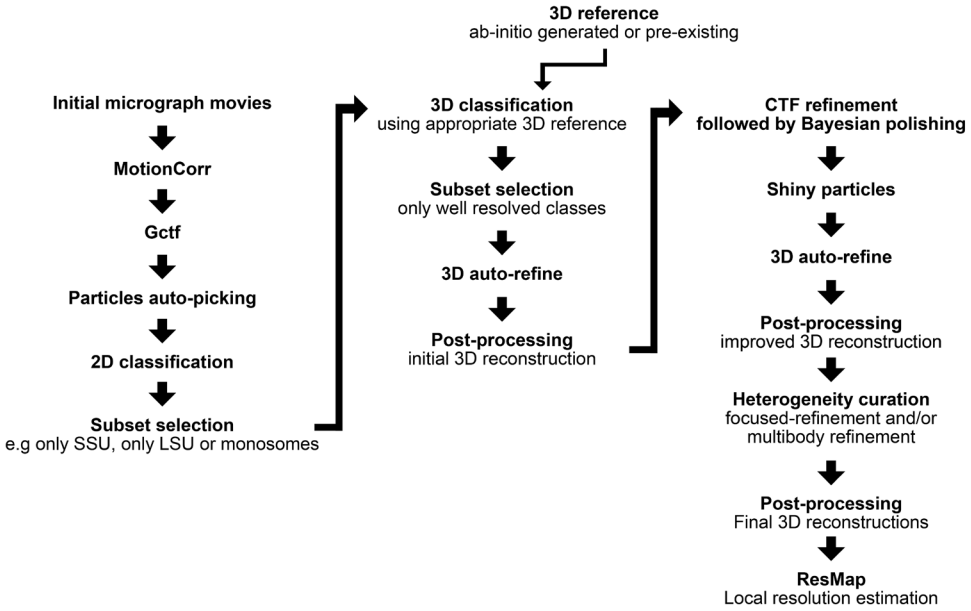

Figure 3. Cryo-EM data processing workflow. The overall workflow for cryo-EM data processing is presented.

The micrograph movies are transferred to a workstation capable of cryo-EM data processing. Using RELION (Nakane *et al.*, 2018; Zivanov *et al.*, 2018), micrographs are drift and gain corrected, and dose weighting is performed using MotionCor2 (Zheng *et al.*, 2017). Gctf (Zhang, 2016) is used to determine the contrast transfer function. Particles are then picked using a Laplacian of Gaussian function (min diameter 260 Å, max diameter 460 Å). The particles are first submitted to reference-free 2D classification, and all non-ribosomal particles are discarded. The more particles, the better, but high resolution can also be achieved with fewer particles depending on the complex. Thus, at the end of this step, we recommend having more than 100,000 particles. The resulting particles are extracted and 3D-classified (5-10 classes are necessary) using an appropriate 3D reference (either monosome or subunit reconstruction obtained from CryoSPARC or RELION). At this step, particles can be binned 2-4 times to speed up the 3D classification. The classes depicting high-resolution features (details) are selected for further processing. These particles are used for 3D-autorefine, and the resulting reconstruction is then post-processed to obtain the final reconstruction.

Further processing can then be performed to improve the resolution of the reconstruction. First, the RELION-integrated CTF Refinement and Bayesian polishing can be applied; this step improved the resolution of our reconstructions by 0.2-1 Å. Then, due to the intrinsic variability of ribosomes, focused refinement and/or multi-body refinement can be performed to improve the local resolution of the reconstructions. To do so, masks are created for the major parts of the ribosome: the LSU, the head of the SSU, and the body of the SSU. Masks are then used during focused refinement and/or multi-body refinement. In the case of the plant mitoribosome, both multi-body and focused

refinement approaches were used, with multi-body refinement yielding better resolution on the highly moving SSU head extension (Nakane *et al.*, 2018; Waltz *et al.*, 2020a). The resulting reconstructions can be visualized in Chimera or ChimeraX (Pettersen *et al.*, 2004; Goddard *et al.*, 2018). Local resolution of the reconstructions can then be estimated using ResMap (Kucukelbir *et al.*, 2014).

Recipes

Note: All buffers are kept cold at 4°C.

Mitochondria purification

1. 3× Extraction buffer
   0.9 M mannitol
   90 mM sodium pyrophosphate (10H_2O)
   6 mM EDTA
   2.4% (w/v) polyvinylpyrrolidone-25
   Adjust pH to 7.5 with 37% HCl
   0.8% (w/v) BSA (add at the end)
   0.15% (w/v) cysteine (add at the end)
   6 mM β-mercaptoethanol (add at the end)
2. Wash buffer
   0.3 M mannitol
   1 mM EDTA
   10 mM phosphate buffer, pH 7.5
3. 28% Percoll gradient
   0.3 M mannitol
   10 mM phosphate buffer, pH 7.5
   28% (v/v) Percoll

Mitoribosome purification

1. Lysis buffer
   20 mM HEPES-KOH, pH 7.6
   100 mM KCl
   30 mM $MgCl_2$
   1 mM DTT
   1.5% (v/v) Triton X-100
   0.3% n-DDM
   100 µg/µl chloramphenicol
   Complete

2. 40% Sucrose cushion
   20 mM HEPES-KOH, pH 7.6
   100 mM KCl
   30 mM $MgCl_2$
   1 mM DTT
   0.25% (v/v) Triton X-100
   0.02% n-DDM
   Complete
   40% (w/v) sucrose
3. Resuspension buffer
   20 mM HEPES-KOH, pH 7.6
   100 mM KCl
   30 mM $MgCl_2$
   1 mM DTT
   0.02% n-DDM
   Complete
4. 10-30% Sucrose gradient
   20 mM HEPES-KOH, pH 7.6
   100 mM KCl
   30 mM $MgCl_2$
   1 mM DTT
   0.01% n-DDM
   Complete
   10% or 30% (w/v) sucrose
5. Final resuspension buffer
   20 mM HEPES-KOH, pH 7.6
   100 mM KCl
   30 mM $MgCl_2$
   1 mM DTT
   0.01% n-DDM
   Complete
6. Complete 25×
   Dissolve 1 tablet complete protease inhibitors EDTA-free in 2 ml RNase-free water
   Aliquot and store at −20°C
7. 10% n-DDM
   Dissolve 10% (w/v) n-DDM in 5 ml RNase-free water
   Aliquot and store at −20°C
8. 50% Triton X-100

Dissolve Triton X-100 to 50% concentration (v/v) in 10 ml RNase-free water. For optimal mixing, heat to 45°C in a water bath.
Aliquot and store at −20°C

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Competing interests

The authors declare no competing interests.

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