Chapter 5

Sample Preparation of Isolated Mitochondria for Cryoelectron Tomography and In Situ Studies of Translation

Lena Thärichen, Robert Englmeier, and Friedrich Förster

Abstract

Cryoelectron tomography is a method to image biological samples three-dimensionally at molecular resolution. This modality provides insights into intracellular processes in their physiological settings. Obtaining a high-quality sample for cryoelectron tomography on mitochondria, however, can be challenging. In this chapter, we describe the crucial steps from sample preparation to data acquisition enabling studies of mitochondrial translation in situ by cryoelectron tomography. We provide detailed protocols for yeast and human mitochondria preparations yielding a high concentration of intact mitochondrial vesicles on cryo-EM grids. In addition, we describe a workflow for particle identification and spatial mapping in context of the organelle.

Key words Mitochondria, Cryoelectron tomography, Translation, Ribosome

1 Introduction

Cryoelectron tomography (cryo-ET) has extended the scope of structural biology studies from isolated molecules to molecules in context of their physiological organellar environment and even native cellular settings allowing to study their molecular sociology [1]. Cryo-ET provides 3D density volumes of an object of interest, typically referred to as tomograms. They are calculated from a set of 2D projection images of the sequentially rotated object, which is called a tilt series. These projections are acquired using a transmission electron microscope (TEM), which provides parallel projections of the sample along the electron beam to very good approximation [2].

Prior to imaging the sample is vitrified. The transition into a vitreous phase of matter is achieved by rapid cooling via plunge freezing into a suitable cryogen like liquid ethane [3]. Importantly, cryo-ET does not involve dehydration of the sample and does not

require any staining or fixation agents, enabling studies under near native aqueous conditions and avoiding preparation artifacts of EM methods relying on resin embedding. Cryo-ET relies on the contrast generated by intrinsic density differences of the sample, which is relatively weak and gives rise to a low signal-to-noise ratio (SNR) of the data [2]. A further limitation of cryo-ET is that sample must not exceed few hundred nanometers in thickness to remain transparent to the electron beam.

The potential of cryo-ET to retain the organelle environment during structural studies makes it attractive for mitochondrial research. Due to the requirement of relatively thin samples, cryo-ET has typically been applied to isolated mitochondria. Early studies revealed the ultrastructure of such isolated mitochondria with relatively modest TEM equipment [4]. High-voltage TEMs (typically operated at 200–300 kV) and advanced direct electron detectors allow insights into the organization of specific macromolecules. Due to the low SNR of tomograms, high-molecular-weight complexes are easier to distinguish in the data due to their larger volume. Thus, cryo-ET has primarily provided information on the intricate spatial organization of large mitochondrial complexes such as the ATP synthetase [5, 6].

Cryo-ET also enables a comprehensive understanding of mitochondrial translation with the large molecular weight mitochondrial ribosome (mitoribosome) as its centerpiece. Two routes are typically pursued to analyze specific particles depicted in tomograms. On the one hand, features of interest such as mitoribosomes can be automatically identified and traced, resulting in a segmentation, as exemplified by Pfeffer et al. and Englmeier et al. for mitochondrial polysomes *in situ* [7, 8]. On the other hand, particle volumes or subtomograms depicting the same type of macromolecule can be aligned in 3D and added up during subtomogram averaging [9]. This step significantly increases the electron density SNR, which manifests in higher-resolution reconstructions of the macromolecule of interest. Thus, subtomogram averaging of yeast and human mitoribosomes *in organello* revealed details of membrane tethering, protein insertion, and translation regulation [7, 8].

Efficient collection of high-quality data relies on a high-quality mitochondria sample. Mitochondria have been isolated from a variety of sources for decades and used in functional studies, examining respiration [10], mitochondrial translation [11], or mitochondrial import [12–14], respectively. However, these protocols include harsh centrifugation steps. For yeast mitochondria preparations, additionally involve extended biochemical treatment for cell wall digestion [15]. Cryo-EM grid preparation from these samples results in a relatively low number of structurally intact mitochondria per grid area, which inevitably leads to extended search time during data collection setup at the cost of expensive and often closely calculated electron microscope time. In this chapter, we

describe gentle and fast protocols for mitochondria preparations from human (HEK) and yeast (*S. cerevisiae*) cells for the purpose of cryo-ET. Furthermore, we complement the sample preparation protocols with a data processing workflow for membrane segmentation and particle mapping in tomograms.

2   Materials

2.1   Isolation of Mitochondria from HEK Suspension Cells

This protocol is focused on obtaining a highly enriched, intact mitochondria preparation for the purpose of cryo-ET and will contain other subcellular vesicle contaminations, such as endoplasmic reticulum (ER) vesicles. The underlying procedure is differential centrifugation for separation of different subcellular vesicles after cell lysis (Fig. 1a) [10]. The centrifugation speeds have been optimized for HEK suspension cells (Fig. 1b) and might need to be optimized for other cell lines (*see* **Note 1**). Since Cytochrome C resides in the intermembrane space, its enrichment in the pellet fraction indicates outer membrane intactness (Fig. 1b). For buffer preparations, use sterile filtered (ø 0.2 μm) Hepes and Sucrose stock solutions.
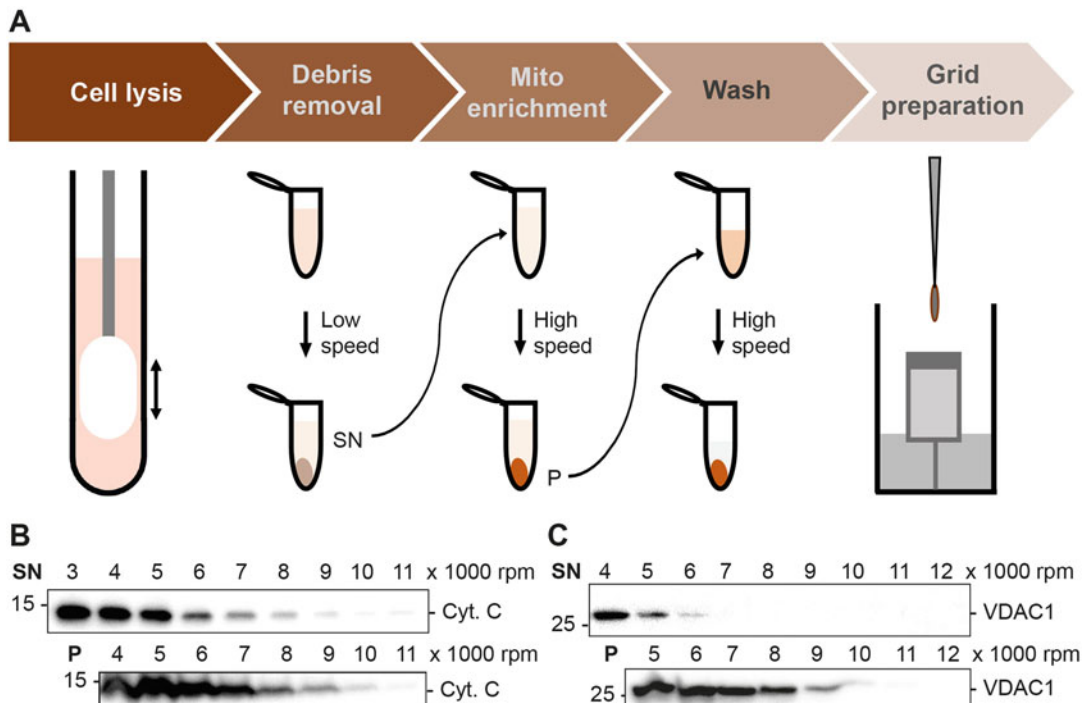

Fig. 1 Enrichment of intact mitochondrial vesicles by differential centrifugation. (**a**) Schematic of the sample preparation protocol ranging from cell lysis to grid preparation. (**b**) Western blot detection of the human mitochondrial marker Cytochrome C in supernatant (SN) and pellet (P) fractions obtained from cell lysates after sequential centrifugation at increasing speeds. (**c**) Western blot detection of the yeast mitochondrial marker VDAC1 in supernatant (SN) and pellet (P) fractions obtained from cell lysates after sequential centrifugation at increasing speeds

1. HEK suspension-adapted cells (e.g., FreeStyle™ 293-F Cells from Thermo Fisher) at 1–1.5 mio cells/mL grown in Free-Style™ 293 Expression Medium (Thermo Fisher) for >2 days.

2. Phosphate-buffered saline (PBS) w/o magnesium and calcium.

3. Glass dounce homogenizer with Teflon piston.

4. Homogenization buffer HEK: 20 mM Hepes pH 7.4, 250 mM Sucrose, 1 mM EGTA, 1 mM PMSF, 0.2% fatty acid-free BSA in ddH$_2$O.

5. Mitochondria buffer HEK: 20 mM Hepes pH 7.4, 250 mM Sucrose.

6. UV/Vis spectrophotometer for OD$_{280}$ measurement (Nanodrop™).

2.2 Isolation of Mitochondria from Yeast Cells

Like the protocol for mitochondria preparations from HEK cells (Fig. 1a), this protocol has been optimized for a high abundance of intact mitochondrial vesicles for cryo-ET and will still contain other subcellular vesicles. The protocol was based on Pfeffer et al. [7], and buffers, cell wall digestion, and centrifugation speeds have been optimized for *S. cerevisiae* spheroplasts (BY4742 [16]) (Fig. 1c). This yeast strain carries modifications to maximize mitochondrial biomass production and reduce loss of mitochondrial DNA that is observed in the wild type S288c background strain. For buffer preparations, use sterile filtered (ø 0.2 μm) buffer, salt, and Sorbitol stock solutions.

1. Yeast strain grown on a YPD agar plate for 3 days at 30 °C.

2. Semisynthetic (SS) medium: Dissolve 0.5 g glucose, 1.0 g H$_2$HPO$_4$, 1.05 g MgSO$_4$·7H$_2$O, 1.0 g NH$_4$Cl, 3.0 g yeast extract, 0.3 mL 1% w/v FeCl$_3$ in 800 mL ddH$_2$O. Add 22 mL 90% lactic acid and adjust the pH to 5.5 using KOH pellets. Then add 0.5 g CaCl$_2$, fill up to 1 L with ddH$_2$O and sterilize by autoclaving.

3. Water bath at 30 °C.

4. Wash buffer: 50 mM Hepes pH 7.4, 50 mM KOAc, 20 mM Mg(OAc)$_2$, 1 mM EGTA.

5. DTT buffer: 50 mM Hepes pH 8.0, 50 mM KOAc, 20 mM Mg(OAc)$_2$, 2 mM DTT.

6. Zymo buffer: 20 mM KH$_2$PO$_4$ pH 7.4, 1.2 M Sorbitol, 50 mM KOAc, 20 mM Mg(OAc)$_2$.

7. Zymolyase 20T (AMSBIO).

8. Glass dounce homogenizer with Teflon piston.

9. Homogenization buffer yeast: 20 mM Hepes pH 7.4, 600 mM Sorbitol, 50 mM KOAc, 20 mM Mg(OAc)$_2$, 1 mM PMSF, 1 mM EGTA.

10. 2 mL Eppendorf tubes.

11. Mitochondria buffer yeast: 20 mM Hepes pH 7.4, 600 mM Sorbitol, 50 mM KOAc, 20 mM Mg(OAc)$_2$.

12. UV/Vis spectrophotometer for OD$_{280}$ measurement (Nanodrop™).

2.3 Grid Preparation

To reduce sample viscosity and beam sensitivity while ensuring membrane intactness, mitochondria samples are diluted with the respective plunging buffer on the mounted grid immediately prior to blotting. Low concentrations of Sucrose or Sorbitol in the plunging buffer will ensure vesicle intactness. Manual one-sided blotting is used to reduce mechanical force on mitochondrial vesicles. BSA-coated gold fiducials are added for tilt series alignment during the tomogram reconstruction process [17].

1. Plunging buffer HEK: 120 mM Sucrose, 20 mM Hepes pH 7.4, 10 nm BSA-coated gold beads (e.g., UMC Utrecht diluted 1:30–1:40).

2. Plunging buffer yeast: 20 mM Hepes pH 7.4, 200 mM Sorbitol, 50 mM KOAc, 20 mM Mg(OAc)$_2$, 10 nm BSA-coated gold beads (e.g., UMC Utrecht diluted 1:30–1:40).

3. Protective goggles and gloves.

4. Manual plunger for single-sided blotting.

5. Ice-free liquid Nitrogen.

6. Pressurized ethane gas.

7. Grid storage boxes for grids.

8. Glow discharger.

9. Quantifoil® Cu 200 Mesh Holey Carbon R2/1 or Cu 200 Mesh Lacey Carbon grids.

10. Blotting filter paper Whatman® 595.

2.4 Image Analysis and Visualization Software

The workflow described here requires already reconstructed tomograms. The software packages required to perform the described segmentation protocol are the following:

1. ChimeraX 1.2.5 [18] for template generation, visualization, and segmentation clean-up.

2. Relion image handler [19] to prepare a template for template matching.

3. PyTOM v0.993 [20] for tomogram deconvolution (`deconv.py`), template matching, and motl file generation (`pytom_xml_to_motl.py`) (https://github.com/FridoF/PyTom).

4. EMAN 2.31 [21] for convolutional neural network (CNN) segmentation [22].

5. TOM toolbox [23].

6. MATLAB vR2018a (The MathWorks Inc.): `plot_particles.m` and `tom_classmask.m` (https://bitbucket.org/FridoF/av3/src/master/utils/).

3 Methods

3.1 Mitochondria Isolation Human

To isolate mitochondria from HEK cells, these need to be grown to logarithmic growth phase (*see* **Notes 2** and **3**). Lysis is performed mechanically while ensuring organelle intactness using a dounce-homogenizer. Gentle differential centrifugation ensures vesicle intactness during the enrichment step (Fig. 1). All buffers should be prepared and cooled prior to the lysis step and the centrifuge and other equipment should be cooled as well to ensure fast execution of the protocol (*see* **Note 4**).

1. Grow suspension-adapted HEK cells in FreeStyle™ medium to a density of approximately 1.5×10^6 cells/mL. 50 mio cells are sufficient for a small-scale purification and preparation of more than 10 grids.

2. Harvest the cells by centrifugation at $400 \times g$ for 5 min.

3. Discard the supernatant, resuspend the pellet in 25 mL ice-cold PBS per 50 mio cells, and repeat **step 2**.

4. Discard the supernatant, resuspend the pellet in 3.5 mL ice-cold homogenization buffer per 50 mio cells, and transfer the suspension to the precooled dounce homogenizer.

5. Lyse the cells by douncing three times with 30 strokes. Shortly cool the lysate on ice in between sets.

6. Transfer the lysate to 2 mL Eppendorf reaction tubes and centrifuge at $800 \times g$ (Heraeus Biofuge 13, 3000 rpm) for 5 min at 4 °C for removal of unbroken cells, nuclei and other cellular debris.

7. Transfer the supernatant into new 2 mL Eppendorf tubes and repeat the centrifugation as in **step 6**.

8. Transfer the supernatant into new 2 mL Eppendorf tubes and centrifuge at $3000 \times g$ (Heraeus Biofuge 13, 6000 rpm) for 5 min at 4 °C to pellet the mitochondria fraction.

9. Remove the supernatant and carefully resuspend each pellet in 1 mL ice-cold mitochondria buffer. Repeat the centrifugation as in **step 8** to wash.

10. Resuspend the mitochondria pellet carefully in ice-cold homogenization buffer to an OD_{280} of 1–1.4 for grid preparation.

3.2 Mitochondria Isolation Yeast

For isolation of yeast mitochondria, the same requirements apply as described under Subheading 3.1. Additionally, DTT buffer and Zymo buffer need to be prewarmed. DTT treatment and zymolyase digestion is kept as short as possible.

1. Streak out the yeast strain onto a YPD agar plate and grow it for 3 days at 30 °C.

2. Use these cells to inoculate 20 mL of SS medium in the morning with an inoculation loop and grow the culture at 30 °C and 160 rpm for 8–10 h.

3. In the evening, inoculate 1 L of SS medium with the preculture, aiming at an OD_{600} of 0.7–1.0 the next day.

4. Harvest the cells at room temperature (RT) and $4000 \times g$ for 10 min.

5. Discard the supernatant and resuspend the cell pellet in 50 mL RT wash buffer. Transfer into a preweighed 50 mL Eppendorf tube and centrifuge at RT, $4000 \times g$ for 5 min.

6. Discard the supernatant and weigh the cell pellet.

7. Per 1 g of cell pellet, add 6 mL prewarmed DTT buffer (at 30 °C), resuspend well and incubate at 30 °C for 5 min. Then harvest the cells as in **step 5**.

8. Per 1 g of cell pellet add 17 mg Zymolyase 20T dissolved in prewarmed (30 °C) Zymo buffer and resuspend well. Incubate at 30 °C for 40 min and mix the suspension by inverting the tube every 15 min. Then harvest the cells as in **step 5**.

9. Discard the supernatant, resuspend the cell pellet in 25 mL Zymo buffer without Zymolyase 20T and repeat the centrifugation as in **step 5**.

10. Resuspend the pellet in 2.3 mL ice-cold homogenization buffer per 1 g cell mass, transfer to the precooled dounce homogenizer and lyse the cells three times with 20 strokes. Shortly cool the lysate on ice between sets.

11. Transfer the lysate into 2 mL Eppendorf tubes and centrifuge for 5 min at 4 °C and $1200 \times g$ (Heraeus Biofuge 13, 4000 rpm). Repeat this step two more times for the respective supernatant sample.
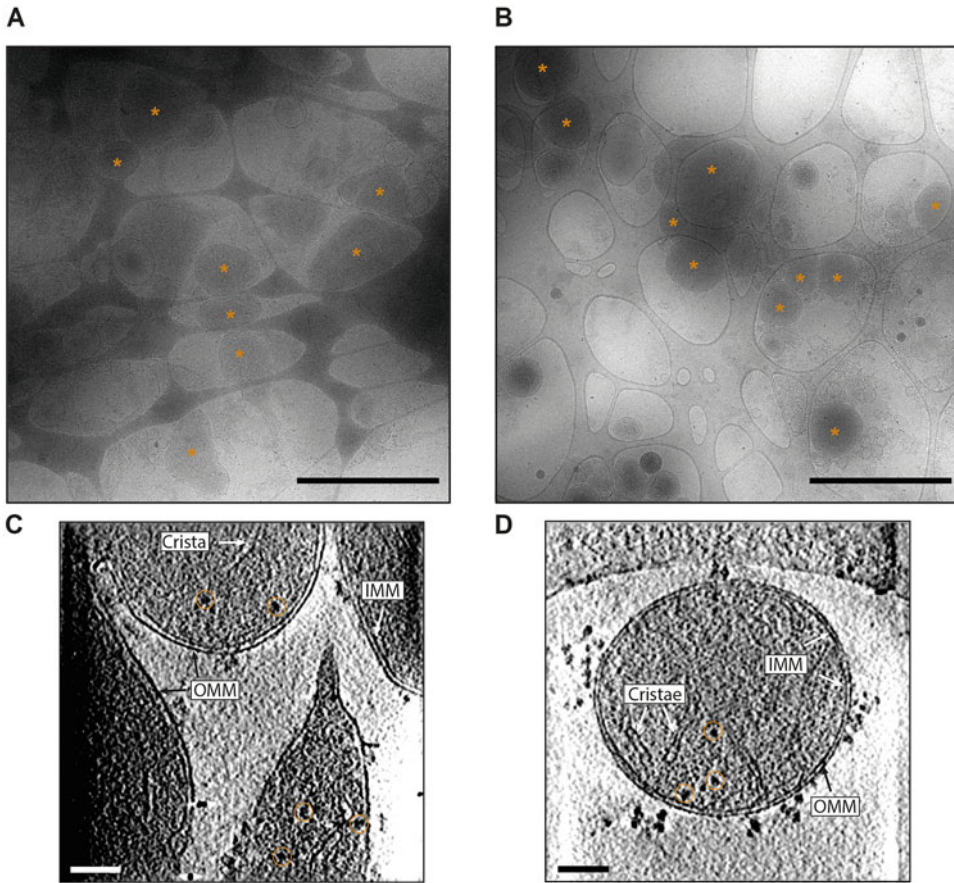
Fig. 2 Cryo-ET sample quality of isolated HEK and yeast cell mitochondria. Electron micrographs of mitochondria samples acquired on a 200 kV Talos Arctica electron microscope from (**a**) HEK cell or (**b**) yeast mitochondria preparations. Intact mitochondrial vesicles are indicated by asterisks and scale bars correspond to 2 μm. (**c**) Example tomogram of HEK cell mitochondria. Intact inner (IMM) and outer (OMM) mitochondrial membranes, Cristae and mitochondrial ribosomes are indicated by arrows or circles, respectively. (**d**) Example tomogram of a yeast mitochondrion with features indicated as in (**c**). Scale bars correspond to 100 nm

12. Pellet the mitochondria fraction for 5 min at 4 °C and 5300 × g (Heraeus Biofuge 13, 8000 rpm) and resuspend the pellet in mitochondria buffer to an OD_{280} of 2.5.

3.3 Grid Preparation   The described procedure yields a good distribution (Fig. 2a, b) of largely intact mitochondria (Fig. 2c, d) on grids suitable for efficient cryoelectron tomography data acquisition.

1. Prepare the grid plunger with liquid nitrogen and ethane using goggles and gloves for protection.

2. Glow discharge the grids for 30 s at 20 mA and 0.39 mBar for Holey carbon grids and 25 s at 15 mA and 0.39 mBar for Lacey carbon grids (*see* **Note 5**).

3. Insert a glow discharged grid into the plunging tweezers and add 2 μL mitochondria sample onto the carbon-coated side of the grid. Then insert the tweezer into the plunging device.

4. Add 4 μL gold fiducial buffer (HEK or yeast, respectively) and carefully mix with the sample on the grid (*see* **Note 6**).

5. Immediately blot away excess sample from the back side of the grid using blotting paper and plunge the grid into liquid ethane (*see* **Note 7**).

6. Store grids in grid boxes in liquid nitrogen.

3.4 Tomogram Segmentation

The following protocol describes all necessary steps to segment mitochondrial membranes using the CNN in EMAN2 and to identify molecules of interest by template matching using pyTOM, as well as plotting them back into the tomographic volume using the TOM toolbox and MATLAB. As mentioned in Subheading 2.4, a reconstructed tomogram is required. Furthermore, we recommend working with 6–8 times down-sampled data (pixel size corresponding to approximately 15 Å). An example for a yeast mitochondrion with visualized inner and outer membranes and cytosolic, as well as mitochondrial ribosomes is shown in Fig. 3c.
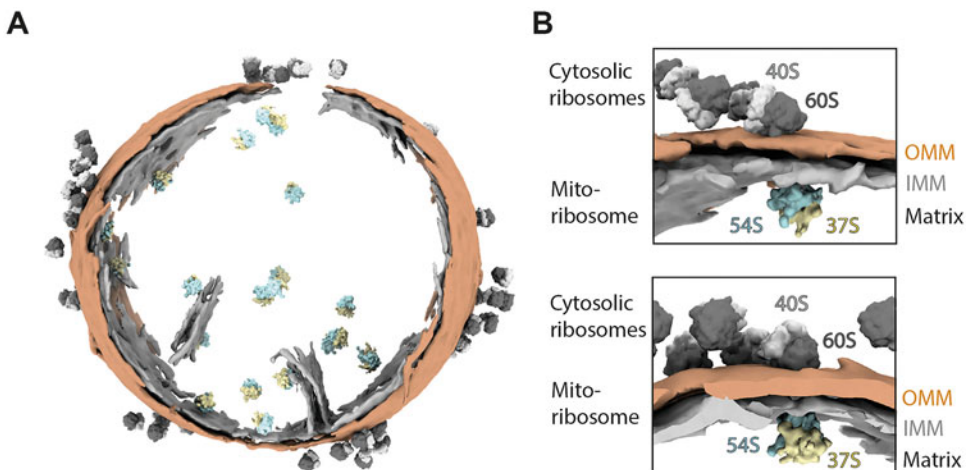

Fig. 3 Visualization of membranes and mitoribosomes in cryoelectron tomograms. (**a**) Tomogram segmentation of a yeast mitochondrion, visualizing cytosolic (40S in light, 60S in dark gray) and mitochondrial (37S in yellow, 54S in blue) ribosomes in the context of the mitochondrial membrane environment (OMM in orange, IMM in gray). (**b**) Cytosolic ribosome clusters coincide with mitochondrial ribosome positions on the IMM

The described segmentation workflow is intended to quickly segment apparent features, such as the outer mitochondrial membrane. For features in dense environments or thick parts of a sample, such as cristae in the central mitochondrial regions, additional manual segmentation in, e.g., Amira (Thermo Fisher) is likely necessary.

1. Make sure the tomogram is compatible with EMAN2 (*see* **Note 8**).

2. In a Linux terminal, load pyTOM 0.993 to execute deconv. py according to the described usage (deconv.py --help). The parameters provided below are an example and should be optimized for each dataset.

```
deconv.py -f <tomogram.mrc> -o <tomogram_deconv.
mrc> -s <pixelsize> -z <defocus (positive value in
m)> --snrfalloff <1.5> --deconvstrength <0.5> --
phaseflipped (in case phase-flipping was used for
CTF correction).
```

3. Load EMAN2 and execute e2projectmanager.py in a terminal. To prevent automatic pixel addition to the tomographic volume (due to --clip option applied during import via EMAN2 Project Manager), create a folder called "tomograms" in the EMAN2 project folder (*see* **Note 9**).

4. Import tomograms into that folder manually as follows:

```
e2proc3d.py <tomogram_deconv.mrc> <tomograms/to-
mogram_deconv.hdf> --process normalize
```

5. Go back to the EMAN2 Project Manager and follow the tutorial as described on the EMAN2 homepage (https://blake. bcm.edu/emanwiki/EMAN2/Programs/tomoseg, accessed 03/03/2022). Segment inner and outer membranes separately.

6. Create a segmentation file using the Segger tool [24] in ChimeraX: Open the segmentation file obtained from EMAN2 and go to *Tools > Volume Data > Segment Map*. Under "*Segmentation Options*" in the *Segger* window "*Display at most 300 regions*" and select "*Group by smoothing 6.*" Click "*Segment*" at the bottom of *Segger* window.

7. Select all regions of interest by right clicking while pressing *Ctrl + Shift*, then group the regions via "*Group*" at the bottom of the *Segger* window.

8. Under Shortcuts Options "*Invert*" the selection, then "*Delete*" all unwanted regions and select the remaining region of interest by *right mouse clicking + Ctrl*.

9. Save the segmentation as an mrc file (*File > Save selected regions to .mrc file . . .*).

10. In the command line resample the segmentation onto the original volume dimensions using "`vop resample #<ID_segmentation> onGrid #<ID_CNN_hdf_file>`" and save the resampled map using "`save <filename.mrc> model #<ID_segmentation_resampled.mrc>`."

11. Clean up the segmentation in ChimeraX: Hide small volumes using the Hide Dust Tool under *Tools > Volume Data > Hide Dust.* Then open *Tools > Volume Data > Map Eraser.* Remove noise or falsely segmented features from the map and save it as a new file.

3.4.2 Mapping Particles in the Segmentation

1. Prepare templates for template matching using the Relion image handler as follows:

```
relion_image_handler --i <EMDBfile.mrc> --o <EMDB-file_temp.mrc> --angpix <Input pixel size(é)> --lowpass <resolution (é)> --rescale_angpix <Desired pixel size(é)>
```

The pixel size needs to match the tomogram used for segmentation.

2. Open the templates in Chimera and, if necessary, scale the density to match the tomogram density values using "`vop scale #<IDEMDBfile_temp.mrc> factor -1.`"

3. Generate a spherical mask around the template in pyTOM (`gen_mask.py`) with the same box size as the template. To adjust the mask position with respect to the template, open both files in in Chimera, center them on each other and use the "`vop resample`" command (*see* Subheading 3.4.1, **step 10**).

4. Perform template matching and extract particles to an .xml file using pyTOM (https://github.com/FridoF/PyTom/wiki).

5. Clean up the particle lists via the pyTOM GUI (`pytomGUI.py`): Create a new project folder and under "*Enable Stage*" select "*Particle Picking.*" In the "*Manual Picking*" tab, open the tomogram and click "*Pick.*" Apply a Gaussian filter to the tomogram, select a suitable particle size, and then load the particle list extracted in **step 4**. Use the left and right keyboard arrows to navigate and right click on particles to remove them. Then save the particle list as a new file.

6. Prepare motl files from the pyTOM particle lists as follows with pyTOM:

```
pytom_xml_to_motl.py <input file name>
```

7. In MATLAB add the path for the TOM toolbox (`addpath (genpath(<path>'));`), then open and execute `tom_classmask.m`. Set paths or add to path if required.

8. Open `plot_particles.m` for plotting molecule references to their positions in a tomogram, enter motl file name, the template file name, the tomogram dimensions and run the script for each molecule type to be displayed.

9. Visualize the segmentation, as well as the plotted particles in ChimeraX.

4   Notes

1. We recommend optimizing the sedimentation settings for differential centrifugation when applying the protocols to other cell lines, yeast strains or other sources of mitochondria.

2. Culture cells for at least 2 days after thawing and do not freeze cells before mitochondria isolation. The freezing process significantly reduces the yield of intact mitochondrial vesicles.

3. Only harvest cells in logarithmic growth phase; otherwise, the yield of mitochondrial vesicles is very low.

4. Working at 4 °C and fast preparation is crucial to yield structurally and functionally intact mitochondrial vesicles.

5. The use of Lacey carbon grids improves the vesicle distribution compared to Holey carbon grids due to a smaller carbon surface. However, holey carbon grids provide more stability during data acquisition.

6. The ratio of mitochondria sample to fiducial buffer can be varied to sample different mitochondria, sucrose/sorbitol or gold fiducial concentrations.

7. Since the sample thickness is one main limiting factor for high-resolution subtomogram averaging, but mitochondrial vesicles are up to 500 nm in diameter, a balance needs to be found between vesicle intactness and sample thickness. Different sample thickness values can be sampled by varying the blotting parameters or by choosing acquisition points depending on ice thickness or vesicle size, respectively.

8. When using tomograms reconstructed with Etomo in IMOD, make sure to untick the "*convert to bytes*" option in both "*Coarse Alignment*" and "*Post-processing*" prior to reconstruction.

9. Coordinate systems can be defined differently in different software packages and need to be considered for combining, e.g., membrane segmentations and back-mapped particles. Especially after a segmentation clean-up in Chimera, the original

tomogram dimensions (box size) are not saved automatically and need to be manually applied prior to saving. Otherwise, Chimera automatically sets the volume dimensions depending on the cleaned segmentation, which might have a smaller box size compared to the initial tomogram.

Acknowledgments

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