

In situ studies of mitochondrial translation by cryoelectron tomography

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Invitation for a chapter in **“Mitochondrial Gene Expression”**

Abstract

Cryoelectron tomography (cryo-ET) enables the three-dimensional (3D) visualization of macromolecular complexes in their native environment (*in situ*). The ability to visualize macromolecules *in situ* is in particular advantageous for complex, membrane-associated processes, such as mitochondrial translation. Mitochondrial translation occurs almost exclusively associated with the inner mitochondrial membrane, giving rise to the mitochondrial DNA-encoded subunits of oxidative phosphorylation machinery. In cryo-ET, the 3D volume is reconstructed from a set of 2D projections of a frozen-hydrated specimen, which is sequentially tilted and imaged at different angles in a transmission electron microscope. In combination with subtomogram analysis, cryo-ET enables the structure determination of macromolecular complexes and their 3D organization. In this chapter, we summarize all steps required for structural characterization of mitochondrial ribosomes *in situ*, ranging from data-acquisition to tomogram reconstruction and subtomogram analysis.

1 Introduction

Cryo-electron tomography (cryo-ET) enables the three-dimensional (3D) visualization of frozen-hydrated biological specimens in a close-to-native state at molecular resolution [1]. In order to obtain a 3D image, the sample is sequentially tilted in the transmission electron microscope (TEM) and projection images are acquired, resulting in a tilt series. In contrast to classical electron microscopy (EM) of dehydrated, heavy metal stained and plastic-embedded specimens, vitrification by rapid cooling (cryo-preparation) preserves the molecular structure of the specimen and enables the detailed analysis of macromolecular complexes [2].

While cryo-EM single particle analysis (SPA) allows for the structure determination of macromolecular complexes at atomic resolution, this method requires purification of the respective molecules, thus isolating them from their native context. In contrast, cryo-ET can be applied to isolated organelles or even whole cells, enabling the visualization of macromolecules *in situ*. Therefore, this imaging modality is suitable for the structural analysis of membrane-associated processes. Mitochondrial translation belongs to this category: mitochondrial mRNA is translated by the membrane-bound mitochondrial ribosome (mitoribosome), and the product is typically co-translationally inserted into the inner mitochondrial membrane (IMM) [3].

Application of cryo-ET to isolated mitochondria can visualize mitoribosomes *in organello*. Due to the beam sensitivity of vitrified biological samples tomograms are acquired with low doses (typically $< 100 \text{ e}^-/\text{\AA}^2$). The resulting low signal-to-noise ratio (SNR) limits the resolution of the tomograms to approximately 5 nm [4]. Key to quantitative analysis of mitoribosomes in noisy tomograms is a process called template matching, which can localize and identify copies of the target macromolecule based on its known structural signature. By aligning and averaging multiple copies of the macromolecule, i.e. subtomogram averaging, the SNR improves, allowing subnanometer and even atomic resolution in selected cases [5] [6]. Classification allows for disentangling different conformational or compositional states, e.g. separating fully-assembled mitoribosomes from smaller species.

In this this method chapter we cover cryo-ET sample preparation, data acquisition and processing in the context of mitochondrial translation. For data processing we also describe the software packages TOM [7] and PyTom [8].

2 Materials

2.1 Isolation of mitochondrial vesicles

The precise purification protocol will ultimately depend on the biological question, which may require different levels of enrichment or ultrastructural preservation. We achieved a fairly pure sample of yeast mitochondria with a previously described protocol [9]. For human mitochondria, we previously used a protocol [10] adapted from Frezza *et al.* [11]. Both protocols employ differential centrifugation, and show sufficient enrichment for cryo-ET analysis despite notable contamination by multivesicular vesicles and ER. Here, the protocol that we employed for the purification of mitochondria from Freestyle™ 293-F cells grown in suspension is described [10].

1. Freestyle™ 293-F cells grown in Freestyle medium.
2. PBS buffer: Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄ and 0.2 g of KH₂PO₄ in 800 ml of H₂O. Adjust pH to 7.4 with HCl and fill up to 1 liter with H₂O.
3. Isolation buffer: 10 mM Tris, 150 mM sucrose, 1 mM EGTA, pH 7.4. Prepare 200 mM Tris stock solution by resolving 2.64 g of Trizma HCl and 0.38 g of Trizma Base in 100 ml. Prepare 0.5 mM EGTA stock solution by resolving 1.9 g EGTA in 8 ml. Adjust pH to 8.0 with KOH and fill up to 10ml. Prepare 2 M Sucrose stock solution by resolving 18g in 35ml H₂O. Fill up to 50 with H₂O. Pipette 5ml of the 200 mM Tris stock solution with 100 µl of 0.5 M EGTA solution, 3.85 ml of the 2 M sucrose solution and 41.15 ml of H₂O
4. Glass-Teflon homogenizer.

2.2 Preparation of cryoEM grids

1. Mechanical or automated grid-plunger (Vitrobot™, Thermo Fisher Scientific) including all the required accessories, i.e. container with separate cup for liquid nitrogen and cryogen and a tweezer-mount with appropriate tweezers.
2. Ethane or Ethane-Propane mixture as a cryogen.
3. Ice-free liquid nitrogen.
4. Holey or lacey carbon coated grids (Quantifoil).
5. Glow discharger (e.g. Pelco Easiglow).
6. Filter paper (Whatman, Grade 595).

7. 10 nm BSA-coated gold beads (e.g., UMC Utrecht). Dilute them at the half of the recommended value (usually 1:40 to 1:50) in 20 mM Hepes pH 7.4 buffer to achieve a 2x gold buffer for on-grid dilution.
8. EM grid storage boxes (e.g., Ted Pella).

2.3 *Electron microscopy*

The crowded environment of the mitochondrial matrix as well as the thickness of mitochondria both result in low contrast in tomograms and make the identification of mitoribosomes difficult. The minimum requirement to obtain interpretable data of the interior of isolated mitochondria is a 200 kV cryo-TEM, equipped with a field-emission gun, energy-filter and direct electron detector. With this equipment, the vitrified mitochondria should not exceed 200 nm thickness along the normal of the EM grid. A 300 kV TEM in combination with a phase plate maximizes penetration depth of electrons and hence the resulting contrast.

2.4 *Software*

The following software is required to perform the protocols described in this method chapter:

- Current version of SerialEM [12] for tilt series acquisition (here we use version 3.6) (<http://bio3d.colorado.edu/SerialEM/>).
- motioncor2 [13], for alignment of dose-fractionated movies.
- TOM and AV3 toolbox [5], [7]; for localization of gold markers required for tilt series alignment (<https://bitbucket.org/FridoF/av3>).
- Matlab R2014a or earlier; to run the TOM/ AV3 toolbox. Add TOM and av3 directories to the Matlab search path, i.e. by the “Set path” button or the ‘addpath’ command.
- PyTOM v0.971 [8]; for tomogram reconstruction, template matching, subtomogram alignment and classification (<http://pytom.org>).
- UCSF Chimera 1.10 or higher [14]; for data visualization.
- “Chooser” plugin for Chimera; available on the PyTom website [15].

Regarding the processing of tomographic data, the TOM/AV3 toolbox is only required for the gold marker clicking. It includes all required functions for the processing of 2D and 3D images (e.g. filtering, downscaling, cropping, reconstruction of tomograms and averaging of subtomograms) and enables the integration of standard Matlab functions and external Matlab packages for data analysis. Template matching, subtomogram alignment and classification are implemented in PyTOM, which provides more functionalities for subtomogram processing, higher interpolation accuracy and higher computational speed. We will therefore focus on the application of PyTom and mention the usage of TOM/ AV3 functions where required.

3 Methods

3.1 Purification of crude mitochondrial vesicle from human suspension cells

1. Grow the cells to a cell density of approximately 1×10^6 cells/ ml.
2. Collect the cells by centrifugation for 5 minutes at 360 g and 4 °C. All subsequent steps should be carried out at 4 °C.
3. Wash the cells twice by resuspending the cell pellet in 1x PBS buffer and repeating the centrifugation from step 2.
4. Resuspend the cell pellet in homogenization buffer (5 ml/g cell pellet).
5. Transfer the cell suspension to a Glass-Teflon Potter and homogenize it by 120 up- and downstrokes (see **Note 1**).
6. Clear the lysate for 10 minutes at 800 g.
7. Transfer the supernatant to a new tube and centrifuge for 10 minutes at 9,000 g.
8. Resuspend the mitochondrial pellet in 1ml/ g homogenization buffer and centrifuge it again at 9,000 g for 10 min.
9. Resuspend the mitochondrial pellet to a concentration of 10 mg/ml in homogenization buffer. Store it at -80 ° upon flash-freezing in liquid nitrogen or use it directly for grid preparation by diluting it to 1.5 mg/ ml (see **Note 2**).

3.2 CryoEM grid preparation of mitochondrial vesicles

To analyze purified biological sample in the cryo-TEM, the sample needs to be applied to a specimen carrier, the EM grid, and vitrified by plunge-freezing into a cryogen (ethane or ethane-propane). The EM grid is a thin, circular (\varnothing 3 mm) specimen container with a regular grid-mesh (e.g. copper or molybdenum), and a support film (usually carbon) to accommodate the sample. Due to the hydrophobicity of carbon, EM grids are generally made hydrophilic by a plasma treatment in a glow-discharger just prior to grid preparation. Due to the relatively short inelastic mean free path of electrons in vitreous ice (~400 nm at 300 kV [16], ~250 nm at 120 kV [17]), the EM grid is blotted after applying the sample and just prior to plunge-freezing resulting in a thin the water-film containing the sample (~200 nm).

1. Glow discharge an adequate amount of Quantifoil holey or lacey carbon coated grids.
2. Cool the container down with liquid nitrogen.
3. Make sure that the container is dry and condense the cryogen in the cooled container (see **Note 3**).
4. Pick up the glow discharged grid with the tweezers and mount it on the manual or automated grid-plunger.
5. Pipette 3 μL of your mitochondrial sample (1.5 mg/ml) on to the EM grid, followed by 3 μL of the 2x gold buffer (see **Note 4**).
6. Blot the EM grid, either one-sided with a manual plunger or from both sides with an automated plunger.
7. Plunge the grid into the cryogen.
8. Transfer the grid into a grid storage box, repeat for the desired number of grids and store them in liquid nitrogen.

3.3 *Data acquisition with SerialEM*

SerialEM is an open-source, academic software, which includes all functionalities required for cryo-ET data acquisition [12]. In addition to a low-dose mode that allows the setup of five different imaging modes and the exposure of separate areas to minimize radiation damage during acquisition, it has an implemented mapping functionality (“Navigator”), supports the execution of scripts, and provides control over all the necessary detector and energy filter parameters. We will briefly cover the data acquisition with SerialEM, but the considerations of imaging parameters apply to any cryo-ET data acquisition irrespective of the software. Before starting, make sure to optically align the TEM and perform all essential calibrations in SerialEM (e.g. pixel size on specimen level, image-, beam-, stage-shift, beam-tilt, etc.; see Serial EM home page [18] for detailed instructions on installation and calibration). Here we will focus on using the Navigator to acquire grid montages and multiple tilt series. For additional details concerning the operation of SerialEM, the reader is referred to the SerialEM documentation.

3.3.1 **Fast and systematic: Using the Navigator and montage-maps in SerialEM**

The initial parameters that need to be defined are the magnifications at which the sample will be screened (View and Search modes), as well as the magnification at which tilt series will be

recorded (Record, Focus and Trial modes). The lateral size of mitochondrial vesicles ranges from 300 nm to 800 nm (depending on the isolation conditions). The field of view (FOV) in Record mode should cover that area. Once the settings are defined, it is useful to map the entire EM grid to obtain a low magnification overview that will aid targeting regions of appropriate ice thickness. SerialEM provides the option to acquire montage maps (i.e. stitched images), at an intermediate magnification that minimizes the electron dose applied to the sample and can be used to interactively select features and move to them from the Navigator window. This function expedites the screening process and also helps to document samples, facilitating the identification of mitochondria within heterogenous samples.

1. Set up the magnification of the five modes (View, Search, Focus, Trial, Record) to cover the useful ranges (the values in the brackets correspond to the approximate pixel sizes of the K2 Summit Direct Electron Detector): View: $\sim 5 \mu\text{m}$ FOV ($\sim 1.5 \text{ nm/pixel}$), Search: $\sim 500 \mu\text{m}$ FOV ($\sim 120 \text{ nm/pixel}$), Record/ Focus/ Trial: $\sim 700 \text{ nm}$ FOV ($\sim 0.22 \text{ nm/pixel}$) (see **Note 5**).
2. Adjust the exposure time and acquisition mode of the detector (Super-resolution mode, Dose fractionation and frame rate) for your five acquisition modes, and the file options for your record parameter (“Setup” in the Camera control panel) so that they match your requirements (see **Note 6**).
3. For a full grid montage, open the navigator and go to “Navigator/ Montage/ Full Grid montage”. Make sure to have the desired low magnification settings ($\sim 500 \mu\text{m}$ FOV) saved to the View parameters. If calibrated correctly, SerialEM will automatically choose the correct number of patches to acquire a full grid montage and the covered area will be displayed below ($\sim 2.5 \times 2.5 \text{ mm}^2$).
4. To map the area around the current location (adjust to above magnification for the View mode if you have changed it for a full grid montage), select “File/ New Montage”. The number of tiles which also defines the covered area can be specified.
5. Start the acquisition of the map from the “Montage” floating window by clicking “Start”.
6. After the acquisition, load the map into the Navigator window by clicking “New map”.
7. To add points of interest to the navigator, click on “Add points” in the navigator floating window and then click on the area of interest on the map. You can add comments to the points in the Navigator and assign them different colors for easy identification on the map.

3.3.2 Tilt series acquisition

During the tilt series acquisition, the specimen is sequentially tilted, and projection movies are acquired. Despite high mechanic accuracy of the stage, the sample is never completely eucentric

during the tilt. To compensate for the residual lateral and horizontal motion, tracking and focusing are usually performed after each tilt. In order to prevent the sample from unnecessary exposure both are performed on user-defined areas that are translated with away from the exposure area along the tiltaxis ('low dose').

1. Once you have found and centered a suitable region at intermediate magnification (View mode), move the sample to eucentric height by running the routine "Tasks/ Eucentric rough". For high magnification also run "Tasks/ Eucentric fine".
2. Define the position of the Focus and Trial area in the "Low Dose" control panel (see **Note 7**).
3. Open the Tilt series dialog box.
4. Check and define all the parameters of the tilt series. The major parameters are the tilt scheme (uni-, bidirectional or dose-symmetric), tilt range, tilt increment, starting angle, settling time and criteria for tracking and focusing (see **Note 8**).
5. Press "GO" to start the acquisition of the tilt series.

3.3.3 Setting up a batch acquisition

The option to set up a batch acquisition is implemented via the Navigator window. SerialEM requires two maps per position, one at low (View mode) and one at high magnification (Record mode). The reference maps are stored in two different files, and the function "Realign to Item" is used by SerialEM to find back areas of interest based on the stored stage coordinates and by cross-correlation of the images.

1. Open two new files for the high and low magnification maps (e.g. "maps.mrc" and "anchors.mrc") by clicking "File/ Open New".
2. Navigate to the area of interest. If you have stored them in the navigator, locate them using the "Go To XYZ" button.
3. Acquire a preview picture of the feature of interest. If necessary, center it and acquire another preview picture. Make sure that the image shift is low (<0.5 FOV).
4. Toggle to the "maps.mrc" file from the "Buffer Control" panel and click "New Map".
5. Acquire a View image.
6. Toggle to the "maps.mrc" file from the "Buffer Control" panel and click "New Map".
7. Check the boxes "Acquire at position" and "New file at item" for the high magnification? item in the navigator window.
8. Repeat this procedure for all your points/ areas of interest.

9. After having created high and low magnification maps at all points, open the batch acquisition window by “Navigator/ Acquire at points...”.
10. Select the desired operations to execute at each point. If the eucentricity has not been refined at all points, this option should be checked.
11. Start the batch acquisition by pressing “GO”.

3.4 Reconstructing a 3D tomogram from 2D micrographs

In order to reconstruct a 3D tomogram from the 2D projections of a tilt series, several mechanical and optical distortions that arise during the acquisition of a projection at near-atomic resolution have to be corrected for. The first major step is the alignment of the subframes, to account for mechanical and beam induced motion of the sample during the exposure. For this task we recommend motioncor2 [13]. The second major step is the alignment of the tilted images to a common 3D coordinate system. If the images contain gold beads (following section 3.1), the following steps will cover the tilt series alignment with TOM/ PyTOM. If this is not the case, we recommend IMOD for alignment by patch tracking (not covered here, see IMOD web site for more information [19]).

1. Run motioncor2 by executing the following command in the terminal (see **Note 9**):

```
motioncor2 -InTif 'path to your frames' -OutMrc 'targetpath' -Patch 5 5
```

2. PyTOM and TOM read projections in the *.em format based on a base-name followed by an index ascending according to the tilt angle (i.e. projection1.em corresponds to -60 °, the maximum negative tilt). To sort the motion-corrected frames according to the tilt-series and the tilt angle in ascending order (e.g. “tomogram1/sorted/sorted[1- 61].em”, run av3_sortTSbymdoc (see **Note 10**):

```
av3_sortTSbymdoc(numtomo, mdocdir, mdocprefix, rawframesuffix, framedir,
projdir, dim, scale)
```

The information of the corresponding angles is derived from the SerialEM *.mdoc file. Here, numtomo is the index of the tomogram (in the above example 1), mdocdir and mdocprefix specify the mdoc logfile, rawframesuffix and framedir the raw frames, projdir the location of the sorted and aligned projections and dim the lateral size of the generated images (in pixels). Define a downscaling factor (e.g. 4: scale=4) to generate downscaled projections, which will will accelerate and facilitate the tilt series alignment.

3. Start the GUI for gold marker by typing 'tom_setmark' in the Matlab shell.
4. Load the tilt series by clicking 'New' and selecting the last projection in the directory of the scaled projections (Fig 1, label 1). Four new windows will open.
5. Choose the first gold marker by clicking 'ADD' in the main panel (Fig 1, label 2), and then clicking on your desired gold marker in the working images (Fig 1, label 3) and the magnified image (Fig 1, label 4).
6. Check the "Turbo" box (Fig 1, label 5) to automatically load the next projection, and click the marker for all projections of the tilt series.
7. After clicking one marker for the whole tilt series, add new markers by clicking 'NEW' and adding them as before. Click at least 3 gold markers in all projections and compute the alignment. The residual error should ideally be below 1.5 pixels.
8. Write a markerfile corresponding to the full-sized images by multiplying the values in the created markerfile with the previously applied downscaling factor from step 4 of this section (e.g. factor 2).
9. Refine the marker positions using `av3_finelocate (matlab)` using the full-sized projections and write out the corresponding markerfile.
10. Run the function 'reconstructTomogram.py' with the desired parameters to align the projections and reconstruct the tomogram by weighted backprojection. Type 'reconstructTomogram.py --help' to display all parameters. Choose the 0° projection as the reference index, the refined markerfile as markerfile ('markerfile_refined.em') and the most central marker as reference marker (see **Note 11**).

3.5 Subtomogram averaging

The resolution of a tomogram is typically not limited by the sampling (=voxel size on specimen level), but rather by the SNR. Similar to SPA, subtomogram averaging (STA) enables the reconstruction of a high-resolution density by aligning and averaging multiple subtomograms each depicting copies of the macromolecule. A common approach to STA is to (i) interactively localize copies of the macromolecule of interest in a few tomograms, (ii) to align and average the corresponding subvolumes by fast rotational matching allowing exhaustive orientation sampling [20], (iii) to localize more copies in the remaining datasets by template matching, and (iv) to determine a higher resolution average of different classes of the molecule using real-space methods. In order to select initial candidates, exclude template bias and select for true positives, the complex of interest should be visible in the tomogram.

For mitochondria, the ATP synthases and mitoribosomes can be identified in a high-quality tomogram (Fig 2).

3.5.1 Creating an initial, reference-free average from manually picked particles by FRM alignment

1. Open chimera and load the reconstructed tomogram (see **Note 12**).
2. Open the Volume tracer in the Volume Viewer Panel ('Tools / Volume Tracer', Fig 3).
3. Choose how to place a marker (e.g. using middle + control button).
4. Set the color of the marker by clicking on it and choose an opacity of 0.1 to 0.2 to facilitate particle selection. Make sure that within the Viewing panel (Favorites/ Side View/ Effects), the box 'single layer' (under transparency) is unchecked.
5. Use the slider to go through your z-slices to locate your particles. For each particle you wish to pick, place a marker on top of it to record its location. When you are done, save the marker file ('File/ Save current marker set as', e.g., tomogram1/reconstruction/man_picked.cmm).
6. Use the matlab script `av3_cmm2particlelist` to read the xyz-coordinates from the '.cmm' marker file (textfile) and write a Pytom compatible particle list in .xml format (see **Note 13**).
7. Run the `reconstruct_subtomos_INFR.py` function to reconstruct subvolumes with the INFR method (see **Note 14**). In this example full-magnification subvolumes of 256x256x256 voxels are reconstructed:

```
pytom PathToPytom/reconstruction/reconstruct_subvolumes_INFR.py -d
[Path_to_unweighted_projections/] -s 256 -b 1 -o 0,0,0 -p
path_to_particleList.xml
```
8. Check that your subtomograms indeed depict particles using with `tom_dspcub` (matlab) or Chimera. In case of a mitoribosome, you should clearly see a central density in the lowpass-filtered subtomogram.
9. Make a directory for your FRM alignment.
10. For an initial alignment, it is advisable to scale down the subvolumes to 5- 10 Å voxel size. Use either `tom_rescale` (matlab) or `resize` (pytom) to write downscaled versions of the reconstructed full-size subtomograms (see point 7.).
11. Generate a particleList for your resized particles (see step 6.)
12. Create an average from your particles created with `tom_average` (matlab) or `average.py` (pytom). Display the average with `tom_dspcub` (matlab) or in Chimera and check that there is a clear density in the center.

13. Generate a mask with `tom_spheremask` (matlab) or `initSphere.py` (pytom). Check that the mask encompasses the structure of interest (Fig 3).

14. The FRM alignment requires a jobfile (e.g., “`job.xml`”) format specifying the parameters (**bold** parameters need to be adjusted to your environment and data). (see **Note 15**). To this end execute ‘`av3_createFRMjobfile`’ in the matlab shell to create the jobfile with the respective parameters. These are the lower (here: 8) and upper (64) limits of spherical harmonics, lowpass for initial iteration (6), number of iterations (30), maximum offset of peaks (30), name of resulting average (‘`average.em`’), mask (‘`maskfile.em`’), pixelsize (5.24 Å), particle diameter (250 Å) and `particleList.xml`):

```
av3_createFRMjobfile('job.xml', 8, 64, 6, 30, 30, 'average.em',  
'maskfile.em', 5.24, 250.0, 'particleList.xml')
```

15. Run the FRM alignment:

```
pytom PathToPytom/frm/FRMAlignment.py -j job.xml -v
```

After each iteration, an unfiltered average, as well as an average filtered to the determined resolution and an updated `particleList` will be written out (see **Note 16**).

3.5.2 Template matching

During the template matching, the cross-correlation of a masked template structure is computed at different orientations and at each voxel coordinate. Therefore, the tomographic volume, a template, a mask and a list of rotations are required. Due to the noise within the tomogram and to speed up the computation, template matching is usually performed on downscaled tomograms. Depending on the macromolecule of interest, we recommend an effective voxel size of 1 - 2nm (the example of the human Mitoribosome was performed at 2 nm).

1. Generate a template by applying the appropriate scaling factor to either your reference-free average or external structure (see step 10, 3.4.1) (see **Note 17**).
2. Generate a mask with same dimensions as the template (see step 12, 3.5.1).
3. If computational time is scarce, crop the tomogram in z to only capture the sample (note down the z offset!). Use either `read` (pytom) or `tom_emread` (matlab) to read and write a subvolume of the tomogram.
4. Execute `av3_createTMjobfile` in the matlab shell to create the jobfile with the respective parameters (tomogram ‘`tomogram.em`’, template ‘`template.em`’, mask

'mask.em', missing wedge of 30°, 7112 angles specified in 'angles_12.85_7112.em')

(see **Note 18**):

```
av3_createTMjobfile('JobInfo.xml', 'tomogram.em', 'template.em', 'mask.em',  
30, 'angles_12.85_7112.em')
```

5. Start the Job with the following function that supports message passing interface (MPI). The precise MPI syntax might vary depending on your system (see **Note 19**):

```
mpiexec --tag-output -n 16 pytom /path_to_pytom/bin/localization.py  
JobInfo.xml 4 4 1
```

6. Inspect the resulting correlation function by opening the "scores.em" file and superimposing it with your tomogram. Adjust the histogram to show the highest scores (Fig 5) (see **Note 20**).

3.5.3 Particle extraction and subtomogram reconstruction

Prior to subtomogram reconstruction, the resulting correlation ("scores.em") and angle ("angles.em") volumes obtained by template matching need to be converted into a list (particleList in PyTom, motiflist in TOM) containing the particle coordinates, orientation and correlation values. During the particle extraction step, the voxels around the high correlation peaks are zeroed within a defined radius to exclude multiple entries of the same particle. To separate false-positives from true-positives particles in your template matching results, it is usually necessary to manually filter the identified correlation peaks. This can be done with "Chooser", a Chimera plugin available on the PyTom website.

1. Extract the particle coordinates into an .xml-file and a motiflist with the following function (see **Note 21**):

```
pytom PathToPyTom/bin/extractCandidates.py -j job.xml -n 700 -s 8 -r  
scores.em -o angles.em -p pl.xml -m targetpath.em -t pathToNewParticles
```

2. Open Chimera and load the tomogram that was used for template matching. Load the corresponding particlelist with the Chooser.
3. The Chooser will display all the entries from the particlelist, starting from the highest correlation values. Inspect all the correlation peaks and assign class indices (e.g. 1 for correct and 0 false-positive matches; more than two class labels can be assigned if desired)see **Note 22**).

4. Save the class labels by clicking “Save” and close the Chooser. Each line in the resulting text file corresponds to the respective entry in the particle-/motifList.
5. Filter the particle-/ motifList by excluding the entries according to the assigned classLabels by executing `av3_filterClassLabels` (see **Note 23**):

```
av3_filterClassLabels(motlin, labelfile, class, motlout, zheight,
scaling, rawsize)
```

6. Run the INFR subtomogram reconstruction (see step 7, 3.4.1), or use the weighted back projection algorithm with the filtered particleList as input (see **Note 24**):

```
reconstructWB.py -p particleList.xml --projectionDirectory
../reconstructTomo/alignedProjections/ -s 128 -b 1 -o 0,0,192
```

7. Check the correct reconstruction by looking at the subtomograms. Depending on the downscaling factor and pixel size, it might be necessary to apply a strong filter to see features (see **Note 25**).
8. Average the particles with the following function (see **Note 26**):

```
pytom PathToPyTom/bin/average.py -p particleList.xml -a Average.em
```

3.5.4 Gold standard subtomogram alignment in real space

In addition to the FRM alignment (see 3.4.1), PyTom also provides the option to perform the subtomogram alignment in real space. The real space alignment tends to be more accurate because it simultaneously samples rotational and translational degrees of freedom, but due to the higher computational requirements the rotational search is restricted. Thus, the method requires initial starting angles, which were at this point either determined from the template matching or by a previous round of FRM alignment. The following steps will cover the gold standard real space alignment, which splits the dataset into two independent halves used for the alignment against independent references to reduce over-fitting.

1. Create a directory for the real space alignment.
2. For an initial alignment, it is advisable to downscale the particles to 5 - 10 Å voxel size. Use either `tom_rescale` (matlab) or `resize` (pytom) to write downscaled particles (see 3.3, step 4), or use a downscaling parameter (“-b”) for reducing volumes on the fly during the alignment (see step 6).
3. Generate a particleList for your resized particles (see 3.4.1, step 6)

4. Create an average from your particles with `tom_average` (matlab) or `average.py`. Open the average and check that there is a clear density in the center (see 3.4.1, step 12).
5. Generate a spherical mask (see step 13, 3.4.1). Check that the mask encompasses the structure of interest (Fig 3).
6. Run the 'gold-standard' Real Space local alignment. In this example, 10 iterations of alignment are performed with the data downsampled on the fly by a factor of 2 using an initial rotational sampling of 3 degrees increment (subsequently adjusted according to resolution) in 3 shells:

```
mpirun -n numberOfCPUs pytom PathToPytom/bin/GLocalJob.py -p
particleList.xml -r Reference.em -m Mask.em --SphericalMask --angleShells 3
--angleIncrement 3. -d ./ -n 10 -b 2 --pixelSize 2.62 --particleDiameter 250
```

3.5.5 Classification

Subtomograms with differing structural features, e.g. due to a particular conformational state or different composition, can be disentangled during subsequent classification. This is usually done by constrained principle component analysis (CPCA), where a pair-wise cross-correlation matrix of the subtomograms is computed and subsequently grouped by principle component analysis and k-means clustering. This allows for the removal of false positives from the dataset, as well as the enrichment of subtomograms showing distinct pronounced structural features such as the small ribosomal subunit or the membrane (Fig. 6A).

1. Generate a mask encompassing the feature of interest within your initial average (Fig. 6A), either with Chimera (shape mask), or with `initSphere` (pytom) for a spherical mask (see 3.4.1, step 13).
2. Compute a cross-correlation matrix of all particles by running the following command (see **Note 27**):

```
mpiexec --tag-output -n numberOfCPUs pytom
PathToPytom/calculate_correlation_matrix.py -p particleList.xml -m Mask.mrc
-f 10 -b 1
```

3. Group the data based on the output 'correlation_matrix.csv' using CPCA and k-means clustering (see **Note 28**). Here, the data are projected to 5 principal components and grouped 7,800 subtomograms into 67 classes (Fig 6A):

```
pytom path_to_pytom/bin/classifyCPCA.py -p particleList.xml -o
pL_classified.xml -c correlation_matrix.csv -e 5 -n 67 -a "Average"
```


4. Open and inspect all the classes (here “Average1-67.em”) in Chimera (Fig 6B) and note down the class numbers that you would like to merge for your further analysis.
5. Filter the particleList to include only the classes that you have picked using the pytom function `splitByClass`:

Load the pytom module and open the interactive shell by typing ‘ipytom’. Then run the following lines to split the particlelist ‘pL_classified.xml’:

```
pl=ParticleList()
pl.fromXMLFile('pL_classified.xml')
lists=pl.splitByClass()
```

Specify the desired classes in the square brackets and write them out to a new particlelist by running the following lines:

```
ribos=lists[1] + lists[2]
ribos.toXMLFile('pickedClasses.xml')
```

For convenience, you can also use the provided bash script `merge_pl_classes.sh` which also allows merging of classes from several particleLists and contains explanations.

6. If desired, apply further rounds of classification with different masks to separate the subtomograms according to variations in specific areas, e.g., membrane density (Fig 6A). Alternatively, repeat the alignment with the filtered set of subtomograms.

4 Notes

1. Lysis efficiency will slightly differ depending on the tightness of the individual Glass-Teflon-Potter. Monitor the cell lysis in a light microscope and adjust the number of strokes to achieve of roughly 60 %.
2. Test at least two dilutions of your sample (e.g. undiluted and 1:3). In the case where you characterize your purification protocol for the first time sample a wider range of concentrations. We found that Nanodrop measurements at 280 nm provide reproducible results with regards to mitochondria and vesicle densities and recommend the following conditions for the mitochondrial sample: 1.5 mg/ mL in 20 mM Hepes pH 7.4 and 150 mM Sucrose (human) or 600 mM Sorbitol (yeast). The concentration of the mitochondrial sample

is a critical parameter to achieve high-quality tomograms since it affects the ice thickness. Plunge freezing of four grids roughly takes 30 min, so the handling time is neglectable compared to mitochondrial isolation protocols, but mitochondria should be kept on ice.

3. Make sure to not contaminate the ethane or ethane-propane with any liquid nitrogen or water, e.g. from your breath. Try to maintain a protective atmosphere of cold nitrogen vapor in your container at all time to prevent contamination of your sample by ice crystals from the air humidity.
4. For iso-osmotic conditions, mitochondria require 150 to 300 mM sucrose (human) and ~600 mM sorbitol (yeast) in the isolation buffer (see 2.1). However, the sugar concentration should be as low as possible in the vitrified sample to provide a good contrast and to prevent beam damage in the TEM.
5. The choice of the magnification is a tradeoff between FOV and the pixel size. A large FOV enables covering large or even multiple vesicles, while the pixel size puts an upper limit to the maximum resolution. In practice, the maximum resolution is limited to approximately 50% of the Nyquist frequency, i.e. the minimal distinguishable distance is four times the pixel size on the specimen level. For *in organello* studies of mitochondrial translation a pixel size of ~2 Å does not limit the resolution, which is rather restricted by the low contrast arising from the sample thickness and the crowded environment of the mitochondrion.
6. It is recommended to test the beam and detector settings in a region of the grid that does not contain useful mitochondrial vesicles, or on a completely different grid to avoid unnecessary radiation damage to the sample. The exposure times for your View/ Search/ Focus/ Trial mode should be adjusted as low as possible but high enough to yield sufficient signal in the resulting image. For your record parameter, the exposure time should be adjusted so that the accumulated dose does not exceed $80 \text{ e}^-/\text{Å}^2$ for 200 kV respectively $120 \text{ e}^-/\text{Å}^2$ for 300 kV EM for thinner samples (accumulated dose = dose per tiltangle * number of images). Saving record frames in super-resolution takes slightly longer than in simple counting, and the files are four times bigger. However, these considerations are negligible for tomography, and more critical for SPA data acquisition, where the fairly small gain in detector quantum efficiency often does not outweigh the lower throughput. Regarding the file options, it is advisable to save the files as .tif files with LZW compression as one stack, which yields compression factors of ~40. Including the tilt angle in the filename is also recommended.
7. The position of the Focus/ Trial area should differ from the Exposure area by more than the beam diameter. To measure the beam diameter, you can over-expose carbon to the beam by

putting the screen down with the Record mode settings. If possible, try to have some carbon or other features in your Focus/ Trial area, since discrete, high-contrast features facilitate the focusing and tracking.

8. For initial or standard data-acquisition, we recommend the following settings: bi-directional tilt scheme, tilt range -60° to $+60^\circ$; starting angle -20° , tilt increment of 2° or 3° . The settling time strongly depends on the stage of the microscope, but 15 sec is a good starting value that can be increased if blurring is observed in the Trial image, or reduced otherwise for higher throughput. For all other parameters the provided standard settings are a good starting point. We routinely employ a dose-symmetric tilt scheme [21], which has been recently implemented in the SerialEM tilt series dialog box. It requires slightly more time per tilt series but provides a better dose distribution and is advantageous for high resolution subtomogram averaging.
9. These are the minimum input requirements for both global and local motion, with the standard patch size of 5 x 5. For more information, e.g. on how to apply gain normalization, see the motioncor2 user manual or type "motioncor2" into your terminal.
10. `av3_sortTSbymdoc(numtomo, mdocdir, mdocprefix, rawframesuffix, framedir, projdir, dim, scale)`: `numtomo` defines the number of tomograms and corresponding mdoc files, `mdocdir` the path to your mdoc file, `mdocprefix` the prefix of the mdoc file, `rawframesuffix` the filesuffix of the unaligned frames (e.g. '.tif' or '.mrc'), `framedir` the directory containing the aligned frames, `projdir` directory of projections, `dim` the desired dimension (cut to square) `scale` the desired scaling factor. The function will create the number of folders defined by `numtomo`, sort the corresponding tiltseries according to the tiltangles into the folder defined in `projdir` and `scale` and cut the projections.
11. Choose a downscaling factor between 4 to 8 to increase the signal in the resulting volume and keep the file size reasonable for subsequent template matching. Pytom also provides an alternative tomogram reconstruction method, called INFR [22]. It requires aligned but unweighted projections, which can also be obtained using `reconstructTomogram.py` with `-weightingType 0`. Run `reconstruct_INFR.py` to perform the tomogram reconstruction with INFR. The resulting tomogram will have better contrast, which facilitates the manual and automated picking (template matching) of particles.
12. Depending on the data quality and binning, it might be necessary to filter the volume. Go to 'Tools/ Volume Filter' and apply a Gaussian filter with width 0.5 to 0.9 to your volume. It might also be necessary to select solid rendering and adjust the histogram for a good contrast.

13. Check the XYZ coordinates in your generated `particleList`. They should be positive values corresponding to the dimension of the tomogram you have picked. If the tilt range is different than -60° to $+60^\circ$, define the opening angle of the missing wedge respectively.
14. `reconstruct_subtomos_INFR.py`: The subtomograms will be written into the specified path in the `particleList.xml`. The option `-d` specifies the input projections, `-s` specifies the box size, `-b` the downscaling factor (be sure to use the same the downscaling factor that corresponds to the tomogram you used for picking your coordinates), and `-o` the offset of the coordinate system in case the positions were picked on cropped tomograms. The function does not have the option to apply a downscaling to the subtomograms. If downscaled subtomograms are intended, create downscaled projections and use them as an input for the subtomogram reconstruction (see 3.3, step 4).
15. The parameter “Frequency” defines the starting spatial frequency (in inverse pixels) for the lowpass filter in the first iteration of alignment, and “BandwidthRange” the bandwidth range that is used throughout all iterations (i.e., a highpass filter is effectively used). 30 iterations should usually result in a converging average.
16. Ideally, the resolution will increase and meaningful structural features will manifest themselves. However, for small particle sizes, the structure can align structures according to the missing wedge than their structures. Nevertheless, for large complexes such as the ribosome this reference-free FRM alignment typically succeeds. If the FRM alignment results in a subtomogram average with distinctive features and details, the gold standard real space alignment with the angles from the FRM alignment might improve the result even further (see 3.3.4)
17. If an external structure as a template is intended, apply the correct scaling and have matching density values (i.e. both positive/ negative values for the electron-density). Also, make sure that handedness corresponds your reference system (left- or right-handed). To generate a template for the human mitoribosome you can use the EMDB structure EMDB-2876. Load it in Chimera (`File/Fetch by ID`), and save the density map as an mrc density file (`'2876.mrc'`). Determine the scaling factor to de-magnify from $1.34 \text{ \AA}/\text{vox}$ to the desired voxel size (e.g. $20 \text{ \AA}/\text{vox}$) and divide the box size by that factor to yield your final box size (e.g. 16). Then run the following command with the value in the square brackets to rescale it:

```
map=tom_mrcread('2876.mrc')
map=tom_rescale(map.Value, [16,16,16])
```

If the map needs to be mirrored or inverted, use the following commands (otherwise skip them):

```
map=tom_mirror(map,'x')
```

```
map=map*-1
```

Save the map by executing the following line:

```
tom_emwrite('5aj4_scaled.mrc',map)
```

18. The angular search of 7,112 angles (12.85° increment) is a good standard parameter. The different angle files are located within `PathtoPytom/angles/angleLists/`.
19. The last three numbers indicate the splitting of the volume in x, y and z. This should ideally correspond to the number of nodes. The division of the volume by the provided factor has to result in full integers.
20. If the results do not correspond to the features, make sure to have checked all the requirements mentioned in note 14. Especially for small, elongated particles, template matching might fail. In this case, apply a higher downscaling to the tomogram.
21. `extractCandidates.py`: Option `-n` defines the number of particles, `-s` defines the radius around the peak which will be zeroed to exclude multiple picking of the same particle, `-p` defines the path to the particle list and `-m` defines the path to write out the results in TOM-compatible motiflist format. A good template matching result will show a gaussian distribution for the determined cross-correlation peaks ("Value" in `particleList.xml` (PyTom), column 1 in AV3-motiflist). However, within the crowded mitochondrial matrix the results of the template matching often show false-positive matches (Fig. 5). In order to filter the template matching results, multiply the correlation volumes prior to particle extraction with a binary mask encompassing the mitochondrion (e.g. by generating the mask with Chimera Volume Tracer). If the particle list is to be used directly for subtomogram reconstruction (see step 7, 3.4.1), provide an option `-t` with an argument, which defines the target directory for the reconstructed subtomograms.
22. At this step it is beneficial to exclude as many false-positives as possible. However, this is not absolutely critical since a classification will also help in removing false-positives (Fig 6B).
23. `av3_filterClassLabels`: `motlin` defines the input motiflist file, `labelfile` the file containing the manual classifications from the Chooser, `class` defines the class index that will be included (e.g. 1) and `motlout` the name of the filtered output motiflist. The last three parameters - `z_height` (defines the z position at which, if applicable, the downscaled tomogram was cropped), `scaling` (scaling factor of the tomogram) and `rawsize` (dimension of the non-downscaled projections) - are optional, and used for the generation of a non-scaled motiflist with the coordinate system of the aligned raw projections.

24. The argument of option `-s` defines the box size, `-b` the downscaling factor of the particle coordinates compared to the projection, and `-o` the offset of the particle coordinates from the coordinate system chosen for the reconstruction, which is used in the `.xml` file, if any.
25. If the box is empty, make sure that the supplied coordinates in the `.xml` file correspond to your tomogram dimension. (0 to 3838/3710 for full-size data from a K2 detector)
26. The resulting average should show distinctive structural features if the template matching and reconstruction have worked. If this is not the case, the template-matching probably resulted in a considerable number of false-positives which have to be removed by repeating steps 2. to 5., checking the template matching results or repeating the template matching. If the results do not improve, manual particle selection might be the only option (see 3.4.1). Mitoribosomes are reliably detected by template matching in very thin tomograms (< 150 nm) with good contrast but are hardly detected in thicker tomograms where they can still be identified by eye.
27. `calculate_correlation_matrix.py`: Option `-f` represents a low-pass filter, and `-b` a downsampling factor to be applied to the subtomograms.
28. `classifyCPCA.py`: Option `-p` defines the path to the particlelist (make sure that the path to the subtomograms within the particlelist is correct), `-o` the targetpath for the output-particlelist that will include the classlabels, `-c` filename of the correlation matrix, `-e` number of eigenvectors; `-n` the number of classes; `-a` basename of the class-averages. Five eigenvectors are usually sufficient to represent the main features of the subtomograms. On the one hand side, the number of classes should be chosen as high as possible in order to separate subtomograms depicting different conformations. On the other hand, the number should be chosen sufficiently small, such that a class-average contains enough subtomograms to yield sufficient SNR to distinguish features. For mitoribosomes, 100 subtomograms/ class should yield enough signal for the reliable assessment of class-averages (Fig. 6B).

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7 Figures

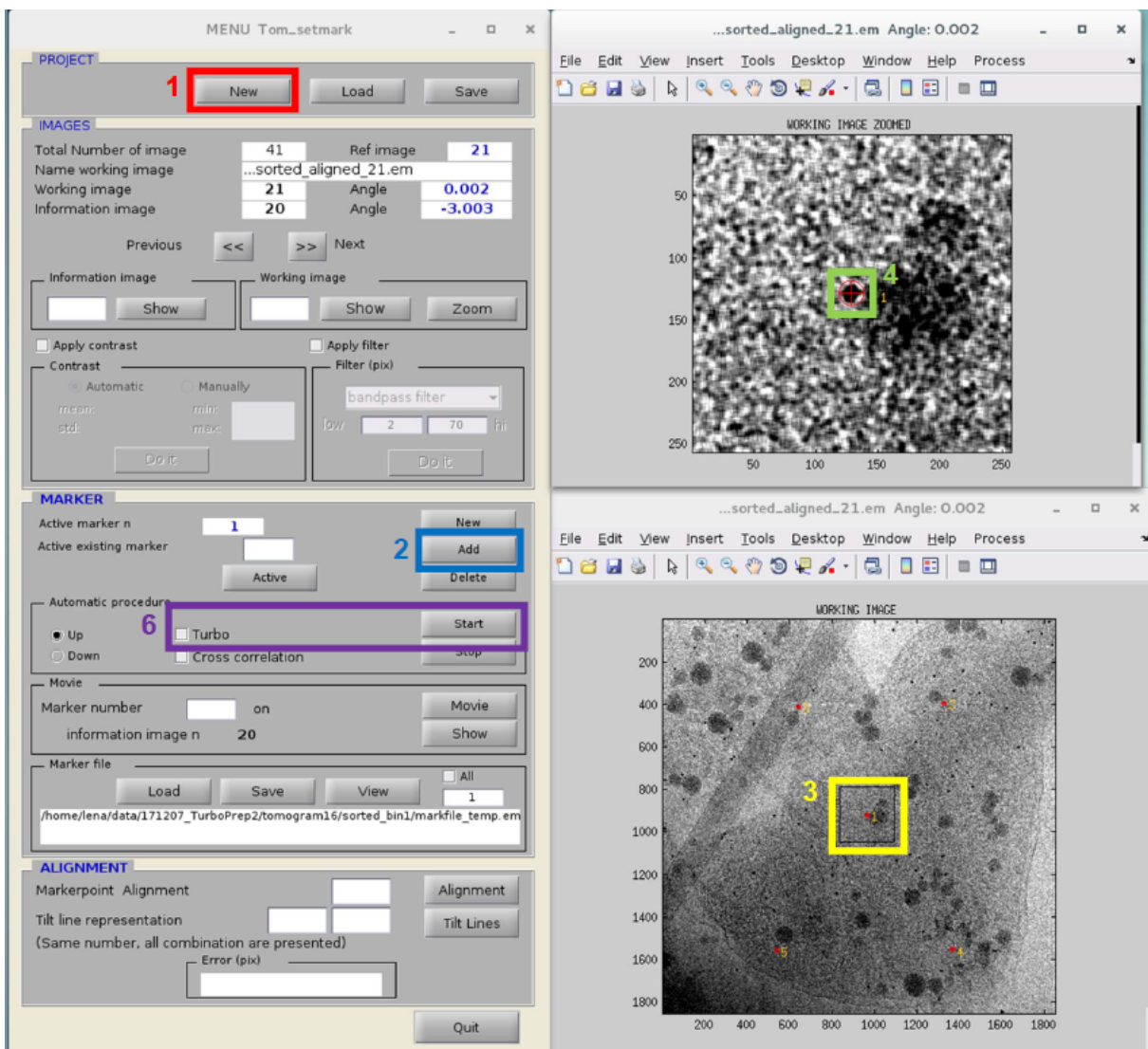


Fig 1: Interface of the tom_setmark GUI. Load a new tilt series from the Project tab (1). Add the first marker by clicking 'ADD' (2), and then selecting the area containing your first marker in the working image (bottom right, 3). Then click on the gold marker in the zoomed image (top right, 4). Check the box 'Turbo' (5) and click 'Start'.



Fig 2: Slice from a reconstructed tomogram of a human mitochondrion (data acquired with the Volta Phase Plate at 300 kV). In the resulting tomogram of the mitochondrion, the outer and inner mitochondrial membranes (OMM and IMM), as well as cristae, membrane-associated mitoribosomes and ATP synthases can be identified.

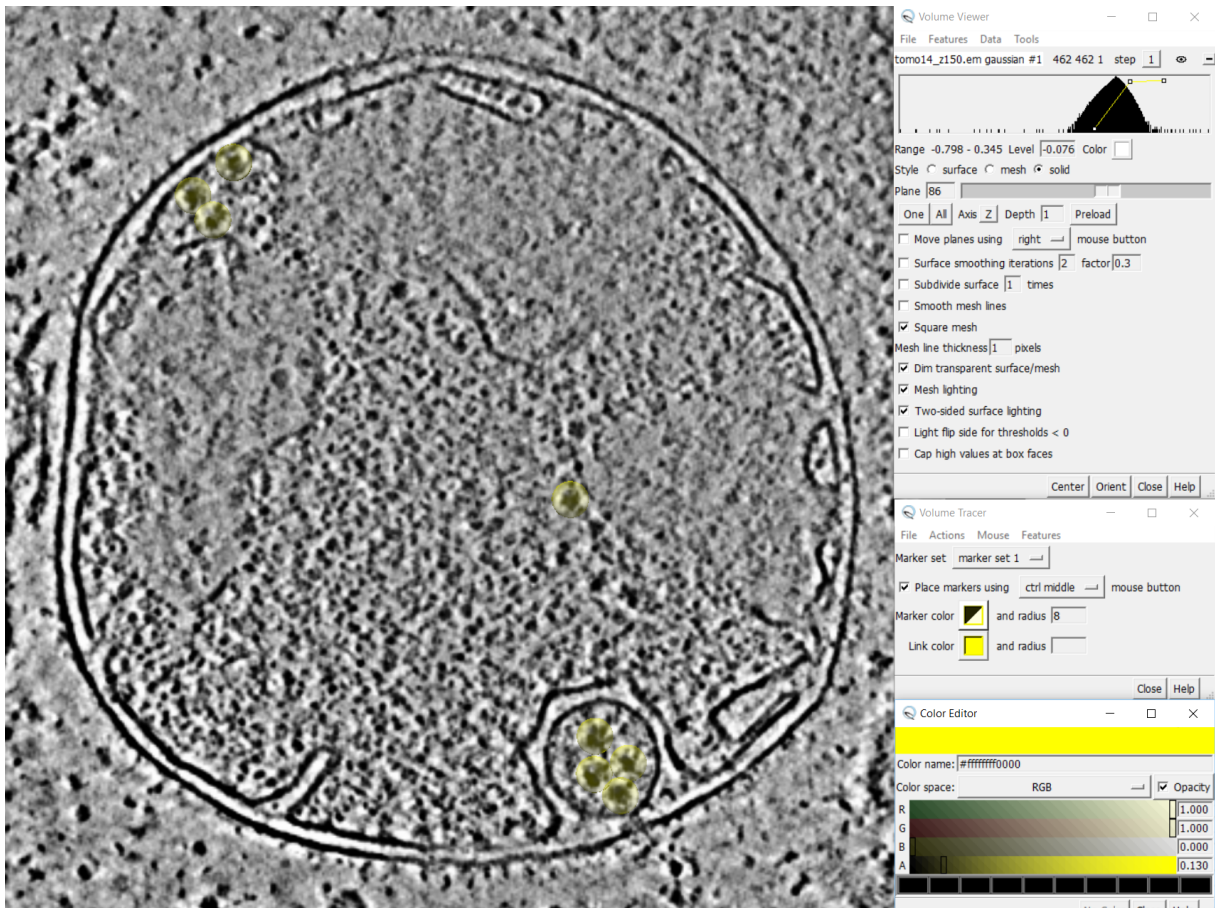


Fig 3: Manual particle picking in Chimera. Use the Volume filter and/ or adjust the histogram to get sufficient contrast. Open the Volume Tracer dialog box (bottom right) and choose the color and radius of your marker. Place markers by clicking on the data planes.

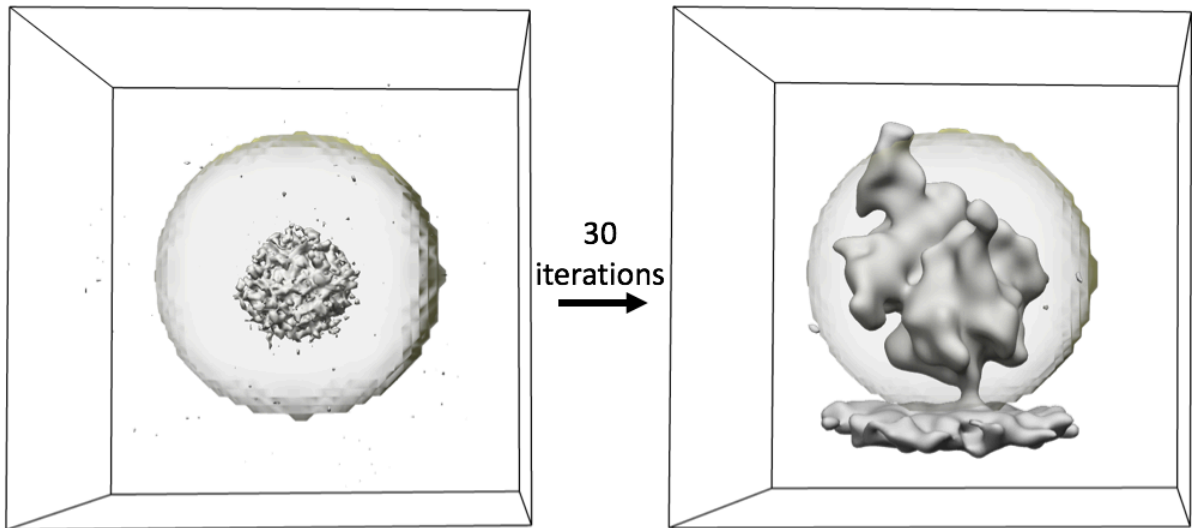


Fig 4: FRM alignment of manually picked particles. a) The average of the subtomograms (grey) with randomly assigned angles should have a spherical shape, and the mask (yellow) should encircle it. b) After 30 rounds of alignment, the spherical shape should ideally converge to a structure resembling your molecule of interest, a human mitoribosome computed from 231 subtomograms in this case.

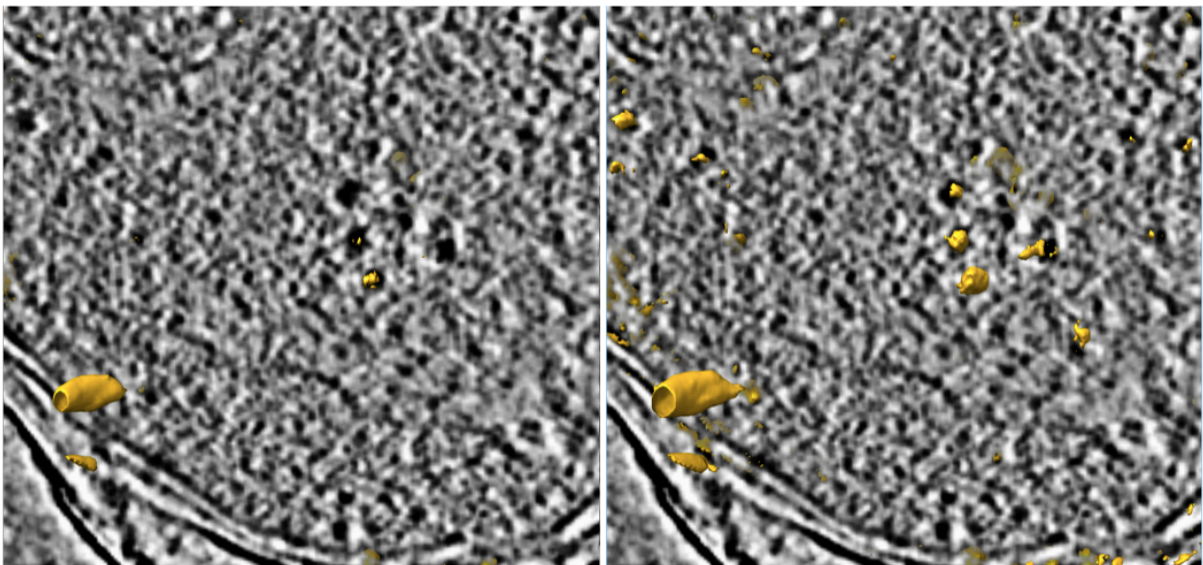


Fig 5: Tomogram with superimposed cross-correlation peaks from template matching. The volume file containing the scores ("*scores.em*", orange density) obtained by template matching was superimposed with the tomogram (shown as slice). At a high threshold (left), the most significant correlation peaks superimpose with mitoribosomes (center) as well ice contamination (left). Reducing the threshold reveals less significant correlation peaks that superimpose with mitoribosomal densities (right image; center and top left part), as well as false positive matches with the membrane (edges).

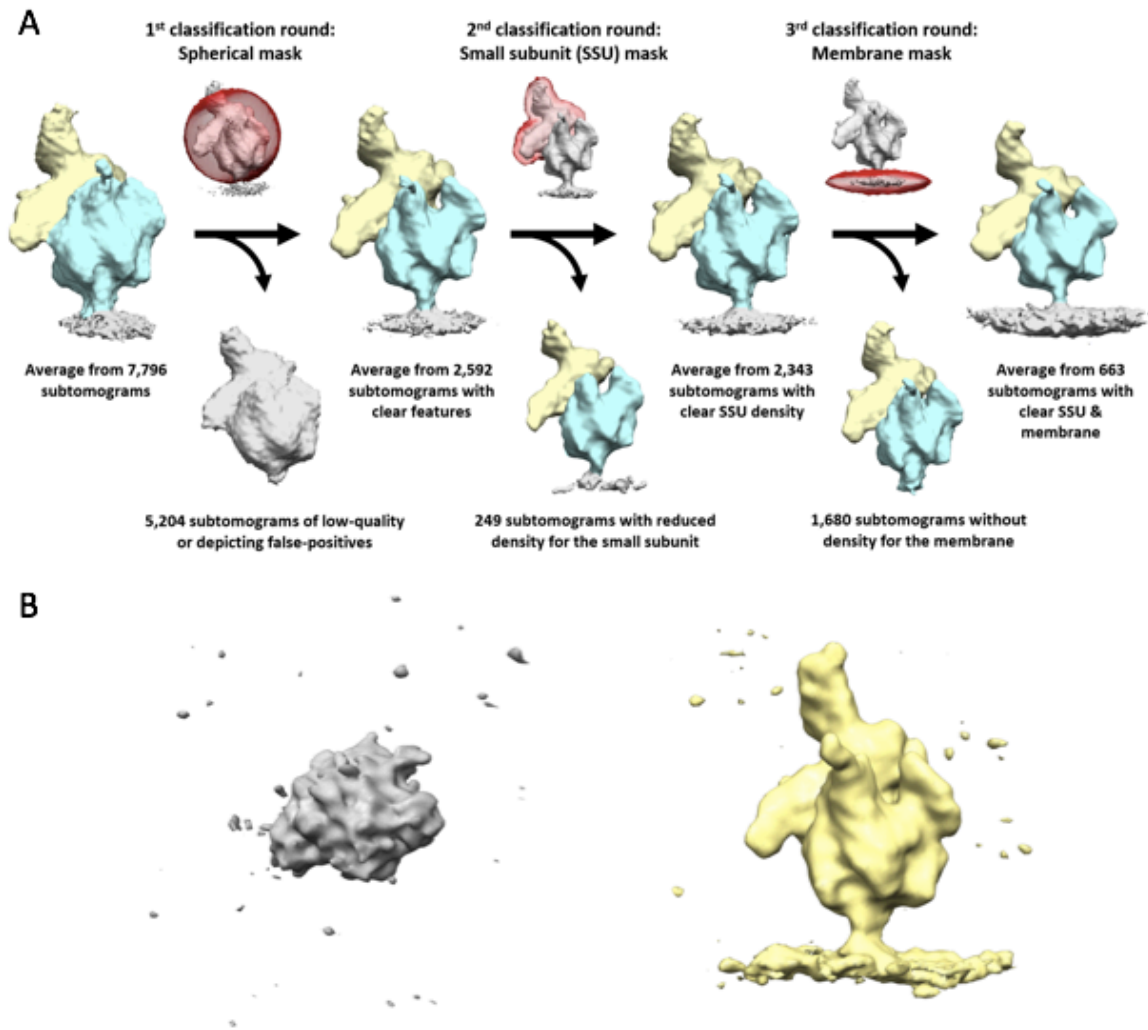


Fig 6: Classification scheme to enrich mitoribosomes with a clear membrane density. A) By performing multiple rounds of classification with different masks (red), subtomograms with a clear membrane density can be separated from subtomograms depicting noise or lacking a clear density for small subunit or membrane. B) Class averages computed from ~100 subtomograms upon the initial classification of 7,796 subtomograms with a spherical mask. The left class-average (grey) represents a class of false-positives matches, e.g. ice, gold markers, or membrane densities that should be discarded. The right class-average (yellow) already shows clear mitoribosomal features, i.e. the small and large subunit as well as the membrane. (both averages were filtered in Chimera with a Gaussian filter of 1.2).