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# iFLM-guided Cryo-FIB milling

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**We use this protocol and it's working**

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## Abstract

Defective mitochondrial quality control in response to loss of mitochondrial membrane polarization is implicated in Parkinson's disease by mutations in PINK1 and PRKN. Application of in situ cryo-electron tomography (cryo-ET) made it possible to visualize the consequences of mitochondrial depolarization at higher resolution than heretofore attainable. Parkin-expressing U2OS cells were treated with the depolarizing agents oligomycin and antimycin A (OA), subjected to cryo-FIB milling, and mitochondrial structure was characterized by in situ cryo-ET. Phagophores were visualized in association with mitochondrial fragments. Bridge-like lipid transporter (BLTP) densities potentially corresponding to ATG2A were seen connected to mitophagic phagophores. Mitochondria in OA-treated cells were fragmented and devoid of matrix calcium phosphate crystals. The intermembrane gap of cristae was narrowed and the intermembrane volume reduced, and some fragments were devoid of cristae. A subpopulation of ATP synthases re-localized from cristae to the inner boundary membrane (IBM) apposed to the outer membrane (OMM). The structure of the dome-shaped prohibitin complex, a dodecamer of PHB1-PHB2 dimers, was determined in situ by sub-tomogram averaging in untreated and treated cells and found to exist in open and closed conformations, with the closed conformation is enriched by OA treatment. These findings provide a set of native snapshots of the manifold nano-structural consequences of mitochondrial depolarization and provide a baseline for future in situ dissection of Parkin-dependent mitophagy.

1 Notched base-clipped EM grids were loaded into an Aquilos 2 with integrated fluorescence (iFLM, ThermoFisher) with the notch facing upwards.

2 Grids were first screened using SEM to identify potential lamellae sites and assess general grid quality. Grids with 20-40 cells in the centers of grid squares were used for lamellae generation.

3 Cells were then screened iteratively using iFLM to target specific regions of clustering mCherry or BFP signal for lamellae site placement. Adjust laser power and exposure time as necessary. Z-stacks were taken at each site cover 5×5 grid squares per Z-stack. These images were mainly used to determine where mitochondria were clustered to in untreated or OA treated cells.

4 Grids were then sputter coated for 15 seconds using 30mA current and 10Pa pressure, and subsequently GIS coated for 1 minute.

5 Auto-TEM was then used to generate a lamellae of approximately 200 nm thickness at each site using the following step-wise protocol for lamellae generation:

6 0.3-0.5 nA of current was used to ablate cell material to 3 µm in thickness.

7 100 pA current thinned cells to 1 µm.

8 50 pA was used to thin cells to 500 nm.

9 30 pA was used to thin lamellae to their final 200 nm thickness.

10 Grids containing lamellae were immediately retrieved from the Aquilos and moved to liquid nitrogen for no longer than 3 days, or loaded directly into a krios with the notch perpendicular to the tilt axis for data collection.