

PC12⁺ cell culture (By Xia Li, Sep, 2019 @ NTU)

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1. Preparation for EM grids.

Quantifoil R2/1 Au-grids (200 mesh with holey carbon film of 2 mm hole size and 1 mm spacing) or Quantifoil R2/1 + 3 nm C Au-NH₂ finder grids (252 mesh with holey carbon film of 2 mm hole size, Alphabets A-Z and Numbers 1-10) were screened under light microscope to assess the integrity of the carbon film. The intact grids were placed in the center of a 35 mm MatTek dish with glass bottom (0.15 mm in thickness and 20 mm in diameter) and preplaced coverslip with 18 mm in diameter and 0.15 mm in thickness to facilitate the subsequent handling of the grids. The carbon side of the EM grids was glow discharged for 25 sec at 15 mA with plasma cleaner (PELCO easiGlow, TED PELLA, USA). EM grids were then sterilized in ethanol for 10 min under UV-light, washed with water six times, coated with human plasma fibronectin (FC010, Millipore, USA) and incubated at 37°C. Subsequently, the EM grids were washed six more times with water and incubated in complete culture media for 6-8 hours (or overnight) before the cells were seeded onto them. During the washes and all subsequent steps, the grids were not being allowed to dry out.

2. PC12 cell culture, differentiation, and transfection.

PC12 cells were gifted from Prof. James Rothman, Yale University. Cells were cultured in DMEM supplemented with 10% normal HS, 5% FBS, sodium pyruvate, NEAA, and penicillin-streptomycin as described. PC12 cells were all maintained at 37°C in a 95% humidified incubator with 5% CO₂. 24 hours before seeding cells onto EM grids, the media was replaced with complete media supplemented with 100 ng/ml NGF (NGF2.5S, Cat: 13257-019, Gibco) to differentiate the cells. Cells were transfected by electroporation and seeded onto the EM grids at a density of 50,000 cells/ml. The cell confluence and grid integrity were analyzed by light microscope every other day (Day 0, 2, 4, and 6) and before freezing to ensure all conditions were appropriate for cryo-ET. All experiments were conducted on cells

cultured for < 10 passages.

PC12 cells were transfected by DNA electroporation with the Amaxa Transfection System (Amaxa Nucleofector II electroporator, Amaxa Biosystems) as described. PC12 cells grown in a 6 cm plate in complete media and supplemented with NGF for 24 hours were washed twice with DPBS without Ca^{2+} and Mg^{2+} (Gibco), trypsinized, and counted. 1×10^6 cells were transferred to a 15 ml polypropylene tube and pelleted at 400 g for 5 min. Cells were re-suspended with 100 μl Buffer, containing 3 mg of a GFP-tagged plasmids, and transferred gently, so as to avoid bubbles, to a transfection cuvette (Amaxa™ cell line Nucleofector™ Kit V, Lonza). Program No. U-029 was selected for PC12 cells, according to the manufacturer's instruction. Immediately following electroporation, 1 ml of complete media was added into the vial and the cells were transferred to a sterile 1.5 ml microcentrifuge tube and centrifuged at 400 g for 5 min. Cells were re-suspended in complete media, diluted to 50,000 cells/ml, and seeded onto the EM grids. 30 min after plating, the media was replaced with complete media, containing 100 ng/ml NGF, and maintained at 37°C. The differentiation media was replenished every 2nd day for 3-7 days. For mitochondrial staining, MitoTracker (M7512, Molecular probes) was added into the culture media at the final concentration of 50 nM for 30 min, followed by a 30 min wash with fresh complete media.

3. Cell vitrification.

The grids with differentiated cells were rinsed with DPBS twice, and then 3-5 ml BSA-coated 10 nm Gold Tracer beads (Cat.25486, Aurion) or reference beads (TetraSpeck™ Microspheres, 4.0 μm) were applied to each grid as fiducial markers immediately prior to vitrification. Grids were then mounted on a manual plunger, blotted from the back side (opposite to cell side or carbon side) for 4 sec using Whatman #1 filter paper (Sigma-Aldrich) and plunge-frozen into liquid ethane as described.

4. Cryo-FIB milling.

The cryo focused ion beam (cryo-FIB) system (Aquilos Cryo-FIB, Thermo Fisher Scientific) was used for this work. The system is equipped with a rotatable cryo-stage cooled by a nitrogen circuit and an Autogrid specimen shuttle designed for FIB preparation under shallow milling angles. The plunge-frozen grids were clipped into cryo-FIB Autogrids (Thermo Fisher Scientific) and mounted into the specimen shuttle under liquid nitrogen. Then the shuttle was loaded into the Aquilos

Cryo-FIB using the cryo-transfer system.

The samples were first sputter-coated with Pt (1 kV, 15 mA, 15 s) to improve the overall sample conductivity, then deposited by an organometallic Pt layer (4-5 μm thick) using the gas injection system for the sample protection. Lamellae were produced using the gallium ion beam at 30 kV with stage tilt angle of 21° , which results in a 14° angle between the final lamella and the grid. The ion beam current was reduced, according to the lamella thickness (t) during the milling process: 0.5 nA for $t \geq 5 \mu\text{m}$, 0.3 nA for $t \geq 3 \mu\text{m}$, 0.1 nA for $t \geq 1 \mu\text{m}$, 0.05 nA for $t \geq 500 \text{ nm}$ and 0.03 nA for $t < 500 \text{ nm}$. Afterwards, a thin Pt layer was sputter-coated (1 kV, 10 mA, 5 s) on the lamella to prevent possible charging issue during cryo-ET investigation.

5. CLEM and cryo-CLEM imaging.

Prior to vitrification, cells transfected with the GFP-tagged plasmid were located by live imaging using fLM. Cells were imaged in the same 35 mm glass-bottom culture dish (MatTek Corp., USA) at a density of $\sim 30\%$ coverage with 37°C warmed live cell imaging solution (Molecular probes by life technologies, Thermo Fisher Scientific) on a Leica DMi8 wide field microscope (Leica, Germany). For correlative microscopy imaging, a stitching map of the whole EM grid was acquired with $40\times$ magnification in a relative focus controller mode (RFC), which was used to auto-acquire the suitable z-focus images in each channel.

After plunge freezing, cryopreserved grids were imaged with the Leica Cryo-CLEM system as described. The MatrixScreener module of Leica LAS X microscope control was developed specifically for cryo-CLEM imaging and allows for the acquisition of 2D mosaic images and 3D stack images of any rectangular shape. 6-8 characteristic apexes of broken carbon film, owing to plunge freezing, were used as grid markers; fluorescent signals were selected as positions of interest (POIs). The coordinates data of both grid markers and POIs were exported as .nav files. A screenshot of the grid markers was saved for manual selection during cryo-EM. The coordinate data files were transferred to the Krios computer and opened with SerialEM Software. After imaging, cryo-preserved grids were carefully recycled back for storage in liquid nitrogen.

6. Cryo-ET data acquisition.

The frozen-hydrated neuritis of PC12⁺ were imaged at $\sim -190^\circ\text{C}$ using a Titan Krios electron microscope (Thermo Fisher Scientific) equipped with a field mission

gun, energy filter, direct detection camera (Gatan K2 Summit), and VPP. The microscope was operated at 300 kV with an effective pixel size of 5.457 Å at the specimen level. SerialEM (Version 3.0) was used to collect low-dose, single-axis tilt series with dose fraction mode at ~ 0 μm defocus and a cumulative dose $\sim 60 \text{ e}^-/\text{\AA}^2$ distributed over 35 stacks, covering an angular range of -51° to 51° with 3° step increments.

The lamellae of cryogenic PC12⁺ were imaged at $\sim -190^\circ\text{C}$ using Titan Krios electron microscope (Thermo Fisher Scientific) equipped with a field mission gun, a Falcon III camera (Thermo Fisher Scientific), with or without VPP. The microscope was operated at 300 kV with an effective pixel size of 4.712 Å at the specimen level. FEI Tomo3 (FEI Tomography software, Thermo Fisher Scientific) was used to collect low-dose, single-axis tilt series with dose fraction mode at ~ 0 μm defocus and a cumulative dose $\sim 60 \text{ e}^-/\text{\AA}^2$ distributed over 35 stacks covering an angular range of -51° to 51° with 3° step increments.

For specimens imaged with the Leica Cryo-CLEM system, a full grid montage was recorded with SerialEM at low magnification (220 \times). The full-montage buffer was saved to register the grid markers, and the EM map was rotated and matched with cryo-EM images. The POIs registered by cryo-fLM were automatically relocated on the cryo-EM map by merging POIs into the full-montage buffer. All POIs were montaged at a medium magnification (3600 \times) and added as the points for cryo-ET acquisition.

7. Usage of Volta Phase Plate.

The optical alignments for the VPP were as such: on-plane condition and correct beam-tilt pivot point were done first. Then the VPP was charged for several minutes to produce a phase shift around $\pi/4$ before taking tilt series. During data acquisition, we took a long exposure image of the focus area before collecting each tilt series, then measured the phase shift of the long exposure image by Gctf to monitor the phase shift change of the VPP spot. Once the phase shift value of the VPP spot was larger than $3\pi/4$, we switched to the next VPP spot.

8. Tomogram reconstruction.

Tilt series were first aligned using MotionCor2 (Version 1.1.0) and then assembled into the drift-corrected stacks by TOMOAUTO. Subsequently, tilt series were aligned using the IMOD (Version 4.9.2, USA), with fiducial markers or fiducial-free cross correlation, and reconstructed by Weighted Back-Projection

(WBP) and Simultaneous Iterative Reconstruction Technique (SIRT).

9. Tomogram segmentation.

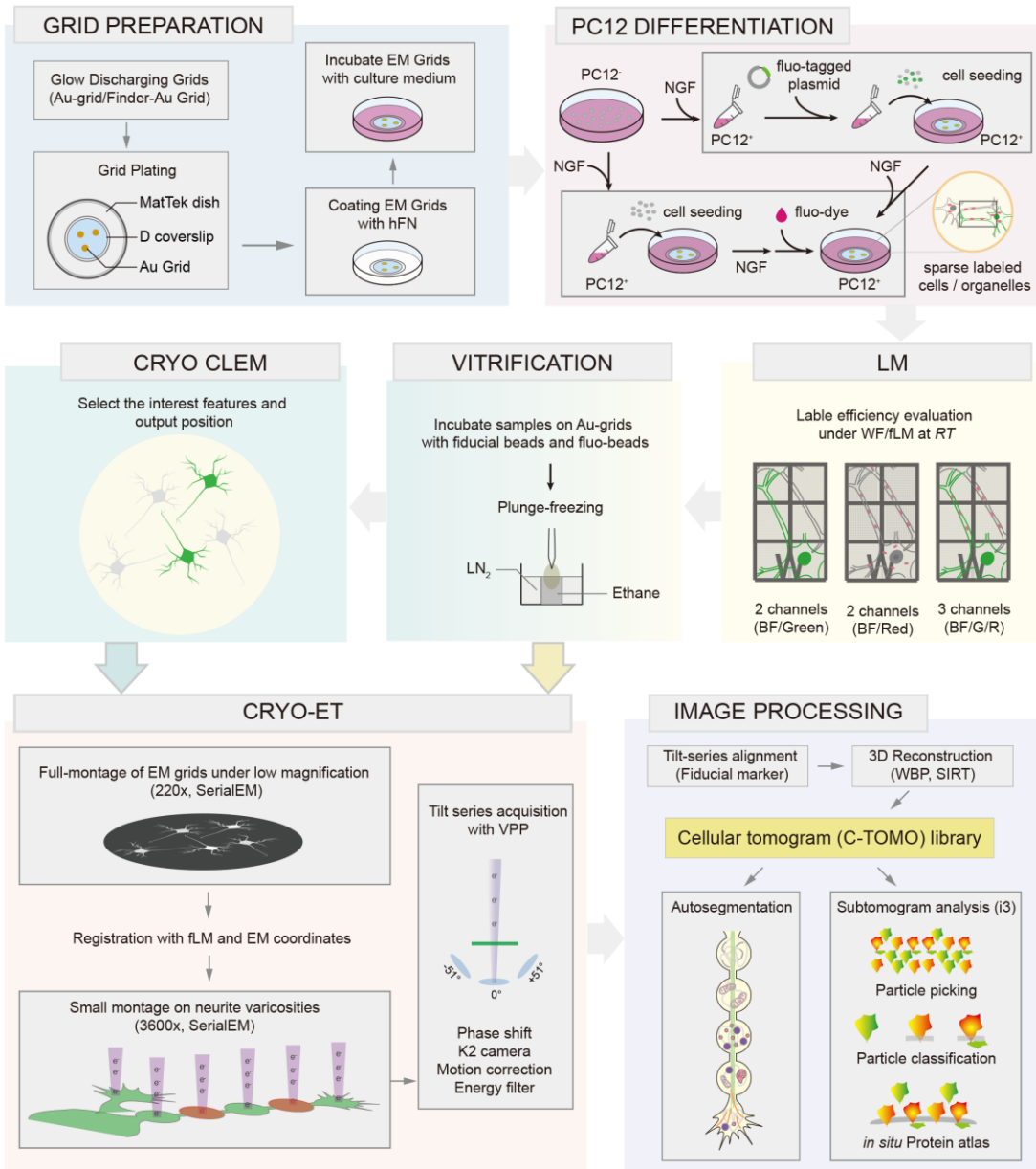
Cryo-ET features in image-processing package EMAN2.2 were used to generate 3D representations of cellular features. Segmentations of ER, mitochondria, and microtubules were performed using the convolutional neural network (CNN) based segmentation tool. Also, sub-tomograms containing ribosomes were selected and extracted using the CNN-based segmentation tool. A total of 1,658 2' 2' 2 binned sub-tomograms, each containing a single ribosome extracted from the tomogram shown in Figure 5, were averaged and mapped back into their original positions. UCSF Chimera and UCSF Chimera X were used to edit and visualize segmentations in 3D.

10. Sub-tomogram averaging.

Tomographic package I3 (0.9.9.3) was used to generate sub-tomogram average structures of xxx. A total of xxx (120' 120' 120 voxels) were identified and extracted from xx tomograms reconstructed using WBP method as described previously. Initial Euler angles were estimated based on the orientation of xxx. Initially, a region containing a xxx was targeted for iterative alignment and classification to remove xxx. Consecutive classifications focusing on the cytoplasmic region resulted in the identification of different sub-assemblies of xxxx.

11. Resolution estimation of average structures.

Resolutions of the structures were estimated as single volume inputs using ResMap.



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