Protocol for cryo-electron tomography of thinned synapses

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Abstract

Here we provide a protocol for cryo-electron tomography (cryo-ET) of thinned synapses within intact rat primary neuron cultures. This workflow relies on cryo-focused-ion-beam (FIB) milling to enable unrestricted access to synapses within neuronal cultures and to achieve samples sufficiently thin (~150 nm) for high resolution cryo-ET imaging. This protocol allows targeting of synapses with and without cryo-fluorescence light microscopy (cryo-FLM) correlation for FIB milling and cryo-ET.

1. Culturing primary rat hippocampal neurons on EM grids

Preparation of the EM grids

Materials: EM grids (e.g. SiO_2 film on Au mesh, R1/2 or R2/2, Quantifoil); 35 mm glass bottom dishes (e.g. Thermo Fisher Scientific); 35 mm dishes with 4 inner rings (e.g. Greiner Bio-One); 1 mg/mL poly-L-lysine in borate buffer; sterile water; plasma cleaner (e.g. PDC-32G-2, Harrick Plasma Cleaner); UV light

1. Glow discharge the EM grids (SiO_2 film on Au mesh, R 1/2 or R 2/2, Quantifoil) with a plasma cleaner (30 s, medium voltage) followed by 30 min under UV light.

2. Place 4 grids in a 35 mm dish. Glass bottom dishes or dishes with 4 inner rings can be used.

3. Coat the EM grids with 1 mg/mL poly-L-lysine 2 h at 37 °C and 5 % CO_2.

4. Wash 3 times with sterile water and keep the grids in sterile water at 4 °C before use.

Primary hippocampal neuron co-cultures with glia

Mixed primary hippocampal cultures are prepared according to the Goslin, Asmussen and Banker protocol (Goslin et al., 1998) and comprise both neuronal and glial cells (mainly astrocytes and microglia). The preparation of these cultures on EM grids is rather simple and fast since the protocol does not deviate much from the conventional preparation on coverslips. However, many glial cells may be present which can be a disadvantage for certain applications (i.e. when live staining for markers that are present in both neurons and glia). Therefore, we also provide a protocol for the preparation of neuronal cultures with almost no glial cells (see accompanying protocol by Domart et al.).

Materials: DMEM supplemented with 10 % FCS, 2 mM L-glutamine and 1 % penicillin/streptavidin (DMEM10%FCS); Neurobasal plus medium supplemented with 2 % B27 plus and 1 % Glutamax

1. Prepare a hippocampal cell suspension from E19 rat embryos.

2. Dilute the hippocampal cell suspension to a concentration of 200-300,000 cells per mL in DMEM10%FCS.

3. For the glass bottom dish, replace the water from the dish with 500 µL of pre-warmed DMEM10%FCS. For the dishes with the 4 inner rings immediately plate the cells after removing the water.

4. Plate 100 µL of cell suspension dropwise on each EM grid. Incubate at 37 °C and 5 % CO_2.

5. After 2 h, add 500 µL of pre-warmed DMEM10%FCS per dish.

6. Incubate overnight at 37 °C and 5 % CO_2.

7. The following day, replace the medium with 2 mL of pre-warmed Neurobasal plus medium supplemented with 2 % B27 plus and 1 % Glutamax.

2. Live staining with Synaptotagmin1 antibodies

Live labelling of neuronal cultures with antibodies directed against the luminal domains of Synaptotagmin1 (α-Syt1-ATTO647N) stains actively recycling pre-synapses and facilitates targeting synapse rich regions for FIB milling. α-Syt1-ATTO647N is taken up into the pre-synaptic terminal during synaptic transmission upon synaptic vesicle (SV) recycling (Fig. 1A). Once a SV fuses with the pre-synaptic membrane, the luminal domain of Synaptotagmin1 is exposed to the extracellular space until the SV is recycled (Rizzoli, 2014). During this time, antibodies can bind. Therefore, live Synaptotagmin1-staining can be used to label the pool of actively recycling synapses (Hua et al., 2011; Kraszewski et al., 1995; Truckenbrodt et al., 2018; Wilhelm et al., 2010).

Materials: Synaptotagmin1 antibody luminal domain coupled to ATTO647N (α-Syt1-ATTO647N, #105 311AT1, Synaptic Systems, c=1mg/ml); Neurobasal plus medium supplemented with 2 % B27 plus and 1 % Glutamax

Note: Live fluorescent labelling is performed immediately prior to vitrification by plunge freezing. When adding or removing medium from culture dishes containing EM grids with primary hippocampal cultures, be careful not to move or flip the grids and pipet slow and gentle.

1. Dilute α-Syt1-ATTO647N at 1:500 in medium from the culture dish containing EM grids.

2. Remove all the remaining medium from the culture dish and replace by the diluted antibody in culture medium.

3. Incubate for 30-45 min at 37 °C and 5 % CO_2 to allow the uptake of α-Syt1-ATTO647N into pre-synapses.

4. Wash twice with Neurobasal plus medium supplemented with 2 % (v/v) B27 plus and 1 % (v/v) Glutamax, pre-incubated at 37 °C and 5 % CO_2.

3. Plunge freezing

Materials: 5 % glycerol in Tyrode solution; automated or manual plunger

1. Remove all the medium and replace it by 5 % (v/v) glycerol in Tyrode solution pre-warmed at 37 °C and incubate for 2-5 min.

2. Immediately after glycerol incubation, plunge freeze grids in a mixture of liquid ethane-propane (37 % ethane, 63 % propane) cooled at -195 °C.

4. Cryo-FLM for identification of synapse rich regions

Microscope: cryo-light microscope (here: THUNDER Imager EM Cryo CLEM, Leica)

1. Acquire an overview of the grid in both bright field and fluorescence channels.

2. Identify squares with cell bodies using bright field and fluorescence data (Fig. 1B).

Note: α-Syt1-ATTO647N-positive puncta localize around the cell body, as well as along the neuronal processes, and together with the outline of the cell body in the bright field channel, serving as a guidance for the identification of neuronal cell bodies.

3. Acquire z-stacks of the squares containing cell bodies deemed suitable for FIB milling (cell bodies should not be localized on the grid bar).

4. Overlay z-stacks (or maximum intensity projections (MIPs) of the z-stacks) with the scanning electron microscopy (SEM) images prior to FIB milling and position the lamella milling pattern according to α-Syt1-ATTO647N positive puncta around the cell body (Fig. 1C, D).

Note: The cryo-CLEM step prior to FIB milling may be omitted by experienced users, and lamellae milling patterns may be placed directly at regions near the cell body with a high synaptic density (neuropil, Fig. 2).

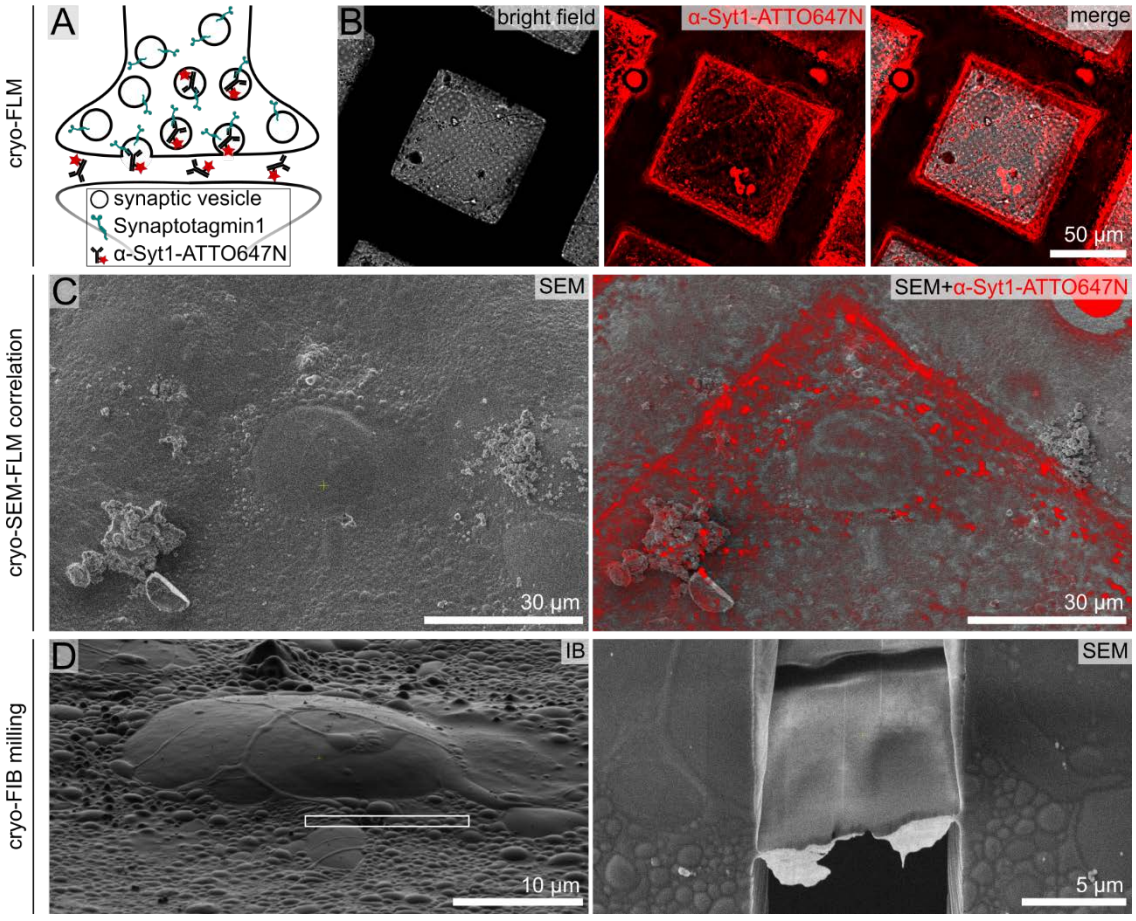

Fig. 1: (A) Schematic representation of α-Syt1-ATTO647N antibody uptake into the pre-synapse during live staining. (B) Representative cryo-FLM images of primary hippocampal cultures live stained with α-Syt1-ATTO647N, acquired with a Leica THUNDER Imager EM Cryo CLEM with a 50x objective (0.9 N.A.) at -195 °C. (C) Scanning electron microscopy (SEM) image of the same neuronal cell body as seen in *(B)* without (left) and with overlayed α-Syt1-ATTO647N-fluorescence (right). (D) Ion beam (IB) image showing the lamella positioning (white rectangle) following α-Syt1-ATTO647N fluorescence at the junction between neuronal cell body and grid surface (left) and SEM image of a fine milled lamella (right).

5. FIB milling

Microscope: cryo-FIB-SEM microscope (here: Aquilos 2, ThermoFisher Scientific)

For the general FIB milling procedure and image correlation using the MAPS software please refer to the following protocols (https://www.protocols.io/view/cryo-fib-milling-protocol-for-mammalian-cells-kqdg3xpyqg25/v1 and https://dx.doi.org/10.17504/protocols.io.btyinpue).

1. Identify neuronal cell bodies in SEM and ion-beam (IB) induced images. Neurons at DIV15 appear as bulges of 15-30 µm diameter with processes surrounding the soma (Fig. 2A).

2. Place the lamella milling patterns in front or to the side of the cell body (Fig. 2A), in the vicinity of the synaptic rich region. This avoids having the lamella going directly through the cell body, thus increasing the chance of capturing synapses (Fig. 2B, C, D).

3. Mill at an angle of 9-12 ° and a lamella width of 12-20 µm.

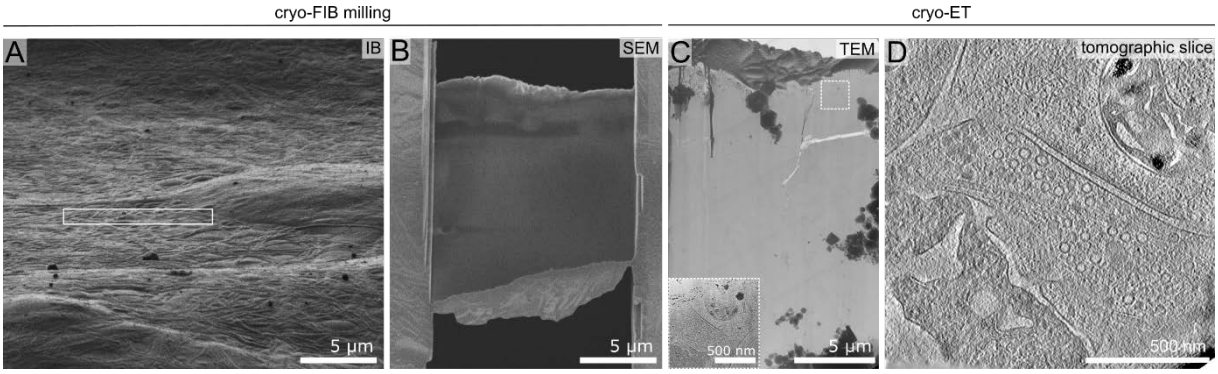

Fig. 2: (A) Lamella positioning (white rectangle) without fluorescence correlation. (B) Fine milled lamella corresponding to the position chosen in *(A)*, approximately 150 nm thin. (C) Low magnification cryo-transmission electron microscopy (TEM) overview of the same lamella as seen in *(B)* with a zoomed inset indicating the position chosen for tomogram acquisition based on synapse characteristics (as described in *6. Tomogram acquisition*) that can be identified on low magnification TEM overviews. (D) Tomographic slice corresponding to the reconstructed tilt series (tomogram) of the position indicated in *(C)* acquired at a pixel size of 2.94 Å/px with a 300 kV Krios G4 Cryo TEM from ThermoFisher Scientific.

6. Tomogram acquisition

Microscope: cryo-transmission electron microscope (here: 300 kV Krios G4 Cryo TEM, ThermoFisher Scientific)

For the general tomogram acquisition procedure please refer to the following protocol (dx.doi.org/10.17504/protocols.io.6qpvr3442vmk/v1).

Note: In general, the cumulative electron dose is kept around 120 $e^-/Å^2$. We acquire tomograms with a magnification of 2.94 or 1.89 Å/px in most cases.

Following tomogram reconstruction using the IMOD software (https://bio3d.colorado.edu/imod/doc/tomoguide.html), the mature synapses should contain the following characteristic features (see Fig. 2D):

(1) the presynaptic terminus should contain densely packed synaptic vesicles with an average radius between 30-40 nm. Often, one should be able to observe the "active

zone", the region containing tethered synaptic vesicles in the close proximity to the plasma membrane. Rarely, membrane fusion events can also be observed, whereby the synaptic vesicle membrane is connected to the plasma membrane. Very often, a synaptic vesicle cluster is associated with microtubules. Additionally, a clearly distinguishable mitochondrion is often seen. Depending on the lamella thickness, actin filaments are also evident.

(2) A synaptic cleft of approximately 20-30 nm containing dense material should also be apparent.

(3) Contrary to the presynaptic terminus, the postsynaptic region is relatively featureless. The postsynaptic density (PSD), often a characteristic feature in the conventional EM studies, is less apparent. The postsynaptic region often appears dense (the grey scale value is darker). Large macromolecular complexes like ribosomes or actin filaments are often present.

7. References

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