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ZAP and Freeze electron microscopy

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We use this protocol and it's working

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Abstract

This protocol describes freezing neurons under high pressure after stimulation and imaging the neurons under electron microscope afterwards. At first, hippocampal neurons were cultured form embryonic tissues, treated with drug for 7 days, frozen in high pressure freezer. After freeze substitution and embedding in Epoxy based resins, thin sections were cut, stained and imaged at 100000x magnification under electron microscope. Images were segmened blinded using FiJi and custom macro and data were analyzed in Matlab.

Materials

Reagents used
DNase I (DN25, Sigma)
Papain(LS003119, Worthington)
Cell strainer (22363548, Fisher Scientific)
Sapphire discs (616-100, Technotrade)
Conduritol B Epoxide (CBE) (15216, Cayman Chemical Company)
Pioloform (0.7% in chloroform,19244, Ted Pella)
Uranyl acetate (19481, Ted Pella)
Methanol (322415-1L Sigma-Aldrich)
Pelco slot grids, Cu (1GC12H Ted Pella)

Dissectionmedia:
1x HBSS (14175095, Gibco) containing 100 U/mL
penicillin-streptomycin (Thermo Fisher 15140122), 1 mM pyruvate (11360070,
Gibco), 10 mM HEPES (15630080, Gibco), 30 mM glucose (G6152-100G, Sigma)
NM5:
Neurobasal medium (21103049, Fisher scientific) containing 5% horse serum
(26050088, Gibco), 100 U/mL penicillin/streptomycin (Thermo Fisher 15140122),
1% GlutaMAX (35050061, Gibco) and 2% B27 supplement (17504044, Gibco).
NeurobasalA media:
Neurobasal A (Fisher Scientific 10888022) containing 2%
B27+ (GibcoA3582801),
1% GlutaMax (35050061, Gibco) and 0.2% penicillin-streptomycin (Thermo Fisher 15140122),.

Physiologicalsaline solution :
140 mM NaCl, 2.4 mM KCl, 10 mM glucose, 10 mM HEPES, (pH 7.5) containing 2 mM Ca^{++} and 3 mM Mg^{++}, 3 µM NBQX (1044, Tocris) and 30 µM Bicuculline methobromide (0109/10, Tocris)

Freezesubstitution solution A:
1% glutaraldehyde (16530, EMS) and 0.1%
tannic acid (403040-100G, Sigma) in anhydrous acetone (100503-622, EMS)

Freezesubstitution solution B:
2% osmium tetroxide (19132, EMS) in anhydrous acetone (100503-622, EMS)
Epon-Araldite 6.2 g of Eponate 12 (18005, Ted Pella), 4.5 g of Araldite (18060, Ted Pella),
12.2 g of DDSA (18022, Ted Pella), 0.8 ml BDMA (18241, Ted Pella)

Hippocampal neuron culture

1 Embryonic day 18 mouse embryos were decapitated and the hippocampi from each brain dissected on ice in 14 ml dissection media.

2 After all the dissections were done, 12 ml of the dissection media was discarded.

3 DNase I (0.01% final concentration) and 10 U/mL papain were added and incubated at 37° C with gentle shaking every 5 min for 20 min.

4 Dissociation media was replaced with 2 ml prewarmed NM5. The tissue was dissociated by gentle titration using three fire polished Pasteur pipettes with decreased opening.

5 The dissociated neurons were filtered through a 70 µm cell strainer and sedimented at 120 *g* for 2 min and gently resuspended in NM5 using a cut tip 1 ml pipette tip.

6 The cells were seeded onto sapphire discs at 125,000 cells/well in Neurobasal A media containing 2% B27+, 1% GlutaMax and 0.2% penicillin-streptomycin.

7 On DIV7 (days in vitro), CBE (10 uM final concentration) was added to the cultured neurons.

ZAP and Freeze

8 ZAP and freeze were performed in EM-ICE manufactured by Leica Microsystem. The system was cooled down with liquid N2 as per manufacturer protocol.

9 On DIV 14 each sapphire disk containing neurons was first immersed in physiological saline solution then was placed in a photoelectric middle plate and assembled in between two half cylinders.

10 The cells were stimulated at 10 Hz for 200 action potentials with a field strength of 10 V/cm and frozen immediately under high pressure.

11 For unstimulated control, the photoelectric middle plate was programmed not to discharge.

12 After freezing, the samples were dropped into a container filled with liquid N2 for temporary storage.

Automated Freeze-substitution

13 Automated freeze-substitution was performed using an EM AFS2 system (Leica Microsystem)

14 AFS2 was filled with liquid N2 and chilled at −90°C prior to use.

15 Freeze substitution solution A was prepared and chilled inside the AFS2 at least for 30 min.

16 A small amount of anhydrous acetone was also chilled inside the AFS.

17 Frozen sapphire disks sandwiches were transferred to a container filled with liq N2 and middle plate was separated from the sandwich.

18 Middle plate was transferred to AFS in a container with liq N2 and dropped in the pre-chilled acetone.

19 Sapphire disk was removed from the middle plate and transferred to freeze substitution solution A and kept at −90° C for at least 36 h.

20 Disks were washed six times in acetone at −90° C, each wash was for 30 minutes.

21 Then freeze substitution solution B was added and temperature was maintained at −90°C for at least 11 hours.

22 The temperature was then increased by 5° C h^{-1} to −20° C, maintained for 12 h at -20° C, and then increased by 10° C h^{-1} to 4° C.

Infiltration and Embedding in Epon-Araldite

23 Freeze-substituted samples were washed six times with anhydrous acetone in room temperature.

24 100 % Epon-araldite was prepared.

25 Samples were sequentially infiltrated in 30% and 70% Epon Araldite for 2 hours and in 90% Epon-Araldite overnight.

26 Next day samples were transferred to 100% Epon-Araldite. After two changes, samples were embedded in 100% Epon-Araldite in beem capsules and cured at 60° C for 48 h.

Sectioning and imaging

27 EM samples were mounted on a dummy block and trimmed with glass knife.

28 40 nm thin sections were cut with an ultramicrotome (UC7, Leica Microsystems) using diamond knife and collected on a pioloform (0.7%) coated single slot copper grid.

29 The sections were stained with 2.5% uranyl acetate in 50% methanol. Sections were washed with 50% methanol followed by water.

30 Electron micrographs were collected in a Hitachi 7600 TEM equipped with AMT XR50 camera
run on AMT Capture v6 (pixel size = 560 pm) at 80 kV on the 100,000x setting.

31 To remove bias, imaging was performed blind to condition and ~100 images were collected per sample.

Image and data analysis

32 For image analysis SynapseEM custom macro and FIJI were used. See https://www.frontiersin.org/journals/synaptic-neuroscience/articles/10.3389/fnsyn.2020.584549/full for
step by step analysis.

33 After annotations, data were analyzed using MatLab (Mathworks) using custom scripts. The
macros and scripts are available via
https://github.com/shigekiwatanabe/SynapsEM.