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Cytochemical staining of endocytosed HRP, flat embedding

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Merja Joensuu[1]

[1]The University of Queensland

Neuronal b-oxidation

Merja Joensuu
The University of Queensland

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We use this protocol and it's working

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Abstract

In this protocol, neurons are pulse-chased with horseradish peroxidase (HRP) to induce activity-dependent uptake of HRP into endocytic compartments such as synaptic vesicles and endosomes. HRP is visualised via diaminobenzidine cytochemistry followed by osmium tetroxide, producing a dark brown, electron-dense precipitate within labeled structures. After staining, neurons are flat-embedded in LX112 directly on the plastic culture dish.

Materials

- Horse radish peroxidase (HRP)
- 2% Paraformaldehyde
- 1.5% Glutaraldehyde
- 0.1 M Sodium cacodylate (NaCac) buffer, pH 7.4
- 50 mM Tris buffer, pH 7.6
- 10 mg DAB tablet
- 30% H_2O_2
- 1% OsO_4
- 6% potassium ferrocyanide
- 2 mL Mq (Milli-Q water)
- 4% OsO_4
- 2% UA (uranyl acetate, aq)
- Ethanol (EtOH) series: 50%, 60%, 70%, 90%, 100%
- LX112 resin
- Beam capsules
- Equipment: microwave (MW), vacuum (VAC), ultramicrotome (e.g., Leica Biosystems, UC6FCS)

HRP-labelling of endocytic compartments

35m

1 For high potassium neuronal depolarisation, stimulate neurons in prewarmed (37°C) high K^+ buffer supplemented with 10 mg/ ml horse radish peroxidase (HRP) and incubate cell incubator at for 5 minutes at 37°C with 5% CO_2. For electric field stimulation assays, add 1 mg/ml HRP diluted in prewarmed (37°C) low K^+ buffer for the duration of high frequency stimulation (300 APs at 50 Hz for 6 s).

5m

1.1 High K+ buffer consists of 0.5 mM $MgCl_2$ (Chem-Supply, MA029), 2.2 mM $CaCl_2$ (Sigma-Aldrich, C5080), 56 mM KCl (Ajax Finechem, 1206119), 95 mM NaCl(Amresco, X190), 5.6 mM D-glucose (Amresco, 0188), 0.5 mM ascorbic acid (Sigma-Aldrich, A5960), 0.1% BSA (Sigma-Aldrich,
A7638), 15 mM HEPES (Gibco, 15630-080), pH 7.4, 290–310 mOsm.

1.2 Low K^+ buffer consists of 0.5 mM $MgCl_2$ (Chem-Supply, MA029), 2.2 mM $CaCl_2$ (Sigma-Aldrich,
C5080), 5.6 mM KCl (Ajax Finechem, 1206119), 145 mM NaCl (Amresco, X190),
5.6 mM D-glucose (Amresco, 0188), 0.5 mM ascorbic acid (Sigma-Aldrich, A5960),
0.1% BSA (Sigma-Aldrich, A7638), 15 mM HEPES (Gibco, 15630-080), pH 7.4
290–310 mOsm

2 Wash away all free HRP from the plates using prewarmed (37°C) low K^+ buffer.

3 Chase the HRP uptake in neurons for 10 or 30 minutes in low K^+ buffer in a cell incubator (37°C with 5% CO_2).

30m

Cytochemical staining of HRP

50m

4 Fix cells with 2% Paraformaldehyde (Electron Microscopy Sciences, 15710), 1.5% Glutaraldehyde (Electron Microscopy Sciences, 16210) in 0.1 M Sodium cacodylate (NaCac, Sigma-Aldrich, C0250) buffer pH 7.4 for 20 minutes at room temperature. NOTE! Use EM-grade, fresh, fixatives only and perform the liquid exchange in a fume hood.

20m

5 Wash 2 times with 0.1 M NaCac buffer, á 3 min. Perform the liquid exchange in a fume hood.

6 Wash 3 times with 50 mM Tris buffer, pH 7.6, á 5 min. Perform the liquid exchange in a fume hood.

7 Incubate in DAB solution (with peroxide) for 30 minutes, room temperature, covered from light 30m
- Dilute 10 mg DAB tablet (Sigma-Aldrich, D4418) into 10 ml of 50 mM Tris-buffer, pH 7.6
- Just before use add 7 µl of 30% H_2O_2 to 10 ml of diluted DAB-solution to final concentration of 0.021%.
NOTE: keep DAB solutions covered from light all the time!

8 Wash 3 times with Tris buffer, á 5 min.

9 Wash 2 times with 0.1 M NaCac buffer. Perform the liquid exchange in a fume hood.

Embedding monolayers in resin using Biowave (Pelco) 50m

10 Osmicate using Reduced 1% osmium Tetroxide (OsO_4), 1.5% potassium ferrocyanide ($K_4[Fe(CN)_6]$) diluted in Millli-q water. Perform the liquid exchange in a fume hood.
Biowave settings: MW 80 W, 2 min ON – 2 min OFF – 2 min ON, VAC on

11 Repeat step 10

12 Water wash:
Biowave settings: MW 250 W, 40 s No VAC. Perform the liquid exchange in a fume hood.

13 Contrast with 2% uranyl acetate (UA) (aq):
Biowave settings: MW 150 W, 1 min ON – 1 min OFF – 1 min ON, VAC on (optional).
Perform the liquid exchange in a fume hood.

14 Dehydration in a fume hood (no Biowave usage):
- 50% EtOH 1min
- 60% EtOH 1min
- 70% EtOH 1min
- 90% EtOH 1min
- Twice: 100% EtOH 1min

15 1st embedding step: 1:1 (v/v) of 100% EtOH: LX112 resin. Perform the liquid exchange in a fume hood.
Biowave settings: MW 250 W, 3 min VAC on

16 2st embedding step: 100% LX112. Perform the liquid exchange in a fume hood.
Biowave settings: MW 250 W, 3 min VAC on

17 3rd embedding step: 100% LX112. Perform the liquid exchange in a fume hood.
Biowave settings: MW 250 W, 3 min VAC on

18 Place beem capsules filled with 100% LX112 upside down on top of the neurons in a fume hood.

19 Polymerization at 60°C 48 h
- Samples are polymerized immediately, no need to wait for 2 h

Thin sectioning

20 Trim 1 mm x 1 mm pyramid, and thin section (80-90 nm) samples using an ultramicrotome (such as Leica Biosystems, UC6FCS).

TEM imaging

21 For HRP-analysis, TEM images are acquired randomly when HRP signal is observed.

Quantification

22 The quantification of the number of HRP-containing synaptic vesicles and endosomes is quantified with Adobe Photoshop Count Tool, and the size (sectional area, μm^2) of the HRP-stained endosomes is quantified manually using ImageJ/Fiji (**https://imagej.nih.gov/ij/**)