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Preparation of organotypic cerebellar cultures

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DISCLAIMER

ABSTRACT

This protocol describes the basics to prepare organotypic cerebellar cultures using the membrane interface method.

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KEYWORDS

organotypic cultures, brain, cerebellum, neural development, immunocytochemistry, live imaging

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GUIDELINES

All procedures must be carried out under sterile conditions.

MATERIALS TEXT

Pentobarbital sodium **Sigma Aldrich Catalog #Y0002194**

D-( )-Glucose **Sigma-aldrich Catalog #G8270**

L-Ascorbic acid **Sigma – Aldrich Catalog #A92902**

Pyruvic acid **Sigma-aldrich Catalog #107360**

N-Methyl-D-glucamine **Sigma-aldrich Catalog #M2004**

Sodium bicarbonate **Sigma Aldrich Catalog #S5761**

Potassium chloride **Sigma Aldrich Catalog #P3911**

Sodium phosphate monobasic **Sigma – Aldrich Catalog #S0751**

Calcium chloride **Sigma – Aldrich Catalog #C1016**

Magnesium chloride **Sigma – Aldrich Catalog #M8266**

Basal Medium Eagle **Sigma – Aldrich Catalog #B9638**

Horse serum **Sigma – Aldrich Catalog #H1138**

Hanks' Balanced Salt solution **Sigma – Aldrich Catalog #H6648**

L-Glutamine solution **Sigma – Aldrich Catalog #G7513**

Antibiotic Antimycotic Solution (100×) Stabilized **Sigma – Aldrich Catalog #A5955**

McIlwain Tissue Chopper with Petri Dish Modification

Tissue chopper

Campden Instruments **Model TC752-PD**

Stereo microscope EZ4, Leica 10447197

Leica EZ4 educational stereomicroscope.

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Certomat CS-18
CO2 incubator
Sartorius **BBI-8863385**

Dissecting tools: universal scissors, fine scissors straight and curved, Adson forceps, student anatomical standard pattern forceps, Dumont #7 forceps, gross anatomy blade (#20) and handle (#4), straight and curved spatulas, razor blades.

SAFETY WARNINGS

Be sure to turn off the UV light when using the laminar flow hoods.

DISCLAIMER:

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BEFORE STARTING

Be sure to have all your tool and solutions ready. Working areas must be clean and sterilized with UV ligh or 70% ethanol.

Preparation of solutions/culture medium 1h 45m

1 Solutions

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1.1 ***Stock solutions:*** [M]**1 Molarity (M)** $CaCl_2$; [M]**1 Molarity (M)** $MgCl_2$; [M]**5 % volume** pentobarbital sodium in ddH_20 30m

1.2 ***Cutting solution composition:*** [M]**130 millimolar (mM)** n-methyl-D-glucamine Cl (NMDG); [M]**24 millimolar (mM)** $NaHCO_3$; [M]**3.5 millimolar (mM)** KCl; [M]**1.25 millimolar (mM)** NaH_2PO_4; [M]**0.5 millimolar (mM)** CaCl2; [M]**5 millimolar (mM)** $MgCl_2$; [M]**10 millimolar (mM)** D-(+)-glucose; [M]**1 mg/mL** ascorbic acid; [M]**2 mg/mL** pyruvic acid.

1.3 ⚠ 1h

Cutting solution preparation: To make **1 L** , pour **850 mL** double-distilled water into a volumetric flask. Add **25.38 g** NMDG, **2.017 g** NaHCO3, **261 mg** KCl, **172 mg** NaH_2PO_4, **1.80 g** D-(+)-glucose, **1 g** ascorbic acid, **2 g** pyruvic acid. After complete dissolution SLOWLY add **5 mL** $MgCl_2$ stock solution and **500 µL** $CaCl_2$ stock solution. pH**7.2** to pH**7.4** with HCl.

> The addition of $MgCl_2$ and $CaCl_2$ is a critical step. If $MgCl_2$ and $CaCl_2$ are added too quickly they precipitate making the solution cloudy. In this case, it must be discharged.

Steril filter and store at **4 °C** . The solution is stable for several months. Discharge if it becomes turbid.

2 Culture medium

2.1 ***Culture medium composition:*** 50% Basal medium Eagle (BME), 25% horse serum; 25% Hank's balanced salt solution (HBSS); 0.5 % D-(+)-glucose; 0.5% L-glutamine (200 mM solution); 1% antibiotic antimycotic solution (100 x)

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2.2 ***Culture medium preparation***: Work under a laminar flow hood and use sterile glassware/plasticware. In a 100 mL cylinder add the components in the order indicated above. For 50 mL: **25 mL** BME, **12.5 mL** horse serum; **12.5 mL** HBSS; **250 µL** D-(+)-glucose; **250 µL** L-glutamine; **500 µL** antibiotic antimycotic solution. Transfer in a glass bottle and protect from light with aluminum foil. Store at **4 °C** . Medium is stable for at least six months. Discharge if color changes and/or it becomes turbid. 15m

Tissue sampling 30m

3 Have ready the following: ice-cooled cutting solution; **50 mL** sterile glass or plastic becker; 150 mm diameter sterile glass or plastic Petri dishes; sterile dissection/slice handling tools; sodium pentobarbital stock solution (**Room temperature**); **500 µL** disposable insulin syringes; sterile razor blades; sterile glass/disposable Pasteur pipettes; sterile filter paper dishes.

Dissection of the brain and separation of individual slices after cutting (see Slice seeding below) should be carried out under sterile conditions as far as possible. If it is not possible to place the stereomicroscope under the laminar flow hood, dissection should be carried out under a simple plastic box opened in the front. The entire dissecting area should be clean and wiped off with **70 % volume** ethanol.

4 Euthanize mice at the required post-natal age with an overdose of intraperitoneal sodium pentobarbital (**60 mg** / **100 g** body weight). 5m

5 Quickly remove the brain from the skull while the head is kept submerged in the ice-cooled cutting solution and isolate the cerebellum under the stereomicroscope. 10m

During the production of slices, all procedures must be carried out in an ice-cold cutting solution.
To keep the temperature a few degrees above °C during the dissection, prepare some blocks of frozen cutting solution to be added to the **4 °C** chilled solution contained in the Petri dish used to dissect the brain.

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6 Before cutting, completely remove the meninges with a pair of N.7 Dupont forceps. Place the cerebellum on the stage of the tissue chopper within a drop of the ice-cooled cutting solution. Operate the chopper and cut 350 µm-thick parasagittal slices. Once terminated slicing, collect slices with a curved spatula (they are usually stuck together) and place them in a sterile 50-mm Petri filled with the ice-cooled solution. Store at **4 °C** until ready to separate slices. Separate individual slices under the stereomicroscope with a spatula and a needle, trying not to damage the tissue. During the entire procedure, slices must be submerged in the ice-cooled cutting solution. Discharge damaged slices. 15m

If the cerebellum is not submerged by an excess of cutting solution, cutting with the chopper is easier. Set section thickness to any value between **200 µm** - **400 µm** after wiping out the solution with a piece of filter paper. Other cutting parameters, such as blade force, must be adjusted based on the type of chopper in use.
Use a spatula with curved edges to collect slices and transfer them from the cutting stage of the chopper to the Petri dish.

Slice seeding 1h

7 Before starting to seed slices onto the Millicell inserts bring the culture medium at **Room temperature** and fill some sterile 35-mm plastic Petri dishes with **1.1 mL** medium. Work under sterile conditions.

The number of dishes required depends on the number of recovered slices. In general, working with the mouse post-natal cerebellum each slice is about 5 mm^2. Therefore, one can easily plate 5-6 slices/insert. Working with older animals or on larger areas of the brain, i.e. the cerebral cortex allows plating a maximum of (roughly) 3 slices/insert.

8 Collect slices one by one and carefully lift them onto the dry Millicell membrane. Once the required number of slices has been plated in the insert, place it inside a 35-mm Petri dish containing the required amount of medium. Be careful to avoid air bubbles forming between the insert membrane and the medium, i.e. check that the membrane's lower surface is completely wet. 1h

9 Incubate at **34 °C** in **5 % volume** CO_2 for up to 30 days in vitro (DIV). Medium has to be changed twice a week. Allow slices to equilibrate to the in vitro conditions for at least 4-6 DIV before treatments (if applicable).

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Slices obtained from cerebellum (and other CNS areas) survive better at temperatures below 37 °C , hence the temperature settings of the incubator are important for culture survival.
However, it should be noted that the neuroprotective effect of mild hypothermia on cultured neurons may obscure the action of certain apoptotic inductors if one is interested in the study of cell death.

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