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Primary Culture of Mouse Mesencephalic Neurons

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ABSTRACT

This protocol details the procedure required for the dissection and collection of primary mouse culture mesencephalic neurons.

ATTACHMENTS

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MATERIALS TEXT

See *Before Start* section for all materials, product numbers, and volumes.

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

ABSTRACT

This protocol details the procedure required for the dissection and collection of primary mouse culture mesencephalic neurons.

BEFORE STARTING

Preparation of solutions for dissociation: *all solution must be prepared fresh*

Papain solution: (5 ml of solution for a maximum of 5 brains and/or region / dissection)

- **2.2 mg** of Cysteine HCl (Sigma C-6852; 2.5 mM; **Room temperature**)
- dissolve in **5 mL** of dissociation solution (stored at **4 °C**)
- adjust the pH to ~7.4 with NaOH 1 N

- add 100 Papain Units[1] (Worthington LS003126; stored at **4 °C**) (volume " x " µl of the stock according to its concentration in Units/mgP)
- incubate at **37 °C** for **00:15:00** without shaking to facilitate activation and solubilization
- filter sterilize using a 0.2 µm/28 mm syringe filter (Sartorius 14555306, SFCA membrane, blue). Use it immediately: the activity decreases with time – if not used within **00:30:00**, trash

Trituration solution: (20 ml of trituration solution for 10 brains)

- **20 mL** of Neurcell+ (**37 °C**)
- **20 mg** of trypsin inhibitor[2] (Sigma T-9253; **4 °C**)
- **20 mg** of BSA (Sigma A-7030; **4 °C**)
- **47.66 mg** of HEPES[3] (Sigma H-3375; 10 mM; **Room temperature**)
- adjust the pH to 7.4 with NaOH 1 N
- filter sterilize and store at **37 °C**

Centrifugation solution: (5ml of solution for a maximum of 5 brains and/or region / dissection)

- **5 mL** of Neurcell+ (**37 °C**)
- **50 mg** of BSA (Sigma A-7030; **4 °C**)
- **11.9 mg** of HEPES (Sigma H-3375; 10 mM; **Room temperature**)
- adjust the pH to 7.4 with NaOH 1 N.
- filter sterilize and store at **37 °C**

*** Prepare a tube with the dissociation solution to rinse the tissues after incubation in papain – this tube also serves as a reference tube for adjusting the pH of the other solutions (the phenol red is pH sensitive, so it allows you to compare the pH). ***

Remember to sterilize enough 35mm petri dishes and Whatman filters in the morning of dissection and leave them in the hood for transfer of coverslips for seeding of neurons.

[1]Papain is a cysteine protease that catalyzes the cleavage of peptide bonds

[2]Protease inhibitor blocks the activity of proteolytic enzymes

[3]Used as a buffer solution and preferred over bicarbonate buffer for cell culture due to better stability at physiological pH

Dissection (preferably in pairs)

1 Prepare two containers of crushed ice.

2 Clean the dissection surface with 70% alcohol. Place the dissection tools in a beakers filled with 70% alcohol and a Kimwipe (to protect the tips of the tools).

3 Prepare **1 petri** dish with **2 mL dissociation medium** for **2 animals (n times)**. Keep **On ice**.

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4 Prepare **1 petri** with **2 mL dissociation medium** for **5 animals (n times)** to collect the tissue blocks. Identify the petri and keep **On ice** .

5 Prepare one 10 ml syringe per series of 5 animals (n times) with dissociation solution. Keep **On ice** .

6 Place the animals **On ice** . Wait **00:02:00** to **00:03:00** (until they are anesthetized). Put a maximum of 5 pups on ice at a time. Once the dissection begins, add the subsequent series of pups. 5m

7 The first person removes the brains from the skulls once the animals are anesthetized (nonresponsive to manipulation and paw pressing). Wipe the skull and neck area with 70% alcohol and dry with a Kimwipe.

8 Hold the animal by the skin under the throat. Cut the skin around top of the skull (starting behind the ear). Take care not to damage the brain, keep the tips of the scissors facing upwards.

9 Remove the cut part of the skin and the skull with the curved forceps (pointing upwards).

10 Rinse the brain thoroughly with cold dissociation medium (2 ml per animal).

11 Using the curved forceps gently remove the brain and place it in a petri dish (start at the olfactory bulbs and finish by cutting the base of the brain stem).

12 The second person, using a binocular magnifying glass, dissects the isolated brains, one by one, preparing the slices and tissue blocks: Place the brain with the ventral region up and hold it gently with the thin forceps at the frontal lobe.

13 Using the scalpel blade, cut a thin coronal slice of the brain at the "midbrain flexure"; using the Willis circle as a reference (slice 1 mm thick).

14 Isolate the VTA and substantia nigra with a scalpel.

15  40m

In the sterile hood, using a 10 ml sterile pipette, transfer the blocks of tissue obtained from a maximum of 5 brains into the 15 ml tube containing papain solution #1, taking care to transfer as little dissociation solution as possible with the tissue blocks, to reduce papain dilution. Incubate with stirring for **00:20:00** at **37 °C** . Quickly continue dissecting the brains of the other mice, transferring the resulting tissue blocks into the tubes containing papain solution #2, #3 and #4 (as needed) and incubate each with agitation **00:20:00** at **37 °C** .

Important: Keep the tissue blocks and solutions on ice for as long possible and perform the entire dissection, from the first skin perforation to the last tissue block being placed in papain **as quickly as your dexterity allows**. The health of cells and their survival depends entirely on this. The procedure for brain extraction and block cutting should be 1 minute or less, per animal.

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Dissociation of Cells 1d 3h 25m

16 Prior to starting, flame polish the tip of a 5 ml glass pipette to a diameter of 1.5 mm and a second to a smaller diameter (~0.5 mm, see annex 1).

17 20m

After 00:20:00 of agitation, replace the papain solution in tube #1 with 2 mL **dissociation solution** . Repeat this rinse a second time.

18

Rinse with 2 mL **trituration solution** , remove and add 1 mL **fresh trituration solution** .

19

Gently triturate 20 times with the 1.5 mm pipette and 40 times with the 0.5 mm pipette (**use a rubber bulb**).

Trituration is not complete if you still see pieces of tissue (cells suspension should be homogeneous without any piece of tissue). If you ask yourself why this might happen, the possible problems are: the size of the tissue blocks, inactivated papain, excessively long dissection time, etc. However, the following can be attempted: let the undissociated pieces fall to the bottom of the tube, transfer the cells in suspension into another sterile 15 ml tube, add 2 ml of trituration solution to the tube of undissociated pieces, and repeat the trituration a second time and finally pair the 2 tubes.

20 Repeat steps 17 to 19 with papain tube #2, #3 and #4 (as needed).

21 **Gently** transfer the dissociated cells, from the different tubes, on top of the centrifugation solution (no more than 2 ml of suspended cells per 5 ml centrifugation tube).

22 5m

Centrifuge for 00:02:00 at position 3 (1150 rpm), then 00:03:00 at position 4 (1400 rpm) on a clinical IEC centrifuge (the centrifuge tubes must be balanced, calibrate the centrifuge with a 15 ml tube filled with water if needed).

23 Remove the supernatant and resuspend the cells in a 500 µl **trituration medium** . Mix 10 µl **cell suspension** with 10 µl **Trypan blue (Gibco, 15250061)** , take 10 µl **of the mixture** and count the cell density using a hemacytometer. Add to the cell suspension the required volume of the trituration medium (supplemented with Neurocell+ if necessary) to achieve the desired concentration. For the volume to add according to the number of cells and the desired concentration, see the calculation table. Combine the tubes of the same cell type in a single tube.

Dilution of the Cell Suspension
by Nicolas Giguère
PREVIEW RUN

23.1 Re-suspend the cell pellet in 500 µl **trituration solution** .

23.2 Take 10 µl of the suspension and dilute it in 10 µl **Trypan Blue Solution (Gibco 15250061)** .

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23.3 Take **10 µl** of this mix to count on the hematocytometer.

23.4 Count the living cells and also the number of dead cells (colored in blue-black, the number should be less than 20% of the total number of cells).

23.5 $$V \text{ to add } = \left[\left(\frac{nb \text{ of cells counted} \times \text{dilution factor}}{\text{dial volume} \times \text{seeding concentration}}\right) \times \text{cell suspension volume}\right] - \text{cell suspension volume}$$

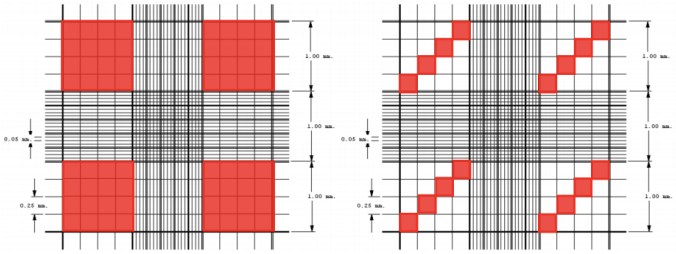
The volume of solution covering 1 millimeter squared of the hematocytometer (see the "How to Count the Cells" section) is 0.1 µl and the dilution factor is 2. If the concentration required for seeding is 240,000 cells/ml, the volume of trituration solution to be added to the 490 µl of cell suspension is:

$$V \text{ to add } = \left[\left(\frac{nb \text{ of cells counted} \times 2}{0.0001\text{ml} \times 240\,000 \text{ cells /ml}}\right) \times 0.490\text{ml}\right] - 0.490\text{ml}$$

# of living cells per quadrant	Volume of solution to be added (ml)	# of living cells per quadrant	Volume of solution to be added (ml)	# of living cells per quadrant	Volume of solution to be added (ml)
5	-0.285	**90**	3.185	**175**	6.655
10	-0.081	**95**	3.389	**180**	6.860
15	0.122	**100**	3.593	**185**	7.064
20	0.326	**105**	3.797	**190**	7.268
25	0.530	**110**	4.00	**195**	7.472
30	0.735	**115**	4.205	**200**	7.676
35	0.939	**120**	4.410	**205**	7.880
40	1.143	**125**	4.614	**210**	8.085
45	1.347	**130**	4.818	**215**	8.289
50	1.551	**135**	5.022	**220**	8.493
55	1.755	**140**	5.226	**225**	8.697
60	1.960	**145**	5.430	**230**	8.901
65	2.164	**150**	5.635	**235**	9.105
70	2.368	**155**	5.839	**240**	9.310
75	2.752	**160**	6.043	**245**	9.514
80	2.776	**165**	6.247	**250**	9.718
85	2.980	**170**	6.451		

23.6 To count your cell sample, dilute **10 µl of your sample** in **10 µl Trypan Blue Solution (Gibco 15250061)** and then load the hematocytometer.

23.7 The counting chamber is covered with a laser engraved grid. Quadrant lines make counting easier. Choose the appropriate quadrant size based on the density of the cells in your sample. For example, for a small number of cells (less than 20 in a quadrant of 16), count all the cells in the 4 quadrants of 16, and divide this number by 4. For samples with a quantity of more than 20 cells, choose the diagonal in the 4 quadrants of 16.



Regardless of the area you choose or the density of the sample, count at least 20 to 50 cells per quadrant.

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23.8

23.9 Not all cells will fall perfectly into the quadrants. For example, you can count the cells that touch the top and left lines but ignore the cells that touch the bottom and right lines. Use a cell counter to keep track of the number of cells counted.

23.10 The volume of solution covering 1 millimeter squared of the hematocytometer is 0.1 µl and the dilution factor is 2. If the concentration required for seeding is 240,000 cells/ml, the volume of trituration solution to be added to the 490 µl of cell suspension is:

$$V \text{ to add } = \left[\left(\frac{nb \text{ of cells counted} \times \text{dilution factor}}{\text{dial volume} \times \text{seeding concentration}} \right) \times \text{ cell suspension volume} \right] - \text{ cell suspension volume}$$

23.11 While counting cells under the microscope, assess the viability of the cells in your sample.

> Trypan blue is a dye used to determine viability. The living cell excludes this dye, but the dead cell has no intact membrane allowing the trypan blue to pass through and mark the cytoplasm. In phase contrast, living cells appear bright and golden and dead cells appear dull and blue. The number of dead cells should be less than 20% of the total number of cells.

23.12 When you have finished counting, clean the counting chamber.

24

Apply **65 µl cell suspension** to the coverslips covered with astrocyte monolayer. Remove 5 petri dishes at a time. Dry the bottom of the coverslips by placing them carefully one by one on a sterile Whatman filter paper. Place the coverslips in pre-sectioned (see annex 2) sterile dry petri dishes, and add the cell suspension at the desired concentrations as quickly as possible to avoid over drying the astrocyte monolayer. Do this step in pairs, the first person removes the coverslips from the old petri dishes, drying them on the Whatman filter paper, the second one recovers them and places them in a new petri, adding immediately **65 µl cell suspension** . Place the petri in the incubator.

> Do not allow the coverslips to touch the edges of the petri dish. Handle the petri dishes with great care – note that the droplet will behave differently if the surface is already wet or not. **When adding neurons to a coverslip with an astrocyte monolayer (that is moist) it is more likely to spread to the edges because of the effect of surface tension on the 65µl cell suspension droplet is reduced.**

25 3h

After **03:00:00** , add **2.5 mL culture medium (Mix Neurocell+/EMEM+ for dopamine neurons, Neurocell+ only if striatal cells)** to each petri dish and put back all the petris in the incubator.

26 1d

After **24:00:00** , add **[M]10 Micromolar (µM) FUDR** (from 2 mM stock; 12.5 µl/2.5 ml) to inhibit glial proliferation.

27

If necessary, following 7 days of culture, add **[M]0.5 Milimolar (mM) kynurenic acid** (from 125 mM stock, 10 µl/2.5 ml) to prevent the toxicity of glutamate release.

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If necessary, add **500 µl culture medium (Mix Neurocell+/EMEM+)** every 5 days to all the petri dishes to compensate evaporation and to feed the cells.

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