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Protocol status: Working
We use this protocol and it's working

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Embryonic/Postnatal Mouse Neuron Culture Protocol

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DISCLAIMER

All procedures need to be approved by the local Institutional Animal Care & Use Committee.

ABSTRACT

This protocol details the embryonic/postnatal neuron culture procedures.

PROTOCOL REFERENCES

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GUIDELINES

*Simplified protocol for neuron culture (hippocampal or cortical)
All procedures on live animals should be performed in accordance with your Institutional Animal Care and Use Committee.

MATERIALS

Need:

- Papain solution, neuron medium, Hibernate medium, Hank's Balanced Salt Solution (HBSS)
- 0.22 µm syringe filters and syringes or SteriFlip
- Dissecting instruments (sterilized with 70% ethanol):
  2 fine forceps and tooth forceps
  fine scissors
  2 large scissors
  spatula
  scalpel with size 23 blade
- Ice in bucket

Media and solutions

Neuron Media (NM)
250 mL

A	B	C	D
Ingredient	**Vendor and Cat #**	**Amount**	**Final Conc.**
Neurobasal Medium (embryonic) or Neurobasal A (postnatal)	Life Technologies; 21103049 or 10888-022	240 mL	
B27	Life Technologies; 17504044	5 mL	1%
GlutaMAX (200 mM)	Life Technologies; 35050-061	2.5 mL	2 mM
Penicillin/Streptomycin	Life Technologies; 15140-122	2.5 mL	1x

Sterile filter and store at 4°C in the dark.

Hibernate Media
250 mL

A	B	C	D
Ingredient	**Vendor and Cat #**	**Amount**	**Final Conc.**

	A	B	C	D
	Hibernate E (embryonic) or Hibernate A (postnatal)	Life Technologies; A1247601 or A1247501	240 mL	
	B27	Life Technologies; 17504044	5 mL	1%
	GlutaMAX (200 mM)	Life Technologies; 35050-061	2.5 mL	2 mM
	Penicillin/Streptomycin	Life Technologies; 15140-122	2.5 mL	1x

Sterile filter and store at 4°C in the dark.

Papain Solution

- *prepare ON the day of culturing. Needs at least an hour in the incubator to equilibrate. Needs to be warmed prior to filtering!*

	A	B	C	D
	Ingredient	**Vendor and Cat #**	**Amount**	**Final Conc.**
	1x HBSS	Life Technologies;14170-112	10 mL	
	Papain	Worthington Biochemical Corporation; LS003126	Varies (see calculation below)	20 U/mL
	L-cysteine		2 mg	0.2 mg/mL
	0.5M EDTA (pH 8.0)	Life Technologies; 15575-038	20 mL	1 mM

Papain needed: (20/(__u/mgP*__mgP/mL))*Total volume (mL)

50 mM Borate Buffer

	A	B	C	D
	Ingredient	**Vendor and Cat #**	**Amount**	**Final Conc.**
	Boric acid	Sigma; B6768	6.2 g	50 mM
	MilliQ H2O		2 L	

	A	B	C	D
	5M NaOH		Adjust to pH 8.5	

Sterile filter and store at room temperature.

Poly-D-Lysine

	A	B	C	D
	Ingredient	**Vendor and Cat #**	**Amount**	**Final Conc.**
	PDL hydrobromide	MP Biosciences; 25988-63-0	100 mg	2 mg/mL
	50 mM Borate buffer, pH 8.5		50 mL	

Sterile filter, aliquot 2.5 mL/tube and store at -20°C.

Reagents

Deoxyribonuclease I, Recombinant, Solution, Animal Free/AF **Worthington Biochemical Corporation Catalog #**LS006353

Coverslip and plate preparation

6h 31m

1 Drop 12 mm coverslips into a 200 ml beaker containing 95% Ethanol (or methanol). (Leave at least 00:01:00). 1m

2 Plate coverslips on a 24-well plate and allow to completely dry (> 00:30:00). 30m

3 Dilute PDL to 100 undetermined with borate buffer (2.5 mL PDL solution + 47.5 mL borate buffer) and coat coverslips with 0.5 mL PDL for 02:00:00 in the incubator or Overnight in the TC hood. Plastic plates can be coated similarly (100 µL for 96-well). 4h

4 Wash coverslips or plates 3-5x in tissue culture water. Remove all culture water from coverslips or plates, then allow to dry for 01:00:00 + in TC hood. 1h

5 Add neuron medium to plates and incubate at 37 °C in incubator > 01:00:00 to equilibrate (50 µL for 96-well, 0.5 mL 24-well, 1 mL 12-well, 2 mL 6-well). If using a 96-well plate, add 200 µL of PBS in periphery wells and 150 µL of PBS between wells. 1h

6 Equilibrate a T25 or T75 flask of neuronal medium in the incubator. Can also warm Neurobasal to Room temperature for wash steps.

Papain Preparation

7 Make the papain dissociation solution (see Media section). Each T25 flask can hold tissue from 1 to maximum 20 mouse preps. Sterile filter (syringe filter or Steriflip, depending on volume) and keep in T25 flask in the incubator.

Note

*Solution needs to be warmed prior to filtering.

Embryonic culture only:

8 Apply CO2 to dam as indicated in IACUC protocol.

9 Dislocate spine.

10 Pin dam, abdomen up, to Styrofoam covered with lab mat.

11 Spray dam with 70% ethanol thoroughly.

12 With dissection scissors, open the abdominal cavity, avoiding internal organs.

> **Note**
>
> These gross dissection tools should not also be used for fine dissection of the sterile embryos.

13 Grasp the uterus with forceps. Lift and cut the uterus out. Transfer to a 10 cm culture dish.

14 Cut the uterus and remove the embryonic sacs to release embryos into a fresh dish On ice .

Pup dissection (embryonic and postnatal): 20m

15 Decapitate pup, allowing head to fall into a 10 cm dish filled with HBSS On ice .

16 Using forceps, hold head by the eyes. Using the other hand and a curved or angled forceps, pinch the scalp just behind the eyes and pull back, tearing it off.

17 Next, use a micro-scissor to cut down the mid-sagittal skull, and gently peel the skull off with forceps, being careful in case any tissue still connects it to the rest of the skull. Gently remove brain (using a spatula or curved forceps) and place into Petri dish filled with ice cold sterile Hibernate Medium.

18 Gently separate hemispheres, removing the thalamus, striatum, and brainstem.

19 Grasp the olfactory bulb and pull caudal, slowly removing the meninges from the cortex. While still grasping the meninges, flip the hemisphere and remove meninges from the hippocampus.

20 Using micro-scissors, cut the hippocampus away from the cortex.

Note

Torn tissue makes for worse culture than cleanly-cut tissue due to excess DNA release.

21 Hippocampi can be left whole. If cortical tissue is to be used, it should be cut into 1 mm^3 segments.

22 Place all segments into a conical with Hibernate medium.

23 Bring to biosafety cabinet. Everything from here on should be done in a sterile biosafety cabinet. Pipette out Hibernate medium and rinse twice with fresh, Room temperature HBSS.

Papain Digestion

24 Use serological pipet to transfer chunks to papain and move to 37 °C incubator, laid flat.

25 Digest for approximately 00:05:00 - 00:20:00 or until chunks have fallen apart and look something like cotton candy. 25m

Trituration 5m

26 Add 50 µL [170 U] DNase/ 10 mL papain solution and gently rotate until pieces of tissue are separated.

> **Note**
>
> If needed, remove as much of this solution as possible, add fresh HBSS and DNase to remove residual DNA.

27 Use 10 mL serological pipet to move tissue from T25 flask into 15 mL conical.

28 Rinse chunks 2-3 times using 5 ml Neurobasal (warmed to 37 °C).

> **Note**
>
> Do NOT use vacuum for these steps since you might vacuum up your tissue.

29 Remove as much media as possible and resuspend cells in 2 mL of neuron medium.

30 Mechanically dissociate cells (do not over triturate!). First, pipet up and down with 1000 µL tips ~10-15 times (when done properly the medium becomes slightly opalescent with dissociated cells). Next, use a 200 µL tip ~20 times. The chunks should now be completely dissociated into individual cells.

31 Bring total volume to 6 mL Neurobasal and strain through a 40 µm cell strainer into a 50 mL conical, rinsing strainer with 2 mL Neurobasal before and after. Transfer cells to 15 mL conical.

32 Centrifuge cells at 1000 x g, 00:05:00 . Resuspend the pellet in 2 mL of neuron media, mix and count. 5m

33 Dilute cell suspension to 1,000,000 cells/mL. For 96-well, cells should be diluted to 170,000 cell/mL.

34 Add the appropriate volume to the well of the neuron media-containing dishes so that each well contains:

- 17,000 cells (96-well)

Note

For 96-well plates cells should be gently agitated in a reservoir before being added directly to the middle of the well.

- 100,000 cells (24-well)
- 250,000 cells (12-well)
- 1,000,000 cells (6-well)

35 Gently agitate plates back and forth in each direction to spread cells. Place in incubator.

36 Cells can be checked the next day for adherence and even distribution. Neurons will start to sprout neurites within the first few days.

37 Add additional media to each well once a week:

- 20 µL (96-well)
- 115 µL (24-well)
- 330 µL(12-well)
- 1 mL (6-well)