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Culturing Primary Hippocampal Neurons from Embryonic Rat Brain

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We use this protocol and it's working

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Disclaimer

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

Abstract

This protocol details the culturing of primary neurons from the embryonic rat hippocampus.

Guidelines

All procedures on live animals should be performed in accordance with your Institutional Animal Care and Use Committee.

Materials

Dissection Media

Hanks' Buffer:

- Dissolve Hanks' Balanced Salts **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H2387 in 1L Water **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**W4502
- Add 0.35g Sodium bicarbonate to obtain pH ~7.2-7.3.
- Filter sterilize with 0.22 μm filter (2× 500 ml bottles)

Hanks' Plus Buffer (500 ml):

- 500 ml Hanks' Buffer
- 5 mL of 1 M HEPES Buffer **Thermo Fisher Scientific Catalog #**15630-080
- 250 μl gentamycin (Final concentration 5μg/ml)

Note

Store at 4 °C for at least 6 months.

FUdR- 5-Fluoro-2′-deoxyuridine/uridine stock solution (10 mM in H_2O):

- 24.62 mg 5-Fluoro-2′-deoxyuridine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F0503
- 24.42 mg Uridine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**U3003
- 10 ml milli Q water
- Sterile filter 0.2 μm, aliquot and freeze stocks at -20 °C.

Neuronal Media (50ml) – Prepare the day before or day of culture:

- 46 ml Neurobasal medium **Gibco - Thermo Fisher Scientific Catalog #**21103049
- 1 mL B-27 Supplement **Gibco - Thermo Fisher Scientific Catalog #**17504044
- 0.5 mL Glutamax (100x) **Gibco - Thermo Fisher Scientific Catalog #**35050-061
- 2.5 ml Fetal Bovine Serum (heat-inactivated)
- 5 μl Gentamycin (10 mg/ml, Gibco 1570060)
- Filter sterilize with 0.22 μm filter

- Store at 4 °C

Note

All ingredients are thawed overnight at 4 °C or Room temperature
Once thawed, keep at 4 °C until ready to prepare the media.
Do not leave at Room temperature for a prolonged period of time!

Papain Solution – Prepare on day of culture:

- 10 mL Hanks' Plus Buffer pre-warmed in 37 °C water bath.
- 200 units Papain suspension (LS003127 Worthington)

Note

- Add papain to buffer, mix by inversion and incubate in 37 C incubator for 20-30 min.
- Once papain is dissolved, mix by inversion and filter sterilize with 0.22 μm filter.
- Equilibrate 37 °C CO_2 incubator for ~30 minutes prior to use.

Feeding Media (5mL) make at 13 or 14 DIV:

- 4.85 mL Neurobasal medium **Gibco - Thermo Fisher Scientific Catalog #**21103049
- 0.1 mL B-27 Supplement **Gibco - Thermo Fisher Scientific Catalog #**17504044
- 50 μL Glutamax **Gibco - Thermo Fisher Scientific Catalog #**35050-061
- 0.5 μL Gentamycin **Gibco - Thermo Fisher Scientific Catalog #**1570060
- 5 μL 10mM FUdR
- Sterile filter 0.2 μm

Poly-DL-Ornithine Stock Solution:

- Dissolve Poly-DL-ornithine hydrobromide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P0421 in sterile PBS at 1 mg/ml and store 1 ml aliquots at -80 °C.

Laminin Stock Solution:

- Thaw Laminin, Mouse **Corning** **Catalog #**354232 at 4 °C overnight and then reconstitute with Hanks' buffer to a final stock concentration of 0.5 mg/ml.
- Make 60-70 µl aliquots and store at -80 °C .

Protocol materials

Ethyl alcohol, Pure **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**E7023

Gibco™ Neurobasal™ Medium **Thermo Fisher Scientific** **Catalog #**21103049

5-Fluoro-2′-deoxyuridine **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**F0503

Neurobasal medium **Gibco - Thermo Fisher Scientific** **Catalog #**21103049

B-27 Supplement **Gibco - Thermo Fisher Scientific** **Catalog #**17504044

Neurobasal medium **Gibco - Thermo Fisher Scientific** **Catalog #**21103049

Hanks′ Balanced Salts **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H2387

Glutamax **Gibco - Thermo Fisher Scientific** **Catalog #**35050-061

HEPES Buffer **Thermo Fisher Scientific** **Catalog #**15630-080

Glutamax (100x) **Gibco - Thermo Fisher Scientific** **Catalog #**35050-061

Water **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**W4502

Gentamycin **Gibco - Thermo Fisher Scientific** **Catalog #**1570060

Poly-DL-ornithine hydrobromide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P0421

Laminin, Mouse **Corning** **Catalog #**354232

Uridine **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**U3003

Coverslip and Plate Preparation

3d

1 Begin cleaning coverslips 3-4 days prior to culture.
We use 18 mm Cover Glasses (Carolina Biological Supply, Catalog# 633013)

1.1 Place coverslips in glass container (beaker or Petri dish) containing 100% ethanol immobilized on a platform shaker and agitate for 3-4 days. 3d

Ethyl alcohol, Pure **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E7023

> Note
>
> Agitating too vigorously can cause coverslip breakage.

1.2 Rinse coverslips with ethanol 2-3 times to remove any glass dust.
Store in 100% ethanol until needed.

> Note
>
> The coverslip cleaning procedure is crucial.

1.3 Transfer coverslips to the biosafety cabinet (BSC).
Remove coverslips from ethanol and let them dry by placing them at an angle on a parafilm coated dish.
Once dried, place coverslips flat on the parafilm-coated Petri dish

2 Coat Coverslips and/or Plates.

2.1 Thaw Poly-DL-ornithine hydrobromide (PO) at Room temperature , then warm for at least 00:30:00 in 37 °C incubator. 30m

> Note
>
> Do not refreeze Poly-DL-ornithine hydrobromide.

2.2 Thaw laminin aliquot at 4 °C, always keep On ice.

2.3 Prepare coating solution of 40 µg/ml PO + 5 µg/ml Laminin in sterile PBS.

> Note
>
> 3 mL of coating solution yields enough for approximately thirty 12 mm coverslips.

2.4 Add 0.1 mL coating solution to each coverslip in the parafilm coated Petri dish.

2.5 Incubate Overnight in 37 °C incubator (5% CO_2). 8h

2.6 Rinse 3-4 times with sterile molecular grade water (e.g., Sigma W4502)

2.7 Remove all water from coverslips or plates.

2.8 Place one coverslip per well of 24-well plate.

2.9 Add 1 ml neuronal media to each well of 24-well plate and return plate to incubator.

> Note
>
> Coating will last at least 8 days at 4 °C.
> If stored for more than a day before plating, add 1 mL
> Gibco™ Neurobasal™ Medium **Thermo Fisher Scientific Catalog #**21103049
> per well and replace with fresh neuronal media the day of dissection prior to cell plating.

Dissection of Hippocampus

5m

3 Anesthetize pregnant rat at 18 days gestation with isoflurane in an anesthesia chamber for 00:05:00 or until it does not respond to toe pinch. Animal is sacrificed according to IACUC guidelines. 5m

4 Clean the region of the rat peritoneum with 70% ethanol.

5 Hold the peritoneum region with forceps and make a small cut with a scalpel. Using scissors, cut open the peritoneum region. Remove the oviduct (10 embryos approx.) without touching the intestines. Put the oviduct in petri dish containing cold Hank's buffer on ice.

6 Take the petri dish with embryos to laminar flow hood. Open each placenta to remove individual embryos. Decapitate with sharp scissors and place heads in a new petri dish On ice containing cold Hank's Plus buffer.

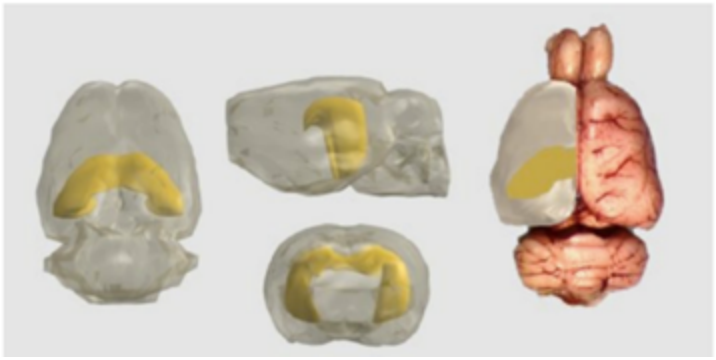
7 Dissect brains and transfer to a new petri dish with cold Hank's Plus buffer On ice .

8 Under a microscope with appropriate cold light source, place the petridish with brain ventral side up. Remove meninges with fine angled (#5/15) forceps.

9 Remove the olfactory bulbs.

10 Flip the brain to dorsal side up. Separate cortical hemispheres through the midline and gently lift the cortex to access the hippocampus.

11 The hippocampi are at the inner edge of each hemisphere. Separate the hippocampi from the cortex using angled (#5/15) forceps.



12 Transfer the hippocampi to new cold Hank's Plus buffer in a 15 mL falcon tube On ice . Repeat until this process has been repeated with all brains.

Tissue Disaggregation (move to biosafety cabinet) 35m

13 Aspirate the Hanks Plus buffer without disturbing the hippocampi. Add 10 mL papain solution, warmed at 37 °C , to the hippocampi and incubate in 37°C incubator with loosened cap for 00:30:00 . 30m

14 After incubation, remove as much papain as possible using serological pipette. Wash three times with approximately 3 mL warm pre-equilibrated neuronal media.

15 Remove the final wash and add 5 mL neuronal media to the hippocampi.

16 Mechanically break up soft tissue by trituration 5-7 times using 10 ml pipette, avoid creating bubbles.

17 Do not over titrate (turbid media indicates many cells dissociated). Any non-dissociated tissue will be removed in a later step.

18 Centrifuge dissociated cells at 250 rcf, 00:05:00 . 5m

19 Aspirate media and resuspend pellet in 1 mL neuronal media. Add additional media to desired volume.

20 Pass the cell suspension through 70 um cell strainer and rinse with neuronal media.

Plating of Hippocampal Neurons

21 Count live cells using a hemocytometer. Averaging counts from at least two quadrants. Dilute 10 µL of cell suspension with 10 µL of trypan blue (dilution factor = 2).

- Count live cells using a hemocytometer. Average number of cells in two quadrants x 2 (dilution factor) x 10^4 cells/ml.

22 Dilute cells to desired concentration in neuronal media and plate the appropriate volume onto by the amount you want on previously coated with Poly-DL-Ornithine / laminin coated coverslips in 24 well plate. Plate 30,000 cells/well in 24 well plates.

Cell culture maintenance

23 Monitor glia abundance 2-3 days after culturing.

- If glia population is too high, treat culture with FUdR.
- Add 1 µL FUdR (final concentration- 10 µg/ml) for 1 mL neuronal media.
- Timing of FUdR addition is determined by inspection under microscope for each culture, typically 2-3 DIV for a culture plated at 30,000 cells/well in a 24-well plate. Delay treatment if the culture does not have excessive glia.

24 At 14 DIV, replace 1/3 of neuronal medium with feeding medium; thereafter, feed cells every 2-3 days by replacing 30% media. Cells can be routinely grown until 28 DIV with minimal to no cell death.

Protocol references

D. Chowdhury and J.W. Hell (2019) ‘Ca^{2+}/calmodulin binding to PSD-95 downregulates its palmitoylation and AMPARs in long-term depression’, ***Frontiers in Synaptic Neuroscience*** 11(6).