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We use this protocol and it's working

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Primary hippocampal and cortical neuronal culture and transfection

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ABSTRACT

Neurons consist some of the most complex cells. The culture of primary neurons from murine brains has advanced the understanding of neuronal function at the cellular and molecular level. This protocol presents an established method to isolate and culture hippocampal and cortical neurons from rat and mouse brains along with a method to sparsely transfect these neurons.

ATTACHMENTS

Ryan Lab Hippocampal Dissection.mp4

GUIDELINES

The animals should be used in accordance with protocols approved by national and institutional regulatory organizations.

MATERIALS

Media Supplies

- ARA-C (Millipore Sigma, Cat. No. C6645)
- Disodium Phosphate (Na_2HPO_4) (Millipore Sigma, Cat. No. 431478)
- DNase (Millipore Sigma, Cat. No. D5025)
- Fetal Bovine Serum (FBS) (R&D Systems, Cat. No. S11550H)
- Glucose (Millipore Sigma, Cat. No. G7021)
- Glutamax Supplement (Thermo Fisher, Cat. No. 35050061)
- Hanks Balanced Salt Solution (HBSS), (Millipore Sigma, Cat. No. H2387)
- HEPES (Millipore Sigma, Cat. No. H3375)
- Insulin (Millipore Sigma, Cat. No. I6634-250MG)
- MEM (Thermo Fisher, Cat. No. 51200038)

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- N-21 Max (R&D Systems, Cat. No. AR008)
- Potassium Chloride (Millipore Sigma, Cat. No. P9333)
- Sodium Bicarbonate ($NaHCO_3$) (Millipore Sigma, Cat. No. S5761)
- Sodium Chloride (Millipore Sigma, Cat. No.S7653)
- Transferrin (Millipore Sigma, Cat. No. 616420)
- Ultra-Pure Water (Thermo Fisher, Cat. No. 10977-015)

Other Supplies

- 0.22 µm syringe filters (VWR, Cat. No. 76479-044)
- 35 mm dishes (D X H 35 X 10 mm) (Falcon, Cat. No. 351008)
- Acetone (LabChem, Cat. No. LC104202)
- Cell strainers (40 µm) (Falcon, Cat No. 352340)
- Cloning Cylinders (6 mm OD) (VWR, Cat. No. 89083-360)
- CNQX (Alomone Labs, Cat. No. C-141)
- Coverslips (22 mm X 22 mm, glass no. 1) (VWR, Cat. No. 16004-094)
- Ethanol (200 Proof) (Koptec, Cat. No. 6175)
- Grease (Millipore Sigma, Cat. No. Z273554-1EA)
- P35 dishes (Falcon, Cat. No. 351008)
- Poly-L-ornithine (Millipore Sigma, Cat. No. P3655-10MG)
- Trypsin (Millipore Sigma, Cat. No. T1005)
- Wash-N-Dry Coverslip Racks (Millipore Sigma, Cat No. Z688568-1EA)
- Sporicidin (Clontec, Cat No. 89176-480)
- 2M Calcium Chloride (in H_2O) (RPI, Cat. No. C25100-50.0)
- Hemocytometer (like VWR, Cat. No. 15170-173)
- Coverslip holder (Millipore Sigma, Cat No. Z688568)
- SYLGARD 184 Silicone Elastomer Kit (Dow, Cat. No. 04019862)
- Carbon powder (Thermo Fisher, Cat. No. 033302)
- Glass petri dishes 60 x 20 mm (Millipore Sigma, Cat No. SLW1480/02D)

Stock Solutions

Heat Inactivating FBS (R&D Systems, Cat. No. S11550H)
FBS can be bought pre-heat inactivated but if not follow the below protocol to heat inactivate.

1. Thaw FBS in water.
2. Aliquot in to 50 mL conical tubes.
3. Place in water bath at 56 ^{o}C for 30 minutes.
4. Filter and aliquot into new 50 mL conical tubes.
5. Store at -20 ^{o}C.
6. When ready to use thaw at 4 ^{o}C.

Ara-C (4 mM in H_2O)

1. Dissolve the bottle in 22.35 mL ultrapure water.
2. Filter and aliquot into 1 mL aliquots and store at -20^{o}C.

Insulin (12 mg/mL in 20 mM HCl)

1. To a 250 mg bottle of insulin resuspend in 20.8 mL Ultrapure water.
2. Add 34.5 µL of 12 N HCl.
3. Make 1 mL aliquots and store at -20 ^{o}C.

Trypsin (40 mg per vial)

1. Measure 40 mg into a 15 mL falcon tube and store at -20 ^{o}C.

DNase (50 mg/mL in H_2O)

1. Dnase is sold by activity units and the mass varies per bottle. Find the amount listed and resuspend in ultra-pure water to a final concentration of 50 mg/mL (1 mg/20 µL).
2. Filter.
3. Make 15 µL aliquots and store at -20 ^{o}C. Do not refreeze aliquots.

Poly-L-Ornithine (1 mg/mL in H_2O, 100x)

1. To a 10 mg bottle add 10 mL of ultrapure water.
2. Filter with a syringe filter and store in 100 µL aliquots at -20 ^{o}C.

CNQX (10 mM Stock)

1. Add 10 mL of ultrapure H_2O to the whole bottle of CNQX.
2. Vortex until dissolved.
3. Make 1 mL aliquots and store at -20 ^{o}C.

Culture Media Recipes

Hank Balanced Salt Solution (HBSS) (4.17 mM $NaHCO_3$, 1.09 mM HEPES added in HBSS pH 7.15 – 7.20)

1. Add one bottle of Hanks Balanced Salt Solution to 900 mL of ultrapure water.
2. Add 0.35 g Sodium Bicarbonate ($NaHCO_3$).
3. Add 0.26 g HEPES.
4. Add water to 1 L.
5. Adjust pH to 7.15 – 7.20.
6. Filter, make 32 mL aliquots and store at 4 ^{o}C.

Hanks Balanced Salt Solution + 20% FBS (20 % FBS, 4.17 mM $NaHCO_3$, 1.09 mM HEPES added in HBSS pH 7.15 – 7.20)

1. Add one bottle of Hanks Balanced Salt Solution to 700 mL of ultrapure water.
2. Add 200 mL of Heat Inactivated FBS.
3. Add 0.35 g Sodium Bicarbonate ($NaHCO_3$).
4. Add 0.26 g HEPES.

5. Add water to 1 L.
6. Adjust pH to 7.15 – 7.20.
7. Filter, make 32 mL aliquots and store at 4 ^{O}C.

Plating Media (~29 mM Glucose*, 0.1 mg/mL transferrin, 1% Glutamax, 24 µg/mL insulin, 10% FBS, 2% N-21 in MEM)
1. Add 400 mL of MEM.
2. Add 2.5 g glucose.
3. Dissolve 100 mg (whole bottle) transferrin in 1 mL MEM. Add 500 µL to the plating media. Discard the extra.
4. Add 5 mL of Glutamax.
5. Add 1 mL of insulin.
6. Add 50 mL of Heat Inactivated FBS.
7. Add 10 mL N-21.
8. Add MEM to 500 mL.
9. Filter, make 5 mL aliquots and store at 4 ^{O}C.

Feeding Media (~29 mM Glucose*, 0.1 mg/mL transferrin, 1% Glutamax, 24 µg/mL insulin, 5% FBS, 2% N-21, 4 µM ARA-C in MEM)
1. Add 900 mL of MEM.
2. Add 5 g glucose.
3. Dissolve 100 mg transferrin (whole bottle) in 1 mL MEM. Add to feeding media.
4. Add 10 mL of Glutamax.
5. Add 2 mL of insulin.
6. Add 50 mL of Heat Inactivated FBS.
7. Add 20 mL N-21.
8. Add 1 mL ARA-C.
9. Add MEM to 1 L.
10. Filter, make 32 mL and 5 mL aliquots and store at 4 ^{O}C.

* Glucose concentration is calculated from the concentration of glucose in MEM (~5.55 mM) the amount added (~27.75 mM) and the amount in FBS (~3.16 mM, though variable by batch) weighted to their respective volumes.

Digestion Solution (238 mM NaCl, 6.24 mM KCl, 8.73 mM Na_2HPO_4, 31.47 mM HEPES in H2O)
1. Add 200 mL of ultrapure water.
2. Add 2 g NaCl.
3. Add 93 mg KCl.
4. Add 248 mg Na_2HPO_4.
5. Add 1.5 g HEPES.
6. Add water to 250 mL.
7. Filter, make 5 mL aliquots and store at 4 ^{O}C.

Dissociation Solution (12 mM $MgSO_4 \cdot 7\ H_2O$ in HBSS)

1. Take 100 mL of HBSS.
2. Add 295 mg $MgSO_4 \cdot 7\ H_2O$.
3. Filter, make 5 mL aliquots and store at 4 ^{O}C.

2X HEBS (274 mM NaCl, 9.52 mM KCl, 1.42 mM $Na_2HPO_4{\cdot}7H_2O$, 15mM D-glucose, 42 mM HEPES, 20 µM CNQX in H2O pH~7.3)

1. Add 150 mL of ultra-pure water.
2. Add 3.2 g NaCl.
3. Add 142 mg of KCl.
4. Add 76 mg of $Na_2HPO_4{\cdot}7H_2O$.
5. Add 540 mg of D-glucose.
6. Add 2 g HEPES.
7. Add 400 µL of CNQX (10 mM stock).
8. Adjust pH to ~7.3.
9. Add water to 200 mL.
10. Confirm pH is correct.
11. Filter and store in ~500 µL aliquots at -20^{O}C.

Note: When initially making HEBS try multiple pH's around 7.30 to see which gives the optimal transfection efficiency.

Preparing Coverslips

1. Using the coverslip holders place coverslips in a beaker.
2. Wash with acetone for 30 min, while shaking.
3. Wash with ethanol for 30 min, while shaking.
4. Rinse with dd$H_2$0 twice.
5. Dry and autoclave.

Preparing Dissection Dishes

1. Mix Part A and Part B from the SYLGARD 184 Silicone Elastomer Kit as per kit instructions.
2. In a chemical hood add the Carbon powder until the mix is dark black.
3. Poor in the Pyrex glass petri dishes.
4. Allow to set.

Animals

An example rat strain is the Sprague-Dawley strain from Charles River Labs (Charles River Strain Code: 400, RRID: RGD_734476). Animals should be used from P0-P2

Equipment

- CO_2 Incubator (like VWR Symphony)
- Stereoscope (like ZEISS Stemi 2000)

- Microscope (like Olympus CK40)
- Student Scalpel Handle #3 Scalpel (like Fine Science Tools 91003-12)
- Surgical blade #10 (like VWR 72044-10)
- Surgical blade #11 (like VWR 72044-11)
- Dumont #5SF Forceps Tweezers (like Fine Science Tools 11252-00)
- Dumont #3 Forceps (like Fine Science Tools 11293-00)
- Dumont Tweezers #5Tweezers (like Roboz Surgical Store RS-4905)
- Ziegler Micro Dissecting Knif Microknife (like Roboz Surgical Store RS-6240)
- Operating Sscissors (like Roboz Surgical Store RS-6828)
- BCL-1 hood (like Forma Scientific 1839 Laminar Flow Work Station)
- BCL-2 hood (like LABONCO Purifier Logic+ Class II, Type A2)
- Heating Blanket (like Harvard Apparatus 557034)
- Refrigerated benchtop Centrifuge (Beckman Coulter Allegra X-30R) with SX4400 swing-bucket rotor
- Water bath (like Corning LSE)

SAFETY WARNINGS

Primary cells constitute a type-2 Biohazard and should be handled according to all national and institutional guidelines in a BSL-2 culture hood.

BEFORE START INSTRUCTIONS

On average, 1 rat pup (2 hippocampi) yields approximately 10 dishes of hippocampal neurons.

Plating media and MEM should be warmed before use in the water bath (37 ^{o}C) and filter-sterilized before addition to cells.
Dissection tools should be kept in sporicidin.
Dissection time should be about 5 minutes per pup.
Pipette solutions slowly on the side of the tubes to prevent bubbles.

Incubators should be set to 5% CO_2 37 ^{o}C.

Preparing dishes, 1 Day before

10m

1 For each dish, place a coverslip in a p35.

2 Take an 100X poly-L-ornithine aliquot and dilute in 10 mL ultrapure water (final conc.

[M] 10000 µg/µL).

3 Add ~ 200 µL of poly-L-ornithine to the center of each coverslip.

4 Place in incubator Overnight .

Preparing for culture

10m

5 Add 8 mL of HBSS + [M] 20 % (v/v) FBS to a dissecting dish.

6 Add 4 mL of HBSS + [M] 20 % (v/v) FBS to a p35.

7 Place in fridge until ready to dissect.

8 Prepare dissection hood and fill a bucket with ice.

9 Turn on centrifuge and cool to 4 °C .

10 Collect rat pups from your animal facility.
You should collect one pup for every 10 dishes. Pups should be P0 – P2.

Dissection (Hippocampal) also see video

30m

11 Perform the dissection on a piece of cold marble. Marble is kept in the -20 °C. Change out the marble every two pups.

12 Decapitate rat pup using the scissors. Place rat carcass in a biohazard bag and dispose of according to your institution's policy.

13 Using tweezers stab through the eyes and use the sharp blade (#11) to cut through the skull.

14 While keeping tweezers in the eyes, using another pair of tweezers, remove skull.

15 Use the scoop to remove the brain and place in a dissecting dish filled with cold HBSS + [M] 20 % (v/v) FBS.

16 Cut the brain in half using the rounded scalpel blade (#10).

17 Stab into the cerebellum and roll the cortex away using the micro knife.

18 Flip over and isolate the hippocampus using the micro knife.

19 Remove meninges with tweezers making sure to remove meninges in the groove between the subiculum and the hippocampus.

20 Stab into the subiculum and use the micro knife the remove the dentate gyrus.

21 Flip over and remove the hippocampus following the dark crescent moon shape.

22 Place in the p35. Store on ice until all hippocampi are dissected.

23 Once all hippocampi are dissected cut each hippocampus into 4 pieces.

Culture

1h 30m

24 Move the dissected hippocampi in HBSS + [M] 20 % (v/v) FBS to a 15 mL falcon tube.

25 Aspirate off the dissection media.

26 Wash with 8 mL of HBSS + [M] 20 % (v/v) FBS.

27 Wash 3 times with 8 mL HBSS.

28 Add 7 µL DNase to 5 mL of pre-aliquoted digestion solution and dissolve the 40 mg trypsin aliquot.

29 Remove HBSS from hippocampi and filter the digestion solution onto the hippocampi.

30 Leave the 15 mL tube at an angle to allow for optimal digestion. Digest for 00:05:00. 5m
(Note: If the sample starts to stick together add more DNase).

31 After 00:05:00, aspirate the digestion solution. 5m

32 Wash 2 times with 8 mL of HBSS + [M] 20 % (v/v) FBS.

33 Wash 3 times with 8 mL HBSS.

34 Add 7 µL DNase to 5 mL of pre-aliquoted dissociation solution. Filter on to hippocampi

35 Using a P1000 pipettor, slowly pipette the cells up and down until fully dissociated. Make sure to not create bubbles.

36 Place a cell strainer in a 50 mL centrifuge tube and pour the cell suspension over the strainer.

37 Wash the 15 mL tube that contained the cell suspension with 6 mL of HBSS + [M] 20 % (v/v) FBS. Add the solution using a serological pipette to the strainer.

38 Take the strained cell suspension and place in a new 15 mL conical and spin at 300 x g 4 °C 10m 00:10:00 .

39 During the spin aspirate off the poly-o from the dishes and wash two times with 0.5 mL ultrapure water.

40 After the spin is complete, remove the media and wash with 6 mL of HBSS. Resuspend the pelle 10m and spin again at 300 x g 4 °C for 00:10:00 .

41 During this spin, add the cloning cylinders. Using heated forceps, place the cylinder in the grease and then place the cylinder in the center of the coverslip.

42 After this spin, aspirate off the media and resuspend in plating media. Our rule of thumb is to resuspend in 0.5 mL of plating media per pup dissected.
When aspirating media after pelleting cells, you can increase yield by removing the final mL of media with the P1000.

43 Then add 10 µL to the hemocytometer and count the cells. You want ~38,000 cells/cylinder. Each

cylinder holds 100 µL.

44 Dilute cells with plating media to the appropriate concentration.

45 Add 100 µL of plating media with cells per cylinder.

46 Add 3 mL of MEM to the outside of each dish.

47 Place cells in the incubator.

Feeding the cells

10m

48 Feeding should be done DIV2.
Replace 60 µL of plating media in the cylinder with 60 µL of warm filtered feeding media.

Transfection

1h 30m

49 Transfection should be done between DIV6 – DIV7.
Warm MEM and Feeding Media in the water bath.

50 Turn on and place heating blanket in the hood.

51 For each dish triple rinse by removing 60 µL media and replacing with 60 µL of warm MEM three times.

52 Replace the MEM outside the cylinder with 3 mL of feeding media.

53 Return cells to the incubator for 00:30:00. 30m

54 Prepare DNA mix as below, but do not add HEBS. Wait at least 00:10:00. 10m

55 Remove cells from the incubator.

56 Add HEBS, while vortexing, to the DNA mix. Immediately add 12 µL of DNA mix to each dish. Pipette quickly and do not add dropwise.

57 Return cells to the incubator for 00:12:00. Do not open the incubator during this time. 12m

58 After 00:12:00, remove cells from the incubator and remove 60 µL of media. 12m

59 Break off the cylinder to flood with Feeding Media.

60 Return to incubator until ready to image.

DNA Mixes (HeBS)

61 These are starting suggestions for your transfection. Optimize as needed. These recipes are good for seven dishes, scale as appropriate. Plasmids for transfection should be from a Midi or Maxi Prep.

62 Single Transfection: 6 µg of plasmid, 4.5 µL $CaCl_2$, Ultrapure water to 45 µL, 45 µL HEBS

63 Double Transfection: 4 µg of one plasmid, 5 µg of the other plasmid (Add 5 µg of the plasmid that gives a lower transfection yield), 4.5 µL $CaCl_2$, Ultrapure water to 45 µL, 45 µL HEBS

64 Triple Transfection: 3 µg of each plasmid, 4.5 µL $CaCl_2$, Ultrapure water to 45 µL, 45 µL HEBS

10. Additional notes

65 This culture protocol can also be used as is for postnatal mouse cultures. The only difference is that feeding the cells should be done later on, typically 4-5 days in-vitro.

66 This culture protocol can be used for postnatal cortical cultures, with the following differences and notes. During dissection, still remove the hippocampus (and olfactory bulb if present).
Make sure to remove the meninges.
Do not use the cell strainer.
Half rat cortex should yield approximately 10 million cells.

Do not do more than one full brain per tube.
Double the amount of DNase.
If you have trouble dissociating leave longer with the trypsin.
Feed cells later once the glia appear more confluent typically 4-5 days in-vitro.