Hippocampal Neuron Dissociation Transfection and Culture in Microfluidics Chambers

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[Abstract] Microfluidics chamber is an ideal tool to study local events that occurring in neuronal projections by perfectly compartmentalizing the cell soma from certain branches. It is very well suited for live cell imaging or immunohistochemistry staining. This protocol has been carefully modified in detail to fit the requirement of primary rat hippocampal neuronal cultures. It can also be applied to a more general neuronal culture purpose in microfluidics.

Materials and Reagents

1. β-mercaptoethanol (Sigma-Aldrich, catalog number: M3148)
2. HBSS (Hyclone, catalog number: SH30268)
3. Boric acid (Sigma-Aldrich, catalog number: B0252)
4. Borax (Sigma-Aldrich, catalog number: B9876)
5. Cysteine (Sigma-Aldrich, catalog number: C7352)
6. EtOH
7. Neural basal media (NBM)
8. Glutamax
9. B27
10. PenStrep
11. Gentamicin
12. Glutamate
13. FBS
14. Laminin
15. Trypan blue
16. Na_2SO_4
17. K_2SO_4
18. $MgCl_2$
19. $CaCl_2$
20. Glucose
21. Phenol Red

22. EDTA
23. 0.1 M borate buffer (pH 8.5) (see Recipes)
24. Dissection media (DM) (see Recipes)
25. Papain activation buffer (see Recipes)
26. Papain (100 mg) (Worthington Biochemical Corp, catalog number: LS003119) (see Recipes)
27. DNase (F. Hoffmann-La Roche, catalog number: 10104159001) (see Recipes)
28. Papain digestion media (see Recipes)
29. Dissociation media (see Recipes)
30. Plating media with phenol red (see Recipes)
31. Growth media without phenol red (see Recipes)

Equipment

1. PDMS devices
2. Microscope
3. Hemacytometer
4. TC hood
5. Incubator
6. -80 °C freezer
7. 37 °C water bath
8. 6 cm cell culture dishes
9. Plasma-bonding machine (model PDC-32G, http://www.harrickplasma.com/)

Procedure

A. Coverslip and Chamber Preparation

Day 1:

1. PDMS devices are fabricated as described in Park *et al.* (2006).

Day 2:

2. Corning No.1, or No.1.5 coverglasses are both suitable for our microscope (24 mm x 40 mm). Or, Carolina coverglasses 24 mm x 50 mm.
    a. Sonicate the cover glasses in autoclaved ddH_2O for 60 min.
    b. Sonicate again in 70% EtOH for 30 min.
    c. Sonicate in 100% EtOH for 30 min.
    d. Wash with autoclaved ddH_2O in TC hood, air dry the coverglasses overnight in the

hood.

Day 3:

3. Assemble the coverglass and PDMS device by using the plasma-bonding machine.
4. Press firmly on the corners but only lightly touch the middle channel. Coat the chamber with PDL (>150K mol wt, prepared in borax boric acid buffer):
   a. 0.5 mg/ml PDL is added to a well on one side of the device and allowed to flow through into the connecting well (leave the other side unfilled).
   b. Allow the PDL to flow through the device for about 10 min and then add more PDL to the wells to fill them up.
   c. Place the devices containing PDL in an incubator at 37 °C for a minimum of 30 min to let the PDL fill the micro-groves.
   d. Fill the wells and major channel on the other side of the chamber with PDL and return the chamber to incubator at 37 °C for a minimum of 4 h, overnight is preferable.

Day 4:

5. Pipette out the excess PDL after treatment, but be careful not to suck all the liquid out of the device.
   a. Add autoclaved ddH_2O to each well on either side of the device, and allow to flow to the other well by capillary action.
   b. Aspirate off the water (again being careful not to fully remove all the liquid from the device), then add another 150 µl of autoclaved ddH_2O to one of each connected well and allow it to flow through to the corresponding well.
   c. Repeat this quick wash twice, then place the device containing ddH_2O in an incubator at 37 °C for 1 h.
   d. Then, repeat the wash step again. Wash the devices at least three times with ddH_2O for one hour each, which is the total.
   e. Add NBM with the necessary factors (glutamax, B27, PenStrep) to the top wells. Let it flow through, incubate at 37 °C overnight.
   f. Prepare 40 ml plating media:

      800 µl B27 + 30 µg/ml gentamicin + 400 µl glutamax + 25 µM glutamate
   g. Prewarm 10 ml dissociation media in 15ml tube: Neurobasal + 5% FBS + gentamicin.
   h. Prewarm 10 ml plain neurobasal media for papain.

Day 5:

6. Add laminin (20 µg/ml) to each well and incubate at 37 °C for at least 30 min: Coat the chamber with laminin: take a 20 µl aliquot out from -80 °C freezer and put it on ice

immediately, wait until it dissolves completely on ice then add cold neurobasal media to 1.8 ml.

a. Turn the UV light on and UV sterilize one 6 cm cell culture dishs for 15 min.
b. Pre-warm 14 ml plating media in this 6 cm cell culture dish, 37 °C incubator.
c. Replace the laminin solution with plating media 1 h before plating the cells.

B. Dissection of hippocampal neurons

1. Activate papain 10 mg aliquot with 750 µl papain buffer at 37 °C for 30 min.
2. Add to prewarmed neurobasal media, filter sterialize, then add one DNase 20 µl aliquot.
3. Wash the tissue with 10 ml cold dissection media in TC hood (wash twice).
4. After the tissue settled by gravity, remove HBSS completely.
5. Add one aliquot of DNase to 10 ml digestion media (prewarmed yesterday).
6. Place half of digestion media (5 ml) onto the tissue for 15 min at 37 °C water bath.
7. Add the remaining half of digestion media into the tube and incubate for another 15 min at 37 °C water bath.
8. Let the tissue settle to bottom by gravity or spin shortly.
9. Re-suspend the pellet with 5 ml papain dissociation media and triturate up and down with 5 ml pipette for 5 times, avoid bubbling the media.
10. Let debris settle slowly, or short spin to 100 g up and down (600 rpm), transfer the supernatant to a new 15 ml tube.
11. Continue triturate the undissociated tissues for 5-10 times, take the supernatant and combine with previous 5 ml.
12. Take 10 µl out and mix with 10 µl trypan blue.
13. Load 10 µl mixture on hemacytometer and count:
    All cells in 16 small squares x 10^4 = cells/ml (A typical yield from 12 E18 pups is ~50 M in total).

C. Amaxa transfection

14. Mix 82 µl nucleofector solution with 18 µl supplement before use.
15. Prepare 50 µl nucleofector with 5 µg total DNA (if using more than 1 plasmid, mix in equimolar ratios).
16. Spin down 5 million (1-6 million for each transfection) neurons at 150 *x g* 5 min. During spinning, transfer 7 ml pre-warmed plating media to a 15 ml tube, add 350 µl FBS, keep at 37 °C. Prepare the 1 ml pipette with a tip.
17. After spinning, remove all media using aspiration pipette with a 200 µl pipette tip.
18. Resuspend the cells in a 50 µl nucleofector solution.
19. Mix 50 µl of the cells with the premix of DNA + neucleofector (a total of 100 µl).

20. Zap with program G-13 (better for Hc) or O-03 (better for Cx). A foam of dead cells will form.
21. Immediately pipette in 1 ml warmed plating medium with FBS and pipette out to a 1.7 ml eppendorf tube. Do not pick up dead foam. Be gentle.
22. Incubate at 37 °C 10 min after rescue.
23. Spin down the cells at 150 *x g*, 5min.
24. Resuspend in 300 µl plating media with FBS.
25. Load 5 µl/well, filling the two upper wells of each microfluidic.
26. Let cell adhere at 37 °C for 10 min.
27. Fill the wells from both ends simultaneously with two pipettes with plating media with FBS
28. Completely change media to fresh plating media W/O FBS 2 h later.

Day 6:

29. Change media once again the next morning with plating media.
30. Change half of the media every 2 days.

Recipes

1. 0.1 M borate buffer (pH 8.5) (borax boric acid buffer)
   1.24 g Boric acid
   1.9 g Borax
   Bring to 500 ml with ddH_2O
   PH should be 8.5 without adjustment
   Filter sterilize
   Store at 4 °C.
2. Dissection media (DM) (for 1L)
   82 ml 1 M Na_2SO_4
   60 ml 0.5 M K_2SO_4
   5.8 ml 1 M $MgCl_2$
   252 µl 1M $CaCl_2$
   8 ml 2.5 M glucose (20 mM)
   1 ml 1 M Hepes (pH 7.4)
   2 ml 0.5% Phenol Red pH with 1 M NaOH to 7.4 filter sterilize and keep at 4 °C.
   OR just use HBSS and add glucose to 20 mM, HEPES:
   500 ml HBSS with Ca^{2+} and Mg^{2+}
   5 ml 1.5 M D-glucose (filter sterilized)
   5 ml 1.0 M HEPES pH 7.2

3. Papain activation buffer (50 ml)
   1.1 mM EDTA 110 µl (0.5 M EDTA, pH 8.0)
   0.067 mM β-mercaptoethanol 0.25 µl (0.0047 µl BME/ml buffer)
   5.5 mM cysteine 33.25 mg
   Store at 4 °C
4. Papain
   Aliquot into 10 mg/1.7 ml eppendorf tube, Store lyophilized powder at 4 °C
   Add 750 µl papain buffer before use
   Activate to 200 U/ml (10 x) - 30 min at 37 °C (shake the tube once in a while)
5. DNase
   1,000 U/µl stock, 20 µl aliquot (20,000 U/tube, used in 10 ml)
6. Papain digestion media (for every ten animals)
   Add the 750 µl activated papain to plain neurobasal media
   Filter sterilize
   Add DNase: 2 U/µl DNase
7. Dissociation media (in 10 ml neurobasal)
   Gentamicin 40 µg/ml
   FBS 500 µl
8. Plating media with phenol red (in 20 ml neurobasal) with serum
   B27 400 µl (Gem21, NS21 all the same)
   Gentamicin 40 µg/ml (10 mg/ml stock, take 80 µl/20 ml media)
   Glutamax 200 µl
   Glutamate 25 µM (100 mM stock, 4,000x, 5 µl/20 ml) (use at 12.5 µM final concentration may be better: 2.5~3 µl/20 ml)
   FBS 200 µl
9. Growth media without phenol red (in 20 ml neurobasal)
   B27 400 µl (Gem21, NS21 all the same)
   Glutamax 200 µl

Acknowledgments

Alternative reference protocol can be found on the Millipore website related to AXIS™ Axon Isolation Devices. The dissection protocol is modified from a protocol developed in Dr. Michael Lin's lab, Department of Pediatrics and Bioengineering, Stanford University, USA.

References

1. Detailed reference protocols can be requested from http://www.xonamicrofluidics.com/about.html.
2. Park, J. W., Vahidi, B., Taylor, A. M., Rhee, S. W. and Jeon, N. L. (2006). Microfluidic culture platform for neuroscience research. *Nat Protoc* 1(4): 2128-2136.

