Primary Neuronal Culture & CRH Treatment

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Abstract

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Protocol

Preparing Coverslips for Primary Cell Culture

Step 1.

Day 1: Poly-D-Lysine (PDL) coating – start ≥20 hrs before culturing

Supplies Needed:

- 24 well plate
- Poly-D-Lysine *2 (0.2 mg/ml) – in freezer
- Autoclaved coverslips (24/plate)
- Sterilized forceps
- 200 µL pipette + tips

Instructions:

1. Sterilize Hood
2. Thaw PDL*2 (0.2 mg/ml)
3. Using sterilized forceps, lay gently coverslips in each well of 24-well dish (1 coverslip per well)
4. Aliquot 80 µl of the PDL*2 to each coverslip, make sure the drop is in the middle of

the coverslip.

5. Let the dishes sit undisturbed, in incubator for 18 hours

Preparing Coverslips for Primary Cell Culture

Step 2.

Day 2: PDL coating – continued

Supplies Needed:

- 24 well plate + treated coverslips from day 1
- Sterile H20
- Disposable 25 ml pipette + pipetter
- Autoclaved glass Pasteur pipette

Instructions:

1. Sterilize Hood

   If culturing on same day, remove ES from freezer to thaw

2. After ≥18 hours of coating (see above), rinse dishes by filling dishes with sterile H20 (using a disposable 25 ml pipette)

3. Remove water immediately using sterile glass Pasteur pipette hooked to the vacuum.

4. Wait 10 minutes

5. Repeat washing one more time.

6. Remove water until dishes are completely dry. Let dry under the hood.

Tissue Dissection & Dissocation

Step 3.

Supplies Needed:

- P0 or P1 rat pup(s)
- 70% EtOH
- Ice Bucket
- Beaker with water (for dirty dissection tools)
- Dissection Microscope
- Microtome + Fresh blade (double sided razor)
- 2x 100 mm petri dishes
- 3x 35 mm petri dishes
- Dissection Tools (all autoclaved):
- Scissors (1x large & 1x small) o 2x forceps
- ≥3x pulled micropipettes
- Brain Scooper/Spatula
- Disposable Pasteur pipette
- Dissection Solution (DS; ≥75 ml) – Make fresh every 2 weeks
- Enzyme solution (ES, 5 ml [1 tube]; stored in -20oC freezer)
- Papain (50 units) – Aliquots in 4oC
- 5x 15 ml conical tubes
- 400 µl BSA/PI stock
- 110µl5mMAPV
- Cortical Plating Medium (50 ml BME + 5 ml FBS + 500 µl Na-pyruvate + 125 µl Glutamax; Filter)
- Conditioned Medium (50 ml aliquots in -80oC freezer
- Slide & Microscope

Instructions:

1. Take ES (without papain) out of freezer to thaw
2. Sterilize working space & all tools
3. Add 50 units papain to the 5 ml thawed enzyme solution, activate at 36oC in the incubator for at least 30 minutes
4. Fill small petri dish with ice-cold dissection solution (DS)

5. Dissection

    1. Spray pup with EtOH and quickly decapitate pup behind ears

    2. Remove brain from skull, place in petri dish with DS, on ice

    3. Remove hindbrain and discard it

    4. Place brain on microtome platform, superior side facing up.

    5. Cut coronal slices of 600 mm and transfer to DS filled 35 mm petri dish

    6. Remove cortex free from rest of brain using autoclaved sharp glass pipettes. Carefully discard fibers and meninges. Cute each part to 600 µm cubes.

6. Dissociation

1. Collect tissue with sterile Pasteur pipette and transfer to new 35 mm petri dish (transfer as little DS as possible)

2. Add 5 ml ES that was previously activated in 36oC (by now the enzyme solution should appear clear instead of cloudy)

3. Place the tissue + enzyme in 36oC incubator for 25 minutes

4. While waiting, prepare all washing solutions and place on ice.

    1. 10mlDS

    2. 2x1.5mlHI (3mlDS,300µlBSA/T1,30µl5mMAPV>aliquot)

    3. 3x2.5mlLI(8mlDS,80µlBSA/T1,80µl5mMAPV>aliquot)

    4. 5 ml Cortical Plating Medium

5. 1.5 ml Cortical Plating Medium/brain in 35 mm petri dish (e.g. 2 pups = 3 ml)

6. 50 ml tube with Cortical Plating Medium for dilutions

5. Collect tissue pieces with sterile Pasteur pipette. Wash in the following order, always placing on ice:

    1. 10 ml DS, swirl and let tissue settle

    2. 1.5 ml HI – wait 2 minutes (on ice)

    3. 1.5 ml HI – wait 2 minutes (on ice)

    4. 2.5 ml LI – wait 2 minutes (on ice)

    5. 2.5 ml LI – wait 2 minutes (on ice)

    6. 2.5 ml LI – wait 2 minutes (on ice)

    7. 5 ml Cortical Plating Medium

6. Transfer tissue to petri dish with 1.5 ml CPM/brain

7. Triturate visually with decreasing tip diameter, 4 times for each piece of tissue (first with 100-1000 µl tip, then with 20-200 µl tip)

8. Transfer cells + medium to new 15 ml tube, leave undisturbed for 3-5 minutes to settle

9. Visualize 80 µl on dish cover under microscope to assess density (take from the top);

    dilute tube if necessary

10. Plate 80 ml in the middle of each coverslip

11. Incubate in 36ºC, 5% CO2 for 2 hours -- also prewarm CPM & Conditioned Medium

12. Add 0.5 ml prewarmed cortical plating medium to each cover slip

13. Incubate in 36ºC, 5%CO2 for 2 more hours

14. Refresh half the volume by removing 250 µl of medium from each well and adding similar amount of prewarmed conditioned medium. Return to incubator

Replace Medium and Add Cyctosine-Arabinoside

Step 4.

Day 5 (72 hours after plating):

• Replace half of the medium, with preheated medium. This time add 2 µl AraC/1 ml NBM+B27 (AraC [Cyctosine-Arabinoside] inhibits glia growth)

Replacing Medium

Step 5.

- Refresh medium twice a week, by replacing half of the volume with fresh conditioned medium that was pre-heated in the incubator to 36°C.

- Occasionally check viability of the culture under microscope to make sure it is not contaminated (but don't do too often, cells are happiest when they are kept undisturbed in the incubator)

- Cultures can be kept like this for at least 3-4 weeks after plating

Add CRH to Cultures (DIV 7-14)

Step 6.

- Per plate for first half-medium change (2x desired concentration CRH) – 250 µl/well
- **CTL/VEH** - 2 mL NBM/B27/AraC
- **200 nM CRH**: 5 µl stock CRH (0.5 µg/ul) + 2.57 ml NBM/B27/AraC
- **20 nM CRH:** 200 µl 200 nM CRH + 1.8 ml NBM/B27/AraC
- **2 nM CRH**: 20 µl 200 nM CRH + 1.98 ml NBM/B27/AraC

- Per plate for following half-medium changes (desired concentration CRH) – 250 µl/well
- **CTL/VEH** - 2 mL NBM/B27/AraC
- **100 nM CRH**: 5 µl stock CRH (0.5 µg/ul) + 5.14 ml NBM/B27/AraC
- **10 nM CRH**: 200 µl 100 nM CRH + 1.8 ml NBM/B27/AraC
- **1 nM CRH**: 20 µl 200 nM CRH + 1.98 ml NBM/B27/AraC

Fix Coverslips (DIV14)

Step 7.

1. Wash in 0.1 M PB briefly at room temp (1 minute)

2. Fix the culture in 4% PFA for 1 hour at room temp (1 ml fixative/well)

3. Remove PFA

Immunocytochemistry

Step 8.

1. Wash with PBS-T (0.01 M PBS + 0.3% Triton)

2. Wash in 2-5% normal goat serum diluted in 0.01 M PBS-T containing 1% BSA for 30 minutes

3. Mouse anti-MAP2 antibody: 1:8000-16,000 in PBS-T – 1-2 days at 4oC

4. Wash in PBS-T 3 times (5 minutes each wash) – 20 minutes total

5. Alexa Fluor 488 goat anti-mouse IgG conjugate – 1:300 in PBS-T containing 1% BSA – room temperature for 2 hours

6. Wash in PBS-T 3 times (5 minutes each wash) – 20 minutes total

7. Place 1 drop of Aqueous mounting medium onto slide (as small as possible), then place coverslip face down on top.