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Protocol for low density primary neuron cultures

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Banker cultures consist of neurons cultured at low density on top of a feeder layer of astrocytes.
Low density cultures are optimal for morphological analysis. However, they are less well suitable for DNA transfection experiments. Their use is time consuming, because feeder layers have to be prepared separately.

Neurons can be grown on glass coverslips placed into 12-well plates containing the feeder layer. Neurons are plated onto the pre-coated coverslips, which are then inserted up-site-down onto the feeder layer. Inclusion of a spacer (small paraffin beads) prevents direct contact between neurons and astrocytes.

Preparing coverslips (in advance)

Coverslips need to be treated with acid to make the surface rough enough for cell attachment. This procedure can be done in advance for multiple preparations as sterile acid-treated coverslips can be stored.

1. Place coverslips in a Teflon rack containing 65% nitric acid
   (nitric acid can be re-used until it becomes yellowish)
2. Incubate overnight on a shaker
3. Wash several times with dH_2O until pH is neutral
4. Let coverslips dry in the Teflon rack in the laminar flow hood (to avoid dust)
5. Autoclave coverslips in glass Petri dishes, in which they can be stored

Coating of glass coverslips with Poly-L-Lysine (ca. 1 day before preparation)

Note: use sterile Poly-L-Lysine that does not need to be filtered. A basic pH is crucial for proper coating. Clumping of cultured neurons is a sign of bad coating.

1. Place pretreated coverslips in a sterile glass rack
2. Dilute Poly-L-Lysine stock solution in sterile sodium borate buffer 150 mM, pH 8.4, to a final concentration of 100 µg/mL
3. Pipet 200 µL Poly-L-Lysine solution onto each coverslip and incubate them overnight in the incubator at 37°C
4. rince 3x with sterile PBS; don't remove the PBS after the last rinse and place the coverslips back in the incubator until they will be used

Preparation of astrocyte feeder layers

Astrocyte cultures are prepared from neonatal rat neocortex and have to be split once a week; they can be kept for about 10 passages.
To remove neurons and microglial cells, flasks can be shaken prior to splitting. The purity of astrocyte cultures is crucial for optimal growth of neurons. The best feeder layers consist mainly of large, translucent astrocytes forming a monolayer, and are devoid of contaminating cells. It is crucial to remove the meninges prior to preparing cells for feeder layers to avoid contamination with fibroblasts.

Isolation of astrocytes

1. Prepare cortices from 2 newborn rats and cut them into small pieces
2. Incubate the tissue in 10-20 mL 0.25 x Trypsin/EDTA with DNase I for 15 min at 37°C
3. Wash twice with DMEM
4. Resuspend in DMEM/10% FCS
5. Dissociate cells with a fire polished Pasteur pipette
6. Let larger tissue pieces sediment; transfer supernatant in a flask (75 cm^2) containing pre-warmed DMEM/10% FCS and incubate at 37°C and 5% CO_2
7. Exchange the medium after 24 h
8. After 8 to 10 days the cells become confluent and can be split. Before using them as feeder layers, split them at least twice to remove all non-astrocytic cells

Expanding astrocyte cultures

1. Thaw trypsin/EDTA (10x) and dilute 1:10 with PBS
2. Warm up PBS and DMEM/10% FCS in flasks at 37°C and 5% CO_2
   (2 flasks for each flask to split plus additional medium for feeder layers)
3. Rinse flasks to be split once with pre-warmed PBS
4. Add 10 mL trypsin/EDTA per flask and incubate 1.5 min at 37°C and 5% CO_2
5. Check cells in the microscope:
   a. If already detached: => rinse cells from the wall and centrifuge 5 min at 1000 rpm. Resuspend with DMEM containing 10% FCS (5 mL out of each flask) and distribute them into two new flasks (or prepare feeder layers; see below)
   b. If only rounded up: => aspirate trypsin and put flask shortly back into the incubator. Add 10 mL DMEM containing 10% FCS and shake gently to detach the cells
6. Keep newly prepared flasks at 37°C and 5% CO_2

Preparation of feeder layers for neuron co-cultures

In advance:

1. Warm up Paraffin on a heater until melting (ca. 60°C)
2. Form a 90° angle in a Pasteur pipette above the Bunsen burner and place 3 small paraffin dots in each well of a 12-well plate
3. Sterilize 12-well plates 30 min under UV light
   Trypsinize astrocytes as described above (1 flask of confluent cells is sufficient for 2 to 3 12-well plates)
4. Suspend cells in DMEM/10% FCS and put a 500 µL drop into each well
5. Incubate for 1/2 h at 37°C and 5% CO_2 to allow cells to attach
6. Add 1.5 mL DMEM/10% FCS and incubate cells at 37°C and 5% CO_2. Allow 2-4 days to reach confluence
7. 24 h before use, stop cell division with 1:100 (in medium end volume) uridin/fluoro-deoxyuridin solution
8. Prior to use, rinse once with PBS

Before starting neuron preparation

1. Wash coverslips 3x with 200 µL sterile PBS; keep the last drop on the coverslips and keep in incubator until use
2. Pre-warm culture NeuroBasal medium
3. Aspirate the medium of the feeder layers
4. Rinse with pre-warmed PBS
5. Add 2 mL of NeuroBasal medium and incubate astrocytes at 37°C and 10% CO_2

Papain solution (fresh): dissolve papain in PBG to final concentration 0.5 mg/mL by heating to 37°C in water-bath for ca.1 h (during the dissection), store at RT until use, immediately before use, add DNase I to final concentration of 10 µg/mL (dilute stock 1:1000), sterilize by filtration.

PREPARATION OF DISSOCIATED NEURONS

Note: for cortical cultures follow the same procedure but only take cortices instead of hippocampi; use 30G needles mounted on 1 mL syringes as knives

1. Anesthetize a time-pregnant rat (Wistar, E18) with methophane or isoflurane and sacrifice it by cervical dislocation
2. Open the abdominal wall and remove embryos
3. Keep embryos on ice (in a laminar flow hood)
4. Decapitate them in ice cold PBS/Glucose
5. Carefully remove the brain and separate the two hemispheres from the brainstem
6. Remove the meninges (crucial step!)
7. Dissect out the hippocampi and transfer them in a 15 mL tube containing PBS/Glucose on ice
8. Carefully aspirate PBS/Glucose, add 0.05% papain solution in PBG and incubate 15 min at 37°C
9. Wash 2 times with DMEM
10. Add 2 mL DMEM/10% FCS
11. Dissociate cells with a fire-polished Pasteur pipette (approximately 5 to 10 times)
12. Count cells (with Trypan blue)
13. Dilute cells to the desired end volume in DMEM/10% FCS (normally 15-20'000 cells per Petri dish in 200 µL medium
14. Aspirate PBS from the glass coverslips
15. Place 200 µL cell suspension on each glass coverslip and incubate them for 1 h at 37°C and 10% CO_2 to allow for cell attachment
16. Take the coverslips out of the medium and place them up-site-down into the 12-well plate containing the feeder layers in NeuroBasal medium
17. Incubate cultures at 37°C and 10% CO_2

Medium change: After approximately two weeks in culture, exchange every week half of the medium with fresh NeuroBasal medium. Note: Never exchange all medium.

BUFFERS, STOCK SOLUTIONS, CELL CULTURE MEDIA

PBS 1x

Dissolve PBS Tablets in the corresponding amount of dH_2O (cell culture grade) and autoclave; store at 4°C

PBG (= PBS, BSA, glucose):

PBS; BSA, 1 mg/mL; glucose, 10 mM

Store at 4°C

Glucose 100x

Glucose-monohydrate (MW = 198.7)
Dissolve 250 mg/mL in PBS, filter sterile, store at 4°C

L-Glutamine (200 mM)

dissolve 1.35 g L-glutamine in 10 mL dH_2O

Glia stopping solution

Compound	**for 10 mL**
Uridin	35 mg
F-deoxyuridin	15 mg
dH_2O	10 mL

Filter sterile, store at 4°C

Cell culture media

Feeder layer culture medium; plating medium

Compound	**for 25 mL**
DMEM	22.5 mL
FCS	2.5 mL
Antibiotics (100x)	250 µL

Cell culture medium

Compound	**Stock**	**for 25 mL**
Neurobasal medium		24 mL
B27	50 x	500 µL
L-glutamine	200 mM, 100 x	250 µL
Antibiotics*	100 x	250 µL

* alternatively: Gentamicin, 50 µg/mL

Materials and chemicals

Cell culture material

- 12-well-plates: Nunclon™, Polystyrene, Cat. 150628
- 6-well plates: Nunclon™, Polystyrene, Cat. 140675
- Petri-dishes: Nunc, Cat. 153066
- Miniflasks: Slideflask, NUNC™, Cat. 170920
- Plastic coverslips: Nunc Thermanox ø 25 mm. Cat. 174985
- Glass coverslips: Assistent Deckgläser, ø 18 mm, thickness 0.17 ± 0.02 mm; Assistent-Hecht, D-97647 Sondheim/Rhön
- Paraffin: Paraplast, Sherwood medical, Cat. 8889-501007

Other chemicals

- PBS Tablets (Gibco, Cat. No. 18912-014)
- Poly-L-Lysine Hydrobromid Sigma, Cat. P-1524
- BSA Sigma, Cat. A-9418
- Trypsin/EDTA Gibco, Cat. 15400-054
- DNase I Sigma, Cat. D-5025
- ARA-C Sigma, Cat. C-6645
- Papain Sigma, Cat. P-4762
  store in aliquots of 2.5 mg or 5 mg powder at -20°C
- L-glutamine Gibco, 25030-024
- Uridin Sigma, U-3003
- F-deoxyuridin Acros, 227601000
- DMEM Gibco, 61965-026
- FCS Gibco, 10270-106
- Neurobasal medium Gibco, 21103-049
- B27 Gibco, 17504-044
- Antibiotics (100x) Gibco, 15240-062
- Gentamicin, 50 μg/mL, Gibco, 15750-037