原代神经细胞培养方法 Neuron Cell Culture

MacDermott Lab

Cell culture protocols

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1. Preparation of coverslips

1.1- Mass culture

Our standard mass cultures are plated on astrocytes. Those, in turn, are plated on glass coverslips pre-coated with poly-D-lysine and laminin.

Materials:

. #1 coverslips

. coverslip racks in a water-tight container (we made ours)

. poly-D-lysine (PDL) stock solution (1mg/ml in dd water)

. laminin stock solution (20 μg/ml in Hank's BSS)

. 35 mm plastic culture dishes

. culture hood equipped with UV lamp

. sterile dd water

Procedure:

. Place the coverslips in the racks and leave them in the culture hood under UV light for 2 hrs.

. Coat the coverslips with 12.5 μg/ml PDL (5ml PDL in 400ml sterile dd water) for 2hrs. in the culture hood.

. Wash the coverslips with sterile dd water five times, in the culture hood.

. Place the coverslips in the sterile 35mm dishes

. Add 0.4ml laminin on top of the coverslips. Wait for 45', then aspirate the excess solution

1.2- Agarose-collagen microislands

This protocol is based on protocols by Segal and Furshpan. Although the following "macro-island" approach has allowed for greater neuronal survival, while still providing a high probability of connection between DRG and dorsal horn (DH)

autapses or DH-DH connections can only be obtained with high probability in the conventional microislands.

Materials:

. #1 coverslips coated with PDL as above

. type II agarose

. Vitrogen 100 collagen, ~3 mg/ml

. 35 mm plastic culture dishes

. culture hood equipped with UV lamp

. sterile dd water

. atomizer

Procedure:

. Place coverslips in 35 mm dishes

. Melt agarose in dd water at 0.2%, and place a drop on the top surface of each coverslip. The height of each drop is diminished as much as possible by removing excess solution with a pipette before the agarose gels.

. Allow coated coverslips to air dry overnight in the culture hood at room temperature to form a thin film. For adequate drying, the dishes must be uncovered.

. Spray the collagen onto the coverslips with the atomizer. We use a glass perfume bottle which can be bought at Macy's in New York. In our experience that makes bigger droplets than the Fisher chromatography atomizer, and is much less expensive (and looks better too). The atomizer is held parallel to the bottom of the dishes, about 25 cm above and away from them. It is then pumped forcefully a few times.

. The collagen islands can be examined with an inverted microscope. Their size should be between 300 and 1000 μm in order to maximize the probability of connection between any two neurons.

. ADD A DROP OF COLLAGEN to the edge of the coverslip. That will serve as support for a "feeder culture" that helps the survival of the insular neurons

. The collagen is also allowed to dry overnight, under ultraviolet light.

1.3- “Macro”-islands

We find that few dorsal horn neurons seem to survive on the “traditional” atomizer-generated microislands, apparently regardless of neuron density. Although we still do not know the reason for that, we developed a variation of the microisland method which generates bigger (est. 1-3 mm) islands. With this method, the probability of finding an usable island in a giver coverslip is very much increased. Although, with such big islands, interconnected DH neurons are about as easy to find as in a mass culture, DRG neurons, which seem to grow extremely long axons, are usually connected to most of the DH neurons present.

Materials:

same as in 2.2, except:

.instead of the atomizer, a fine painting brush (#3 or smaller)

Procedures:

. All identical to 2.2, except for the collagen spraying.

. Dip the paint brush in the collagen solution, then shake the excess solution off the brush.

. Gently tap the brush on the edge of each dish, rotating the dish a few degrees after each tap.

. Control the quality of the islands by looking at the dishes through an inverted microscope. Some practice is required to optimize the islands.

2. Dissection, plating, and maintenance of cells

2.1- Media

In our system, Neurobasal + B-27 seems to improve the survival of the neurons, as well as increase neurite extension/branching. However, it does not favor astrocyte survival. Overall, we tend to use it for the co-cultures in “macro-islands” and for the

mass cultures in general, but not for the microislands on astrocytes.

A. IMDM for Astrocytes

Prepare stocks:

. For Glucose/Glutamine/Pen-Strep solution, mix

60 g of glucose in 200 ml dd water

100 ml Glutamine 200 mM

100 ml Penniciline-Streptomycine

. Filter through a 0.22 μm filter, separate in 20 aliquots of 20 ml each and freeze

. For FVM, mix

15 mg 6,7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride

75 mg glutathione

1.5 g ascorbic acid

into 300 ml dd water

. Adjust the pH to 5.5, filter through a 0.22 μm filter, divide in 60 aliquots of 5ml each and freeze

Final medium preparation

. Add

375 ml IMDM

100 ml Fetal Bovine Serum (20 %)

20 ml Glucose/Glutamine/Pen-Strep

5 ml FVM

. Filter through a 0.22 μm filter and store in fridge after use

B. MEM for Neurons

Prepare stocks:

. Heat-inactivate Horse Serum at 60 C for 30 min; then, freeze in 10 ml aliquots

. Freeze 8ml aliquots of Glucose (200mg/ml dd water)

. Freeze 2 ml aliquots of Vitamins for MEM

. Freeze 1 ml aliquots of ……UFDU

. Freeze 500 µl aliquots of NGF (10 µg/ml Hank's BSS)

Final medium preparation

. Add

180 ml MEM

10 ml Heat-inactivated Horse serum

8 ml glucose stock

2 ml Vitamins for MEM

. Add U/FDU and NGF as directed below, after plating the cells

. Filter through 0.22 µm filter

C. Neurobasal + B-27

Prepare stocks

. Freeze B-27 supplement in 4 ml aliquots

. Freeze 0.5 ml Glutamine 200 mM aliquots

Final medium preparation

.Add

195.5 ml Neurobasal

4 ml B-27

0.5 ml Glutamine 200 mM

2.2- Cortical astrocytes

A. Dissection and plating

Materials:

. 70% ethanol

. Three 60 mm culture dishes with cold L-15 medium (keep on ice)

. One 35 mm culture dish with 2 ml S-MEM

. Large scissors

. Small iris scissors

. Coarse dented forceps

. Two fine forceps

. Small spatula

. 2.5% trypsin

. IMDM for astrocytes

. Sterile 15 ml centrifuge tube, with cap, and culture centrifuge

. 0-1 day old rat pups (21-22 days from the plug date)

Procedures::

. Take out dissection instruments and place them in a tray full of ethanol (we recommend covering the bottom of the tray with paper towel to preserve the tips of the fine forceps). Place 35mm dish with S-MEM in the culture incubator

. Wipe pup's neck with 70% alcohol, cut off the head

. Add 70% alcohol to the head, hold it with lab tissue

. Cut open the skin longitudinally in order to see the whole superior face of the brain

. Carefully cut open the skull longitudinally and pull open the two flaps to expose the brain

. Carefully scoop brain out of the skull with a spatula

. Place the brain into a dish with L-15

. Cut off the brainstem, and separate the two hemispheres

. Clean out the hemispheres (i.e. remove the hippocampus, basal nuclei, etc.)

. You should only remain with the convexity of the hemispheres

. Remove the meninges and the blood vessels

. Place the two hemispheres into the 35mm dish with S-MEM

. Mince the two hemispheres with the small iris scissors

. Add 200 μl of Trypsin (2.5%), return dish to the incubator, and let incubate for 20-25 minutes

. Add 2 ml of IMDM to the dish

. Place all 4 ml into a 15 ml centrifuge tube and spin for about 8 min

. Discard the supernatant and add 2 ml of IMDM to the pellet

. Disperse the cells by repeatedly aspiring them with a 5ml pipette

. Place the cells into a flask that has been coated with PDL

. Add 11 ml of IMDM for a total of 13 ml in the flask

B. Splitting of the astrocytes

Materials:

. 2.5% trypsin-EDTA

. IMDM for astrocytes

. Sterile-filtered Ara-C (1mM in Hank's BSS)

Procedures:

Monitor the growth of the astrocytes :

. When the astrocytes have grown confluent they are ready to be shaken and split

. If the medium turns yellow remove it and add 13 ml of fresh medium

Shaking and Splitting the Astrocytes:

. Add Ara-C to the flask (130 μl for 13 ml of IMDM in flask)

. Close the cap tightly

. Place flask in a heated shaker, or in a shaker in an incubator or hot room

. The speed of rotation should be high, but not so much that the medium will splash

. Shake for 24 hrs

. Remove the medium and add 13 ml of Trypsin-EDTA (2.5%)

. Incubate 20-25 minutes at 37 C

. Add 13 ml of IMDM to stop the trypsin

. Place the total 26 ml into a 50 ml centrifuge tube

. Spin for about 10 minutes

. Remove the supernatant and add 5 ml of IMDM

. Disperse the cells gently by repeatedly pipetting the medium

. Add 8 ml of IMDM for a total of 13 ml

. Gently mix the cells by repeated pipetting, then split the medium into four flasks that have been previously coated with PDL (3.25 ml per flask)

. There will be 1/4 of brain per flask

. Feed the astrocytes every two weeks or whenever there is a discoloration of the

medium

. Once the astrocytes have again grown confluent they are ready to be plated

C. Astrocyte plating

Materials:

. Trypsin-EDTA

. IMDM for astrocytes

. PDL- and laminin-coated coverslips in 35 mm dishes (see above for coating procedure)

. 50 ml sterile centrifuge tubes

Procedures:

. Remove the medium from the flask

. Add 13 ml of Trypsin-EDTA

. Incubate 20-25 minutes at 37 C

. Check to see that the astrocytes have lifted off the bottom by holding flask up to the light: the Trypsin-EDTA solution should be cloudy with astrocytes that have lifted and are floating in the solution

. If there are still some stuck to the bottom, gently tap the sides or wait a little longer

. Add 13 ml IMDM to stop the trypsin

. Place the 26 ml into a 50 ml centrifuge tube and spin for approx. 10min.

. Remove supernatant and add 5 ml of IMDM

. Gently disperse cells with a 5 ml pipette

. Add enough IMDM so that you can add 0.5 ml of cell-containing medium per 35 mm dish

. Add 0.5 ml to each PDL- and laminin-coated coverslip (see above for coating procedure)

. Go back to each dish and add 1.5 ml more of IMDM for a total of 2 ml per dish

2.3- Dorsal horn neurons

A. Dissection and plating

Materials:

. MEM for neurons OR Neurobasal +B-27

. 70% ethanol

. Three 60 mm culture dishes with cold L-15 medium (keep on ice)

. One 50 ml tube with ~35 ml cold L-15 (keep on ice)

. One 35 mm culture dish with 2 ml S-MEM

. Large scissors (set of 3, largest is for cervical dislocation)

. Small iris scissors

. Small regular scissors

. Coarse dented forceps

. Two fine forceps

. 2.5% trypsin

. U/FDU (Uridine/5'-fluoro-2'-deoxyuridine) 1mM stock solution

. Sterile 15 ml centrifuge tube, with cap, and culture centrifuge

. Pasteur pipettes

Procedures:

Preparation:

. Look at the astrocyte dishes under the microscope to pick out the healthiest ones

. Remove all of the IMDM from the dishes and replace with MEM for NEURONS or with Neurobasal + B-27

. Thaw a 200 μl aliquot of 2.5% trypsin and reserve

. Add 2 ml of S-MEM into a 35 mm dish and place in the incubator

. Clean dissection area and wipe with ethanol

. Take out dissection instruments and place them in a tray full of ethanol

Remove the embryo:

. Select a pregnant rat in the 16th day of gestation

. Anesthetize the rat by placing it in an air-tight chamber and saturating the atmosphere with CO2

. Use the large scissors to kill the rat by cervical dislocation

. Wipe the abdomen of the rat with ethanol

. Cut through the skin, separate it from the peritoneum, and move the flaps of skin away from the surgical area

. Squirt ethanol in the exposed peritoneum

. Cut the peritoneum, and use coarse forceps to suspend the uterine tube, being careful to avoid the external surfaces of the rat

. Cut a section of the tube containing 2 or 3 embryos (seen as bulges in the uterus), and place in the L-15-containing centrifuge tube

. Appropriately dispose of the dead rat

Remove the spinal cord:

. Cut open the uterine tube, then remove two embryos by cutting the placenta

. Place embryos into a 60mm dish with cold L-15

. Remove embryo from its sac

. Transfer embryos to a new 60 mm dish with cold L-15 (use forceps to transfer the embryo by its head)

. Cut off the head, the umbilical cord and the tail

. Using fine forceps and the smallest iris scissors place the embryo on its back, and pin the embryo down by its shoulders with the forceps

. Using the small iris scissors, cut down the ventral length of the embryo

. With the embryo still pinned down, remove viscera with another pair of forceps (be careful not to puncture the spinal cord while you are removing the unwanted tissue)

. The ventral side of the vertebral column should now be visible; using the small iris scissors CAREFULLY cut down the length of the column to fully expose the spinal cord

. With iris scissors closed, free the cord from the surrounding tissue by pressing down off each side of the cord, as close to the cord as possible without actually touching it

. Transfer cords to a new 60 mm dish

Dissect the dorsal horns:

. Make sure the cord is on its ventral side (cord will curl in towards the ventral side,

and DRG's are closer to the dorsal side)

. Remove any DRG's

. Use two pairs of fine forceps to pull apart the meninges at the rostral end of the cord

. Gently press down the length of the medial aspect (ventral horn) of the spinal cord with the bottom of the closed iris scissors: the cord will open like a book, with the ventral horns in the center and the dorsal horns on each side

. Cut off the tip of the cord that curls inwards

. Using the iris scissors cut the cord longitudinally on each side to remove the lateral (dorsal) 1/3 of the cord

Dissociate the spinal cord:

. Place the four strips of dorsal cord into the 35mm dish with S-MEM

. Add 200 μl of trypsin (2.5%) and incubate for 20-25min at 37 C

. Remove all the S-MEM from the dish, carefully avoiding the dorsal horns

. Add 2 ml of MEM for NEURONS or Neurobasal + B-27 and transfer the dorsal horns to a 15ml tube

. Use a fire-polished Pasteur pipette to dissociate the tissue.

Count neurons and dilute to appropriate density:

. Mix cells in the tube and withdraw a small amount with a Pasteur pipette

. Place a small drop of the cells into each of the wells of the hemocytometer, the coverslip should already be in place (also wet the two rails that the coverslips sits on so that it won't fall off)

. Place hemocytometer upside down on the microscope

. Count all the phase-bright neuron sized objects in 9 squares (use the smaller gridded squares)

. Total number of cells equals:

number of cells counted x25/9x10,000x2mls

e.g. 125 cells x 25/9 x 10,000 x 2mls = 6,944,444.5 cells in 2mls

. Dilute dissociated cells to the appropriate volume with MEM for NEURONS or Neurobasal + B-27

(cells are usually plated at 250,000 per dish or 25 times less for microisland cultures; for microislands intended for autapse studies, we usually include a very small number of DRG neurons, since they seem to improve survival of the DH neurons)

Plate the neurons:

. Place 1ml of cell suspension into each dish

. Add 20 µl U/FDU to each dish the following day (do NOT add again to the same culture)

B. Maintaining the cultures

. Feed cells once a week by replacing 1 ml of the medium with fresh medium

. When feeding the cells, frequently change the pipette used for suction, to reduce cross-contamination

2.4- Dissociated dorsal root ganglion (DRG) neurons

A. Dissection and plating

Materials:

. MEM for neurons (see end of section for recipe)

. 70% ethanol

. Three 60 mm culture dishes with cold L-15 medium (keep on ice)

. One 50 ml tube with ~35 ml cold L-15 (keep on ice)

. One 35 mm culture dish with 2 ml S-MEM

. Large scissors (set of 3, largest is for cervical dislocation)

. Small iris scissors

. Small regular scissors

. Coarse dented forceps

. Two fine forceps

. 2.5% trypsin

. U/FDU (Uridine/5'-fluoro-2'-deoxyuridine) 1mM stock solution

. NGF 10 µg/ml stock solution

. Sterile 15 ml centrifuge tube, with cap, and culture centrifuge

. Pasteur pipettes

Procedures:

For co-cultures, we usually dissect the DRGs after the dorsal horn, as they seem to be less sensitive. Remove and dissect the embryos in the same way as for DH dissection, including removal of the viscera. From then on, there are differences:

Cut open the backbone

. Insert the lower blade of small iris scissors in the vertebral cavity, through the neck opening, and, by alternately cutting at either side of the midline, make two parallel cuts all the way down to the tail stub.

. The cuts should be made as lateral as possible to the midline.

. Use fine forceps to lift the “strip” of bone with care not to damage the underlying spinal cord.

. Using 2 fine forceps, gently pull spinal cord away from the vertebrae, at each side. Do not pull the spinal cord out yet. This is just to loosen the DRGs from the vertebrae. I have found that this considerably improves DRG recovery.

Remove the spinal cord with the attached DRGs

. Using 2 fine forceps, pull the spinal cord from the vertebral cavity. Hold the spinal cord by its most rostral aspect, being sure to take hold of some dura, and pull it away, slowly, while holding the body down with the other forceps.

. Transfer each spinal cord with attached DRGs to the previously prepared 35 mm Petri dish containing 2 ml S-MEM.

Extract DRGs

. Hold the spinal cord down with one fine forceps and, with the other, pluck the DRGs away like grapes (holding them by roots).

. After removing all possible DRGs, discard the spinal cord in some other dish

Enzymatic treatment and dissociation of DRGs

. Add 200 μl of trypsin (2.5%) and incubate for 20-25min at 37 C

. Remove all the S-MEM from the dish, carefully avoiding the dorsal horns

. Add 2 ml of MEM for NEURONS and transfer the dorsal horns to a 15ml tube

. Use a fire-polished Pasteur pipette to dissociate the tissue.

. Centrifuge the tube for 5 min at 500-1000 rpm

. Remove the supernatant and add re-suspend the pellet in 2ml MEM for neurons.

Count neurons and dilute to appropriate density:

. Mix cells in the tube and withdraw a small amount with a Pasteur pipette

. Place a small drop of the cells into each of the wells of the hemocytometer, the coverslip should already be in place (also wet the two rails that the coverslips sits on so that it won't fall off)

. Place hemocytometer upside down on the microscope

. Count all the phase-bright neuron sized objects in 9 squares (use the smaller gridded squares)

. Total number of cells equals:

number of cells counted x25/9x10,000x2 ml

e.g. 125 cells x 25/9 x 10,000 x 2 ml = 6,944,444.5 cells in 2 ml

. Dilute dissociated cells to the appropriate volume with MEM for NEURONS (cells are usually plated at 50,000 per dish for mass cultures, or 25 times less for microisland cultures; for microislands intended for autapse studies, we usually include a very small number of DRG neurons, since they seem to improve survival of the DH neurons)

Plate the cells

. Either mix DRG neurons with DH neurons or add 40 µl of concentrated cell suspension to dishes previously plated with DH neurons

. Add 10µl NGF per dish.

. Add 20µl U/FDU per dish

B. Maintaining the cultures

. Same as for DH monocultures, except that NGF must be added to the fresh medium at each feeding

2.5- Dorsal root ganglion (DRG) explants

This culture is identical to the dissociated DRG except in the plating procedures: the cells are not dissociated, and special care must be taken to recover as many explants as possible and to appropriately place them in the coverslips. A glass Petri dish is used for the final DRG removal because the DRG explants readily attach to the plastic culture dishes, reducing the final yield.

A. Dissection and plating

Materials:

Similar to dissociated DRG culture, except::

. add a 60 mm or smaller glass Petri dish with cold L-15, on ice

. add wide-bore 1-200 μl pipette tips

. add Pipetman or similar automatic pipettor

. no dish with S-MEM is necessary

. no trypsin is necessary

Procedures:

. Add U/FDU to dishes BEFORE adding the explants

Similar to dissociated DRG culture, until removal of spinal cord

. Place spinal cords with DRGs in the glass Petri dish with L-15

. Remove DRGs one by one, so that they will be well separated in the end

. After discarding the bare spinal cords, take Petri dish to the hood

Plating the DRG explants:

. Aspirate the medium from the Petri dish, rinsing the dish with medium if necessary to recover as many DRGs as possible

. Centrifuge the tube for 5 min at 1000 rpm

. Remove the supernatant, and re-suspend the cells in a final volume equivalent to 45 μl/dish

. Using the wide-bore pipette tips, aspirate and eject medium several times to re-suspend explants and remove 40 μl of explant-containing medium

. Carefully aim the tip at the center of the coverslip, and gently eject the medium. You have to actually touch the medium in the dish in order to get optimal placement

of the explants in the center of the coverslip.

. Repeat procedure, including re-suspending the explants, for every dish to be plated with explants.

3. Supplies & Suppliers

Ascorbic acid: Gibco #850-3080

B-27 supplement: Gibco #17504 -044

Fetal Bovine Serum: Gibco #263-00061

Hank’s BSS (Balanced Salt Solution) :Gibco #24020-125

L-Glutamine 200 mM: Gibco # 25030-081

MEM: Gibco # 11090-057

Neurobasal: Gibco # 21103-0495

Penicillin-Streptomycin: Gibco #1514-0122

S-MEM (Modified Eagle Medium) : Gibco #320 -1385 AJ

Trypsin-EDTA (2.5%): Gibco# 25300-054

Trypsin (2.5%): Gibco 610-5090 PE

Trypsin: Gibco #25300-054

Vitamins for MEM (Gibco #11120-052)

IMDM : Cellgro :#10-016-LV

Horse Serum: JRH Biosciences #12449-77p (test sample before purchasing)

Laminin: Collaborative Biomedical Products #40232

6,7-Dimethyl 5,6,7,8-tetrahydropterine HCl: Calbiochem #31636

Glutathione: Calbiochem #3541

ARA-C (Cytosine-B-D arabino-furanoside) Sigma # C1768

5’-Fluoro-2’-Deoxyuridine: Sigma #F0503

Glucose: Sigma #G7528 (highest grade)

PDL (poly-D-lysine): Sigma #P7886

Uridine: Sigma # U3750

NGF (Nerve Growth Factor): Boehringer Mannheim #1362-348

Vitrogen 100 collagen: Collagen Biomaterials #PC0701

4. Useful references

Agarose-collagen microisland cultures:

. Segal, M.M (1994) J.Neurophysiol.72:1874-83

Neurobasal:

. Brewer et al., (1993) J. Neurosci. Res. 35:567-576