Preparation and Coculture of Neurons and Glial Cells

In the development and maintenance of the nervous system there is a complex interdependency between neurons and glial cells. This relationship is vital for their individual differentiation, development, and functionality but also seems to play an important role in progressive neurodegeneration and in the modulation of neurotoxic effects.

Glial cells maintain normal functioning of the nervous system both by controlling the extracellular environment and by supplying metabolites and growth factors. Neurons may interfere with the proliferation and maturation of glial elements (Steward et al., 1991; Gegelashvili et al., 1997) and dynamically regulate the glial signaling pathway through the release of substances such as glutamate (Bezzi et al., 1998). There is also evidence that glia can be neurotoxic both in vivo and in vitro and can exacerbate neuronal damage caused by a variety of agents (Brown et al., 1996; Meucci and Miller, 1996; Rogove and Tsirka, 1998; Viviani et al., 1998). This effect may be attributable not only to an altered supply of trophic factors to neurons, establishment of contacts, and an altered buffering of the extracellular microenvironment by glia, but also to direct release of substances toxic to neurons such as reactive oxygen species (ROS), glutamate, and some cytokines—e.g., interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α).

Physiologically, the bidirectional communication existing between glia and other cells of the nervous system (i.e., neurons, other glial cells, and blood vessel cells) allows glia to link cells and structures that are not functionally connected and to continuously monitor and modulate their activity as a function of local needs (for a review, see Volterra and Meldolesi, 2005). The consequences of such interactions are the modulation of synaptic transmission, neuronal synchronization, and the regulation of cerebral blood flow. The recognition of this connection led to the definition of a new function—gliotransmission. The term gliotransmission describes the release of factors from physiologically stimulated glia; these factors are able to activate a rapid response in neighboring cells (Volterra and Meldolesi, 2005).

Cocultures of different cells of the nervous system (i.e., neurons, astrocytes, and microglia) represent the easiest approach to: (1) study intercommunication between the different cell populations of the nervous system, (2) evaluate its relevance in several physiological responses and/or propagation of the damage, and (3) study the molecular mechanisms involved. Other possible approaches are to use aggregate cultures of neural and glial cells and the more complex organotypic slices of hippocampus (Harry et al., 1998).

This unit describes procedures to set up a sandwich coculture system with a combination of neurons and glial cells (see Basic Protocol 1) or astrocytes and microglia cells (see Basic Protocol 2), as well as methods for the preparation of hippocampal neurons (see Support Protocol 1), glial cells (see Support Protocol 2), coated glass coverslips (see Support Protocol 4), and the separation of astrocytes and microglia from glial cells (see Support Protocol 3). A sandwich coculture is an in vitro cell system formed by two different cell populations growing on different surfaces, usually a coverslip and a petri dish. These surfaces are separated by small paraffin dots at the edges of the coverslip, on which one of the cell populations is seeded. In this way the two cell populations face each other without touching (Fig. 2.7.1), and soluble substances can diffuse between them. This cell system is therefore suitable for the study of biological responses that are due to the release of soluble mediators but not of those dependent on the contact of the two cell types.

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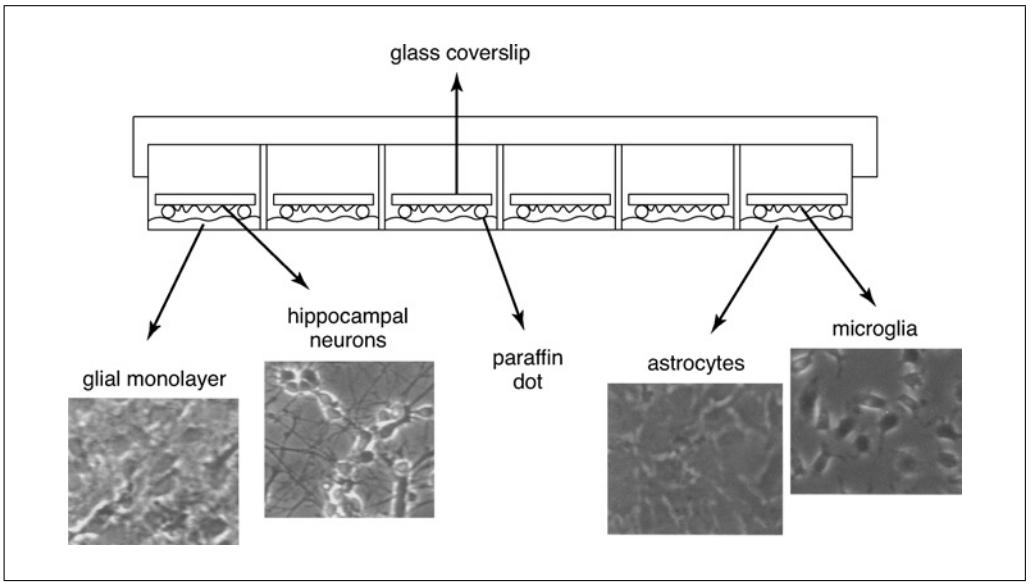

Figure 2.7.1 Scheme of a typical coculture (i.e., hippocampal neurons–glia or microglia-astrocytes) in a 24-well plate. Bright-field photographs are at 10× magnification. Note that cell populations are separated by paraffin dots on the edges of 12-mm round glass coverslips.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

NOTE: Working conditions must ensure the highest degree of sterility. Thus, brain tissues should be dissected and cells plated and grown in a laminar flow hood. Equipment must be sterilized before use and stored in 95% ethanol when not in use. All solutions and reagents that come into contact with tissues or cells must be sterile.

NOTE: All culture incubations should be performed in a 37°C, 5% CO_2 incubator with 95% relative humidity unless otherwise specified. As soon as prepared, cultures must be maintained in this type of incubator.

BASIC PROTOCOL 1

PREPARATION OF HIPPOCAMPAL NEURON-GLIA SANDWICH COCULTURES

In the author's laboratory, the neuron-glia sandwich coculture is used not only to study the interaction between glia and neural cells, but also to obtain a highly differentiated neural culture. Since glial cells are a valuable source of neurotrophic substances, cultures of cortical glial cells (see Support Protocol 2), consisting mainly of astrocytes and a small number of microglial cells, are prepared and allowed to reach confluence (∼10 days). These monolayers can be used for up to 1 month from their preparation. Next, cultures of hippocampal neurons are prepared by plating onto glass coverslips. These coverslips are then placed above the glial monolayer as shown in Figure 2.7.1. In the author's laboratory, the coculture is set up in 24-well plates, with one coverslip for each well. This avoids overlapping of different coverslips, which can occur if petri dishes are used. The main steps in preparing a neuron-glia sandwich coculture are summarized in Table 2.7.1. The dissection and culture techniques described here are largely based on the method of Goslin and Banker (1991) but have undergone minor changes in the author's laboratory.

Table 2.7.1 Preparation of Neuron-Glia Sandwich Coculture

Day	Action
1[a] (Thursday)	Prepare primary glial cultures (see Support Protocol 2)
8 (Friday)	Add paraffin dots and coat glass coverslips (see Support Protocol 4)
10 (Monday morning)	Wash and condition glass coverslips
	Substitute DMEM with SFM in glial cells
10 (Monday afternoon)	Prepare and seed hippocampal neurons (see Support Protocol 1)
11 (Tuesday)	Transfer coverslips with neurons over glial cells and add cytosine arabinoside to the coculture
18 (Tuesday)	Perform experiments

[a]Note this is 10 days before hippocampal dissection.

Materials

Confluent cortical glial monolayer in 24-well plates (see Support Protocol 2)
Serum-free medium (SFM; see recipe)
Coated glass coverslips seeded with hippocampal neurons (see Support Protocol 1)
2 mM cytosine arabinoside (see recipe)
Sharpened forceps

1. On the day hippocampal neurons are to be prepared (see Table 2.7.1), replace the medium in confluent cortical glial monolayers with 1 ml SFM and return them to the incubator overnight.

   The addition of SFM to glia prior to adding glass coverslips with hippocampal neurons allows conditioning to favor early phases of neural maturation. SFM is chosen because the ingredients are totally defined, and the absence of serum limits the growth of glial cells together with neurons on the glass coverslip.

   Since the author's laboratory usually prepares a number of 24-well glial plates, an amount sufficient for cocultivation with neurons produced over 1 month (i.e., more than will probably be used in one experiment), the medium is replaced only in wells that will receive a coverslip plated with neural cells the following day.

2. The next morning, use one tip of a pair of sharpened forceps to lift the edge of a coated glass coverslip plated with hippocampal neurons. Seize the coverslip with the forceps, and transfer and turn it over the glial monolayer. Repeat for each well to be used.

3. To each well containing neurons and glia, add 2 mM cytosine arabinoside to a final concentration of 5 μM to reduce the proliferation of glial cells.

   Addition of cytosine arabinoside, toxic to dividing cells, is important to block glial proliferation in the neuronal culture.

   In other workers' hands, cytosine arabinoside is usually added on the second to fourth day of culture. Under the experimental conditions used in the author's laboratory, such delayed addition of arabinoside did not satisfactorily reduce glial contamination. This is probably because of the large number of neural cells seeded by the author (160,000/coverslip), which enriches the cell suspension with rapidly dividing astrocytes. Addition of cytosine arabinoside immediately after neuronal cells are attached allows this laboratory to obtain a 98% pure neuronal culture on the glass coverslip, as assessed by immunocytochemistry of microtubule-associated protein 2 (a marker for neurons) and glial fibrillary acidic protein (a marker for astrocytes).

   Under these experimental conditions cytosine arabinoside is not toxic to neurons.

4. Maintain the cocultures, routinely feeding once every 7 to 10 days with SFM.

Neurons are very sensitive to temperature and environmental conditions. Thus, if the incubator is opened often during the day it is better to keep the plates towards the bottom where the temperature, percentage CO_2, and humidity are better preserved.

During feeding, it is important not to change the culture medium completely, since neurons depend upon glial cells to condition the medium for long-term survival. Thus, replace $\sim\frac{1}{3}$ of the medium each time. Under such conditions neurons survive for several weeks and become richly innervated.

These cells reach a high degree of maturation (i.e., developed neuronal network, functional glutamatergic system, and almost complete development of the post-synaptic density) after ~7 to 9 days of culture.

SUPPORT PROTOCOL 1

ISOLATION AND SEEDING OF HIPPOCAMPAL NEURONS

The author prepares hippocampal cultures from 18-day-old fetal rats (Table 2.7.1, day 10). At this stage, the generation of pyramidal neurons is complete while the generation of dentate granule cells has only just begun. Thus, the culture obtained will consist mainly of pyramidal neurons, which constitute the principal cell type in the hippocampus (85% to 90% of total neurons). From one litter of 18-day-old embryos (i.e., nine to twelve embryos), $\sim 7 \times 10^6$ cells for 30 to 40 coverslips 12 mm in diameter (160,000 cells/coverslip) are obtained. The number of coverslips is of course related to the plating density, which has to be chosen according to the experiment to be performed and the techniques available in the laboratory.

Low-density cultures suitable for the study of the development of individual cells and their synaptic interactions or the distribution of antigens within single cells require the application of microscopic techniques because of their sparse distribution. The author's group found that a plating density of 160,000 cells/coverslip yielded a highly differentiated hippocampal culture with a sufficient number of cells for the independent measurement of several parameters of neurotoxicity by nonmicroscopic and quantifiable techniques (Table 2.7.2).

Table 2.7.2 Parameters Measured in Hippocampal Cultures at Low and High Density

Parameter	Low-density cultures	High-density cultures
Neural cell death	Vital dye exclusion and count of living cells	MTT test (Denizot and Lang, 1986)
Apoptosis	Conformation of nuclei using Hoechst 33258 or propidium iodide dyes (Zhivotovsky et al., 1999)	Quantification of oligonucleosomal fragments
Intracellular Ca^{2+} homeostasis	Confocal microscopy (*UNIT 4.5*), video imaging	Spectrofluorimetric measurement in cell population stained with Fura-2 AM (Gunter and Gunter, 1999)
Reactive oxygen species production		Spectrofluorimetric measurement in cell population stained with 6-carboxy-2′,7′-dichlorohydro-fluorescein diacetate
Production of cytokines	Immunohistochemistry (Watkins et al., 2000)	Quantification by ELISA and/or RT-PCR (Bookout et al., 2006)

The procedures performed for isolating hippocampal neurons from rat embryos in the author's laboratory are similar to those described by Goslin and Banker (1991). This section provides an alternative for the dissociation of the hippocampi obtained and for growth in order to set up a sandwich coculture. The highest number of healthy neurons will be obtained by minimizing the total time between removal of uterine horns and dissociation of neurons, which must not exceed ~2 hr.

Materials

Pregnant female rats (Sprague-Dawley) at gestational day 18 (E18)
95% ethanol
HBSS (see recipe)
1× trypsin/EDTA solution (Sigma)
10 mg/ml DNase I stock solution (see recipe)
High-glucose MEM/10% (v/v) FBS (see recipe)
0.04% (w/v) trypan blue

Dissecting tools, sterile:
- Stainless steel scissors with ~4- and ~2-cm blades
- Curved forceps (2 pairs)
- Dumont forceps, no. 3c and no. 5

100- (two), 60- (one) and 35-mm (six) petri dishes
Dissecting microscope, e.g., Zeiss Stemi DV4
Anesthetizing chamber connected to a CO_2 tank
1.5-ml microcentrifuge tubes, sterile
1-ml and 200-µl pipet tips, sterile
24-well tissue culture plate containing coated coverslips (see Support Protocol 4)
75-cm^2 canted-neck flasks with screw caps

Additional reagents and equipment for determination of cell number and viability with a hemacytometer (*UNIT 1.1*)

Prepare for dissections

1. Arrange all the sterile dissecting tools on a sterile surface (e.g., sterile lid of a petri dish).
2. Fill all the petri dishes required for the preparation with the appropriate amount of HBSS (10 ml for 100-mm dishes, 5 ml for 60-mm dishes, and 2 ml for 35-mm dishes).
3. Set up the dissecting microscope.

Remove uterine horns and isolate embryo head

4. Sacrifice the day 18 pregnant animal by an officially approved procedure.

   Anesthetization is often required prior to decapitation. Anesthetize the pregnant rat in an anesthetizing chamber filled with CO_2. Wait until the rat has stopped breathing for a few seconds.

   Longer anesthesia is unnecessary and may result in damage to fetal neurons.

5. Remove the rat from the anesthetizing chamber and transfer it to a dissecting table, ventral side up, and sterilize the abdomen by pouring 95% ethanol over it.
6. Rinse forceps and scissor in ethanol, grasp the abdominal skin with forceps, and cut the abdomen completely open from the vagina to the thoracic cavity. Cut the diaphragm.

7. Gently grasp uterine horns at one of the constrictions and lift up. Remove the horns by cutting the attachments to the abdominal cavity and place them in a 100-mm petri dish filled with 10 ml cold HBSS. Keep on ice.

   Keeping embryos, brains, and later the hippocampi cold reduces metabolic activity and associated cellular damage.

   Typically there are ten to twelve fetuses in a Sprague-Dawley litter.

8. Remove the fetuses from the uterine horns and place them in a 100-mm petri dish with cold HBSS. Keep on ice.

   To remove fetuses grasp at the upper constriction of each yolk sac and cut along one side, the fetus will slip out of it.

9. Decapitate the fetuses with scissors and place the heads in a 60-mm petri dish with cold HBSS. Keep on ice.

Dissect out embryo brain

10. Place one head in a 35-mm petri dish (2 ml HBSS), with the neck down and the skullcap towards the operator. Under the dissection microscope, gently grasp the cut edges of skin and skull with both the Dumont no. 3c and no. 5 forceps and pull in opposite direction to expose the brain.

    Place anterior end of the head towards the top side of the petri dish and posterior end towards the bottom. This orientation will greatly facilitate the dissection.

11. Sever the cerebral hemispheres from the cerebellum and the spinal cord with Dumont no. 5 forceps.

12. Remove the brain by lifting upward out of the skull with curved-tip forceps and place it in a 60-mm petri dish with 5 ml HBSS. Keep on ice.

    Place the curved tips of the forceps under the brain and gently lift it upward.

13. Repeat steps 10 to 12 for each head.

Isolate hippocampi

14. Place the brain in a 35-mm petri dish (2 ml HBSS), with the dorsal side up and the posterior end toward the operator. Working under the dissecting microscope, place the two tips of Dumont no. 5 forceps along the brain midline under the cortices, one at the anterior and the other at the posterior end. Use a single cut to isolate the cortices from the brain stem. Repeat for the other hemisphere.

15. Discard the brainstem and orient one of the hemispheres with the medial surface upwards.

    The cerebral hemisphere is defined by a dorsal side that is much more rounded than the ventral side and usually by the olfactory bulb that projects anteriorly. The hippocampus is located in the medial-posterior part of the cerebral cortex on the back of the olfactory bulb. When the medial cortex is perfectly clean, the hippocampus is clearly visible as a C-shaped structure, with its dorsal margin separated from the adjoining cortex by a fissure while the ventral margin is free.

16. Gently remove meninges by pulling them off with Dumont no. 3c and no. 5 forceps.

17. To obtain the hippocampus, make a curving incision with Dumont no. 5 forceps along the dorsal hippocampal fissure. Release the hippocampus by cutting at the end of the curved incision.

18. Transfer the hippocampus to a 35-mm petri dish with 1 ml HBSS and keep on ice.

19. Repeat steps 14 to 18 for each brain.

Prepare hippocampi

20. Collect hippocampi isolated from rat embryos in a sterile 1.5-ml microcentrifuge tube.

21. Centrifuge 4 min at 100 to 150 × *g*, room temperature. Carefully remove the supernatant.

    Aspiration with a pipet is recommended over a vacuum system to avoid aspirating the hippocampi along with the medium.

Trypsinize hippocampi

22. Add 400 µl of 1× trypsin/EDTA solution and 80 µl of 10 mg/ml DNase I solution. Shake gently (do not vortex) and incubate 5 min at 37°C.

    DNase is added to prevent the DNA released by damaged cells from making the dissociation medium too viscous during digestion.

23. While hippocampi are incubating, prepare four 1.5-ml microcentrifuge tubes with 400 µl high-glucose MEM/10% FBS each.

24. Gently aspirate trypsin/DNase solution with a sterile 1-ml pipet (do not use vacuum). Add 400 µl MEM/10% FBS and shake until hippocampi are floating in the medium.

    The serum in the MEM inhibits residual trypsin and prevents overdigestion of the cells.

***Wash out trypsin and DN*ase**

25. Allow the hippocampi to pellet at the bottom of the tube.

26. Gently collect the pelleted hippocampi with a 1-ml pipet in the smallest possible volume of medium and transfer to the first of the four tubes containing MEM/10% FBS (step 23).

    Take care not to destroy the hippocampi during this and the immediately following steps.

27. Gently shake and allow the hippocampi to pellet at the bottom of the tube.

28. Repeat steps 26 and 27 another three times using the remaining tubes in succession.

    Repetitive passage through microcentrifuge tubes with fresh medium washes the hippocampi free from trypsin and DNase I.

Disaggregate and assess cell number and viability

29. In the last microcentrifuge tube, disaggregate the hippocampi by gently drawing up and down in a 1-ml pipet first and then a 200-µl pipet tip. Adjust the volume of the cell suspension to 1 ml using MEM/10% FBS.

    It is important to be very gentle in pipetting: too vigorous or extensive pipetting causes significant cell death. Pipetting in sequence through a 1-ml pipet and then a 200-µl pipet tip gradually yields a single-cell suspension. Note that pipet tips must be sterile.

30. Gently mix 10 µl cell suspension with 10 µl of 0.04% (w/v) trypan blue. Determine the total cell number and viability with a hemacytometer and an inverted phase-contrast microscope (see *UNIT 1.1*).

    Trypan blue is used to distinguish viable from dead cells. Viable cells exclude trypan blue while dead or damaged cells are stained (dark blue).

Plate and prepare hippocampal neurons for the sandwich coculture

31. Pipet 160,000 cells into each well of a 24-well plate containing a coated coverslip.

32. Incubate plates overnight and place the coverslip over the confluent glial monolayer the next morning.

Remember to keep the plates towards the bottom of the incubator where temperature, percentage CO_2, and humidity are better preserved. Neural cells will attach to the coverslip surface during this incubation.

Since seeding medium is not suitable for hippocampal neuron survival and differentiation, remember to transfer the coverslips the day after plating.

BASIC PROTOCOL 2

PREPARATION OF ASTROCYTE-MICROGLIA SANDWICH COCULTURES

Glia include three cell types: astrocytes, microglia, and oligodendrocytes. All glial subtypes can greatly influence not only neuronal, but also neighboring glial cell activity. Astrocytes, long considered to be primarily supportive of neurons, are now thought to be active participants in neural circuit functions. Recently it has been observed that astrocytes are organized in distinct territories and may influence the surrounding cells (including other glial cells) due to the release of diffusible extracellular signals (Volterra and Meldolesi, 2005). Thus, astrocytes influence spontaneous astrocytic excitation by releasing glutamate (Nett et al., 2002), which in turn may result in the excitation of neighboring neurons. The astrocytes may control the release of several modulators (i.e., TNF-α, SDF-1α, and glutamate) through the production of eicosanoids (Bezzi et al., 1998).

Under physiological conditions microglia have a "resting" phenotype adapted to the microenvironment of the central nervous system. However, microglia are able to respond quickly to a variety of signaling molecules. Activation at a very early stage in response to damage or alterations to the microenvironment that precede pathological changes is characteristic of microglia, and its activation often precedes reaction of any other cell type in the brain. The author has recently observed that microglial cells can influence the response of astrocytes in producing neurotoxic substances. For instance, the production of tumor necrosis factor-α (TNF-α) after a neurotoxic insult is much higher in a culture of astrocytes and microglia than with astrocytes or microglia alone (Viviani et al., 1998; Bezzi et al., 2001). Thus, the progression of neurodegenerative events is also modulated by glia-glia interactions.

On the basis of the experience gained by the author's group in coculturing neurons and glial cells (Basic Protocol 1), a similar coculture system of astrocytes and microglial cells has been set up in order to study the molecular mechanisms involved in communication between these two cell population. First, the MEM/FBS medium present in a 24-well plate seeded with astrocytes (see Support Protocol 3) is replaced with SFM (see recipe) 1 to 2 days after the preparation of the culture. Next, the tip of a pair of sharpened forceps is used to lift the corner of a coated glass coverslip seeded with microglia (see Support Protocol 3), seize it, and transfer it over the astrocyte monolayer. In this fashion, microglial cells are juxtaposed, without direct contact, with a monolayer of astrocytes. Note that in the author's laboratory, the astrocyte-microglia coculture is usually used within 48 hr of its preparation.

SUPPORT PROTOCOL 2

ISOLATING AND CULTURING CORTICAL GLIAL CELLS

Glial cells (astrocytes, microglia, and oligodendrocytes) are obtained by mechanical and enzymatic dissociation (e.g., trypsin) of cerebral tissue from 1- to 2-day-old old rat pups (Sprague Dawley; day 1, Table 2.7.1). The use of such young rats ensures the absence of viable neurons in the cell suspension. From one 2-day-old pup, the author usually obtains $\sim 5 \times 10^6$ cells.

The author uses confluent cultures of glial cells both to obtain astrocytes and microglia, and to cocultivate hippocampal neurons. Shaking a confluent glial culture makes it possible to separate astrocytes from microglial cells and obtain two purified cultures (see Support Protocol 3). For this purpose the author's laboratory seeds 4×10^6 glial cells in 75-cm^2 culture flasks. To obtain a glial monolayer for neuron-glia coculture, cells are seeded in 24-well plates at a density of 50,000 cells/ml per well. Both the cells seeded in the wells and those seeded in the flasks reach confluence in ~10 days.

Materials

1- to 2-day-old Sprague-Dawley rat pups.
95% (v/v) ethanol
HBSS (see recipe)
10× trypsin/EDTA (Sigma)
10 mg/ml DNase I (see recipe)
High-glucose MEM/10% and 20% (v/v) FBS (see recipe)
0.04% (w/v) trypan blue

Dissecting tools, sterile:
- Stainless steel scissors with ~4- and ~2-cm blades
- 2 pair curved forceps
- Dumont forceps, no. 3c and no. 5
- Bistoury (Aesculap)

100- (one), 60- (two) and 35-mm (four) petri dishes
Dissecting microscope, e.g., Zeiss Stemi DV4
100-μm nylon cell strainer (Falcon)
50-ml conical polystyrene centrifuge tubes
24-well tissue culture plates
75-cm^2 canted-neck tissue culture flasks with screw caps

Additional reagents and equipment for determining total cell number and viability with a hemacytometer (*UNIT 1.1*)

Prepare for dissections

1. Arrange all the sterile dissecting tools on a sterile surface (e.g., sterile lid of a petri dish).
2. Fill all the petri dishes required for the preparation with the appropriate amount of HBSS (10 ml for 100-mm dishes, 5 ml for 60-mm dishes, 2 ml for 35-mm dishes).
3. Set up the dissecting microscope.

Isolate heads from neonatal pups

4. Transport 1- to 2-day-old Sprague-Dawley pups from the cage to the laboratory.

   The authors order a number of pups, usually three, adequate to provide the amount of glial cells needed for 1 month of experiments.

   Neonatal rats can become hypothermic very quickly. To avoid this, place the pups in a nest of cotton in a small open box and cover them as much as possible to help maintain their body temperature.

5. Sacrifice the animal by an officially approved procedure.

   Anesthetization is often required prior to decapitation. To anesthetize pups, place a small piece of cotton wool soaked in halothane or other approved anesthetic in a tube. Place the pup into the tube until it is anesthetized. Replenish the anesthetic between uses if necessary.

6. Gently hold the pup with thumb and forefinger and rinse it with 95% ethanol.

7. Decapitate the pup with scissors over a 100-mm petri dish with 10 ml HBSS. Keep head on ice.

   Keeping head, brains, and later the cortices cold reduces metabolic activity and the associated cellular damage.

8. Repeat steps 5 to 7 for each pup.

   Sterile scissors should be placed on a sterile petri dish lid or in a beaker with 95% ethanol.

Dissect out brains

9. Place one head in a 35-mm petri dish with 2 ml HBSS. Secure the head by holding down the snout with a curved-tip forceps and cut skin and skull along the midline from the base of the skull to the snout with sharpened scissors. To ease removal of the brain, cut left and right side of the skull in the center perpendicularly to the midline.

   Place the head in the petri dish with neck down and skullcap towards the operator. Place anterior end of the head towards the top side of a petri dish and posterior end towards the bottom. This orientation will greatly facilitate the dissection.

   To avoid damaging the underlying brain tissue, place one scissors tip just under the skull bone and lift upward with the scissor blade while cutting.

10. Still keeping the head secured, remove the skull with a curved-tip forceps and expose the brain. Sever the olfactory bulbs at the anterior end of the brain and the spinal cord at the posterior end with Dumont no. 5 forceps.

11. Remove the brain with a curved-tip forceps and collect it in a 60-mm petri dish with 5 ml HBSS. Keep on ice.

    Place the curved tips of the forceps under the brain and gently lift it upward.

12. Repeat steps 9 to 11 for each head.

Isolate cortices

13. To separate the cortices from the rest of the brain, move one brain to a 35-mm petri dish with 2 ml HBSS. Working under the dissecting microscope, place the two tips of Dumont no. 5 forceps along the brain midline under the cortices, one at the anterior and the other at the posterior end. Use a single cut to isolate the cortices from the brain stem.

14. Repeat this step for each brain and store all the cortices on ice in a 60-mm petri dish with 5 ml HBSS.

15. Place one cortex in a 35-mm petri dish (2 ml HBSS) and, still working under the dissecting microscope, gently remove any extraneous tissue as well as the meningeal coverings on the cortical surface by pulling them off gently with Dumont no. 3c and no. 5 forceps. Take care not to destroy the tissue by pressing too hard with the forceps.

    A semitransparent tissue, the meninges, surrounds the surface of the cortex. They are recognizable for their slightly pink color due to the presence of blood vessels. When the meninges are completely removed, the surface of the cortex appears completely white.

16. Repeat step 15 for each cortex.

Disassociate cells

17. Place cortices from 1- to 2-day-old rat pups in a 35-mm petri dish on ice (no medium). To obtain a homogenate of finely minced cortices, accumulate them together in the center of the dish and cut several times in different directions with a bistoury.

18. Add 2 ml HBSS and resuspend the minced cortices by drawing the mixture in and out of a disposable, sterile, 2-ml plastic pipet.

19. Remove the suspension and transfer to a 50-ml centrifuge tube.

20. Repeat steps 2 and 3 twice more to collect all of the minced cortices from the dish. Resuspend minced cortices in 6 ml HBSS.

Trypsinize cells

21. Add 750 µl of 10× trypsin/EDTA and 750 µl of 10 mg/ml DNase I to the suspension.

    DNase is added to prevent the DNA released by damaged cells from making the dissociation medium too viscous during digestion.

22. Seal the capped tube with Parafilm. Vigorously agitate 15 min in a 37°C water bath to favor enzymatic digestion of the tissue. While the tube is being agitated, prepare a 50-ml centrifuge tube containing 12 ml high-glucose MEM/10% FBS.

23. After agitation, remove the tube from the shaking water bath and allow the undissociated tissue to collect at the bottom.

    Avoid centrifugation; dissociated cells must remain suspended so they can be collected.

24. Collect 5 ml dissociated cells with a disposable, sterile, plastic 12-ml pipet and transfer to the 50-ml centrifuge tube containing MEM/10% FBS (step 22).

    Take care not to include undissociated tissue pieces.

Repeat trypsinization and consolidate products

25. Add 6 ml HBSS, 750 µl of 10× trypsin, and 750 µl DNase I to the 50-ml tube containing the remaining undissociated tissue.

    The FBS in MEM (step 22) inhibits trypsin and thus prevents overdigestion of the dissociated cells.

26. Repeat steps 22 and 23, and add these dissociated cells to the 50-ml tube containing the cells from the first digestion as described in step 24.

***Remove trypsin and DN*ase**

27. Filter dissociated cells through a 100-µm nylon cell strainer and collect in a fresh 50-ml centrifuge tube.

    Filtration eliminates any pieces of tissue accidentally collected with dissociated cells.

28. Pellet pooled cells by centrifuging in a swinging-bucket rotor 5 min at 200 to 300 × *g*, room temperature.

29. Carefully remove the medium from the pelleted cells.

Disaggregate and assess cell number and viability

30. Disaggregate the pellet by first adding 2 ml MEM/10% FBS and then gently drawing in and out of a 2-ml disposable plastic pipet until the solution becomes homogeneous. Add an additional 3 ml MEM/10% FBS for a total resuspension volume of ~5 ml.

31. Gently add 100 µl cell suspension to 200 µl of 0.04% (w/v) trypan blue. Determine total cell number and viability with a hemacytometer and an inverted phase-contrast microscope (*UNIT 1.1*).

    Trypan blue is used to distinguish viable from dead cells. Viable cells exclude trypan blue while dead or damaged cells are stained (dark blue).

Plate and prepare glia for the sandwich coculture

32. From the 5-ml cell suspension (step 30) aspirate a volume containing a sufficient number of viable cells to prepare the desired number of 24-well plates at a ratio of 50,000 cells/well. Dilute the cell suspension to 50,000 cells/ml with high-glucose MEM/20% FBS.

    The author usually plates a sufficient number of wells for the cocultivation of neurons prepared over 1 month.

    Glial cells are plated and kept in MEM/20% FBS for 5 days, then the medium is changed to MEM/10% FBS. The cells are maintained in this medium up to 1 month.

33. Pipet 1 ml cell suspension into each well of a 24-well plate.

Plate and prepare glia to obtain astrocytes and microglia

34. From the 5-ml cell suspension (step 30), aspirate a volume containing a number of cells sufficient to prepare the desired number of 75-cm^2 canted-neck flasks with screw caps at a ratio of $\sim 4 \times 10^6$ cells/flask. Dilute 4×10^5 cells/ml in MEM/20% FBS.

    Astrocyte and microglial cells are obtained by shaking the glial culture to divide the two cell populations (see Support Protocol 3). Thus, it is important to use culture flasks having screw caps that can be completely tightened to prevent medium spillage during the shaking period.

35. Pipet 10 ml cell suspension into each flask.

Grow cultures to confluence

36. Incubate plates and flasks 24 hr.

37. To eliminate undetached cells, remove the medium and add 1 or 10 ml fresh MEM/20% FBS for each well or flask, respectively. Repeat 5 days later.

38. Five days later, remove MEM/20% FBS, replace with MEM/10% FBS, and continue to grow to confluence, changing the medium twice a week (e.g., Monday and Thursday or Tuesday and Friday).

    Confluence will be reached in ~10 days.

SUPPORT PROTOCOL 3

ISOLATION AND SEEDING OF CORTICAL ASTROCYTES AND MICROGLIA CELLS

The techniques described here to prepare and separate astrocytes and microglial cells are based on the methods of McCarthy and DeVellis (1980) and Giulian and Baker (1986), which have undergone some changes in this laboratory. The method is based on the selective detachment of microglial cells from astrocytes by shaking mixed glial cultures on an orbital shaker; taking advantage of the difference in degree of attachment of the two cell types to tissue-culture plastic. Astrocytes are then further depleted of microglial cells by treatment with L-leucine methyl ester (L-LME), which selectively kills microglia.

Both astrocytes and microglial cultures appear >97% pure, as assessed by immunocytochemistry of glial fibrillary acidic protein (GFAP), a cytoskeletal protein found only in astrocytes, and with *Griffonia simplicifolia* isolectin B4, a selective marker of both resting and activated microglia (Cheepsunthorn et al., 2001). From each flask of confluent glial cells the author's group usually obtains $\sim 5 \times 10^6$ astrocytes. The number of microglial cells separated from each flask is extremely variable and changes with different preparations (i.e., from $\sim 5 \times 10^5$ to $\sim 3 \times 10^6$ cells).

Materials

High-glucose MEM/10% and 15% FBS (see recipe)
0.04% (w/v) trypan blue solution
1× PBS (Sigma; *APPENDIX 2A*)
High-glucose MEM/10% FBS containing 5 mM L-LME (Sigma)
1× trypsin/EDTA solution (Sigma)
24-well plate containing coated glass coverslips (see Support Protocol 4)
Additional reagents and solutions for preparing confluent glial cultures in 75-cm^2 cantered-neck flasks (see Support Protocol 2) and determining cell number and viability with a hemacytometer (*UNIT 1.1*)

Detach microglia

1. Prepare confluent glial cultures in 75-cm^2 canted-neck flasks (see Support Protocol 2).

   Use culture flasks since the caps can be completely tightened to prevent medium spill during the shaking period.

   In the author's laboratory, usually ~4 × 10^6 cells are seeded per flask. Under these conditions glial cells reach confluence after 10 days (i.e., to obtain a confluent monolayer on Monday prepare glial cells on the Thursday two weeks before).

2. Use Parafilm to seal the flask caps and vigorously agitate on an orbital shaker 1.5 hr at 260 rpm, 37°C.

   Within 2 hr of shaking, most of the microglia can be detached from the glial monolayer and subcultured. Astrocytes remain adherent to the flask.

3. Collect the medium from the flasks in one or more 50-ml centrifuge tubes.

   Collect up to 50 ml in each tube; one 50-ml tube is enough for six flasks.

Equilibrate astrocytes and pellet microglia

4. Add 10 ml fresh high-glucose MEM/10% FBS to each flask with a sterile pipet.

5. To pellet the suspended cells, centrifuge the 50-ml tubes 5 min in a swinging-bucket rotor at 200 to 300 × *g*, room temperature. Meanwhile, transfer the flasks to an incubator for at least 1 hr.

   To obtain purified astrocytes, the flasks have to be shaken further overnight. Since it is usually impossible to shake the flasks in an incubator, it is better to allow the fresh medium to equilibrate before shaking again. This period usually suffices for the preparation of microglial cultures.

Disaggregate and assess cell number and viability

6. At the end of the centrifugation discard the supernatant, add 2 ml MEM/10% FBS to microglial-cell pellet using a sterile pipet and disaggregate by drawing up and down. Add an additional 3 ml MEM/10% FBS for a total of 5 ml and resuspend to obtain a homogeneous suspension.

7. Add 100 µl cell suspension to 200 µl of 0.04% trypan blue solution and mix gently. Determine total cell number and viability with a hemacytometer and inverted phase-contrast microscope (*UNIT 1.1*).

   Trypan blue is used to distinguish viable from dead cells. Viable cells exclude trypan blue while dead or damaged cells are stained (dark blue).

Prepare and plate microglia for the sandwich coculture

8. Gently resuspend sedimented cells, then dilute to 200,000 cells/ml with MEM/10% FBS.

9. Add 1 ml suspension to each well of a 24-well plate containing coated glass coverslips.
10. Incubate 30 min.

    This short incubation period allows the adhesion of chiefly microglial cells to the substrate.

11. Gently shake the plate, remove the medium, and wash once with 1× PBS.

    By shaking and washing, loosely adhering cells like oligodendroglia are removed.

12. Add 1 ml high-glucose MEM/15% FBS to each well and transfer plates to an incubator.

Purify astrocytes

13. Transfer the flasks containing partially purified astrocytes (step 5) to an orbital shaker and agitate overnight at 260 rpm, 37°C.
14. The next morning, remove the medium and wash the monolayer three times with 1× PBS.
15. Add 10 ml high-glucose MEM/10%FBS/5 mM L-LME to each flask.

    L-LME selectively kills microglial cells and is used to reduce any microglial contamination of astrocytes.

16. Incubate 2 hr.
17. Wash twice with PBS.

Trypsinize astrocytes

18. Add 2 ml of 1× trypsin/EDTA to each flask to detach astrocytes and gently rotate so that the trypsin can spread over the surface where astrocytes are attached. Incubate 5 min.

    Do not exceed this time, otherwise there is danger of overdigestion. If it is necessary to detach astrocytes from several flasks, do not add trypsin to all of them at the same time.

    To detach cells without using trypsin, incubate the monolayer 5 min in 5 ml PBS without calcium and magnesium, supplemented with 5 mM EDTA; however, this procedure gives lower cell recovery than trypsinization.

19. Detach astrocytes by gently shaking and washing the surface of the flask.
20. Transfer the trypsin solution containing astrocytes to a tube containing 2 ml MEM/10% FBS.

    The serum contained in the medium inactivates trypsin. Thus, to avoid overdigestion the cells resuspended in trypsin are immediately added to an equal volume of cell culture medium. If more than one flask is to be trypsinized, the cells can be pooled in a single tube containing cell culture medium. Add 1 ml of cell culture medium for each milliliter trypsin.

Prepare and plate astrocytes for the sandwich coculture

21. To pellet cells, centrifuge 10 min in a swinging bucket rotor at 100 to 200 × *g*, room temperature. Aspirate the medium and resuspend the pellet in 2 ml of MEM/10% FBS.
22. Repeat steps 7 and 8 with the astrocyte suspension.
23. Add 1 ml cell suspension to each well of a 24-well plate.
24. Transfer to an incubator.

    Astrocytes can be maintained up to 1 month.

PREPARATION OF GLASS COVERSLIPS FOR SANDWICH COCULTURES

SUPPORT PROTOCOL 4

Hippocampal neurons and microglial cells do not readily adhere to glass. Thus, to promote their attachment, maturation, and survival, coverslips are coated using poly-L-ornithine, or poly-L-lysine. In addition, three dots of paraffin are placed on each coverslip to create a narrow gap between the two cultivated cell populations.

The use of 24-well plates and 12-mm coverslips to obtain a coculture does not allow an open-face sandwich coculture. This is basically because the cell monolayer below the coverslip degenerates. In 24-well plates, where the well is slightly bigger than the coverslip, this means the destruction of the glial or astrocyte monolayer.

The coverslips are prepared at least one day before the hippocampal neurons (see Support Protocol 1) or microglial cells (see Support Protocol 2) are dissociated. In the author's laboratory, if hippocampi are dissected on Monday, coverslips are prepared the Friday before and kept in polyornithine at room temperature until Monday morning. Next they are washed and incubated in growth medium before being seeded with cells.

Materials

Paraffin wax
1× poly-L-ornithine solution (see recipe)
1× PBS (Sigma)
High-glucose MEM/10% FBS (see recipe)

12-mm glass coverslips
24-well tissue-culture plates
Microwave oven
5- to 10-ml sterile syringe with 0.95 × 40–mm needle
Germicide lamp (e.g., as equipped on a flow hood)

1. Place one 12-mm glass coverslip in each well of a 24-well tissue-culture plate.

   Each time, prepare the exact number of coverslips to be used. This will reduce the possibility of contamination.

2. Sterilize the coverslips by microwaving 10 min at the highest power setting.

3. Place the plates next to a beaker half full of water in order to avoid melting the plastic plates.

4. Heat paraffin wax to ∼100°C. Take an ∼2-ml aliquot in a 5- or 10-ml syringe and apply three small drops near the outer edge of each coverslip at roughly equal distances from each other.

   To melt paraffin, place four pieces in a beaker and place the beaker on a hot plate.

   By working rapidly, it is possible to place several dots over two to three coverslips. The temperature of the paraffin is very important. If it is too hot, it spreads too thin and wide, while if it is too cool, the dots do not adhere to the coverslip and will subsequently detach.

5. Resterilize the coverslips by UV irradiation for 30 min with a germicide lamp.

   The germicide lamp of a flow hood is sufficient. Avoid exposing coverslips to UV after they have been coated with polyornithine.

6. In a laminar flow hood, add 1 ml of 1× poly-L-ornithine solution in each well containing the coverslips.

   Check that the coverslips do not float in the well but are completely covered by polyornithine. To prevent floating, be sure to eliminate any air bubble under the coverslip by gently pressing over it.

7. Incubate the plates up to 2 days at room temperature or 2 hr at 37°C.

   When plates are out of the laminar flow hood, store them sealed with Parafilm in the dark.

8. Immediately before isolating cells (see Support Protocol 2), remove polyornithine and rinse twice with 1× PBS.

   To obtain the best result in the attachment of cells and their development do not let the coverslips dry at any stage.

9. After the final rinsing add 1 ml high-glucose MEM/10% FBS and incubate until the end of cell preparation.

   When isolated and dissociated, cells will be seeded in this same medium.

   The coverslips are now ready to be seeded. Add the desired number of cells over the coverslips without changing the incubation medium.

   In the author's laboratory unused coverslips are discarded.

REAGENTS AND SOLUTIONS

Use deionized, distilled water or equivalent for all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cytosine arabinoside, 2 mM

Dissolve 2 mg cytosine-1-β-D-arabino-furanoside (cytosine arabinoside) in 5 ml water. Store up to 6 months at −20°C.

DNase I, 10 mg/ml

Dissolve 100 mg of 536 Kunitz units/mg DNase I (Sigma) in 10 ml HBSS (see recipe). Store up to 6 months in 1.5-ml aliquots at −20°C.

HBSS

To 850 ml H_2O add:
Hanks Balanced Salts powder (Sigma), enough for 1 liter
10 ml 10 mM HEPES (Sigma)
10 ml penicillin/streptomycin stock solution (Sigma)
Adjust volume to 1 liter with H_2O
Sterilize using a 0.22-μm cellulose-acetate disposable vacuum-filtration system (Millipore)
Store up to 1 month at 4°C

The stock solution of penicillin/streptomycin contains 10,000 U/ml penicillin and 10 mg/ml streptomycin. Store this stock solution up to reported expiration date in 5-ml aliquots at −20°C.

High-glucose MEM/10%, 15%, or 20% FBS

500 ml minimal essential medium with Earle's salts (MEM; Sigma)
0.6% (w/v) D(+)-glucose (Sigma)
5 ml penicillin/streptomycin stock solution (Sigma)
5 ml of 200 mM L-glutamine
Shake vigorously to dissolve glucose
10%, 15%, or 20% (v/v) FBS (Sigma)
Sterilize using a 0.22-μm cellulose-acetate disposable vacuum-filtration system (Millipore)
Store up to 1 month at 4°C

Do not add FBS before shaking, otherwise there is excessive foam formation.

continued

The stock solution of penicillin/streptomycin contains 10,000 U/ml penicillin and 10 mg/ml streptomycin (100 U and 100 μg/ml final, respectively). Store this stock solution up to indicated expiration date in 5-ml aliquots at −20°C.

See UNIT 1.1 for more information regarding preparation of medium.

Insulin, 5 mg/ml

100 mg insulin from bovine pancreas (Sigma)
20 ml sterile H_2O
100 μl glacial acetic acid
Store up to 6 months at 4°C

Poly-L-ornithine solution, 1×

Prepare a 100× stock solution by dissolving 10 mg poly-L-ornithine (Sigma) in 6.67 ml water. Store up to 6 months in 1-ml aliquots at −20°C. Dilute to 1× with water.

Progesterone, 0.1 mM

Dissolve 0.314 mg progesterone in 10 ml of ethanol. Store up to 6 months at −20°C.

Putrescine, 1 M

Dissolve 1.6 g putrescine (Sigma) in 10 ml water. Store up to 6 months at −20°C.

Serum free medium (SFM)

To 850 ml H_2O add:
Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham powder (Sigma), enough for 1 liter
3.7 g $NaHCO_3$
0.11 g sodium pyruvate (1 mM final; Sigma)
1 ml 5 mg/ml insulin (see recipe)
100 mg >97% human apotransferrin (Sigma)
100 μl 1 M putrescine (1 μM final; see recipe)
120 μl 0.25 M sodium selenite (30 nM final; see recipe)
200 μl 0.1 mM progesterone (20 nM final; see recipe)
10 ml penicillin/streptomycin (100 U/ml and 100 μg/ml final, respectively; Sigma)
Adjust pH to 7.2
Adjust volume to 1 liter with H_2O
Sterilize using a 0.22-μm cellulose-acetate disposable vacuum-filtration system (Millipore)
Store for up to 1 month at 4°C.

See UNIT 1.1 for more information regarding preparation of culture medium.

Sodium selenite, 0.25 mM

Dissolve 1 mg sodium selenite (Sigma) in 10 ml water. Store up to 1 month at room temperature.

COMMENTARY

Background Information

Coculture systems provide an easy controlled way to study how a cell population can influence the function, viability, and response of another cell population. Other in vitro systems suitable for the study of the interactions between different cell populations are brain reaggregate cultures or organotypic explants. Brain reaggregate cultures are rotation-mediated aggregating cultures constructed from single-cell suspensions of fetal brain characterized by an organotypic cell association.

A more complex situation is represented by the organotypic cultures. These are derived from explants of undifferentiated embryonic brain and retain some of the structural and functional characteristics of the area of origin.

Both organotypic and reaggregate cultures differ from cocultures since they retain a three-dimensional organization. While a flat coculture system is particularly well suited to detect changes in cell function or differentiation and the interference of a compound on these parameters, organotypic and reaggregate cultures are designed to detect changes in cellular organization by morphological and electrophysiological features. Thus, the choice of the cell system largely depends on the question to be answered.

The great advantage of a sandwich coculture system in studies over the other in vitro systems is the possibility of separating the two cell populations at any time (e.g., prior to or after a treatment) while retaining their integrity. This allows the investigator to (1) manipulate the cell types differently before they are treated together, thus providing information on the involvement of specific mediators or biochemical pathways, (2) perform different biochemical measurements on the two cell populations separately at the end of the treatment, and (3) evaluate the activity on highly differentiated neurons in the presence or absence of the glial feeder layer. For example, the author used this system to study the molecular mechanisms involved in the glia-mediated neuronal death triggered by the human immunodeficiency virus glycoprotein 120 (gp-120). The question of whether the production of reactive oxygen species (ROS) induced by gp-120 in glial cells could be responsible for an increased production of IL-1β from glia and subsequent neural death was assessed (Viviani et al., 2001). For this purpose, glial cells were loaded with an antioxidant, washed, and exposed together with hippocampal neurons to gp-120. In this way, the ability of glial cells to produce ROS as a consequence of gp-120 exposure was blocked without altering the neural cells. As a control, hippocampal neurons and unloaded glia were exposed to gp-120. At the end of the treatment, glia and neurons were separated, neural cell death was assessed by the MTT test, and the results compared with the death rate of neurons exposed to gp-120 in the presence of an unloaded glial monolayer. The synthesis of IL-1β in glial cells was also measured by RT-PCR. It was observed that antioxidant pretreatment of glial cells reduced gp-120-induced production of IL-1β and neural cell death.

This model has also been used to monitor the influence of neural degeneration on glial response in modulating neural cell death (Viviani et al., 2000; Villa et al., 2003).

Because there is no contact between the cell populations, sandwich coculture allows one to study how different cell populations can reciprocally influence their functions/viability through the release of soluble mediators. The lack of contact between neurons and glia, or astrocytes and microglia may, however, represent a disadvantage since it does not mirror physiological conditions. Hippocampal neurons can be plated directly onto glial cells, as can microglia onto astrocytes, but the advantage of manipulating one of the two cell populations forming the coculture would be lost.

Critical Parameters and Troubleshooting

Three very important parameters to obtain successful primary cultures are sterility, rapidity in dissecting brain tissues and plating the obtained cells, and satisfactory reagents. Problems encountered during cell isolation and growth, their possible causes, and possible remedies are reported in Table 2.7.3.

Sterility

Working conditions must ensure the highest degree of sterility. This means that, wherever possible, cell isolation, culturing, and preparation of all solutions should be performed in a laminar flow hood. If nonsterile ingredients are added to a solution, the product has to be filtered through a 0.22-μm filter. To maintain sterility, solutions must never be opened outside the laminar flow hood and must be stored in small aliquots. The addition of antibiotics such as penicillin and streptomycin to the culture medium helps to avoid bacterial contamination.

The use of disposable materials for culturing cells reduces the possibility of contamination and is strongly recommended. All disposable materials (even plastic pipets) should be used only once.

Autoclave surgical instruments (i.e., 21 min at 121°C) to sterilize and store in 95% ethanol when not in use. If, during dissection, tools touch nonsterile surfaces or materials, spray them with 70% ethanol and let the ethanol evaporate before using again.

If any contamination occurs, it is wise to eliminate the contaminated cultures, prepare fresh reagents, and clean the laminar flow hood

Table 2.7.3 Troubleshooting Guide for Preparation and Coculture of Neurons and Glial Cells

Problem	Possible cause	Solution
Low cell viability	Prolonged dissection time	Never exceed 2 hr to obtain brain and dissect hippocampi or cortices
Poor neuronal differentiation	SFM incomplete (e.g., without apotransferrin)	Prepare fresh SFM and check that all ingredients are added
	L-Polyornithine lot unsatisfactory	Test different lots and choose the best
	Glial feeder layer too old	To cocultivate hippocampal neurons use a glial feeder layer within 3 to 4 weeks
	Damaged glial feeder layer	Check the morphology of glial cultures every time before adding neurons
Floating cells in cultures	Contamination	See Critical Parameters and Troubleshooting, Sterility

prior to new culture preparation. To clean the laminar flow hood, wash with a detergent first and then with 70% ethanol. Contaminated solutions can be filtered, but sterility has to be checked prior to use by incubating in culture dishes for a few days at 37°C.

Tissue dissection

Rapid dissection of both hippocampi and cortices is essential to obtain a successful neural or glial culture. In the author's experience the shorter the time of dissection the better the culture obtained. The author's group never exceeds 2 hr to obtain brains and dissect hippocampi and cortices. Preparation time for cultures can be considerably shortened by using a team of two people, one removing brains from the skull and the other cleaning, removing, and dissociating hippocampi or cortices.

Reagent lots

Lot-to-lot variability of reagents such as trypsin, poly-L-ornithine, and all the ingredients of SFM as well as FBS, influences the viability, development, and functionality of glia and particularly of hippocampal neurons. Wherever possible, it is better to test different lots of the same reagent before using it for the routine cultivation of glia and neurons. Measurements to identify the best lot are: the number of cells obtained after tissue dissociation (which can depend on the lot of trypsin used), growth rate of glial cells (dependent on serum), cell viability, and differentiation of neural cells (dependent on the poly-L-ornithine or the ingredients of SFM). If poor neural differentiation is observed, also check that all the requisite ingredients have been added to the culture medium.

Anticipated Results

Healthy hippocampal neurons readily attach to coated glass coverslips and are initially characterized as small round shapes. Within a day of coculture they extend short processes (Fig. 2.7.1). A well-developed network is clearly evident at the third or fourth day of coculture. In the following weeks, even if great morphological changes are not evident any more, hippocampal neurons develop a functional glutamatergic system and a complete post-synaptic density. Hippocampal neurons become sensitive to glutamate, showing an increase in cell death and intracellular calcium, starting from the eighth day of culture.

Glial cultures at confluence are characterized by the presence of an overwhelming percentage of astrocytes and ~5% of microglia. Astrocytes have a flat polygonal appearance and form a uniform layer over which small, dark microglial cells can be observed. Microglial cell numbers can increase considerably with aging of the culture. Once detached from astrocytes and plated on a glass coverslip, microglial cells acquire a round shape that tends to ramify as the number of days in culture increases. A "ramified" morphology is typical of resting microglia. The author's group observed no morphological difference in microglia or astrocytes cultivated alone or in coculture <48 hr from their preparation.

Time Considerations

Preparation of hippocampal neuron-glia sandwich cocultures (Basic Protocol 1) or of astrocyte-microglia sandwich cocultures (Basic Protocol 2) requires 35 to 45 min. Hippocampal neuron-glia cocultures can be maintained up to 3 to 4 weeks. In the author's laboratory, the astrocyte-microglia coculture is used within 48 hr from its preparation.

Isolation and seeding of hippocampal neurons (Support Protocol 1) require 1.5 hr to 2 hr. Hippocampal neurons seeded by following the described protocol can be maintained without glial cells up to a couple of days.

Isolating and culturing cortical glial cells (Support Protocol 2) require 2 hr. Cortical glial cells can be maintained up to 1 month.

Isolation and seeding of cortical astrocytes (Support Protocol 3) require 19 hr. Astrocytes can be maintained up to 1 month. Isolation and seeding of microglial cells (Support Protocol 3) require 2 hr. In the author's laboratory, microglia are maintained up to 48 hr from preparation.

Preparation of glass coverslips for sandwich cocultures (Support Protocol 4) requires 2 days when glass coverslips are incubated with poly-L-ornithine at room temperature or 3 hr when glass coverslips are incubated with poly-L-ornithine at 37°C.

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Harry et al., 1998. See above.

An extensive overview on several in vitro methods to study neurotoxicity, their advantages and disadvantages. A description of organ, slice, and aggregate cultures is also provided.

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