Chapter Two

Interface Organotypic Hippocampal Slice Cultures

Dominique Muller, Nicolas Toni, Pierre-Alain Buchs, Lorena Parisi, and Luc Stoppini

1. INTRODUCTION

In complex tissues such as the central nervous system, differentiation and functional activity often require temporally and spatially dynamic epigenetic cues that cannot be reproduced in dissociated cell cultures. Organotypic cultures and, among them, hippocampal slice cultures represent in vitro models that keep the different cell types and retain the complex three-dimensional organization of the nervous tissue. In addition, much of the appropriate synaptic circuitry, physiology, and neurotransmitter receptor distribution of the intact hippocampus is preserved in this type of culture (Bahr, 1995; Gahwiler et al., 1997). Functional activities of neurons in slice culture were found to be similar to their \textit{in situ} counterparts. These cultures can be grown for many weeks without any passages. More mature neurons (until 21—23 d postnatal) can be grown with slice culture, but only embryonic or neonatal neurons can be grown with dissociated cell culture techniques. Finally, if needed, even more complexity can be achieved by co-culturing different brain target areas.

Two classical techniques have been previously described for preparing organotypic cultures, the Maximov chamber procedure (Toran-Allerand, 1990) and the roller tube method. Gahwiler (1981) has characterized the latter in detail. In both of these techniques, the basic procedure involves the attachment of a tissue explant onto a glass coverslip.

The principle of the interface type of culture we will described below is to maintain nervous tissue on a porous and transparent membrane at the interface between the culture medium and the atmosphere (Stoppini et al., 1991). No plasma clots or roller drums are used. The important factor to ensure good tissue survival is to add the culture medium only below the membrane of the culture insert. By capillarity, the culture medium crosses the membrane and covers slices by a thin film of medium. In this condition, explants do not dry out, and remain well-oxygenated (Fig. 2A).
2. SLICE CULTURES ON MEMBRANES

2.1. Animals

Brain tissue from both rats and mice (wild-type or transgenic mice) is routinely used for the preparation of slice cultures. The best results, in terms of reproducibility, survival, and morphological organization of hippocampal slice cultures, have been obtained with animals between 0 and 15 d old for rats, and between 0 and 5 d for mice.

2.2. Materials

**Scissors (1), large, 16 cm.**
**Scissors (1), fine, 9 cm.**
**Forceps (1), curved, fine (Dumont-type, no. 7).**
**Forceps (1), straight, fine (Dumont-type, no. 5).**
**Holder (1), for razorblade knives, curved (Aesculap, cat. no. BA 290).**
**Razorblades.**
**Spatulas (4), metal with polished edges, straight or angled.**
**Minimum essential medium (MEM) (Gibco-BRL, Eur. cat. no. 11012-010).**
**Horse serum, mycoplasma-screened (Gibco-BRL, Eur. cat. no. 26050-047).**
**Note: Serum is stored at —20°C for up to 18 mo and thawed slowly at room temperature.**
**Horse serum is heat-inactivated in a water bath at 56°C for 30 min, before using to inactivate complement.**
**Penicillin streptomycin solution (10,000 U penicillin 10,000 µg streptomycin; Gibco-BRL, Eur. cat. no. 15140-114).**
**Hanks balanced salt solution (HBSS) (Gibco-BRL, Eur. cat. no. 042-0460M).**
**Insert, culture with 3-mm membrane (Millipore, cat. no. PICMOR G50) or Insert, 25-mm, 4.15 cm² (Nunc, cat. no. 161395).**
**Multiwell plates, 6-well (Falcon, cat. no. 3046).**
**Petri dishes, 35-mm, plastic, sterile (Falcon, cat. no. 1008).**
**Petri dishes, 100-mm, plastic, sterile (Falcon, cat. no. 1029).**
**Membrane punch (8-mm).**
**Syringes (2, 5, and 10 mL).**
**Needles (20-gage).**
**Pasteur pipet (1), fire-polished, cut.**
**Note: Cut pipets should have openings of 2—5 mm; the size of the opening depends on the age of the animal.**
**Pasteur pipet (1), curved, fire-polished (to unroll hippocampus).**
**Stereo microscope.**
**CO₂ incubators (2), one set at 36°C, the other at 33°C.**
**Tissue chopper (McIlwain, Brinkmann, Westbury, NY).**

2.3. Preparation for Slice Cultures on Membranes

1. Media:
   a. Serum-based medium (100 mL).
      25 mL 2X MEM, with 25 mM HEPES. 2X MEM may be stored at 4°C for up to 6 mo.
      25 mL Horse serum.
      25 mL 10X HBSS. HBSS may be stored at room temperature for up to 6 mo.
1 mL 100X penicillin streptomycin (if needed).
60 mg Tris (Fluka, cat. no. 93349) (5 mM final concentration).
7.5% NaHCO₃ (Fluka, cat. no. 71627 (460 µL).
Sterile, double-distilled water (DW) of high quality to 100 mL.
b. Dissection medium.
For the dissection of brain tissue and the preparation of the cultures, the authors’ experience has been that the pH of the regular culture medium is not sufficiently stable. Therefore, a dissection medium of the following composition is used: MEM 100% + Tris (10 mM), pH 7.2.
c. Filtering.
Dissecting and culture media are filtered into 500-mL sterile bottles, using bottle-top filters (Millipore Steritop 0.22-µm SCGPT05RE). Sterile dissecting medium is made fresh for each experiment; culture medium can be kept refrigerated at 4°C for 2 or 3 wk, or stored at —20°C for several months.

2. Preparation of the culture inserts.
One millileter culture medium is added to each well in a multiwell plate. A culture insert is placed into each well. Both the Millipore and Nunc culture membranes should become completely transparent when wetted. The multiwell plate is placed in the CO₂ incubator 1 h prior to explantation, to warm up and to adjust the pH of the culture medium.

2.4. Dissection
Decapitation of the rat and dissection of brain tissue are carried out under sterile conditions.
1. Using small scissors, make an incision along the midline of the head, to simultaneously cut the skin and the skull to expose the brain. Move away the sectioned skull with forceps. Remove the brain with a spatula, while cutting the cranial nerves (Fig. 1B,C).
2. Drop the brain into precooled dissecting medium. Make a sagittal cut through the inter-hemispheric sulcus, and separate the two hemispheres (Fig. 1D).
3. Hold one hemisphere with forceps placed in the frontal part of the brain. Cut off the neighboring thalamus and basal ganglia situated on top of the hippocampus. Remove the pia carefully with two forceps (Fig. 1E).
4. Unroll the hippocampal structure by placing a curved, fire-polished glass pipet underneath the hippocampus and the underlying cortex, and sever the septo-hippocampal connections with a scalpel (Fig. 1F and G).

2.5. Hippocampal Slices Using the Tissue Chopper
Although the vibratome approach can be used to slice brain tissue, we found that, for the hippocampal structure, the chopper device is more appropriate.
1. Place one or two hippocampi on a Teflon stage of a McIlwain-type tissue chopper. Aspirate the excess buffer medium. The hippocampi will adhere to the Teflon plate.
2. Align the hippocampus perpendicular to the chopper blade. Slices (250—400 µm) thick are rapidly cut (2/s) by the tissue chopper.
Note: The thickness of the slices will depend on the age of the animal. The older the animal, the thinner should be the slice, with a compromise around 300 µm.
3. Using a cut, fire-polished Pasteur pipet, transfer the slices to a Petri dish containing dissection medium. Place the Petri dish under a dissecting microscope, so that slices can be gently separated under visual control.
Fig. 1. Dissection protocol. The sectioned skull is moved away with forceps (A). The cerebellum (B) and the olfactory bulbs (C) are cut with a curved spatula. The brain is removed with a spatula and dropped into precooled dissecting medium. A sagittal cut is made through the interhemispheric sulcus to separate the two hemispheres (D). One hemisphere is held with forceps placed in the frontal part of the brain, while neighboring thalamus and basal ganglia, situated on top of the hippocampus are cut off (E). The hippocampal structure is unrolled by placing a flat spatula underneath the hippocampus (F), and the underlying cortex and the septo hippocampal connections are severed with a scalpel (G) to detach the hippocampus (H).
4. Transfer selected individual slices (using a cut, fire-polished Pasteur pipet), with a drop of dissection medium, onto a Millipore insert, or on a 25-mm Nunc filter, in one of the wells of the prepared multiwell plate (Fig. 2A). Note: Four hippocampal slices can be plated on a single insert.

Remove the excess dissection medium on the insert, using a regular Pasteur pipet (Fig. 2A).

5. Immediately transfer the multiwell plate into an incubator equilibrated with 5% CO$_2$ in air at a temperature of 37°C.

2.6. Maintenance of Cultures

During the first 4 d, slice cultures are maintained in a CO$_2$ incubator at a temperature of 37°C. The medium is changed the day after preparation of the cultures, then twice a week. For long-term cultures, we found that maintaining the cultures after the first week in a CO$_2$ incubator set at 33—34°C yields better results. No irradiation or antimitotic treatments are necessary.

Horse serum is required during the first few days, to allow the slice cultures to recover from the explantation trauma. Serum-free media can then be used to maintain slice cultures for several days. The recovery processes (microglial cell phagocytic activities) are speeded up at 37°C, although, when growing slice cultures from older animals (>15-d-old), tissue cultures should be transferred directly into an incubator, with the temperature set at 33°C, to ensure a better survival.

From a thickness of 400 µm, slice cultures will flatten to 150 µm after 1 wk in culture. Pyramidal neurons will spread out, and will remain organized into a 3—4-cell layer surrounded by a dense network of apical or basal dendrites and glial processes (Fig. 2E).
3. PRECUT PATCHES OF MEMBRANE

3.1. Precut Patches of Membrane in Culture Insert

For experiments in which slice cultures will later be transferred to specific chambers, such as for electrophysiological recordings or for histological studies, we use small patches of polytetrafluoroethylene (PTFE) membranes (6, 8, or 10 mm in diameter; Millipore, cat. no. FHLC0477). Other types of membrane can also be used, e.g., Whatman Cyclopore Polyethylene terephthalate (PET) (1.0 µm, Whatman, cat. no. 70690310) and Whatman Anodisc membranes (13 mm, 0.2 µm; Whatman, cat. no. 68097023).

Note: Precut patches of PTFE and PET membranes, 6—10 mm in diameter, can only be purchased from BioCell-Interface (www.BioCell-Interface.com).

1. At the time of preparation of the cultures, the patches of membranes are humidified with culture medium and placed inside a Petri dish.
2. The slice of brain tissue (normally, one slice per disk) is then transferred, with a drop of dissection medium, onto the disk of membrane, and the excess of medium is removed while keeping the slice on the membrane (Fig. 2B). Do not let slices dry out.
3. The patch of membrane bearing the hippocampal slice is then transferred onto the membrane of the culture insert (Fig. 2C).
4. Comment: Within a day, the slice cultures will adhere to the membrane patches, then can easily be transferred to any place by simply picking up the precut patches with fine tweezers. The patches, as well as the membrane culture inserts, can be treated with poly-L-ornithine or poly-L-lysine to increase adherence of the slice culture to the membrane. We find, however, that this is not routinely necessary.

3.2. Interface Slice Cultures on Disks of Membrane onto Solid Agarose Medium

Instead of using culture inserts, the precut patches of membrane bearing tissue slices can also be transferred onto solid medium, using, e.g., agarose to gel the culture medium. The procedure to transfer the hippocampal slice onto the patch of membrane is similar to that described above. The disk of membrane with the slice of hippocampus is laid down onto the surface of the solid agarose medium. Transfer slice cultures on patches onto a new agarose medium every 2 wk (see Appendix 7.5).

4. ELECTROPHYSIOLOGY

The viability and functional connections in hippocampal slice cultures on membranes can be analyzed either in submerged or in interface types of chambers. While the regions of the slice cultures enriched in glia cells are getting thinner, neuronal layers remain thick, and thus allow recordings of large field potentials. See Section 7 for preparation of artificial cerebrospinal fluid.

4.1. Electrophysiological Chambers

1. Slice cultures are placed in an interface type of chamber (Dunwiddie and Lynch, 1978), gassed with a humid atmosphere composed of 95% O₂/5% CO₂, and continuously perfused with the artificial cerebrospinal fluid.
2. The temperature is maintained at 34°C by warming a bath filled with dH₂O. The cultures can be kept for several hours in this chamber.
3. The stimulation electrodes, made of twisted nichrome wires, are usually placed on a group of CA3 neurons of the hippocampus and synaptic responses are recorded extracellularly, using pipets filled with saline (about 5 Mohm resistance) in the CA2—CA1 region.
4. The maximal size of evoked synaptic responses recorded in the cell body or dendritic layer are used as an index of the number of functional synaptic contacts that were activated by stimulation of a group of neurons.

Note: Figure 3 shows an example of evoked synaptic activities recorded in a co-culture of four hippocampal slice cultures.

4.2. Comment

After 2—3 wk in culture, some slice cultures can present spontaneous epileptical activities. These rhythmic activities may induce neuronal loss, a phenomenon that can be precluded by adding glutamate receptor antagonists (Pozzo-Miller et al., 1994).

5. IMMUNOSTAINING

5.1. Solutions

1. Paraformaldehyde 4%.
2. Methanol (biochemistry grade, Fluka, cat. no. 65535).
3. Phosphate-buffered saline (PBS) 0.1 M.
4. PBS 0.1 M with 0.2% Triton X-100 (Merck, cat no. 12298).

5.2. Fixation of Tissue

1. Paraformaldehyde 4%.
   a. Dip the tissue for 30 min into a 4% paraformaldehyde solution.
2. Rinse 2× with PBS (0.1 M) for 10 min at room temperature.
3. Rinse 1× with PBS 0.1 M containing Triton X-100 detergent for 10 min, to make the membrane permeable to antibodies (Abs).

   Dip the tissue for 5 min into pure methanol precooled at —20°C.
   **Note**: This type of fixation works well for cytoskeleton staining.
3. Paraformaldehyde 4% and methanol (—20°C).
   This is a combination of the two above-described methods that allows some double staining.
   a. Dip the tissue for 30 min into a 4% paraformaldehyde solution.
   b. Rinse the slice culture with PBS 0.1 M for 30 min.
   c. Dip the tissue into methanol —20°C for 5 min.

5.3. **Antibody Incubation**

Incubation can last from a few hours to several days, depending on the type of antibodies and the thickness of the slice.
1. Primary antibodies.
   a. As examples, slice cultures were incubated overnight at 4°C with neurofilament antibodies (Sigma, 68 kDa monoclonal antibody, dilution 1:50 or neurofilament antibody,
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Boehringer, cat. no. 814326) (Fig. 4A) or with glial fibrillary acidic protein antibodies (Dako, cat. no. Z0334; dilution 1:50) (Fig. 4B).

b. Slices were left on the membrane during the whole immunostaining procedure.
c. A 50 µL drop of antibodies was placed on both sides of the membrane and trapped with parafilm.

2. Secondary antibodies.
   After three rinses with PBS, reactive sites were visualized by an antimouse rhodamine secondary antibody (Boehringer, cat. no. 1214608; dilution 1:10, incubation time 2 h, protected from light).

3 After the last wash in PBS, the slice culture grown on a membrane patch is placed on a glass slide and mounted with SlowFade antifade kit (Molecular Probes, cat. no. S-2828). The whole mount is then glued with nail polish.

6. TRANSMISSION ELECTRON MICROSCOPE PROCESSING OF HIPPOCAMPAL ORGANOTYPIC CULTURES

A simple method is described here to allow observation of hippocampal organotypic cultures at the level of electron microscopy. Tissue slices cultured on hydrophilic PTFE membrane disks can be easily manipulated individually for classical fixation, and a step of flat embedding allows precise transverse, as well as longitudinal sectioning.

6.1. Materials

- Tweezers (1), small (Regine S.A.).
- Scissors (1).
- Razor blades.
- Potassium hydroxide (KOH), granules.
- Bottles (2), glass, 25 mL, with waterproof caps.
- Glass slides.
- Petri dish, glass, paraffin-filled, with a black cover.
- Petri dish, glass, paraffin-filled.
- Pasteur pipets.
- Copper grids (200-mesh).
- Empty gelatin capsules.
- Plastic foil (3), approx 10 × 10 cm, transparent, heat resistant.

**Note:** One 10 × 10 cm sheet will have a hole of about 2 cm diameter in the middle. The plastic foils must be tested before the first use. To test, put 1 mL resin epoxy between two plastic foils, and let polymerize at 60°C. The plastic foils should not undulate.

- Magnetic mixing bar.
- Drying oven.
- Ultramicrotome (we use Reichert-Jung) with glass and a Diatome diamond knife.

6.2. Preparation of Solutions (Quantities for One Culture)

1. Fixation solution (10 mL): glutaraldehyde, 1.5%, and paraformaldehyde, 1.0%, in PBS 0.1 M, pH 7.4, 4°C.
2. Wash solution (50 mL): PBS 0.1 M, pH 7.4.
3. Postfixation solution (5 mL): Osmium tetroxyde 1% in PBS 0.1 M, pH 7.4.
4. Dehydration alcohols.
a. Ethanol solutions (15 mL each) at 25, 50, 75, and 95%.
b. 100% Ethanol, 50 mL.

5. Pre-embedding solution:
a. 20 mL Propylene oxide (Fluka, cat. no. 82320) 100% to 26 mL EPON.
b. To prepare 26 mL EPON, add 16 g epoxy embedding medium (Fluka, cat. no. 45345), 5.7 g dodecenyl succinic anhydride (Fluka, cat. no. 45346), and 9 g MNA (Fluka, cat. no. 45347) into a glass bottle. Mix gently with magnetic stir bar, add 0.45 g 2,4,6-tri(dimethylaminomethyl) phenol-30 (EMS, cat. no. 13600), and mix again. EPON can be conserved for 1 mo at —20°C.

6. Sectioning solution: EPON colorant solution (400 mL): Mix 2 g methylene blue, 2 g Azur II, and 2 g Borax in 400 mL dH₂O. Filter and conserve at room temperature.

7. Staining solutions:
a. Uranyl acetate, 5%.
i. 1 g uranyl acetate in 20 mL water.
ii. Store at 55°C in a sealed metal box. The solution is light-sensitive and radioactive.
b. Lead citrate.
i. 1.33 g Pb(NO₃)₂ (lead nitrate), 1.76 g Na₃(C₆H₅O₇)·2H₂O (sodium citrate), 30 mL dH₂O.
ii. Shake vigorously for 30 min, then add 8 mL NaOH 1 N and dilute the suspension to 50 mL with dH₂O.
iii. Centrifuge if any faint turbidity is present.
iv. Store at 5°C.

6.3. Protocol

1. Fixation.
   Take a slice culture on its membrane (if needed, cut the surrounding membrane), and put in a glass bottle containing 1.5% glutaraldehyde and 1% paraformaldehyde in PBS 0.1 M, pH 7.4, at 4°C for 1 h 30 min.
2. Wash 3× for 10 min each, with PBS 0.1 M, pH 7.4, at 4°C.
3. Postfixation.
   Postfix in the dark for 1 h at 20°C with 1% osmium tetroxide in PBS 0.1 M, pH 7.4.
4. Wash 3× for 15 min each, with PBS 0.1 M, pH 7.4 at 20°C.
5. Dehydration (all steps at 20°C):
   25% Ethanol: 5 min.
   50% Ethanol: 5 min.
   75% Ethanol: 10 min.
   95% Ethanol: 10 min.
   100% Ethanol 3× for 20 min each.
6. Pre-embedding:
a. 50% Ethanol in propylene oxide: 5 min.
   100% Propylene oxide: 3× for 5 min each.
   **Note: These steps must be completed as quickly as possible to avoid drying.**
b. Then pre-embed into:
   i. A mixture of 50% propylene oxide in EPON for 2 h.
   ii. Then in a mixture of 30% propylene oxide in EPON for another 2 h.
   iii. Then overnight in a new bottle containing pure EPON.
7. Flat-embedding (Fig. 5):
   a. Place the culture on the first plastic foil (Fig. 5A).
   b. Place the second, perforated foil on top of the culture, and add a drop of EPON on the culture (Fig. 5B).
   c. Finally, place the third plastic foil on top, press gently to flatten the culture (Fig. 5C), and let polymerize at 60°C overnight.

8. Embedding:
   a. Remove the plastic foil numbers 1 and 3. The culture will be surrounded by polymerized EPON and stay in the hole of the second plastic foil.
   b. Cut the surrounding membrane of the culture, and dip the culture for approx 5 min in EPON.

Note: For longitudinal sections, place the culture on a glass slide, membrane down, and cover with a gelatin capsule filled with EPON (Fig. 6). For transverse sections, traditional embedding in a silicone matrix can be used. Polymerize at 60°C for 48 h. The gelatin capsule and the tissue can be removed from the glass slide by heating at about 70°C for 5 min.

9. Sectioning:
   a. Trim the block in the gelatin capsule, using a clean razorblade.
   b. Sections 2 μm-thin can be stained with the EPON colorant solution for 1—5 min at 50°C for light microscopic observations of the tissue and final trimming in the region of interest.
   c. Ultrathin sections of approx 60 nm are realized on an ultramicrotome and mounted on uncoated copper grids (200-mesh).

10. Staining:
    a. Place drops of uranyl acetate on the surface of a paraffin-filled Petri dish. Stain the grids on top of a drop for 25 min under light protection of a black cover, then wash with jets of dH2O and allow to dry for about 30 min.
    b. Place drops of lead citrate on the surface of a paraffin-filled Petri dish in the presence of KOH granules, to avoid CO2 dissolution in lead citrate and carbonate precipitation. Stain the grids for 35 s and wash them with 0.02 N NaOH in dH2O, then with dH2O alone, and allow to dry.

Note: Figure 7 shows an example of an electron microscopic picture of a hippocampal slice culture neuropil taken in the stratum radiatum region.
7. APPENDIX

7.1. MEM (Gibco-BRL, Eur. cat. no. 11012)

<table>
<thead>
<tr>
<th>Components</th>
<th>mg/L</th>
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</thead>
<tbody>
<tr>
<td>Inorganic salts:</td>
<td></td>
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<tr>
<td>CaCl₂ (anhydrous)</td>
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<tr>
<td>KCl</td>
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<tr>
<td>KH₂PO₄</td>
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<tr>
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<td>Na₂HPO₄ (anhydrous)</td>
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<td>Other components:</td>
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<tr>
<td>D-Glucose</td>
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<td>Amino acids:</td>
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<tr>
<td>L-Glutamate</td>
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</table>

Fig. 7. Electron microscopy picture showing the parenchyma of a hippocampal slice culture in the stratum radiatum region. The neuropil is dense and the different types of synapses can be found (arrows), as well as glial cell processes (stars). Bar = 2 µm.
l-Histidine HCl H₂O 42.00
l-Isoleucine 52.00
l-Leucine 52.00
l-Lysine HCl 72.50
l-Methionine 15.00
l-Phenylalanine 32.00
l-Threonine 48.00
l-Tryptophan 10.00
l-Tyrosine, disodium salt 52.09
l-Valine 46.00

Vitamins:
D-Calcium pantothenate 1.00
Choline chloride 1.00
Folic acid 1.00
i-Inositol 2.00
Nicotinamide 1.00
Pyridoxal HCl 1.00
Riboflavin 0.10
Thiamine HCl 1.00

7.2. HBSS (10X) (Gibco-BRL, Eur. cat. no. 14060)

Components g/L
Inorganic salts:
CaCl₂ (anhydrous) 1.40
KCl 4.00
KH₂PO₄ 0.60
MgCl₂ 6H₂O 1.00
MgSO₄ 7H₂O 1.00
NaCl 80.00
Na₂HPO₄ 7H₂O 0.90

Other components:
D-Glucose 10.00
Phenol Red 0.10

7.3. PBS (Gibco-BRL, Eur. cat no. 20012)

Components g/L
KH₂PO₄ 0.21
NaCl 9.00
Na₂HPO₄ 7H₂O 0.726

7.4. Artificial Cerebrospinal Fluid (aCSF)

1. Prepare 10X (10-fold concentrated) stock solution.
   a. The composition for 1 L is as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (mol wt 58.44)</td>
<td>124.0 mM 72.5 g</td>
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<tr>
<td>KCl (mol wt 74.56)</td>
<td>1.6 mM 1.2 g</td>
</tr>
<tr>
<td>NaHCO₃ (mol wt 84.01)</td>
<td>24.0 mM 20.16 g</td>
</tr>
</tbody>
</table>
KH$_2$PO$_4$ (mol wt 136.09) 1.2 mM 1.7 g
Ascorbic acid (mol wt 176.13) 2 mM 3.52 g
Glucose 1.0 g
b. QSP: 1 L with mono-DW.
c. Keep at 4°C.
2. Prepare 10 mL 1 M solution of CaCl$_2$.
   Component Final concentration
   CaCl$_2$ (mol wt 219.08) 2.5 mM 2.19 g
3. Prepare 10 mL 1 M solution MgCl$_2$.
   Component Final concentration
   MgCl$_2$ (mol wt 203.31) 1.5 mM 2.033 g
4. Working solution (100 mL).
   Add 250 μL 1 M CaCl$_2$ and 150 μL 1 M MgCl$_2$ to 10 mL stock solution, and complete to 100 mL with mono-dH$_2$O. The final pH is 7.4 after its saturation with a 95% O$_2$ / 5% CO$_2$ gas mixture.

7.5. **Solid Agarose Medium (100 mL)**
1. Autoclave 25 mL regular agarose (Argarose type II; Sigma, cat. no. A-6877) 3.9% in dH$_2$O, keep warmed at 56°C.
2. Mix the following compounds, and warm the solution to 56°C:
   2X MEM with 25 mM HEPES 2X (Sigma, cat. no. H-3375) 25 mL
   Tris (Fluka, cat. no. 03349), 5 mM 60 mg
   Penicillin streptomycin (100X) 1 mL
   NaHCO$_3$ (7.5%) 460 mL
   Horse serum (Gibco-BRL, cat. no. 26050-047) 25 mL
   Hanks balanced salt solution 25 mL
3. Add 25 mL warm agarose, while stirring at 56°C (final concentration of agarose: 0.9%).
4. Pour the mixture into Petri dishes to a depth between 8 and 10 mm of culture medium.
5. Let the solution solidify at room temperature, then put the Petri dishes into the CO$_2$ incubator (prepare the solid medium 1 d before explantation).

**FURTHER READING**


Protocols for Neural Cell Culture
Fedoroff, S.; Richardson, A. (Eds.)
2001, XXII, 262 p., Softcover
A product of Humana Press