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Slice Culture Modeling of Central Nervous System (CNS) Viral Infection

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Abstract

The complexity of the central nervous system (CNS) is not recapitulated in cell culture models. Thin slicing and subsequent culture of CNS tissue has become a valued means to study neuronal and glial biology within the context of the physiologically relevant tissue milieu. Modern membrane-interface slice culturing methodology allows straightforward access to both CNS tissue and feeding medium, enabling experimental manipulations and analyses that would otherwise be impossible in vivo. CNS slices can be successfully maintained in culture for up to several weeks for investigation of evolving pathology and long-term intervention in models of chronic neurologic disease.

Herein, membrane-interface slice culture models for studying viral encephalitis and myelitis are detailed, with emphasis on the use of these models for investigation of pathogenesis and evaluation of novel treatment strategies. We describe techniques to (1) generate brain and spinal cord slices from rodent donors, (2) virally infect slices, (3) monitor viral replication, (4) assess virally induced injury/apoptosis, (5) characterize "CNS-specific" cytokine production, and (6) treat slices with cytokines/pharmaceuticals. Although our focus is on CNS viral infection, we anticipate that the described methods can be adapted to address a wide range of investigations within the fields of neuropathology, neuroimmunology, and neuropharmacology.

Keywords

Organotypic; Ex vivo; Brain slice; Spinal cord slice; Virus; Encephalitis; Myelitis; Cytokine; Apoptosis; Caspase

1 Introduction

In the mature brain, ~100 billion neurons interconnect to form >10^{15} synapses [1]. There are another 100–5,000 billion glial cells (i.e., astrocytes, oligodendrocytes, microglia, and ependymal cells), which not only provide spatial organization to the nervous system, but also support neurological homeostasis, participate in signaling events, and mediate immunologic responses [2, 3]. Due to its inherent complexity, the organizational and functional aspects of the central nervous system (CNS) cannot be completely recapitulated in cell line and primary cell culture models. While such reductionist approaches are amenable to a wide range of experimental protocols, these systems often fail to reveal the fundamental nature of in vivo neurobiology. Although in vivo studies provide highly relevant information about systems biology, animal experimentation is procedurally challenging, time consuming, and—in the case of disease models—debatably inhumane.

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Ex vivo culturing of CNS tissue is a uniquely powerful method that provides neuroscientists with in vitro-like experimental versatility and in vivo-like experimental validity. Culture of nervous tissue dates back to 1907, when neuronal outgrowth was observed from embryonic frog explants affixed to coverslips [4]. Successful long-term culture of explanted mammalian CNS tissue, however, was first achieved 40 years later with the introduction of the Roller-tube method [5–8], which was described in detail by Gähwiler et al. [9]. This method results in a dramatic thinning of the slice over time resulting in the sacrifice of three-dimensional cytoarchitecture, which is reminiscent of cultures prepared from dissociated cells. Another classic technique for culturing CNS tissue utilized Maximov-type chambers rather than rolling test tubes, in order to achieve "organotypic" cultures that were many cell layers thick [10]. While Roller-tube and Maximov-chamber methodologies offered distinct advantages over culturing primary cells, these ex vivo culturing systems have fallen out of favor because such preparations are technically cumbersome, provide only limited access to slices/ medium, and often result in experimental variability [11].

The original membrane-interface method of brain slice culture [12] was brought into practical use by Stoppini et al. [13]. In short, this procedure involves placement of explanted rodent brain slices upon a semiporous membrane insert. The insert, itself, sits in a medium-containing well, such that the slices are suspended at the interface between medium and a humidified atmosphere (36 °C, 5 % CO_2) (*see* Fig. 1). A thin film of medium is formed above each slice through capillary action, allowing a sufficient level of hydration and nutrient absorption (without sacrifice of gas exchange) for successful maintenance of tissue survival over several weeks in culture [14]. Slices become progressively thinner over this time span, but remain "organotypic" and will not reach the minimal thickness attained via Roller-tube preparation.

Underscoring the impact it has had on modern neuroscience, the Stoppini et al. [13] membrane-interface culturing method has been utilized with steadily increasing frequency over the last two decades and has been cited in ~2,000 primary publications to date. The popularity of membrane-interface cultured slice methodology is largely due to the fact that in vivo neurobiology (i.e., neuronal differentiation, dendritic arborization, spine formation, neurotransmitter release, and receptor distribution) are remarkably well replicated in these systems and, as a result, local synaptic circuitry (mini currents, excitatory postsynaptic potentials [EPSPs], inhibitory postsynaptic potentials [IPSPs], long-term potentiation [LTP], long-term depression [LTD]) can be functionally intact [13, 15–20]. The utility of membrane-interface slice models is probably best demonstrated by pharmacological and genetic studies that yield similar experimental results in both ex vivo and in vivo settings [21–23]. Unlike the in vivo CNS, however, the extracellular environment (tissue surface, medium, and atmosphere) is readily accessible when culturing on membrane-interface inserts. This allows unmatched control over experimental conditions (physical, chemical, and electrical) and precise monitoring of medium and tissue parameters over extended time periods.

In addition to yielding mechanistic insight into fundamental aspects of normal neurobiology, such as learning/memory [16], brain development [17, 18], and neurogenesis [24], organotypic slice culture techniques have been utilized to develop highly relevant systems

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for the in vitro study of neurologic disease. Insults to cultured brain slices, such as oxygen-glucose deprivation (OGD), mechanical disruption, and pharmaceutical application, have become established models for studying stroke [21, 25], traumatic brain injury [26–28], and epilepsy [14, 29, 30]. Multiple ex vivo slice models of neurodegenerative disease, including Alzheimer and Parkinson diseases, have been developed [31–35]. In addition, bacterial [36, 37], parasitic [38, 39], and viral [40–45] infections of the CNS have been modeled in slice cultures.

Not surprisingly, several of the established slice models of neurologic disease have become valuable in the drug discovery process. Pharmaceuticals can be directly applied to slices or slice medium to initially screen for efficacy, in the absence of complications related to the drug's metabolic stability or brain penetration. Given that multiple brain slices can be made from a single animal, ex vivo pharmaceutical screening is relatively high-throughput compared to whole animal studies. It is also relatively high-content when compared to cell culture studies, in that a positive “hit” is more likely to translate to animals because the system is inherently more contextual to in vivo tissue and disease biology [46].

Methods for the production and long-term culturing rodent brain and spinal cord slices are outlined in the following sections. We also describe methods for viral infection of slice cultures and subsequent assessment of viral replication, injury, and apoptosis. Notably, injury can be accessed over time in a single sample via detection of released lactate dehydrogenase (LDH) in feeding medium. In addition, ELISA-based quantification of cytokine release into the medium is presented, as a sensitive technique that allows investigation of the innate immunological response mounted by the CNS during viral infection. In this system, CNS tissue is completely isolated from systemic, cell-mediated immunity, thus, measured cytokine responses are considered “CNS-specific.” Finally, cytokine and pharmaceutical application to slice cultures are discussed. A possible work flow diagram is depicted in Fig. 2, though these methods can readily be customized according to the questions posed by individual investigators.

2 Materials

2.1 Slice Preparation (See Note 1)

2.1.1 Brain

1. 2–3 day old mice (*see* **Note 2**).
2. Razor blades.
3. Surgical tools for d issection: large scissors, small scissors, large forceps, small forceps.
4. Tools for slice manipulation: weighing spatula (*see* **Note 3**) and paintbrush.
5. Vibratome (Leica; Buffalo Grove, IL).
6. Slicing medium: MEM supplemented with 10 g/L D-glucose and 1 mM HEPES (pH 7.2). Can be stored at 4 °C for 3 weeks. Optional: equilibrate medium with 95 % O_2/5 % CO_2 immediately prior to use.

7. 10 % FBS plating medium: Neurobasal supplemented with 10 mM HEPES, 1× B-27, 400 μM L-glutamine, 600 μM GlutaMAX, 60 U/mL penicillin, 60 μg/mL streptomycin, 6 U/mL nystatin, 10 % FBS.
8. Membrane inserts (Millipore #PICMORG50, Billerica, MA).
9. 6-well cell culture plates.
10. Humidified incubator set at 5 % CO_2 and 36.5 °C.
11. Laminar flow hood (optional).
12. 95 % O_2/5 % CO_2 tank (optional).

2.1.2 Spinal Cord

1. 4–5 day old mice (*see* **Note 2**).
2. 5 mL syringe with Luer lock tip.
3. 26G needle.
4. Ca^{2+}- and Mg^{2+}-free PBS.
5. Agarose slicing medium: MEM supplemented with 10 g/L D-glucose, 1 mM HEPES (pH 7.2), and 2 % low melt agarose (Bioexpress, E-3112; Kaysville, UT).
6. Disposable plastic cryomolds.

2.2 Viral Infection

1. Purified viral stock.
2. Ca^{2+}- and Mg^{2+}-free PBS.

2.3 Culture Maintenance

1. 5 % FBS plating medium: Neurobasal supplemented with 10 mM HEPES, 1× B-27, 400 μM L-glutamine, 600 μM GlutaMAX, 60 U/mL penicillin, 60 μg/mL streptomycin, 6 U/mL nystatin, 5 % FBS.
2. Serum free plating medium: Neurobasal supplemented with 10 mM HEPES, 1× B-27, 400 μM L-glutamine, 600 μM GlutaMAX, 60 U/mL penicillin, 60 μg/mL streptomycin, 6 U/mL nystatin.
3. Humidified incubator set at 5 % CO_2 and 36.5 °C.

2.4 RT-PCR Quantification of Viral or Host Genes

1. Ca^{2+}- and Mg^{2+}-free PBS.
2. RLT buffer (Qiagen; Germantown, MD).
3. β-mercaptoethanol.
4. QIAshredder spin column (Qiagen; Germantown, MD).

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5. RNeasy spin kit (Qiagen; Germantown, MD).
6. Tabletop centrifuge.
7. Primers to gene/s of interest and housekeeping gene (i.e., β-actin or GAPDH).
8. iScript™ One-Step RT-PCR Kit with SYBR® Green.
9. Bio-Rad CFX96 thermocycler with compatible hardware and software (Hercules, CA).

2.5 Cryosectioning and Immunofluorescence Imaging

1. Ca^{2+}- and Mg^{2+}-free PBS.
2. 4 % paraformaldehyde (PFA).
3. 15 % sucrose in PBS.
4. 30 % sucrose in PBS.
5. Tissue Freezing Medium (Triangle Biomedical Sciences; Durham, NC) (*see* **Note 4**).
6. Metal cylinder (~1 in. diameter).
7. Aluminum duct tape.
8. Cryostat.
9. ColorFrost™ Plus microscope slides (Fisher; Pittsburgh, PA).
10. Antigen unmasking solution (Vector Laboratories; Burlingame, CA).
11. Fetal bovine serum (FBS).
12. Normal goat serum (NGS).
13. 1° antibodies of interest.
14. Fluorescent 2° antibodies.
15. Hoechst stain.
16. ProLong® Gold mounting medium (Life Technologies; Grand Island, NY).
17. Epifluorescence microscope and compatible imaging software.

2.6 Propidium Iodide (PI) Quantification of Tissue Injury

1. Propidium iodide (Sigma; St. Louis, MO).
2. Dounce homogenizer.
3. 96-well plate.
4. Plate centrifuge.
5. Spectrofluorometer.

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2.7 Lactate Dehydrogenase (LDH) Quantification of Tissue Injury

1. LDH-Cytotoxicity Assay Kit II (Biovision; Mountain View, CA).
2. Microplate reader.

2.8 MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Visualization of Tissue Injury

1. MTT (Life Technologies; Grand Island, NY).
2. Digital camera.

2.9 Western Blotting

1. Ca^{2+}- and Mg^{2+}-free PBS.
2. Lysis Buffer (1 % Triton-X, 10 mM triethanolamine–HCl, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1× Halt protease and phosphatase inhibitor cocktail [Thermo Scientific; Rockford, IL]).
3. Tabletop centrifuge.
4. Polyacrylamide gel reagents.
5. Western blot gel apparatus.
6. Protein transfer apparatus.
7. Nitrocellulose membrane.
8. 1° antibodies of interest.
9. HRP-conjugated 2° antibodies.
10. Chemiluminescence reagent.
11. Chemiluminescence detection equipment.

2.10 Caspase Activity Assay

1. Ca^{2+}- and Mg^{2+}-free PBS.
2. Caspase fluorogenic assay kit (B&D Biosciences; San Jose, CA).
3. Spectrofluorometer.

2.11 Cytokine Analysis

1. Multi-Analyte ELISArray plate (SABiosciences, Frederick, MD).
2. Single-Analyte ELISA plate/s (SABiosciences, Frederick, MD).
3. Microplate reader.

2.12 Pharmaceutical/Cytokine Treatment

1. Ca^{2+}- and Mg^{2+}-free PBS.
2. Pharmaceutical/s or cytokine/s of interest.

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3 Methods

3.1 Brain and Spinal Cord Tissue Preparation

1. Place slicing medium on ice and, if possible, bubble slicing medium with 95 % O_2/5 % CO_2.
2. Pipette 1.2 mL 10 % FBS plating medium into each well of 6-well plates. Place plate into incubator (36.5 °C, 5 % CO_2) for at least 1 h prior to explantation to allow the medium to warm and pH adjust.
3. Prepare razor blade for placement in Vibratome chuck by carefully removing the blade's back guard with the weighing spatula or flat-head screw driver. Spray and wipe blade with 70 % ethanol to remove manufacturing oil.
4. Mount razor blade into Vibratome chuck and assemble Vibratome. Set Vibratome settings (*see* **Note 5**).
5. Clean all tools and Vibratome with 70 % ethanol. If possible, sterilize equipment with ~20 min exposure to UV light in laminar flow hood.
6. Obtain mice.
7. Harvest mouse brains or spinal cords (*see* **Note 6**).

   (a) Brains (*see* Fig. 3a–g): rapidly decapitate 2–3 day old animal with large scissors. Spray the head with 70 % ethanol. Tent the scalp with large forceps and remove with a single transverse cut made with large scissors. Stabilize the anterior skull with large forceps placed between eyes and nose. Insert small dissection scissors into foramen magnum and carefully cut the skull as far anteriorly as possible (point the scissors upward toward the skull to avoid damaging the underlying brain). Use the small forceps to carefully pry each half of the skull away from the brain. Then gently lift the brain out of the skull, severing the nerve tracts in the process. Place the brain in a Petri dish. Use a new razor blade to make a single coronal cut to separate cerebrum from cerebellum. This forms a flat surface on the cerebrum that can be glued to the Vibratome stage.

   (b) Spinal cords (*see* Fig. 4a–g): rapidly decapitate 4–5 day old animal with large scissors. Spray the body with 70 % ethanol. Cut the tail away from the body at its most proximal point (the sacral spine should be severed in the process). Insert the 26G needle into the spinal column, such that the bevel is just buried (*see* **Note 7**). With steady force, push a PBS stream through the spinal column until the spinal cord is forced out of the spinal column. The spinal cord often remains connected at the anterior portion of the body and can be gently manipulated into a Petri dish containing slice medium or PBS. Upon collecting multiple spinal cords, pour a 37 °C 2 % low melt agarose into a cryoform. Immediately, suspend spinal cords in the agarose, with cervical spinal cord oriented

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upwards. Manipulate the cords such that they are suspended upright in the block with the cervical cord providing buoyancy (*see* Fig. 5). Allow the block to cool/solidify (*see* **Note 8**).

8. Glue specimen to Vibratome stage (*see* **Note 9**).

   (a) Brains (*see* Fig. 3h, i): Place a small amount of super glue onto the stage and place the brain onto the glue, with frontal lobes oriented upwards (newly cut, flat side down on the glue spot). Wait for ~30 s for glue to dry, place the stage inside the Vibratome bath, then pour ice-cold, oxygenated slicing medium into the Vibratome bath until the point where the olfactory bulbs/frontal lobe are just submerged.

   (b) Spinal cords (*see* Fig. 4h, i): Carefully remove the agarose block from the cryomold. The block can be trimmed with a razor blade. Place a small amount of super glue onto the stage and place the agarose block onto the glue, with sacral regions oriented upward and cervical regions oriented closest to the stage. Wait for ~1 min for glue to dry, place the stage inside the Vibratome bath, then pour ice-cold, oxygenated slicing medium into the Vibratome bath until the point where the agarose block is just submerged.

9. Slicing (*see* **Notes 10** and **11**).

   (a) Brains: Initial coronal slices will be of olfactory bulbs, followed by slices of frontal lobes. We generally discard these slices. When the bi-lobar shape of the frontal lobes is no longer apparent, sections will contain both hippocampus and thalamus. We generally save these slices for culture. Four, 400 μM slices containing hippocampus/thalamus can be obtained from each 2–3 day old mouse brain. By gentle pickup with a paintbrush and bent weighing spatula, collect each nascent slice into 6-well plates containing ice-cold, oxygenated slicing medium. If further dissection of tissue slices is desired *see* **Note 12**.

   (b) Spinal cords: Initial coronal slices will be of sacral and lumbar sections, followed by thoracic and cervical sections. We generally discard sacral sections (too small to work with) and cervical slices (not true coronal slices). Approximately 20, 400 μM slices can be obtained from each 4–5 day old mouse. By gentle pickup with a paintbrush and bent weighing spatula, collect each nascent agarose slice into a Petri dish containing ice-cold, oxygenated slicing medium. Individual spinal cord slices can easily be dislodged from agarose by holding the agarose slice in a stationary position and gently "blowing" slicing medium toward the slices with a disposable pipette.

3.2 Plating Brain and Spinal Cord Slices

1. Place a Millipore insert into each of the 6-well plate wells with sterile forceps.

2. Carefully transfer slices onto the Millipore insert.

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(a) Brain slices (*see* **Note 13**): Gently coax the slice onto the bent weighing spatula with the paintbrush. Once secure, place the spatula above the insert at a steep angle. Pick up a large droplet of slicing medium by briefly submerging the paintbrush, then place the droplet at the higher end of the spatula. Ideally the droplet will roll down the incline of the spatula and wash the slice onto the membrane. It is important that the slices lay perfectly flat on the membrane.

(b) Spinal cord slices: Using a disposable pipette, pick up slices (and minimal slicing medium) and transfer to the membrane.

3. Remove any slicing medium that is transferred along with the slices by Pasteur pipette. The slices should not be submerged in large volumes of liquid because slices need exposure to incubator atmosphere for optimal survival.

4. Place plates in humidified incubator set at 5 % CO_2 and 36.5 °C.

3.3 Viral Infection

1. Immediately after plating or following slice recovery (*see* Fig. 6) add purified virus (diluted in PBS) dropwise to the top (air interface) of each slice. A volume of 15–20 μL covers a single brain slice and a volume of 5–10 μL covers a single spinal cord slice. Minimized inoculation volumes will ensure that slices are not submerged in liquid for long periods of time. Small volumes will be absorbed into the slice tissue and enter the underlying medium in <5 min.

2. Mock inoculations should be performed in a similar manner with PBS alone.

3. Time points need to be determined by each individual investigator. If in vivo time points are known, these should be used as starting points. It is recommended that all experimentation is completed prior to the 21st day post-plating.

3.4 Culture Maintenance (See Note 14)

1. At ~12 h post-plating, pipette 1.2 mL 5 % FBS plating medium into each well of new 6-well plates. Place plates into incubator (36.5 °C, 5 % CO_2) for at least 1 h prior to slice transfer to allow the medium to warm and pH adjust.

2. With sterile forceps, transfer each membrane insert into wells containing fresh medium.

3. Replace medium every 2 days thereafter with serum-free medium.

3.5 RT-PCR Quantification of Viral or Host Genes

3.5.1 Harvest Slices Into RLT-βME Buffer

1. Add 10 μL β-mercaptoethanol to each 1 mL of RLT buffer.

2. Pipette 600 μL of RLT-βME buffer into labeled 1.5 mL microcentrifuge tubes.

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3. Rinse slices two times by briefly submerging the membrane insert into the wells of 6-well plate filled with PBS.
4. Lift slices away from the membrane by gently sweeping under slices with the weighing spatula (*see* **Note 15**). Put up to four brain slices or 20 spinal cord slices directly into 600 μL of RLT-βME buffer. Triturate the tissue through a pipette tip until it is completely disassociated into the buffer.
5. Freeze the lysate at −80 °C or proceed immediately to the next step.

3.5.2 Purify Slice RNA

1. Homogenize the lysate by pipetting it into a QIAshredder and spinning at 12,000 × *g* for 2 min.
2. Add 600 μL of 70 % ethanol (in DEPC water) to each homogenized lysate and mix by gentle trituration through a pipette tip.
3. Pipette half of the homogenized lysate/ethanol (600 μL) into a labeled Qiagen RNeasy MINI spin column.
4. Spin at 12,000 × *g* for 1 min, discard flow through.
5. Repeat **steps b** and **c** with the remainder of the sample.
6. Add 700 μL of RW1 buffer to the column, spin at 12,000 × *g* for 15 s, discard flow though.
7. Add 500 μL of RPE buffer, spin at 12,000 × *g* for 15 s, discard flow though.
8. Add another 500 μL RPE, spin at 12,000 × *g* for 2 min, discard flow though.
9. Spin again at 12,000 × *g* for 1 min to dry column.
10. Carefully place spin column to a new 1.5 mL microcentrifuge tubes. Add 50 μL of water to each column.
11. Wait 1 min and spin again at 12,000 × *g* for 1 min.
12. Store purified RNA at −80 °C.

3.6 RT-PCR Quantification

1. Design and synthesize primers to gene/s of interest and a housekeeping gene.
2. Mix purified RNA template, primers, SYBR® Green RT-PCR master mix, and iScript™ reverse transcriptase into a total volume of 20 μL.
3. Perform 40 cycles of PCR amplification on thermocycler as follows: cDNA synthesis at 50 °C for 10 min, reverse transcriptase inactivation at 95 °C for 5 min, denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 30 s.
4. Check melt curve to confirm the absence of nonspecific products and primer dimers.

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5. Convert raw *C*(t) values into relative expression values with compatible analysis software.

3.7 Cryosectioning and Immunofluorescence Imaging (See Note 16)

3.7.1 Fix and Cryoprotect Tissue

1. Rinse slices two times by briefly submerging the membrane insert into a the wells of 6-well plate filled with PBS.
2. Transfer membranes into a 6-well plate filled with 4 % PFA and allow ~12 h fixation at 4 °C (make sure slices are completely submerged in fixative).
3. Rinse slices two times by briefly submerging the membrane insert into a the wells of 6-well plate filled with PBS.
4. Lift slices away from the membrane by gently sweeping under slices with the weighing spatula (*see* **Note 15**). Put loose slices directly into 15 % sucrose for cryoprotection.
5. Store slices for ~1 day in 15 % Sucrose at 4 °C to cryoprotect tissue.
6. Store slices for ~1 day in 30 % Sucrose at 4C to cryoprotect tissue.

3.7.2 Form a "Face-Off" TFM Block (Used to form a Surface That Is Parallel to Cryostat Blade)

1. Create a small well by running aluminum tape around the end of a metal cylinder, such that the tape edge is higher than the edge of the cylinder.
2. Fill well with TFM and place metal cylinder in dry ice until TFM solidifies (this forms a "face-off" block that does not contain slices).
3. Take the cylinder out of dry ice, remove the tape, remove the "face-off" block from the metal cylinder, and store the "face-off" block in aluminum foil at −80 °C.

3.7.3 Cryoembed Slices

1. Place slices in room temperature TFM for at least several minutes.
2. Carefully lift slices out of room temperature TFM with a paint-brush and place them in a metal well identical to that created in **step 2**(a). The slice should be laid flat against the surface of the metal cylinder, without the creation of bubbles.
3. Gently apply a thin layer of TFM over the tissue slices and place the metal cylinder in dry ice until TFM solidifies (this forms a thin block that contains slices at the extreme bottom).
4. Take the cylinder out of dry ice, remove the tape, remove the "slice block" from the metal cylinder, and store the "slice block" in aluminum foil at −80 °C.
5. Repeat until all tissue slices have been cryoembedded in "slice blocks."

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3.7.4 Cryosectioning

1. Attach the "face-off block" to the cryostat chuck and make sections until the entire surface of the block is removed with each pass of the blade (i.e., the block face is perfectly parallel to the blade path).
2. Mark orientation of the "face-off block" with a small mark with a marker.
3. Remove the "face-off block" from the cryostat chuck.
4. Coat the "face-off block" with a thin layer of TFM and immediately place a "slice block" (with slices oriented downward, so that the slices are sandwiched inside the two blocks).
5. Attach the "sandwich block" to the cryostat chuck.
6. Make sections until the slices are clearly visible (*see* **Note 4**).
7. Ensure that the blade is sharp and recent sections are of high quality.
8. Make serial 10–20 μm sections through the tissue and mount on ColorFrost™ Plus slides (*see* **Note 17**).
9. Store slides at room temperature until immunohistochemical processing.

3.7.5 Immunohistochemistry

1. Desiccate slides at 50 °C for 15 min.
2. Rehydrate slides in PBS over 30 min.
3. Perform antigen retrieval with antigen unmasking solution according to manufacturer's instructions.
4. Permeabilize tissue and block antigen by submerging tissue with 5 % fetal bovine serum and 5 % normal goat serum in 0.3 % Triton/PBS for 2 h at room temperature.
5. Incubate with primary antibodies overnight at 4 °C.
6. Incubate with secondary antibodies for 2 h at room temperature.
7. Stain nuclei with Hoechst stain according to manufacturer's instructions.
8. Mount sections with ProLong® Gold mounting medium.
9. Image slides on an epifluorescence microscope.

3.8 Propidium Iodide (PI) Quantification of Tissue Injury

1. Replace serum-free medium, with serum-free medium containing 3 μM propidium iodide.
2. Return cultures to incubator for a 1 h incubation.
3. Rinse slices two times by briefly submerging the membrane insert into the wells of 6-well plate filled with PBS.

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4. Homogenize four brain slices or spinal cord slices into 500 μL PBS with a Dounce homogenizer.
5. In triplicate, pipette 150 μL of homogenate into 96-well plate.
6. Spin down cells at 1,000 × *g* for 5 min.
7. Desiccate cells by incubating in a dry incubator set at 37 °C for 24 h.
8. Quantify tissue injury with a fluorescence plate reader, with an excitation wavelength of ~493 nm and emission wavelength of ~630 nm.

3.9 Lactate Dehydrogenase (LDH) Quantification of Tissue Injury

1. Take samples (40 μL) of feeding medium at sequential time points.
2. According to manufacturer's instructions, detect LDH in triplicate 10 μL samples by colorimetric assay and quantification on a 96-well microplate reader.

3.10 MTT (3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Visualization of Tissue Injury

1. Replace serum-free medium, with serum-free medium containing 0.5 mg MTT/mL medium.
2. In 30 min to 1 h, take photographs to characterize regionality of tissue injury (blue/purple indicates live tissue, whereas white/brown indicates otherwise).

3.11 Western Blotting

1. Pipette lysis buffer (50 μL/brain slice or 10 μL/spinal cord slice) into labeled 1.5 mL microcentrifuge tubes.
2. Rinse slices two times by briefly submerging the membrane insert into the wells of 6-well plate filled with PBS.
3. Lift slices away from the membrane by gently sweeping under slices with the weighing spatula (*see* **Note 15**). Put slices directly into lysis buffer. Triturate the tissue through a pipette tip until it is completely dissociated into the buffer.
4. Freeze the lysate at −80 °C or proceed immediately to the next step.
5. Place homogenates on ice until thawed. Then allow cell lysis to occur for 5–10 min, while vortexing frequently.
6. Clear lystates, by spinning at 16,000 × *g* for 10 min at 4 °C.
7. Transfer supernatant to fresh tube containing equal volume of 2× Laemmli buffer.
8. Boil for 5 min.
9. Load and run samples on polyacrylamide gel (*see* **Note 18**).
10. Transfer to nitrocellulose membrane.

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11. Block membrane with 5 % milk.
12. Immunoblot with primary antibody overnight at 4 °C.
13. Wash membrane and incubate with appropriate HRP-conjugated secondary antibody.
14. Obtain film or digital images following addition of chemiluminescence reagent.

3.12 Caspase Activity Assay

1. Rinse slices two times by briefly submerging the membrane insert into a the wells of 6-well plate filled with PBS.
2. Lift slices away from the membrane by gently sweeping under slices with the weighing spatula (*see* **Note 15**). Put slices directly into lysis buffer provided by manufacturer. Triturate the tissue through a pipette tip until it is completely dissociated into the buffer.
3. Freeze the lysate at −80 °C or proceed immediately to the next step.
4. Quantify caspase activity according to manufacturer's instructions.

3.13 Cytokine Analysis

3.13.1 Cytokine Screen on Multi-Analyte ELISArray Plates

1. Collect samples of medium at sequential time points.
2. Pool medium from experimentally similar wells together for cytokine screening (it is suggested that some medium is left unpooled, such that medium from individual wells can later be used for cytokine quantification on single-analyte ELISA plates).
3. Screen pooled medium on multi-analyte ELISArray plates according to manufacturer's instructions.

3.13.2 Cytokine Quantification Single-Analyte ELISA Plates

1. Obtain single-analyte ELISA plates for each cytokine that was positive on the ELISArray.
2. With the un-pooled medium collected in **step 1a**, quantify cytokine of interest in each medium sample through serial dilutions fit to a standard curve according to manufacturer's directions.

3.14 Pharmaceutical/Cytokine Treatment

1. Obtain in vitro dosage information, if available.
2. Dilute pharmaceutical/cytokine so that it may be applied directly to the top (air interface) of each slice. The initial concentration must be non-toxic and should contain 10 % DMSO. A volume of 15–20 μL covers a single brain slice and a volume of 5–10 μL covers a single spinal cord slice. Small volumes will be

absorbed into the slice tissue and enter the underlying medium in ~5 min. Ideally, the final concentration should be above the effective concentration as established in vitro.

3. Apply pharmaceutical/cytokine following each medium change.

4 Notes

1. Slices are vulnerable to fungal infection. During preparation, we recommend wearing a mask and sterilizing gloves frequently with 70 % ethanol. Nystatin is added to medium to alleviate problems with fungal infection.
2. Slices can be prepared from both mice and rats using described methodology. While we recommend using young postnatal animals, due to the resilience of tissue at this age, other investigators have successfully cultured CNS tissue derived from animals that are several weeks old at the time of sacrifice. In our experience, P2–3 seems ideal for brain slices model, or P4–5 for spinal cord model.
3. Bend the end of the spatula to a 45° angle for easier manipulation of slices.
4. Usage of colored TFM (red, green, blue) helps tremendously with the visualization of slices during cryosectioning.
5. Our laboratory uses the following settings on a Leica VT1000S Vibratome: advance speed = 7–10 and amplitude = 7–10. Exact settings need to be determined by individual investigators. Our brain and spinal cord slices are made at 400 μm thickness. Slices can be made thinner (i.e., 250 μm), though thin slices will have a larger percentage of the total slice damaged by the slicing procedure. It is not advisable to make slices thicker than 400 μm, because the top of thick slices will not have adequate contact with feeding medium.
6. It is important that this step be performed quickly. With practice, it should not take any longer than 5 min. With practice, up to six brains or spinal cords may be harvested and sliced at once.
7. Aim for the small blood spot that immediately forms when the sacral spinal cord is severed. Gentle side-to-side movement of the needle can be used to confirm that it has indeed entered the spinal column. Furthermore, correct needle placement is confirmed if limb flexion is induced by mechanical stimulation of the spinal cord when PBS is forced through the spinal column. Incorrect needle placement, on the other hand, will cause the body cavity to fill with PBS.
8. Cooling of the agar block can be hastened by briefly placing the form on ice or at 4 °C.
9. Size of glue spot should be approximately the same size as the “footprint” of the brain or spinal cord-containing agarose block. Controlling the glue volume

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is a critical part of the slicing procedure: if too little glue is applied the specimen will become dislodged from the Vibratome stage, whereas too much glue will coat the outside of the specimen, harden, and induce damage to the tissue and razor blade.

10. Replace the razor blade often (i.e., every time the stage is loaded with new specimens). Small imperfections in the razor blade can result in unnecessary damage to tissue slices, whereas, usage of a sharp blade will minimize tissue tearing.

11. Use a paintbrush to hold the nascent slice against the blade; this keeps it from rolling up and allows for a cleaner cut.

12. If isolated hippocampal cultures are desired, individual hippocampi may be dissected out of nascent slices with a dissecting scope prior to plating. If many experimental conditions will be applied (e.g., drug screening), each slice may be split into right and left hemispheres with a scalpel prior to plating.

13. The plating procedure for brain slices is technically challenging. Accordingly, mastery of this step will take significant patience.

14. We prefer to culture in a serum-free system to minimize variables. However, providing FBS to nascent slices helps with tissue recovery. So, we tend to initially plate cultures with 10 % FBS, and wean to 5 % FBS at ~12 h post-plating, then serum-free medium by 3 days post-plating.

15. Separating slices from culture membranes will become more difficult with increased time in culture due to the incorporation of glia into the membrane.

16. As an alternative to labor-intensive cryosectioning and epifluorescence imaging, confocal imaging of the surface of the whole slice may be performed. Confocal microscopy also makes live imaging possible.

17. Tissue will not bind well to uncoated slides and will wash off during immunofluorescence processing. We recommend usage of coated slides.

18. In our experience, most proteins are detectable (yet not over-loaded) when the equivalent of one brain slice or five spinal cord slices are loaded per well.

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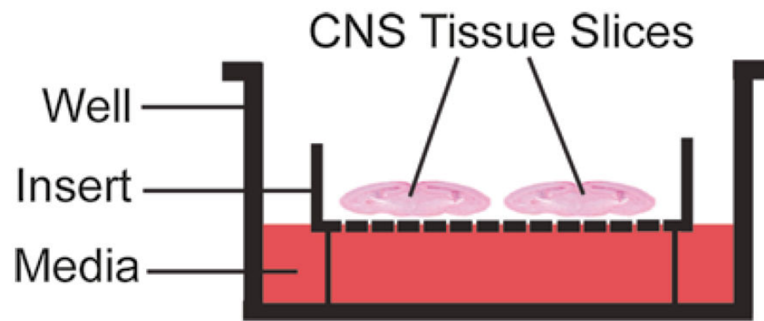

Fig. 1.

Schematic of membrane-interface culturing system. Cutaway view of a single well depicts CNS tissue slices suspended at the interface of media and the incubator atmosphere. This configuration allows both nutrient absorption and gas exchange for long-term viability

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Fig. 2.

Possible workflow for production of CNS tissue slices, subsequent infection/treatment, and multiple endpoints. Analysis of media is useful for the quantification of tissue injury, virus release, and cytokine release and does not require destruction of slice tissue. Membrane insert picture is reprinted with permission from Millipore Corporation

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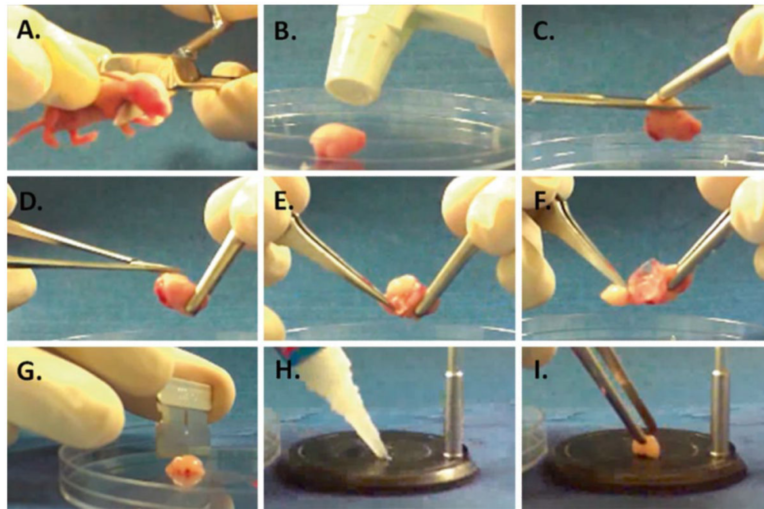

Fig. 3.

Steps to harvest brains and mount to Vibratome stage. (**a**) Rapid decapitation is followed by (**b**) ethanol rinse of pup's head. (**c**) Scalp is removed by tenting skin and making a transverse cut. (**d**) The skull is hemisected starting from foramen magnum and moving rostrally. (**e**) Each half of skull is peeled away from underlying brain and (**f**) the brain lifted brain out of cranium. (**g**) Cerebrum is separated from cerebellum with razor blade. (**h**) A glue spot of approximately the same size as the freshly cut cerebrum surface is placed on the Vibratome stage and (**i**) the cerebrum is placed on the glue spot, with olfactory bulbs oriented upwards

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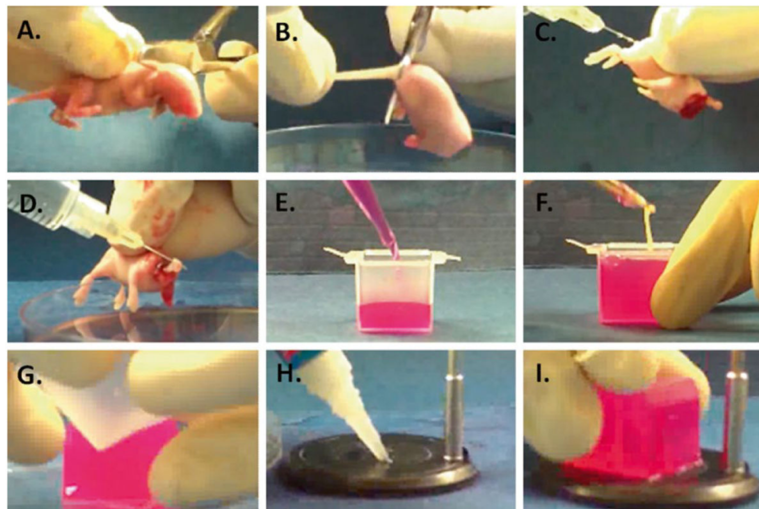

Fig. 4.

Steps to harvest spinal cords, agarose embed, and mount to Vibratome stage (**a**) Rapid decapitation is followed by ethanol rinse of the pup's body. (**b**) The tail removal at its most proximal point. (**c**) The bevel of a 25G is inserted into the spinal column and a stream of PBS is forcefully injected through the column, impelling the spinal cord from the anterior body. (**d**) The spinal cord is gently teased away from the body. (**e**) Upon collecting several cords, pour a 2 % agarose form (**f**) and transfer each cord into the filled form. (**g**) The agarose is solidified and removed from the form (**h**) A glue spot of approximately the same size as the freshly cut agorose block is placed on the Vibratome stage and (**i**) the widest base of the agorose block is placed on the glue spot, with the spinal cords oriented perpendicular to the Vibratome stage

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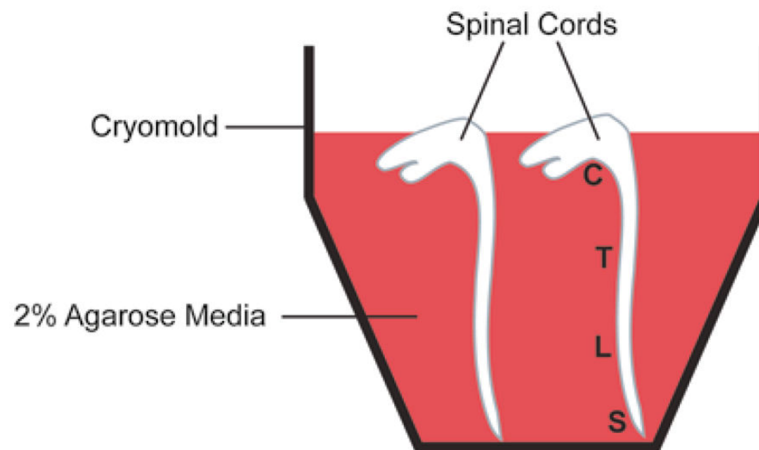

Fig. 5.

Schematic of spinal cord placement in 2 % agarose. Cutaway view depicts a cryomold containing spinal cords, which are supported by the density of agarose. Cervical (C) 20 spinal cord provides buoyancy so that thoracic (T), lumbar (L), and sacral (S) spinal cord regions are hung vertically

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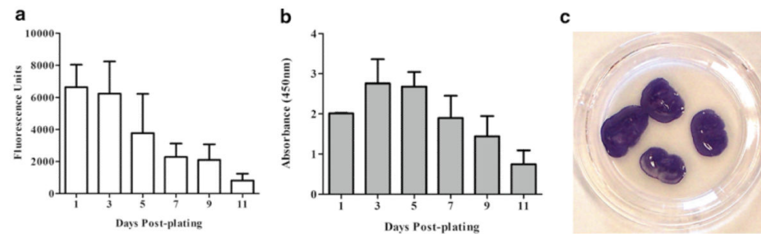

Fig. 6.

Prior to experimentation, CNS slices may need time to recover from axonal/cellular injury induced by the slicing procedure. (**a**) Brain slices were harvested into lysis buffer at indicated times post-plating ($N = 3–4$). To quantify apoptosis, Caspase 3 activity was determined by fluorogenic activity assay. Apoptosis occurs during the first week in culture, but is negligible at later time points. (**b**) Media was taken at specified time points ($N = 3–12$). To quantify tissue injury, LDH was detected by colorimetric assay. Tissue injury is evident in the week following slicing, but then progressively decreases over time. (**c**) At 11 days post-plating, BSC media was replaced with fresh media containing MTT and a representative photograph was taken 45 min later. Live tissue cleaves the substrate to form *purple* formazan crystals through mitochondrial activity. This demonstrates the long-term viability of brain slice tissue, despite an initial apoptosis and tissue injury caused by slicing