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Corresponding Author:	Adriano Sebollela, PhD Universidade de Sao Paulo Ribeirao Preto, Sao Paulo BRAZIL
Corresponding Author's Institution:	Universidade de Sao Paulo
Corresponding Author E-Mail:	sebollela@fmrp.usp.br
Order of Authors:	Artur Fernandes
	Niele D. Mendes
	Glaucia M. Almeida
	Giovanna O. Nogueira
	Carla M. Machado
	Jose A.C. Horta-Junior
	João A. Assirati
	Norberto Garcia-Cairasco
	Luciano Neder
	Adriano Sebollela, PhD
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TITLE:

Short-Term Free-Floating Slice Cultures from the Adult Human Brain

AUTHORS AND AFFILIATIONS:

Artur Fernandes[1,2], Niele Dias Mendes[1,3], Glaucia Maria de Almeida[1], Giovanna Orlovski Nogueira[1], Carla de Moraes Machado[4], Jose de Anchieta de Castro Horta-Junior[4], João Alberto Assirati Junior[5], Norberto Garcia-Cairasco[2], Luciano Neder[3], Adriano Sebollela[1]

[1]Department of Biochemistry and Immunology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil
[2]Department of Physiology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil
[3]Department of Pathology and Forensic Medicine, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil
[4]Department of Anatomy, Institute of Biosciences, São Paulo State University, Botucatu, São Paulo, Brazil
[5]Clinical Hospital at the Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

Corresponding author:

Adriano Sebollela (sebollela@fmrp.usp.br)

Email addresses of co-authors:

Artur Fernandes (arturfernandes@usp.br)
Niele Dias Mendes (niele@usp.br)
Glaucia Maria de Almeida (glaucia.maria.almeida@usp.br)
Giovanna Orlovski Nogueira (giovanna.orlovski.n@usp.br)
Carla de Moraes Machado (carla.demoraes@yahoo.com.br)
Jose de Anchieta de Castro Horta-Junior (anchieta@ibb.unesp.br)
João Alberto Assirati Junior (assiratijamj@uol.com.br)
Norberto Garcia-Cairasco (ngcairas@usp.br)
Luciano Neder (neder@fmrp.usp.br)

KEYWORDS:

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SUMMARY:

A protocol to prepare free-floating slice cultures from adult human brain is presented. The protocol is a variation of the widely used slice culture method using membrane inserts. It is simple, cost-effective, and recommended for running short-term assays aimed to unravel mechanisms of neurodegeneration behind age-associated brain diseases.

ABSTRACT:

Organotypic, or slice cultures, have been widely employed to model aspects of the central

nervous system functioning in vitro. Despite the potential of slice cultures in neuroscience, studies using adult nervous tissue to prepare such cultures are still scarce, particularly those from human subjects. The use of adult human tissue to prepare slice cultures is particularly attractive to enhance the understanding of human neuropathologies, as they hold unique properties typical of the mature human brain lacking in slices produced from rodent (usually neonatal) nervous tissue. This protocol describes how to use brain tissue collected from living human donors submitted to resective brain surgery to prepare short-term, free-floating slice cultures. Procedures to maintain and perform biochemical and cell biology assays using these cultures are also presented. Representative results demonstrate that the typical human cortical lamination is preserved in slices after 4 days in vitro (DIV4), with expected presence of the main neural cell types. Moreover, slices at DIV4 undergo robust cell death when challenged with a toxic stimulus (H_2O_2), indicating the potential of this model to serve as a platform in cell death assays. This method, a simpler and cost-effective alternative to the widely used protocol using membrane inserts, is mainly recommended for running short-term assays aimed to unravel mechanisms of neurodegeneration behind age-associated brain diseases. Finally, although the protocol is devoted to using cortical tissue collected from patients submitted to surgical treatment of pharmacoresistant temporal lobe epilepsy, it is argued that tissue collected from other brain regions/conditions should also be considered as sources to produce similar free-floating slice cultures.

INTRODUCTION:

The use of human samples in research is unequivocally a great option to study human brain pathologies, and modern techniques have opened new ways for robust and ethical experimentation using patient-derived tissue. Methods like organotypic/slice cultures prepared from adult human brain have been increasingly used in paradigms such as optogenetics[1], electrophysiology[2–5], plasticity[6–9], neurotoxicity/neuroprotection[10–13], cell therapy[14], drug screening[15–17], genetics and gene editing[12,18–20], among others, as a strategy for better understanding neurological diseases during adulthood.

The comprehension of mechanisms underlying human brain pathologies depends on experimental strategies that require a large number of subjects. Conversely, in the case of slice cultures, although access to human samples is still difficult, the possibility of generating up to 50 slices from a single cortical sample partially circumvents the requirement of recruiting multiple volunteers by increasing the number of replicates and performed assays per collected tissue[21].

Several protocols for brain organotypic/slice cultures have been described, ranging from the classical oculo drafts[22,23] to roller tube[24–26], semi-permeable membranes interface[27–30], and free-floating slices[31,32]. Depending on the particularities of an experimental design, each technique has its own advantages and disadvantages. Short-term, free-floating slices cultures from adult human brains is in some cases advantageous over the method used by Stoppini et al.[27], if considering the fact that although long-term cell survival in vitro is usually a major concern when evaluating a culture method, in many experiments only short periods of time in culture are needed[12,31–35]. Under these conditions, the use of free-floating cultures presents the advantage of being simpler and more cost-effective, as well as more accurately resembling the original

human tissue condition than slices kept in culture over 2−3 weeks.

Despite the potential of slice cultures to neuroscience, studies using adult nervous tissue to prepare such cultures are still scarce, particularly from human subjects. This article describes a protocol to use collected brain tissue from living human donors submitted to resective brain surgery to prepare free-floating slice cultures. Procedures to maintain and perform biochemical and cell biology assays using these cultures are detailed. This protocol has been proven valuable for analyzing viability and neuronal function in investigations on the mechanisms of neuropathologies linked to adulthood.

PROTOCOL:

Live adult brain tissues were obtained from patients undergoing resective neurosurgery for the treatment of pharmacoresistant temporal lobe epilepsy (**Figure 1A**). All procedures were approved by the Ethics Committee from the Clinics Hospital at the Ribeirão Preto Medical School (17578/2015), and patients (or their legal responsible person) agreed and signed the informed consent terms. Collection of the tissue was done by the neurosurgery team at the Epilepsy Surgery Center (CIREP - Clinics Hospital at the Ribeirão Preto Medical School, University of São Paulo, Brazil).

1. Sterilization of materials

NOTE: All material and solutions must be sterilized prior to use.

1.1. Sterilize all surgical tools and vibratome slicing material (knife holder, specimen disk, buffer tray) in a dry sterilizing oven for 4 h at 180 °C.

1.2. Sterilize temperature-sensitive material or equipment by UV or gamma irradiation.

1.3. Sterilize media and solutions by autoclavation or filtration through 0.22 µm pore membranes.

2. Preparation of solutions

2.1. Prepare 15−20 mL of transport solution: 50% v/v Hanks' balanced salt solution (HBSS), 50% v/v basal medium for maintenance of post-natal and adult brain neurons (**Table of Materials**), supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 3 mg/mL glucose, and 33 µg/mL gentamicin.

NOTE: Transport solution must be refrigerated and oxygenated (bubbling with carbogen gas) for at least 20 min prior to sample collection.

2.2. Prepare 300 mL of slicing solution (HBSS supplemented with 10 mM HEPES and 3 mg/mL glucose) and cool it down in a freezer to the point of initial crystal formation.

2.3. Prepare 20 mL of culture medium: basal medium for maintenance of post-natal and adult brain neurons (**Table of Materials**) supplemented with 1% L-glutamine derivative (**Table of Materials**), 2% supplement for neural culture (**Table of Materials**), 1% penicillin/streptomycin, and 0.25 µg/mL amphotericin B.

3. Setting up the slicing apparatus

NOTE: This protocol is ideally performed with the assistance of a colleague due to the logistics of sample collection in the surgical room.

3.1. In a bucket of salt-added ice, let the slicing solution rest under carbogen mixture bubbling (95% O_2, 5% CO_2) for at least 20 min prior to use.

3.2. Prepare a block of 3% agarose (approximately 2 cm x 2 cm x 2 cm) and superglue it to the vibratome specimen disk in order to create additional mechanical support to the tissue sample during slicing (**Figure 1E**).

3.3. Set the vibratome for slicing: section thickness of 200 µm, frequency of vibration of 100 Hz, and speed of slicing between 0.05−0.15 mm/s.

3.4. Add the slicing solution to the vibratome buffer tray and keep it refrigerated and oxygenated prior to receive the sample and throughout the slicing procedure.

4. Sample collection

NOTE: In this protocol, human neocortical tissue was collected in the surgical room and transported to the laboratory.

CAUTION: When dealing with human samples, follow the appropriate safety protocols established by the Institution.

4.1. Set up the transport apparatus (**Figure 1C**) that consists of: a portable gas cylinder with carbogen mixture connected to a pressure/flux valve that controls gas output connected to a silicon tubing that connects gas output to the transport vessel; a transport vessel, usually a 50 mL conical centrifuge tube with perforated lid for gas input, containing the transport solution; and ice for sample cooling during transport.

4.2. Collect and transport the specimen (**Figure 1B**) immediately to the lab. Submerse the specimen in cold transport solution (constantly bubbled with carbogen mixture).

5. Slicing

5.1. Transfer the specimen to a Petri dish (100 mm x 20 mm) containing slicing solution and, with

fine surgical tools, carefully remove as much as possible of the remaining meninges in the sample (**Figure 1D**).

5.2. Choose the best specimen orientation for producing slices with the particular characteristics of the experimental design, and with a no. 24 scalpel blade, trim a flat surface to be the base glued to the specimen disk.

5.3. Using a disposable plastic spoon and delicate paintbrushes, collect the fragment from the Petri dish and dry excess solution using filter paper (dry by capillarity and avoid touching the tissue fragment with paper).

5.4. Using superglue, attach the tissue to the vibratome specimen disk until it is firmly adhered to the disk and in contact with the agarose block (**Figure 1E**).

5.5. Place the vibratome specimen disk (with tissue properly attached) in the vibratome buffer tray filled with slicing solution that must be bubbling during the whole process.

5.6. Lock the knife holder in place with the razor blade firmly fixed.

5.7. The slicing solution must cover both the specimen and the blade, only then start slicing (**Figure 1F**).

5.8. Cut the specimen into 200 µm slices.

NOTE: Although some vibratomes cut the specimens automatically, the close observation and minor adjustments in slicing speed during the process may help producing better slices. Discard initial irregular slices.

5.9. Transfer the slices from the buffer tray to a Petri dish with slicing solution and trim loose edges and excess white mater to a proportion of around 70% cortex/30% white matter.

6. Culture

NOTE: Perform this step in a laminar flow cabinet under sterile environment.

6.1. Add 600 µL of culture medium per well (in a 24 well plate) and incubate for at least 20 min at 36 °C and 5% CO_2 prior to plating the slices.

6.2. Plate one slice per well using a paintbrush (**Figure 1G**).

6.3. If there are any unused wells in the plate, fill them with 400 µL of sterile water.

6.4. Incubate the plate at 36 °C, 5% CO_2.

NOTE: First medium replacement must be done in between 8−16 h after plating depending on the size of the slice.

6.5. Supplement 10 mL of the previously prepared culture medium with 50 ng/mL brain derived neurotrophic factor (BDNF).

NOTE: During the first 8−16 h, the slices are incubated in 600 µL of medium to avoid nutrient deprivation and acidification, since medium consumption in this phase is accelerated. From the next step, the volume of medium per well is adjusted to 400 µL.

6.6. Remove 333 µL of the conditioned medium from each well and add 133 µL of fresh BDNF-supplemented medium.

6.7. Repeat the process of medium replacement every 24 h by replacing one-third of the conditioned medium with fresh BDNF-supplemented medium.

7. Evaluating health, morphology, and function in cultured slices

NOTE: To induce cell death for the purpose of illustration in the representative results, some slices were submitted to a 24 h treatment with the oxidative stress inducer H_2O_2. Steps 0 and 7.1.3 describe use of H_2O_2 to induce cell death.

7.1. Cell viability

NOTE: A simple, straightforward method to evaluate neurotoxic/neuroprotection in challenged slice cultures is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay[36], which measures the percentage of metabolic active cells under normal conditions compared to treated samples.

7.1.1. In the laminar flow cabinet, replace one-third of the medium with fresh medium.

7.1.2. From a 30% H_2O_2 stock solution, add a volume of H_2O_2 per well to reach the intended final concentration (30 mM or 300 mM).

NOTE: H_2O_2 was added after the daily change of the medium to guarantee the proper supply of fresh nutrients prior to the challenge.

7.1.3. Incubate the plate for 24 h at 36 °C, 5% CO_2.

NOTE: After H_2O_2 treatment (or other toxic stimulus of interest), the following steps describe cell viability determination by the MTT assay.

7.1.4. Add 40 µL of MTT solution to a final concentration of 0.5 mg/mL to each well in the plate.

7.1.5. Incubate the plate at 36 °C, 5% CO_2 for 3 h.

7.1.6. Wash the slices with phosphate-buffered saline (PBS) and transfer to a microtube.

7.1.7. Carefully remove any remaining solution by pipetting.

7.1.8. Weigh the microtubes containing the slices to determine the mass of each slice (this is key for normalizing the absorbance readings obtained).

NOTE: If needed, samples can be frozen (-20 °C) at this stage for later processing.

7.1.9. Homogenize the slices in 200 µL of isopropanol/HCl using a motorized pestle.

7.1.10. Centrifuge at room temperature (RT) for 2 min at 2600 x *g*.

7.1.11. Collect the supernatant and measure the absorbance at 540 nm.

7.2. KCl-induced neuronal depolarization

NOTE: Phosphorylation of the mitogen activated protein kinase (MAPK) signaling cascade protein ERK, followed by western blotting, can be used for the quantification of the neuronal response to KCl-induced depolarization[37] .

7.2.1. At the flow cabinet, replace the culture medium by 300 µL of HBSS previously equilibrated to 36 °C.

7.2.2. Replace the HBSS with 300 µL of fresh HBSS previously equilibrated to 36 °C.

7.2.3. Incubate the plate at 36 °C, 5% CO_2 for 15 min.

7.2.4. Replace the HBSS with either the fresh HBSS or with 80 mM KCl depolarizing solution (both at 36 °C) and incubate at 36 °C, 5% CO_2 for 15 min.

7.2.5. Transfer the slices from the plate to microtubes. At this step, slices can be stored at -20 °C for later processing.

7.2.6. Prepare tissue extracts in 150 µL of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl; 150 mM NaCl; 1 mM EDTA; 1% nonionic surfactant; 0.1% sodium dodecyl sulfate; pH 7.5). Centrifuge extracts at 4 °C for 10 min at 16000 x *g*, collect supernatant, and determine total protein concentration using the Bradford method.

7.2.7. Load 30 µg of total protein onto a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

7.2.8. After electrophoresis, transfer the gel content to a nitrocellulose membrane.

7.2.9. After blocking the membrane with 5% non-fat dry milk in TBS plus 0.1% Tween, incubate with mouse anti-pERK (1:1,000) for 16 h at 4 °C. After washing, incubate for 2 h at RT with rabbit anti-ERK1/2 (1:1,000).

7.2.10. Incubate with the appropriate HRP-conjugated secondary antibody at RT for 1 h.

7.2.11. Reveal with the preferred HRP substrate.

7.3. Morphological evaluation

NOTE: In addition to cell survival and functional evaluations, it is important to analyze tissue morphology. Be aware that resectioning the cultured slice is an important step to producing as much high-quality material as possible for morphological analysis.

7.3.1. Fixing, cryoprotecting and resectioning the slices

7.3.1.1. Transfer the slices from the wells with culture medium to a new 24 well plate containing PBS.

7.3.1.2. Remove the PBS and add 1 mL of 4% paraformaldehyde (PFA). It is important that slices be kept flat prior to adding PFA. Incubate overnight at 4 °C.

7.3.1.3. Carefully remove the PFA solution and add 1 mL of 30% sucrose solution. Incubate for 48 h at 4 °C.

7.3.1.4. Set the freezing microtome to -40 °C.

7.3.1.5. Prepare a sucrose base on the microtome stage where the slices should be placed (**Figure 2A**). Let it freeze completely and carefully cut some of the frozen sucrose to produce a flat surface on which the slice will be placed.

7.3.1.6. Place each slice over a stretched plastic film and use a paintbrush to flat the tissue.

7.3.1.7. Transfer the stretched slice to the frozen sucrose base in one single move.

NOTE: It is not possible to move the slice once it is over the frozen sucrose base. Perform this transfer step carefully.

7.3.1.8. Let the slice rest for 5−10 min for proper freezing.

7.3.1.9. Cut the slice into 30 µm sections.

7.3.1.10. Transfer the 30 µm sections to a Petri dish containing PBS.

7.3.1.11. Proceed to the histology protocol more adequate to the experimental design.

NOTE: The 30 µm sections can be readily used for free-floating immunohistochemistry, mounted onto microscopy slides for further histology or stored in antifreeze solution at -20 °C.

7.3.2. Immunohistochemistry

NOTE: Immunohistochemistry and immunofluorescence standard protocols vary among labs. For a detailed version of the protocol used here, refer to Horta et al.[38]. Primary antibodies used for immunostainings presented in **Figure 2** include neuronal nuclei (NeuN), healthy mature neuron marker, glial fibrillary acidic protein (GFAP), astorcytes marker, ionized calcium binding adapter (Iba-1), and microglia marker.

7.3.2.1. Incubate the slices in blocking solution (e.g., 2% normal donkey serum in PBS) for 40 min.

7.3.2.2. Incubate overnight with primary antibody under mild agitation at 4 °C.

7.3.2.3. After washing with PBS, incubate for 120 min with biotinylated secondary antibody under mild agitation at RT.

7.3.2.4. After washing with PBS, incubate at RT for 120 min with avidin-peroxidase conjugate (**Table of Materials**).

7.3.2.5. Reveal with DAB + 0.04% nickel ammonium.

7.3.2.6. Mount the stained sections on gelatin coated microscopy slides and let them air dry. Dehydrate in ethanol, diaphanize in xylene, finish with mounting medium (**Table of Materials**), cover with a coverslip, and image.

REPRESENTATIVE RESULTS:

A critical aspect to evaluate the quality and health of cultured slices is the presence and typical morphology of the expected neural cell types, neurons, and glial cells. The typical architecture of the human cortical lamination was observed in a slice at DIV4, revealed by neuronal immunolabeling (**Figure 2D**). In addition, the expected presence of microglia and astroglia (**Figure 2B,C**) was also observed. These results demonstrate that tissue architecture is not significantly affected either by the surgical procedure/sample processing or by the short-term period in vitro. In accordance with previous findings, it was shown that NeuN immunoreactivity was not altered between D0 and D4[32]. Based on these results, the free-floating culture format, associated to the reduced thickness of the slice to 200 µm (compared to the widely used 300−400 µm when membrane inserts are used), contributed to better diffusion of oxygen and nutrients to inner cell layers in slices, which has been previously demonstrated to be critical to the health of cultured

slices[39,40].

Quantification of cell death in slices is also a valuable approach in ex vivo models of neuropathologies, such as slice cultures (reviewed by Lossi et al.[41]). In a previous study, we used the MTT assay to determine cell death levels along the period in culture[32]. In addition to viability, that same study also showed that cultured slices (up to D4) preserved the capacity to release neurotransmitters upon KCl-induced depolarization[32]. Here, those findings were expanded by investigating the neuronal response to KCl-induced depolarization on ERK phosphorylation, a central kinase involved in processes such as synaptic plasticity and memory[42,43]. Interestingly, a clear increase in ERK phosphorylation was seen in KCl-treated slices at D4 (**Figure 3A,B**).

Finally, the response of slices at DIV4 to a toxic challenge was evaluated with a known oxidative stress inducer, H_2O_2. The rationale was that the extent of cell death should be proportional to the level of cell viability in the cultured slices. As shown in **Figure 3C**, exposing the slices to 300 mM H_2O_2 for 24 h led to a robust decrease in MTT reduction. Taken together, the massive cell death observed in DIV5 after the H_2O_2 challenge and the KCl-induced depolarization results indicate that the preserved general health of slices at DIV4 responds adequately to a toxic stimulus such as oxidative stress.

FIGURE LEGENDS:

Figure 1: Sample collection, transport, slicing, and culturing of cortical tissue from adult humans. The procedure starts at the surgical room with collection of cortical tissue from temporal lobectomy for the treatment of pharmacoresistant epilepsy (**A,B**). (**C**) Tissue fragment (n = 1) is immediately transferred to a tube containing ice-cold oxygenated transport medium (see below). (**D**) In the lab, meninges are removed using fine ophthalmic tweezers. Excess liquid is dried using filter paper, and the fragment is superglued (**E**) to the vibratome specimen disk with the white matter facing down and pial surface facing up. (**F**) Using a commercial shaving razor, the specimen is cut into 200 µm slices that are collected with a delicate paintbrush and transferred back to the Petri dish for further trimming of excess white matter and loose ends (not shown) prior to (**G**) plating and culturing in a free-floating format. (**H**) Slices cultures are kept viable for several days and can be used in a variety of experimental protocols.

Figure 2: Morphological analysis of neural cells in slice cultures from adult human brain. Slices were fixed at the fourth day in vitro. (**A,B,C**) Representative steps of the sectioning procedure prior to immunohistochemistry. Tissue was digitally colored to improve visualization. (**D**) Normal distribution of neurons in cortical layers (Roman numerals). (**E**) Microglia and (**F**) astrocytes were also clearly observed (n = 1 slice per cell type labelling). All slices were obtained from tissue from a single donor. Scale bars = 100 µm.

Figure 3: Functional and cell viability assays in adult human brain slice cultures. Neuronal activity was evaluated in slices at day in vitro 5 (DIV5) by KCl-induced depolarization and consequent increase in ERK phosphorylation. (**A**) A representative immunoblot result obtained with homogenates from one slice. Bands corresponding to phospho ERK (Perk) and total ERK

(tERK) are indicated. (**B**) Quantification of pERK/tERK ratio in three independent slices from a single human donor. (**C**) Hydrogen peroxide (H_2O_2) toxicity was evaluated by the MTT assay in slices at DIV5. Optical density values obtained were normalized by the mass of each slice. Slices were challenged with H_2O_2 at the indicated concentrations (No H_2O_2; 30 mM H_2O_2; 300 mM H_2O_2) for 24 h. Images of representative slices after incubation with MTT are shown above the bars. Results are presented as average ± standard error from data obtained from three independent slices from a single donor.

DISCUSSION:

This protocol for producing free-floating, short-term slice cultures is an alternative method for culturing adult human neocortical slices. Such a protocol for slice cultures may be amenable for studies on (but not restricted to) optogenetics[1,44,45], electrophysiology[2–5], short-term plasticity[46,47], long-term plasticity[48,49], neurotoxicity/neuroprotection[10–13], cell therapy[14], drug screening[15–17], cancer[50,51], genetics, and gene editing[12,18–20].

The use of samples from adult human tissue is particularly important to understanding human neuropathologies, due to unique properties typical of the human brain lacking in slices produced from rodent nervous tissue[52]. Moreover, slice cultures from rodent brains are commonly prepared from neonatal, immature brains, which are highly plastic and contain migrating cells that may change the original slice cytoarchitecture to adapt to the in vitro environment[26,53–55]. Such plastic events lead to changes in circuitry that should be avoided when the goal is to mimic the in vivo condition, as stated by Ting et al.[30]: "We opted to focus on cultures of less than one week, where structural and functional properties are reasonably maintained with minimal perturbation, to be as comparable as possible to measurements obtained using gold-standard acute slice preparations". Therefore, although a method devoted to short-term culturing may initially be seen as limited, long-term culturing is not needed to produce relevant results in many experimental designs[32–35,56,57].

Two critical steps of the protocol are the reduced thickness of the slices (200 µm) and supplementation of the culture medium with BDNF. In previous work[32], we have shown preservation of cell viability up to 4 DIV and discussed the likely contribution of the reduction of slice thickness to 200 µm compared to the more often used 300−400 µm slices[12,29]. Basically, reducing slices thickness may contribute to a better oxygenation and nutrients uptake in free-floating slices, decreasing the chances of core hypoxia and neurodegeneration[31,32,57–61]. In addition, it is recommended to keep tissue in cold, oxygenated media from the surgical room to the slicing step at the vibratome, considering the high demand for O_2 by the adult human central nervous. Supplementation of medium with BNDF has been previously seen to slightly increase viability in adult human brain slices cultured free-floating[32], in line with recommendations for medium supplementation with neurotrophic factors by other authors[62,63].

In conclusion, this protocol describes methods for preparing and running assays with short-term, free-floating slice cultures from adult human brains. This model should be amenable for investigations on the mechanisms of toxicity/neuroprotection relevant to age-associated brain

diseases. The use of human resected tissue presents the advantage of preserving brain cytoarchitecture and local circuitry, adding translational power to obtained findings. Moreover, bursting the use of human-derived samples from neurosurgery in neuroscience may help reduce the need for animal experimentation. Although this protocol is centered around the use of tissue collected from patients submitted to neurosurgery for pharmacoresistant epilepsy treatment, it is suggested that tissue collected from other brain regions/conditions also be considered as sources for producing slice cultures in a simple and cost-effective way.

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DISCLOSURES:

The authors have nothing to disclose.

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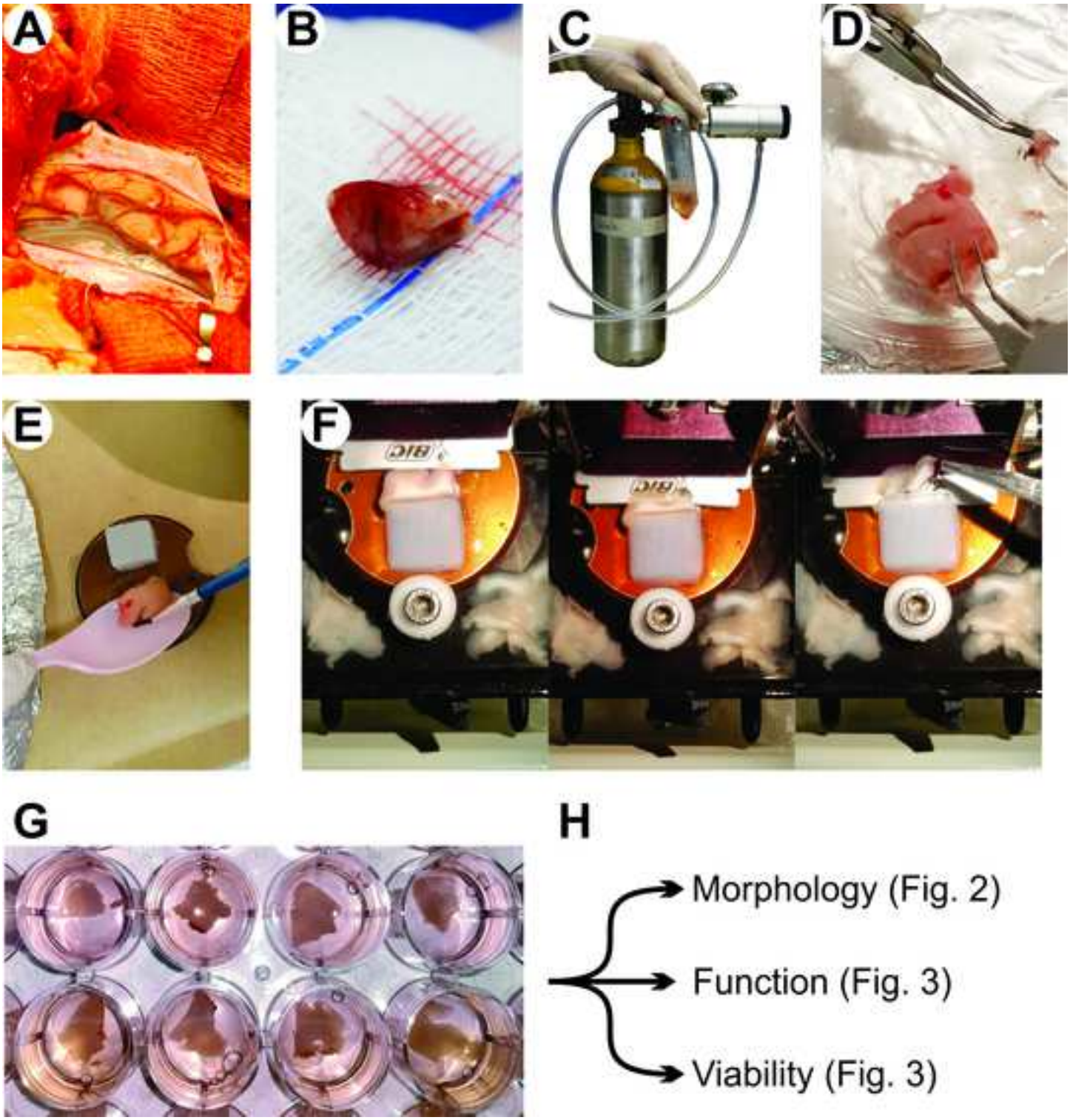
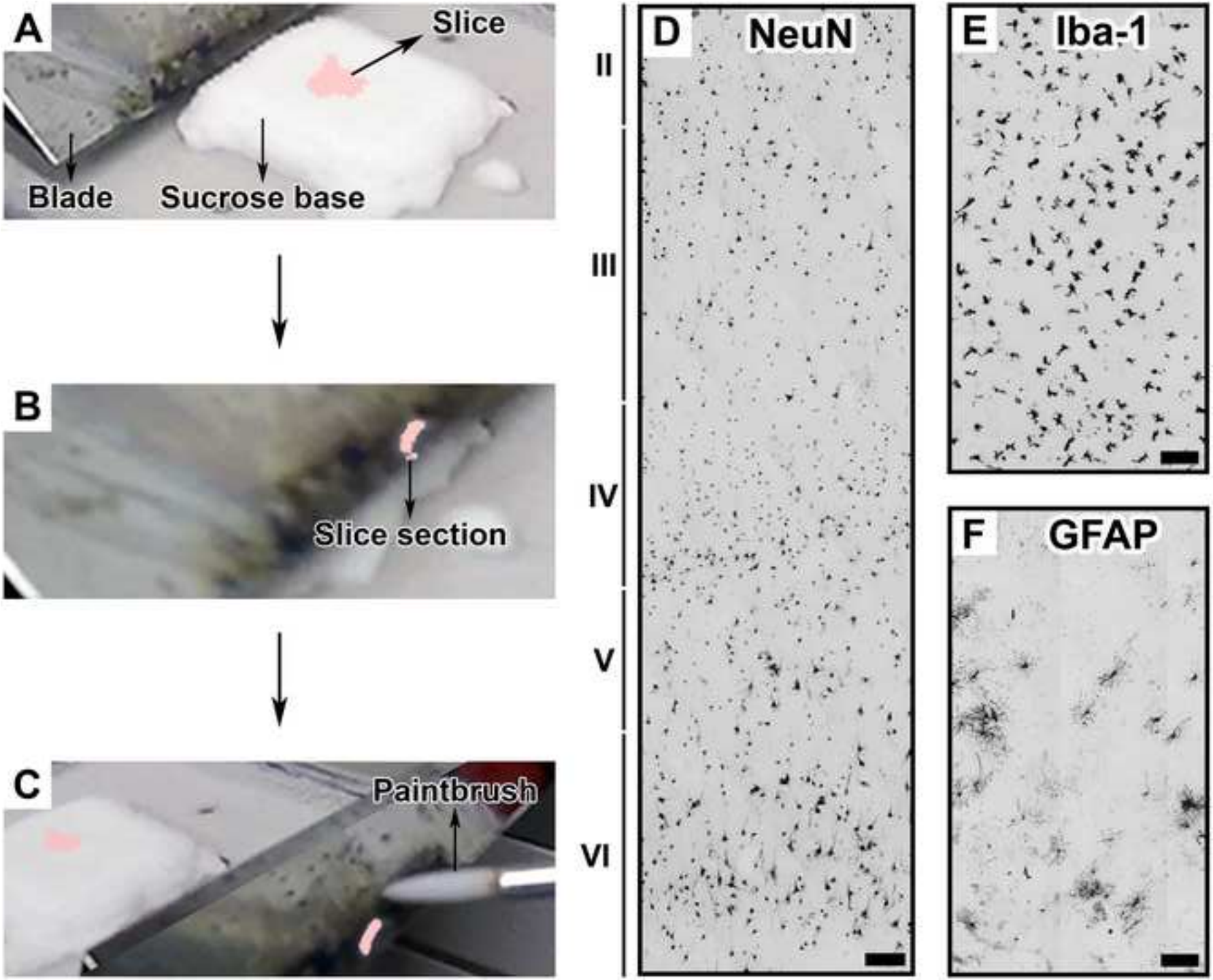


Figure 2



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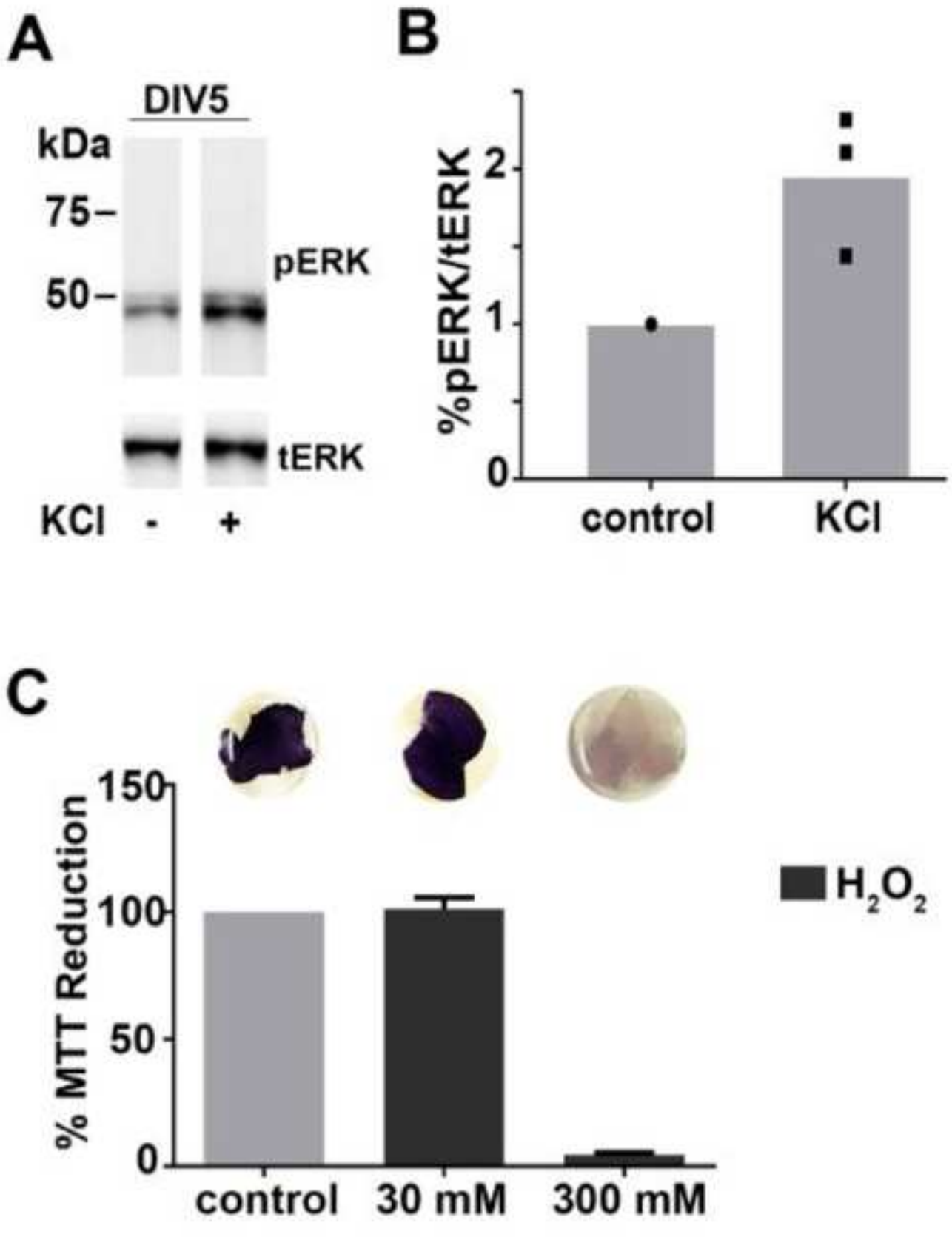

Solutions, Reagents and medium components

2-Propanol

Acrylamide/Bis-Acrylamide, 30% solution

Agarose

Ammonium persulfate

Amphotericin B

Antibody anti-ERK 2 (rabbit)

Antibody anti-pERK (mouse)

B27

BDNF

Bovine Serum Albumin

Bradford 1x Dye Reagent

EDTA

Glucose

Glutamax

Hank's Balanced Salts

Hepes

Hydrochloric acid

Hydrogen Peroxide (H_2O_2)

Mouse IgG, HRP-linked whole Ab (anti-mouse)

NaCl

Neurobasal A

Non-fat dry milk (Molico)

PBS Buffer pH 7,2

Penicilin/Streptomicin

Potassium Chloride

Prime Western Blotting Detection Reagent
Rabbit IgG, HRP-linked whole Ab (anti-rabbit)

SDS

TEMED

Thiazolyl Blue Tetrazolium Bromide (MTT)

Tris

Triton x-100

Ultrapure Water

Equipment and Material

24-well plates

Amersham Potran Premium (nitrocellulose membrane)

Carbogen Mixture

CO2 incubator

Microplate Reader

Microtubes

Motorized pestle

Plastic spoon

Razor Blade

Scalpel Blade

Superglue (Loctite Super Bonder)

Vibratome

Name of Material/ Equipment for Immunohistochemistry

Antibody anti-NeuN (mouse)

Antibody anti-GFAP (mouse)
Antibody anti-Iba1 (rabbit)
Biotinylated anti-mouse IgG Antibody (H+L)
DAB
Entellan
Ethanol
Gelatin
Microtome
Normal Donkey Serum
Paraformaldehyde
Rabbit IgG, HRP-linked whole Ab (anti-rabbit)
Slides (Star Frost)
Sucrose
Vectastain ABC HRP Kit (Peroxidase, Standard)
Xylene

Company	Catalog Number/Model
Merck	1096341000
Sigma Aldrich	A3449
Sigma Aldrich	A9539
Sigma	A3678-25G
Gibco	15290-018
Santa Cruz Biotecnology	sc-154
Santa Cruz Biotecnology	sc-7383
Gibco	17504-044
Sigma Aldrich	SRP3014
Sigma Aldrich	A7906
BioRad	500-0205
Sigma	T3924
Merck	108337
Gibco	35050-061
Sigma Aldrich	H1387-10X1L
Sigma Aldrich	H4034
Merck	1003171000
Vetec	194
GE	NA931-1ML
Merck	1064041000
Gibco	10888-022

Nestlé	
Laborclin	590338
Sigma Aldrich	P4333
Merck	1049361000
GE	RPN2232
GE	NA934-1ML
Sigma	L5750
GE	17-1312-01
Sigma Aldrich	M5655
Sigma	T-1378
Sigma	X100
Millipore	

Company	Catalog Number/Model
Corning	CL S3526
GE	29047575
White Martins	
New Brunswick Scientific	CO-24
Molecular Devices	
Greiner	001608
Kimble Chase	
Bic	

Becton Dickinson (BD)	Number 24
Henkel	
Leica	14047235612 - VT1000S

Company	Catalog Number/Model	
Millipore	MAB377	
Merck	MAB360	
Abcam	EPR16588 - ab178846	
Vector	BA-9200	
Sigma Aldrich	D-9015	
Merck		107960
Merck	1.00983.1000	
Synth	00G1002.02.AE	
Leica	SM2010R	
Jackson Immuno Research	017-000-121	
Sigma Aldrich	158127	
GE	NA934-1ML	
Knittel Glaser		
Vetec	60REAVET017050	
Vector	PK-4000, Kit Standard	
Synth	01X1001.01.BJ	

Comments/Description
Dilution 1:1,000 in BSA 2.5%
Dilution 1:1,000 in BSA 2.5%
Used in RIPA buffer
Used in RIPA buffer

Used for membrane blocking

Used in RIPA buffer

Used in RIPA buffer

Used in RIPA buffer

Sterile water, derived from MiliQ water purification system

Comments/Description

Flat Bottom with Lid

95% O_2, 5% CO2

Incubation of slices 5% CO2, 36ºC

1,5mL microtube

Size of a dessert spoon

Chrome Platinum, used in slicing with vibratome

Used for slicing of tissue; recommended same size or smaller
Composition: Etilcianoacrilato; 2-Propenoic acid; 6,6 -di-terc-butil-2,2 -metilenodi-p-cresol; homopolymer

Comments/Description

Dilution 1:1,000 in Phosphate Buffer

Dilution 1:1,000 in Phosphate Buffer
Dilution 1:2,000 in Phosphate Buffer

Used for coating slides
Equipped with Freezing Stage (BFS-10MP, Physiotemp), set to -40ºC

Gelatin coated slides

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CORRESPONDING AUTHOR

Name:	ADRIANO SEBOLLELA
Department:	BIOCHEMISTRY AND IMMUNOLOGY
Institution:	RIBEIRAO PRETO MEDICAL SCHOOL - UNIVERSITY OF SAO PAULO
Title:	ASSISTANT PROFESSOR
Signature:	
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UNIVERSITY OF SÃO PAULO

May 3, 2019

Nam Nguyen, Ph.D.
Manager of Review
JoVE

Dear Dr. Nguyen:

Please find attached the files corresponding to the revised version of the manuscript JoVE59845 entitled “Short-term Free-Floating Slice Cultures from Adult Human Brain”, by Dr. Artur Fernandes, myself and colleagues.

We are grateful to the JoVE Editorial Staff and the external reviewers for the careful and constructive revision, which significantly improved the quality of our work. In the revised manuscript we have addressed all the concerns raised, and have made a number of changes in the text (highlighted in bold) according to their criticisms and suggestions.

In addition, we also submit a rebuttal letter in which we provide a point-by-point analysis to each comment raised by both Editor and Reviewers.

We thank JoVE for the opportunity to improve our manuscript, and look forward to receiving your feedback as soon as possible.

Cordially,

Adriano Sebollela
Assistant Professor
Dept. of Biochemistry and Immunology
Ribeirao Preto Medical School
University of Sao Paulo
Brazil

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been carefully revised as advised and all detected spelling or grammar issues have been addressed.

2. Authors and affiliations: Please provide an email address for each author.

The revised version of the manuscript presents all authors' e-mail addresses.

3. Please add a Summary section before the Abstract section to clearly describe the protocol and its applications in complete sentences between 10−50 words: "Here, we present a protocol to …"

A summary section has been included as requested.

4. Abstract: Please provide a more detailed overview of the method and a summary of its advantages, limitations, and applications.

The abstract has been expanded to cover the suggestions.

5. Introduction: Please expand to include the advantages of the presented method over alternative techniques with applicable references to previous studies, description of the context of the technique in the wider body of literature and information that can help readers to determine if the method is appropriate for their application.

In the revised manuscript, "Introduction" has been significantly expanded, including additions of citations to key publications in the field missing in the original version. A comparison between previously published and our protocols has been added as requested.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Neurobasal A®, Leica, Glutamax®, etc.

Manuscript is now free of commercial language. This is now restricted to the table of materials. For instance, Neurobasal A is now referred to as "basal medium for maintenance of post-natal and adult brain neurons", and Glutamax has been replaced by "L-glutamine derivative".

7. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

We have added the following ethics statement before the numbered protocol steps.

"Live adult brain tissues were obtained from patients undergoing resective neurosurgery for the treatment of pharmacoresistant temporal lobe epilepsy (Fig. 1A). All the procedures were approved by the Ethics Committee from the Clinics Hospital at the Ribeirão Preto Medical School (17578/2015) and the patients (or their legal responsible person) agreed and signed the informed consent term. The collection of the tissue was done by the neurosurgery team at the Epilepsy Surgery Center (CIREP - Clinics Hospital at the Ribeirão Preto Medical School, University of São Paulo, Brazil)".

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Topics are now in sequential numbers.

9. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Personal pronouns have been removed as suggested.

10. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Manuscript has been revised accordingly.

11. Everything in the protocol (except for the introductory ethics statement) should be in a numbered step (in the imperative tense and with no more than 4 sentences), numbered header, or a "NOTE". Please move the introductory paragraphs of the protocol to the Introduction, Results, or Discussion (as appropriate) or break into steps.

Manuscript has been revised accordingly.

12. Lines 83-92, etc.: The Protocol should contain only action items that direct the reader to do something. Please either write the text in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.), or move the solutions, materials and equipment information to the Table of Materials.

We revised the manuscript accordingly. The Table of Materials has been carefully revised and expanded.

13. Lines 93-94: Please describe how to remove pia mater and uneven edges in the imperative tense.

In the revised manuscript we have attempted to better describe this procedure in the imperative tense, as requested. We believe though that this procedure will be more efficiently demonstrated in the video article.

14. Please organize the sections/steps properly so that the protocol can be followed in chronological order.

We have changed the order of appearance of some steps, in particular the initial steps of each protocol, where the preparation of solutions is now in a separate initial section.

15. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

We revised the manuscript accordingly.

16. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.

All text is now in single line spacing.

17. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

The steps selected for the video article have been highlighted in yellow.

20. Please add a Discussion section to explicitly cover the following in detail in 3-6 paragraphs with citations:

A Discussion section has been added following the all the Jove recommendations.

21. JoVE article does not have a Conclusion section. Please move information in the Conclusion section to Results or Discussion (as appropriate).

We revised the manuscript accordingly.

22. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

All figures were removed from the manuscript, which now only contains the Figures legends.

23. Figure 3: Please include a space between numbers and their corresponding units (30 mM, 300 mM).

Figure 3 is now modified with spaces between numbers and units.

24. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment. Please remove trademark (™), registered (®) and copyright symbols.

We updated the Table of Materials in order to provide all the information on relevant supplies and equipment.

Reviewers' comments:

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded. Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

In the present study JoVE59845 Fernandes et al. give a detailed methodological protocol to culture human brain sections free-floating. This is a nice protocol to be published in a low impact journal. The use of adult tissue is important and exciting but the authors could have made more out of it. But there are some issues which must be considered before acceptance.

1. The authors aim to suggest that this technique is related to organotypic brain slice cultures. This is wrong. The authors make thin 200 µm brain sections and culture them free-floating. This has nothing to do with the original protocol published by Gähwiler and Stoppinis groups and others.

We agree with the reviewer that the classical concept of organotypic culture is associated with thicker slices cultured on membrane inserts. Based on this notion, in the revised version we have referred to our model as "slice cultures" instead of organotypic cultures. However, it seems important to mention here that there are some examples in the literature in which thinner slices were also termed as organotypic cultures (Verwer et al. 2002; González-Martínez et al. 2008; Mendes et al. 2018).

I wonder why the authors did not culture on membrane inserts, as this would be clearly be more comparable with others. Instead they culture free-floating as acute short-term slices, which is not really a novel and innovative idea.

In fact, we did use membranes inserts in earlier experiments. However, we have found no significant improvement in viability after 4 days in culture (see Mendes et al. 2018 for details). We concluded that the conditions we found in the protocol described here could be of interest to groups that, like us, routinely perform assays using short term cultures (some examples are given in the next answer), and therefore would not need to use membrane inserts in their cultures, what turns the procedure simpler and more affordable.

2. I also must criticize the short culture period of 5 days; usually cultures for 2 weeks are more interesting and give more information. Such acute slice cultures only have limited information.

We agree with the reviewer that long-term cultures are more informative when the aim is, for instance, to follow the long-term effects of drug treatments on

synapses. However, we respectfully disagree with the reviewer's point of view about the potential of long term cultures in general. There are several examples of relevant findings obtained using short-term organotypic cultures (e.g. Bruce, Malfroy, and Baudry 1996; Finley et al. 2004; Bsibsi et al. 2006; Schoeler et al. 2012; Mendes et al. 2018; Ting et al. 2018;). Importantly, in the later paper is stated: *"We opted to focus on cultures of less than one week, where structural and functional properties are reasonably maintained with minimal perturbation, to be as comparable as possible to measurements obtained using gold-standard acute slice preparations"*. Moreover, the following criticism to long term human brain-derived cultures used in Eugène et al. (2014) is also present in Ting et al. (2018): *"This is in contrast to recent work that explored optimal conditions for long-term cultures of human ex vivo brain slices for up to one month in vitro, with the earliest time point systematically examined being six days in vitro".* Part of this relevant discussion has been included in the revised manuscript ("Discussion").

3. I have no idea why the authors mention Alzheimer-associated Ab petide aggregates; this has nothing to do here.

We have deleted the statement on Abeta aggregates, as requested. The original idea was to illustrate an application of the model. But we understand that the readers interested in AD pathology may refer to our previous paper (Mendes et al., 2018) for details on the experiments using Abeta aggregates.

4. The citing of references is limited, especially regarding brain slice cultures, the author miss many important reviews in this field.

We have added new references in "Introduction" and "Discussion" sections.

In summary, the authors must mention in Title and Abstract, that they made and used acute short-term cultures (5 days), and that these slices are not organotypic cultures in the classical sense.

Title and Abstract have been changed according to the reviewer's suggestions and now refer to our protocol as "short term slice cultures".

The authors should add 4 new paragraphs in Discussion where they discuss (a) comparison to classical organotypic brain slices, (b) problems for long-term culture > 2weeks, (c) limits and outlook of their free-floating technique and (d) troubleshooting for the present protocol.

A "Discussion" section has been added, which cover the topics suggested by the reviewer.

Reviewer #2:

Manuscript Summary:

The paper by Fernandes et al summarises a protocol for short-term culturing of human ex vivo human brain tissue slices derived from surgical dissectates. The origin and handling of the tissue is first summarized, followed by details of material requirements for supporting tissue when transferred to the tissue culture lab, and for continued maintenance of the cultures. Description of a protocol for assessing the viability of the free-floating tissue slices using an MTT assay was described. Representative effects on viability of overnight exposure of slices to two doses of H2O2 were further summarised.

Correlating tissue functionality with the ability of neurons to depolarize, the authors provided representative data which demonstrated that the 4d-old ex vivo slices were indeed able to depolarize when KCl was added to the cultures, as evidenced by increased expression of phosphorylated ERK in treated tissue samples. Finally, sample evidence of the structural integrity of cultured tissue was presented in the form of images of tissue stained for NeuN, Iba1 and GFAP. There is a clear need for robust protocols supporting the maintenance of human tissue samples in vitro, such that studies such as the one described are welcome.

Major Concerns:

The current protocol summary lacks depth. This reviewer didn't get the sense that the group had extensive experience of producing the cultured human tissue slices i.e. the authoritative voice was lacking.

We agree and thank the reviewer for the criticism. The revised manuscript has been modified to follow the recommendations from the reviewer and the editorial staff regarding the communication of the protocol.

More commentary on variation and limitations, including mention of expected yields would be helpful and more persuasive. Justification for each step in the protocol would also be useful. For example - did the authors compare the effects of oxygenated versus non-oxygenated solution on slice viability?

Since JoVE instructs the authors to avoid discussing throughout the "Protocol" section, we opted to comment on limitations (including the effects of oxygenation) and potential of our method in the "Discussion" section.

Some modifications are required in order to turn the protocol described into a 'go to' reference for those who wish to maintain human organotypic slice cultures.

1. Abstract

The abstract is too vague. Only one third of max word count is used. More detail of results obtained should be provided.

We have expanded the abstract as suggested.

2. Authors did not follow recommended guidelines

a. Summary is missing

A summary has been added as recommended.

b. There is no proper discussion section. There is no mention of critical steps or limitations etc?

A Discussion section has been added, in which critical steps, advantages and limitations are commented, as suggested.

c. The recommended guidelines for the materials description were not followed. In its present format, the summary of materials is confusing and unusable.

While preparing the revised manuscript we have made use of the JoVE template, which we believe allowed us to efficiently address all reviewers' criticisms concerning formatting of the text. The Table of Materials originally submitted got

confusing due to misformatting during the process of conversion to PDF. Nevertheless, we have both added information and optimized formatting in the revised Table.

3. What is the role of the carbogen gas in the slice culture solution? Is O2 level critical at all time-points? Was this tested?

We opted to include the use of carbogen gas during transport and slicing based on some examples in the literature that support the advantages of oxygenation in help supporting neuronal viability. Considering how much oxygen is necessary in brain normal physiology (20% of total oxygen consumption or the normal pressure of oxygen in the cerebral cortex 27 ± 6 mmHg in the cerebral cortex (Zhang, K. 2011)) it is imperative that the tissue remain well oxygenated after loss of blood support to avoid cell loss by hypoxia. In 1991, Stoppini, L. executed electrophysiological tests in cultured slices keeping the tissues embedded in solution enriched with gas containing 95% O_2 and 5% CO_2 (carbogen gas), thus demonstrating the survival of these tissues for long hours outside the culture and also the ability to return these tissues to culture also currently used during preparation (Douglas, H. A. 2011) or to incubate the slices (Verwer, R.W.H. 2002). Additionally, in a recent review, Jones et al., (2016) are categorical in the advantages of this step in the protocol: "It is therefore essential that the collection aCSF remains chilled and continually carbogenated (95% O_2/5% CO_2) to ensure that the tissue does not become anoxic". Part of this important discussion has been included in the revised manuscript (Discussion section).

4. Line 88 - specify size/type of scalpel blades - what shape is the blade?

The scalpel blade used in our protocol is a number 24. This commentary was added to the revised text

5. What kind of plastic spoon? (line 89)

We have used a disposable dessert spoon. This information has been added in the revised text.

6. Need for sterilization of instruments isn't mentioned? Also - presumably all solutions should also be sterile?

We have now added detailed instructions on sterilization of both media/solutions and tools/equipment.

7. Lines 93-94 - is pia matter removed while viewing under a dissecting microscope? Why is it important to remove this prior to slicing?

No. Dissection was done with the naked eye, but we believe it would be of some help if a stereomicroscope is available. In fact we have modified the revised manuscript and provided a better description of this procedure as follows: "with fine surgical tools, carefully remove as much as possible of the meninges left in the sample". Regarding the need of removing meninges, we have seen in our preliminary experiments that slicing tissue still containing meninges produces slices highly irregular in thickness. This step should be detailed shown in the video article.

8. In general, it would be helpful to explain the reasoning behind a particular protocol detail.

We have added notes throughout the protocol providing relevant information and the rationale behind some protocol steps. Moreover, some details not covered in the "Protocol" section are commented in "Discussion".

9. Line 145 - supposed to be 37 degrees C? The use of a temperature that is one degree lower than standard temperature used in cell culture facilities is puzzling. Can authors justify choice of this temperature?

It is common sense to culture adult brain slices at 36°C (Gähwiler 1981; Stoppini L, Buchs A, and Muller D 1991; Noraberg et al. 2005) or even lower temperatures in between 32-35°C (O'Connor et al. 1997; Verwer et al. 2002; Douglas et al. 2011). We should keep in mind here that the main goal in a brain tissue culture is to maintain cells alive, rather than stimulate proliferation or exacerbated responses to injury.

10. Total volume info not consistent? - see lines 157-158. Line 158 - should that read 'remove 133 ul'?

We agree with the referee that this was not clear in the original text. We have modified the manuscript as follows: "Note: In the first medium replacement, the initial volume of 600 µL is adjusted the final volume of 400 µL that will be maintained throughout the days in vitro with daily medium refreshment. Therefore: 6.6. Remove 333 µL of the conditioned medium and add 133 µL of fresh BDNF-supplemented medium; 6.7. Repeat the process of medium replacement every 24 h by removing 1/3 of conditioned medium and adding an equal volume of fresh BDNF-supplemented medium".

11. No source information has been provided for the cell viability or depolarization assays i.e. supplier info or antibody detail. Control conditions not mentioned etc. Same comment applies to the immune protocol.

The revised manuscript brings detailed information about the antibodies used in the study (in the revised Table of Materials). Detailed information on cell viability or depolarization assays, or on immunostaining can be found in our original publication (Mendes et al., 2018).

12. Normalisation of data according to slice weight is not properly explained. Data should be presented as MTT/ug tissue, or something similar.

To make normalization by mass clearer to the readers, we have added the following statement in the Legend for Fig. 3: "*(Optical density values obtained on spectrophotometric measurements, in triplicates, were normalized by the mass of each slice)".*

13. How were microglia and astrocytes observed? - Stained images are presented - but no protocol for staining is given.

Neurons, microglia and astrocytes were observed under a light microscopy in re-sectioned slices submitted to standard DAB-Nickel immunostaining. Antibodies used are listed in the revised Table of Materials. Since we found that preparing the thin sections (from the 200µm cultured slices) for immunostaining is more critical than the immunostaining itself (which should work with any established protocol once nice sections are obtained), we opted to not include details on our staining protocol (which can be found in our previous publication; Mendes et al., 2018).

14. No info on 'n' numbers for any of the assays? Variability in slice conditions etc? How reproducible is the data presented?

15. If possible, the authors should indicate the variability is slice quality, from prep to prep. How consistent is the data?

On this protocol article, 'n', which refers to the number of donors and of slices/condition tested, is now presented in the legend for the Figures. In our previous paper (Mendes, et al., 2018), we have obtained viability and functional data using tissue from more than 30 subject donors. Therefore, variability between culture batches can be depicted from those data. It seems important to make clear that the protocol described here has been established after collecting/culturing tissue from dozens of subjects.

16. Why was DIV4 chosen for experimentation?

17. Did authors ever track viability of slices at time-points before or after DIV4? This detail would be of great interest to the readers.

In our previous study (Mendes, et al., 2018) we have found no significant reduction in viability up to DIV4. Since that article motivated the invitation to submit this JoVE article, we decided to keep using DIV4 as a reference. However, we have already obtained data showing partially preserved viability at time points after DIV4 (e.g. DIV9). This information has been included in the revised manuscript ("Discussion"). Data on viability and tissue integrity at time points earlier than DIV4 can also be found in Mendes et al. (2018).

18. There is no discussion of adequate oxygenation in floating slice cultures. Most current slice culture protocols exploit well inserts, creating a medium-air interface. Can authors explain advantages of using floating slices over well inserts?

Although we agree with the reviewer that in most studies the slice culture protocols use inserts, there are also relevant studies relying on free-floating culturing protocols. For instance, Verwer et al., (2002) successfully maintained adult brain slices from post-mortem patients in a free-floating culture for up to 50 days. Free-floating cultures were also used in other studies (L. Wu et al. 2008; H.-M. Wu et al. 2014).

19. Inherent variability in the system should be discussed.

Variability has been already discussed in a previous answer.

20. Although authors state that 200um thickness is better than the thicker slices used by others, they have produced no evidence to support this statement. Were different slice thicknesses analysed for viability?

The impact of different thickness to culture healthy has been discussed in our previous article (Mendes et al., 2018). There we argue that preservation of cell viability up to 4 DIV in our free floating cultures is likely a consequence of the reduction in the thickness of the slices to 200 µm, compared to often used 300-400 µm slices (Sebollela, Freitas-Correa et al. 2012; Eugene, Cluzeaud et al. 2014), which may have a positive impact on diffusion of O_2 and nutrients through the tissue". This is in conformity with (Masamoto and Tanishita 2009; Hadjistassou, Bejan, and Ventikos 2015) for oxygen consumption and maximum

diffusion distance for O_2 in nervous tissue. A commentary on this matter has been added to the "Discussion" section in the revised MS.

Reviewer #3:

Manuscript Summary:

The authors describe a very interesting and relevant technique establishing a protocol to culture and prepare adult human brain slice cultures. However, at the current stage I cannot recommend publication in Jove.

Major Concerns:

In the current manuscript the discussion section is missing: The critical steps in the protocol, modifications and troubleshooting of the method, limitations of the method, the significance of the method with respect to existing/alternative methods, future applications or directions of the method are not proper discussed. Further important publications like Eugene et al 2014, establishing adult human brain slices surviving up to 4 weeks and more, Jung et al, 2002; Chaichana et al, 2007; Gonzalez-Martinez et al, 2007 are not mentioned

We thank the reviewer for the constructive criticism and suggestions. In the revised manuscript we have expanded "Introduction" and added a "Discussion" section. In both sections we have added citations to important publications missing in the original submission including those mentioned by the reviewer.

Figure 2: Pictures of cortical lamination, where neurons, astrocytes and microglia are stained should be included for day 1, 2, 3 and 5 in small and higher magnification. Without it one cannot judge morphology and organotypic structure.

In the current manuscript we focus to show the longest time point in vitro, assuming that morphology/structure at earlier time points should be equal or even better preserved. Nonetheless, readers may refer to our previous publication (Mendes et al., 2018) for more details on this regard.

Microglia cells look highly activated in the image, possibly impacting experimental outcomes. Please discuss this issue.

We agree with the reviewer that in the field presented, some cells display a morphology resembling activation. However, this was not observed in the entire slice. In fact, in other fields the microglial morphology observed was considerably different. In the present work we have chosen to include that field since the goal was to unequivocally demonstrate the presence of this cell type in cultured slices at DIV4. A comprehensive analysis of the microglia status along the period in culture is currently under investigation in our Lab, including its possible relationship with the patient donor medical condition.

Since pia mater consists of a one- to two-cell-thick layer of leptomeningeal cells, how did the authors removed specifically only the pia mater and nothing else? Were membrana limitans gliae superficialis and molecular layer etc removed too?

We thank the reviewer for raising this important issue. This procedure was poorly and erroneously described in the original manuscript. In the revised manuscript we have modified the text to "*Transfer the specimen to a Petri dish containing*

***slicing solution and, with fine surgical tools, carefully remove as much as possible of the remaining meninges in the sample*".**

Why the authors chose day 5 for KCl and H_2O_2 treatment, since the text stated that viability and organotypic structure were only examined up to day four? No images of slices at day 5 are present.

How is the NeuN morphology, release of neurotransmitter on day 5? If the slices already degenerate at day 5, huge damage would be obvious.

H_2O_2 treatment started at DIV4 and lasted 24h. The result presents the difference between treated and control (untreated) slices at DIV5. Therefore, the observed H_2O_2-induced toxicity clearly indicates that control slices preserved (at least partially) its viability and functionality at DIV5. Nonetheless, it seems important to mention here that we have analysed viability at longer time points (e.g. DIV9), and observed high percentage of viability, although some degree of cell death was also detected.

Further assays for cell viability, like PI uptake would improve the statement, since MTT assay alone is highly unspecific.

Although we agree that it could be interesting to compare our MTT data with an alternative viability determination assay, we also reinforce that MTT assay has been widely employed to evaluate in vitro viability (Tobita, Izumi, and Feinberg 2010; Paterniti et al. 2013) being considered even more sensitive than the LDH assay (Mewes, Franke, and Singer 2012).

Minor Concerns:

Do the authors know how the slices change after treatment with BDNF? and how would it impact the studies on the mechanisms of neuropathologies linked to adulthood?

We previously showed in Mendes et al (2018) a slight positive effect of BDNF addition on the viability of slices cultured in free floating. Regarding a possible protective effect of BNDF, we think this is a relevant topic that needs to be considered in a case-to-case manner. We may say though that we have tested NGF as an alternative survival factor and got promising results. This opens the possibility of customization of medium supplementation according to the toxic stimulus under investigation. We have included this discussion in the revised manuscript ("Discussion").

The table (unnumbered) is chaotic and listing is unstructured, the product names are not mentioned. General formatting is chaotic and some spelling mistakes are present.

We have revised and updated the Table of Materials.