

Optimising of Immunohistochemical Protocol for Organotypic Brain Slice Cultures

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Abstract:

Organotypic slice cultures of brain tissue is an ex-vivo technique that been used increasingly to study neuronal death induced by hypoxia, hypoglycaemia, oxidative stress and brain disorders such as; Alzheimer and Parkinson's disease. Most of previous studies have used propidium iodide as a marker for dead or dying cells. This study aimed to evaluate existing immunohistochemical methods, in order to improve detection of

organotypic slice culture of postnatal mice at 4-5 days of age, then fluorescence labelling conducted and examined by a confocal microscope. In the current study, Glial Fibrillary Acidic Protein (GFAP) dye was used for astrocyte detection instead of propidium iodide. The organotypic slice culture is very sensitive when placing the coverslip, so, the thickness of the mounted slices was optimized with accurate casket bridge. It was shown that a bridge mounting method after overnight incubation at 4°C in 1% Triton X- 100 in PBS provided high quality for the immunohistochemical technique. Various detergent concentrations of permeabilization were examined as well. Results are reflecting clear morphology of astrocytes and the accuracy of the permeabilization concentration.

KEYWORDS; *Permeabilization, confocal microscopy, immunohistochemistry, organotypic slice culture, casket bridge*

Introduction

There is an increasing awareness of the importance and understanding of the mechanism of the neurodegenerative diseases where the inflammatory response is unclear ([Abushofa, 2014](#)). Neurodegenerative diseases including Parkinson's, Alzheimer's, and Huntington's occur as a result of neurodegenerative changes ([Abushofa 2016](#)). Organotypic slice cultures are a widely accepted model system for study the rodent central nervous system (CNS). This technique provided a good facility to mimic the nerve system. The nervous system is divided into two main sections, the central nervous system and the peripheral

nervous system. The central part contains; the brain and spinal cord. The peripheral consists of sensory neurons, collections of neurons called ganglia which provide the connections between different neurological structures and nerves. This study focused on glial cells, especially astrocytes which the biggest glial cells in size, and considered essential cell type that undergoes proliferation during the neurodegeneration ([Abushofa, 2014](#)). There is variety of astrocytes; which perform a several tasks, mainly an axon guidance and synaptic support, to the control of the blood brain barrier and blood flow ([Burda, Bernstein et al. 2016](#)).

Immunohistochemistry is routinely used to define the morphological characteristics of the slice cultures and to quantify the changes in protein expression levels ([Tillberg, Chen et al.](#)). Whole mount immunohistochemical labelling preserves the organization *in vivo*, allowing imaging of 3D relationships between cell types and organization of connectivity ([Humpel, 2015](#))([Tillberg, Chen et al. 2016](#)), but none of these really take into account the thickness of the slice when mounting. This article illustrates some of the drawbacks of current methods for immunohistochemical labelling of cultured slices and the optimization steps that can overcome these technical issues to improve the quality of data obtained.

The current study has focused on improved a permeabilization, it is a very importance step that the antibody proteins are enormous and ionic in nature to pass through cell membranes without prior permeabilization. The detergents are offer differing degrees of membrane proved levels of access to subcellular compartments. Triton-X-100 is a non-ionic detergent widely used for permeabilization as it efficiently dissolves cell

membranes without disrupting protein-protein interactions and it allows access to the nuclear interior. It is usually used at 0.1 %-0.2 % in PBS buffer for 10 minutes. Longer incubations or higher amounts of the detergent are not recommended due to its somewhat harsh nature. Others have used saponin compounds which are milder detergents used instead of Triton-X-100 to disrupt membranes ([Liu, 2018](#)).

Materials and Methods:

Immunohistochemistry Materials:

Glial fibrillary acidic protein (GFAP) primary antibodies from thermo scientific were used to identify the astrocytes. GFAP is a common marker use to distinguish astrocytes via intermediate filament proteins of astrocytes.

Culture mediums:

Hanks' Balanced Salt solution containing sodium bicarbonate, without phenol red, sterile-filtered, pH 7.1-7.5, glucose concentration 0.9 - 1.1 g/l from Sigma was used as a basic salt solution in all sterile procedures. Culture medium was Dulbecco's Modified Eagle Medium containing s (D-MEM) - 1X, liquid (containing 4500 mg/L; D-glucose), sodium pyruvate, L-glutamine and 25 mM HEPES buffer, Sodium Bicarbonate (PH 7.2) but without sodium pyruvate or phenol red.

Chemicals and Instruments:

Normal goat serum was purchased from Vector, bovine serum albumin (BSA) from Sigma. Acavity slides (depression slide) were used

for organotypic slices. The slides measures about 1.0× 1.2 mm were purchased from FisherScientific. Brain slice culture was prepared and a McILWAIN tissue chopper was used to produce the organotypic brain slices. Brains were processed in a Leica-TP 1020 tissue processor. The organotypic slices were incubated in Galaxy S Co₂ Incubator. Axiovert 25 PCR machine was used for amplification of DNA release of pups.

Inserts and slides types:

TransWell® permeable supports 0.4 µm polyester membrane – 12 mm inserts, 12 well plates was used for organotypic brain slice cultures (Corning Incorporated- Coring, NY 14831 the USA). See section 7-2-3. Micro-plate 96 well purchased from IWAKI / EZ-BirdShut®.

Three types of slides were used in the study: cavity slide, APES (2% 3-Aminopropyltriethoxysilane) and Gelatin-coated (Gelatinised) slides.

Mounting medium:

DABCO (1-4-Diazabicyclo-2-2-2-octane) in 90% glycerol in phosphate buffered saline (PBS) used to mount the fluorescent brain tissue slides. (100 mg of DABCO in 5 ml of PBS and then add 45 ml of Glycerol and max well, and store at 4⁰C).

Microscopes:

DMRB Fluorescence Microscope LeicaEM was used to capture fluorescent images of tissues which were detected by using DAPI, wavelength (350/50 – 460/50) and mCharry filters; wavelengths of excitations and emissions are (480/40 – 535/50) and Rhodamine RedTM

(TRITC, 545/20 – 610/75). Light microscope; Leica DM4000B microscope fitted with MicroPublisher 3.3RTV camera. The images were captured with a Confocal Microscope (Leica TCS SP2) and analysed off line on a workstation equipped with Volocity software.

Methodology :

Sample preparation:

For whole brain fixation, the mice were injected with heparin, anesthetized using isoflurane inhalation and then perfused transcardially under deep anesthetia. The perfusion was initiated by washing out the blood with 0.9 % saline solution at 37⁰C until the perfusate became clear. This was then followed by perfusion of 4% paraformaldehyde ([Abushofa 2014](#)), in 0.1 M PBS, pH 7.4 at 40C and post-fixed in 4% PFA overnight. The brains of mice were dissected out and fixed in Eppendorf tubes with 4% PFA overnight ([Beaudoin III, Lee et al. 2012](#)).

In vivo, postnatal pups of different ages (1-5 days) were used to prepare organotypic slices at thickness of 300 µm and cultured for period up to 6 weeks. The fixative slices were then washed 4 times in PBS, fixed 20 minutes in 4% PFA and stored in 4⁰C until used for immunohistochemistry with rhodamine red stain ([Holtzclaw, Pandhit et al. 2002](#)).

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Organotypic Slice Culture:

There was reasonable grounds for using postnatal mice, that they can be maintained in culture from a couple of weeks to a few months ([Abushofa, 2014](#)). Thus, brain slices of new-born C57 black 6 modified (Psmc1^{fl/fl}; CaMKII α -Cre) mice at ages 1-5 day old were used.

The experiments were performed in accordance with protocols from Scientific Procedures (Act 1986) and the home office recommendation regarding to killing by schedule 1 method at the designated establishment (BMSU, QMC at the University of Nottingham). Mouse pups were decapitated, the skull was removed and the brain cut into 200, 300 and 325µm thick tissue slices using a McILWAIN tissue Chopper. The different thickness was checked to select the best for the long term survival in culture. The slices were transferred to a sterile petri dish containing Hanks' Balanced Salt solution and then dissected slices are detached from each other under the microscope using sterile needles and carefully transferred to the transwell membrane inserts.

Organotypic slice cultures were grown for periods of 2, 3, 4, 5 and 6 weeks in culture harvested from control and mutant pups. 1 or 2 brain slices were placed into each transwell insert with 500µl medium

(Dulbecco's Modified Eagle Medium) in the base of a 12 multi-well plate. The tissues need to be strictly controlled in terms of sterilization, temperature and rate of carbon dioxide availability as well as a regular changing to medium. The slices were incubated at 36-37 °C in 5% CO₂ and the medium changed every second day. The culture medium contained the following components: 89 ml DMEM, 10 ml FCS and 1 ml Glutamen. All procedures were carried out under strict sterile conditions ([Beaudoin III, Lee et al. 2012](#)).

Immunohistochemical procedure with optimisation of permeabilization to organotypic slice cultures

Organotypic slice cultures were fixed in 4% PFA for 20 minutes and then washed with PBS 3x5 minutes. The slices were immersed in PBS and stored at 4°C until required which should be within two weeks of fixation. The slices were rewashed with PBS x 3, and then permeabilized by incubation with 0.5% Triton X-100 in PBS in humid chamber at room temperature, and another tissues were permeabilized an overnight incubation in 1% Triton X- 100 in phosphate-buffered saline (PBS) at 4°C. Non-specific protein binding was blocked with 10% Bovine serum albumin was optimized as well (0.5% Triton X-100 in PBS) for 2 hours at RT, and then washed with PBS x3. The primary antibody 1 µl/150 µl (anti-GFAP) in solution of 10% with 0.5% triton-X100 in PBS was incubated overnight at 4°C and then washed with PBS for 20 minutes. Rhodamine- conjugated secondary antibodies - goat anti-mouse (1 µl/400 µl) of 10% NGS in 0.5% triton-X100 in PBS was applied and incubated for 3 hours at room temperature. The tissue was

then washed with PBS X 4 for 20 minutes, and finally incubated with DAPI stain for 20 minutes at room temperature. After washing in PBS X 4 for 20 minutes a drop 5µl of mounting medium was added to the slide and the cover slip then sealed with nail varnish.

Bridge Mounting:

The thickness of the cultured slices, a bridge mounting technique was used as follows: a 22 mm x 26 mm (150 µm-ticks) coverslip was secured onto each end of a glass slide with nail varnish. The slice insert in the 200, 250 and 300-µm deep space underneath. To avoid damaging of slices, cavity slide and normal slide were both used with frames. The dealing with brain tissue during immunohistochemistry protocol in terms of position of membrane and the brain tissue was very accurate. Using of the gasket to avoid any pressure on brain slice due to the cover slips. The coverslip should be resting gently on the specimen with little or no gap between the specimen and the coverslip. The mounts were sealed around the edges and stored in the dark at 4°C for at least 8 hours to allow the mounting medium penetration.

Tissue fixation of organotypic slices:

The cultured slices were washed in PBS and fixed in 4% paraformaldehyde for 20 minutes. Organotypic slice cultures were grown for period's up to 6 weeks in culture harvested from control and mutant pups. Organotypic slices cultures were re-washed three times in PBS. The slices were immersed in PBS and stored at 4°C until required for use. Storage time was kept to a minimum to avoid loss of immunogenicity of the samples ([Gogolla, Galimberti et al. 2006](#)).

Confocal Fluorescence Microscopy:

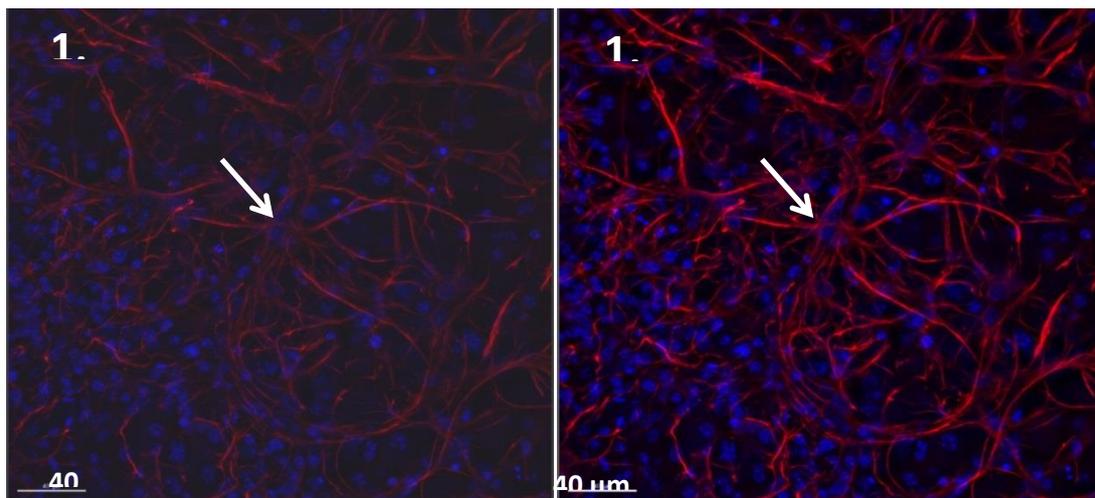
The fluorescent sections were examined with confocal DMIRE microscope (Leica SP2). Confocal images were collected and saved in TIFF format using HAMAMATUS digital Camera C4742-95([Abushofa, 2014](#)).

Results:

We have optimised the impact of thickness of gasket on clarity of image resolution for organotypic slice cultures. To avoid coverslip pressure damage to the organotypic slice cultures during mounting for microscopy, it was necessary to use a gasket that serves as a bridge between the coverslip and the organotypic slice to protect it from damage. However, it was necessary to find out the exact gasket thickness that would provide protection and still retain a minimal working distance from the objective lens. Immunohistochemistry protocols provided clear and reliable results without gaskets. However, with using gaskets, the astrocyte resolution was lower as seen in Figure (1; A). To avoid any chance of altering the slice morphology by coverslip pressure, it was chosen to use gaskets of thickness 0.25 mm. A justification of this decision is that the slice thins down from 300 μm to approximately 200 to 150 μm after the culture period.

It may a blank space formed caused by inadequate antibody penetration or may resulted from a labelling artifacts. Subsequently, varying the amounts of Triton X-100, a permeabilization agent, and

different durations of incubation were tested. The blank space was observed with and without a permeabilization step a Triton X-100 at 1% for 20 minutes and 0.5 % overnight both improved antibody penetration but signals were still weak with Triton X-100 at 1%, but much better permeabilization step at 0.5% Figure (1, B) show that overnight incubation in 0.5% Triton X-100 vastly improved antibody penetration and provided clear immunolabelling through the entire thickness of the slice.



Micrographs; Organotypic slice cultures of postnatal mice at 3-5 days of age.

A1; Mouse brain sections at five days of age were grown in an organotypic slice culture system up to 6 weeks. Overnight in 1% Triton X-100 Immunohistochemistry performed using anti-GFAP and Rodamine Red marker. Micrographs show that clear of astrocytes in red and its nucleus in blue (white arrow), but the ratio of the difference cells is dwindling. B1; Same Mouse brain section shows astrocytes in good resolution after improving the permeabilization, overnight in 0.5% Triton

X-100. Micrographs were captured by confocal fluorescent microscope at magnification 63X.

Discussion:

Immunohistochemical techniques for brain slice cultures do not fully take into account the thickness of the tissue when preparing the specimens for microscopical examination ([Yoon, Suri *et al.* 2011](#)). In the current experiment, the thickness maintained with the bridge mounting method. Coverslip is typically 150 μm thick and in our case cultured slices (200, 250 and 300) were processed attached to the 50 μm -thick membrane. There was gap between the tissue and the coverslip which overcome by enhancement of permeabilization also; gasket at 250 μm height has showed good resolution with tissue slices at 300 μm thick, Figure (1, B). The blocking step is very important, where the sections were blocked in 20% bovine serum albumin with 0.1% TritonX-100 in PBS for one hour at room temperature ([Warford, Akbar *et al.* 2014](#)). This step has improved the Clarity of micrographs.

Conclusions:

In summary, the optimal steps for whole mount immunohistochemistry involved the bridge mounting technique to avoid slice cultures from being squashed and overnight incubation in 1% Triton X-100 which enhanced antibody penetration. The protocol established here has produced continuous triple immunolabelling through the full thickness of cultured slices and represents an improvement for

investigating cellular morphology of organotypic brain slice culture models.

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