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Short communication

A method for objectively quantifying propidium iodide exclusion in organotypic hippocampal slice cultures

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HIGHLIGHTS

- Organotypic hippocampal cultures are a great tool to investigate excitotoxicity.
- A simple method to quantify cell death using propidium iodide is proposed.
- This facilitates comparison of responses to drug administration on cell viability.
- Increasing doses of NMDA resulted in a dose-dependent increase in PI uptake.

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ABSTRACT

Background: Organotypic hippocampal slice cultures (OHSCs) are an attractive *in vitro* model to examine mechanisms of neuronal injury, because the normal hippocampal architecture, function and cellular diversity are mostly preserved. The effects of exposure to excitotoxins such as *N*-methyl-D-aspartate (NMDA) on cell viability can be determined by propidium iodide (PI) staining.
New method: We describe a simple method to objectively quantify cell death in NMDA exposed slice cultures using PI that provides a standardized means of quantifying cell death in hippocampal subfields without the need to induce maximal cell death in each slice. The method employs separation of subfields using simple landmarks and densitometric quantification of PI intensity in 10 template-oriented counting fields.
Results: We show that exposure to increasing concentrations of NMDA results in a dose-dependent increase in PI uptake. Additionally, our method facilitates the comparison of cell death in different hippocampal subfields, such as dentate gyrus, CA1 and CA3. Our results show marked differences of PI uptake in the hippocampal regions with the CA1 area being most sensitive to NMDA-induced injury.
Comparison with existing method(s): The method provides a standardized format for quantifying PI exclusion in OHSCs that can be applied to cultures of differing shapes and sizes, permits comparisons between hippocampal subfields and does not require induction of maximal cell death.
Conclusion: The method of quantifying PI uptake described herein allows for an objective, quantitative and reproducible analysis and comparison of cell death in distinct regions of OHSCs.

1. Introduction

Organotypic hippocampal slice cultures (OHSCs) have become a valuable *in vitro* preparation to study a wide variety of central nervous system functions including mechanisms of neurotoxicity. OHSCs preserve most elements of normal hippocampal cytoarchitecture and function (Stoppini et al., 1991), and therefore present an attractive and more easily manipulated alternative to studying the hippocampus *in vivo*. Among other uses, OHSCs have been used increasingly for the investigation of mechanisms of neuronal damage underlying neuropathological diseases such as stroke by

Abbreviations: CA, cornu ammonis; DG, dentate gyrus; NMDA, *N*-methyl-D-aspartate; OHSC, organotypic hippocampal slice cultures; PI, propidium iodide; PND, postnatal day.

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subjecting OHSCs to either oxygen-glucose-deprivation or exposure to excitotoxins such as *N*-methyl-D-aspartate (NMDA) (Laake et al., 1999; Ring et al., 2010).

The fluorescent dye propidium iodide (PI) is commonly used as a quantitative marker for cell integrity and neuronal death. PI diffuses rapidly into cells with compromised membrane integrity. Once inside the cell, PI can bind to nucleic acids causing the dye to fluoresce brightly red. Cell death in OHSCs has been found to correlate with PI fluorescence intensity. Thus, PI fluorescence can offer an effective estimate of neuronal cell death.

Despite the use of PI exclusion as a measure of cell viability in OHSCs (and other preparations) for decades, a review of the literature reveals considerable variability in the methods used to measure PI. Subjective evaluations of fluorescence intensity or the use of nominal scales (*e.g.*, 0–3) are common, often making the measurement of PI exclusion qualitative or only semi-quantitative at best (Bruce et al., 1996; Liu et al., 2003). Other authors use quantitative densitometric methods but there is a lack of consistency in the reported methods (Radley et al., 2012; Katayama et al., 2012).

The aim of the present study was to develop a simple and reproducible method to objectively quantify cell death in PI-exposed OHSCs. To demonstrate the utility of the method we used exposure to varying concentrations of the excitotoxin NMDA.

2. Material and methods

2.1. Preparation of organotypic hippocampal slice cultures (OHSCs)

Slice cultures were prepared according to the interface method of Stoppini et al. (1991) with minor modifications. Sprague-Dawley rats of both sexes were born "in house" to untimed pregnant dams purchased from a commercial supplier (Charles River, Quebec, Canada). On postnatal day (PND) 5/6, pups were quickly decapitated and the brain removed and hippocampus dissected under aseptic conditions. The two hippocampi were straightened and transversely sliced throughout the entire length at a thickness of 400 μm using a mechanical tissue slicer (Stoelting, IL, USA). Slices were immediately transferred into ice-cold dissection buffer containing 1% penicillin-streptomycin solution (Gibco, NY, USA), 25 mM HEPES, and 10 mM Tris (Fisher Scientific, NJ, USA) in Minimum Essential Medium (Gibco, USA). Under a light microscope, slices were selected for clear hippocampal morphology, and only slices with intact cornu ammonis (CA) regions and dentate gyrus were transferred onto 0.4 μm porous Millicell® membrane inserts (Millipore, MA, USA). Membrane inserts containing three slices each were placed in individual 35 mm cell culture dishes (Fisher Scientific, USA) filled with 1 ml of serum-based medium containing 50% Minimum Essential Medium, 25% Hanks' balanced salt solution (Gibco, USA), 12 mM HEPES, 25% heat-inactivated horse serum (Gibco, USA), and 1% penicillin-streptomycin solution. Tissue slices were incubated over medium in a humidified chamber with 5% CO_2 at 37 °C for 13 days and medium was exchanged every three days. All animal use was approved in advance by the University of Prince Edward Island Animal Care Committee and in accordance with the Canadian Council on Animal Care guidelines. All possible efforts were made to minimize animal suffering and the number of animals used.

2.2. Propidium iodide staining

At 13 days *in vitro* and before any experiments, OHSC viability was assessed after 30 min exposure to PI (5 μg/ml) (Sigma–Aldrich, MO, USA). Using a Fluoroarc exciter lamp with a Zeiss Axioplan2 microscope equipped with a standard rhodamine filter, PI uptake was recorded using an AxioCam HR digital camera (Carl Zeiss Canada Ltd., ON, Canada) at this and subsequent stages. All images were taken with a 5× objective using the manufacturer's Axiovision software. To ensure consistency and proper comparability of experimental results, the same exposure settings were set during all experiments. Slice cultures displaying distinct PI fluorescence were excluded from further experiments.

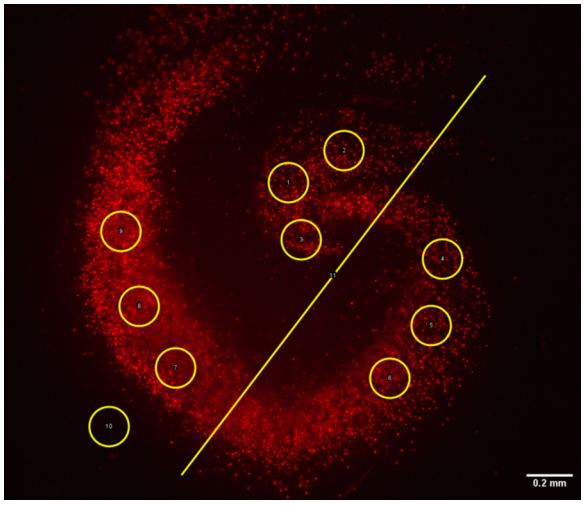

Fig. 1. Representative illustration of placement of measurement windows in the hippocampal areas (dentate gyrus, CA3 and CA1) of propidium iodide-stained organotypic hippocampal slice cultures.

2.3. NMDA-induced excitotoxic injury

PI-negative slices were exposed to increasing concentrations of NMDA (Sigma–Aldrich, MO, USA) (1 μM, 10 μM, 100 μM, and 1 mM) in saline, or to normal saline as a control, for 4 h. OHSCs were then transferred to fresh culture medium for 24 h, after which slices were re-evaluated by propidium iodide exclusion to determine the effects of drug exposure on cell viability. Additionally, light microscopy pictures of each slice were recorded to identify hippocampal structures. Negative control slices were exposed to the same amount of 0.9% saline. To serve as a set of positive controls, slice cultures were exposed to ice cold 70% ethanol for 24 h at −20 °C with the resulting PI uptake (see below) representing maximal (100%) cell death. A minimum of 6 slices were analysed and averaged per treatment with rat pup as the unit of variance.

2.4. Quantification of PI fluorescence

Digital pictures of propidium iodide uptake obtained using the fluorescence microscope (1300 × 1030 pixels) were analyzed by densitometry with the public domain Java image processing program ImageJ® inspired by NIH Image (National Institute of Health, MD, USA) for Macintosh.

To ensure consistency of the analysis, a template of 9 circular regions of interest, with three in each of three hippocampal regions (dentate gyrus, CA3 and CA1), was developed (Fig. 1). Within the ImageJ software, the circles were specified as 80 × 80 pixels. Using conventional landmarks, the hippocampal subfields were defined. A straight line was drawn connecting the two arms of the dentate gyrus (DG) and extending through the cornu ammonis area. This facilitated the optical separation of the hippocampal subfields as well as the standardization of the placing of the circles in the three different regions. The first circle in each subfield was placed at the midpoint of the region, with the other two circles placed equally distant from the midpoint and boundaries. Additionally, a tenth