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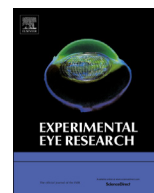

Methods in eye research

Preparation of embryonic retinal explants to study CNS neurite growth

Sonia T. Hanea [a], Ushananthini Shanmugalingam [a], Alyson E. Fournier [b], Patrice D. Smith [a,*]

[a] Department of Neuroscience, Carleton University, 1125 Colonel By Drive, Ottawa, ON, K1S 5B6, Canada
[b] Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, BT-109, 3801 Rue University, Montreal, Quebec, H3A 2B4, Canada

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ABSTRACT

This protocol outlines the preparation of embryonic mouse retinal explants, which provides an effective technique to analyze neurite outgrowth in central nervous system (CNS) neurons. This validated *ex vivo* system, which displays limited neuronal death, is highly reproducible and particularly amenable to manipulation. Our previously published studies involving embryonic chick or adult mouse retinal explants were instrumental in the preparation of this protocol; aspects of these previous techniques were combined, adopted and optimized. This protocol thus permits more efficient analysis of neurite growth. Briefly, the retina is dissected from the embryonic mouse eye using precise techniques that take into account the small size of the embryonic eye. The approach applied ensures that the retinal ganglion cell (RGC) layer faces the adhesion substrate on coated cover slips. Neurite growth is clear, well-delineated and readily quantifiable. These retinal explants can therefore be used to examine the neurite growth effects elicited by potential therapeutic agents.

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1. Introduction

1.1. Overview

Adult mammalian central nervous system (CNS) neurons fail to regenerate after injury. Both the extracellular environment and the intrinsic growth state of neurons affect their ability to regenerate. Studies on the visual system, a region of the CNS, have been informative on neuronal regenerative ability and the corresponding underlying mechanisms (Aguayo et al., 1987, 1990; Benowitz and Yin, 2008; Fournier et al., 2001, 2002; Moore et al., 2009; Park et al., 2008; Smith et al., 2009; Sun et al., 2011; Vidal-Sanz et al., 1987). Overall, the visual system is easily accessible and amenable to scientific investigations. The retina is located at the back of the eye and contains the retinal ganglion cells (RGCs) in its innermost layer. The RGCs are CNS neurons that extend their axons through the optic nerve, which projects to different brain regions. The relatively few cell types in the retina and their precisely organized layered structure permit not only the examination of a particular cell type but its interactions with connecting cells (London et al., 2013). The visual system has been widely adopted as a model system to study axonal growth and regeneration. The protocol outlined here includes the evaluation of the growth of RGC projections using retinal explants – small pieces of retinal tissue that can be cultured and analyzed in an *ex vivo* setting. Overall, retinal explantation involves the isolation of the retina and its maintenance intact in culture. This *ex vivo* culture serves as an intermediate between *in vitro* dissociated cell cultures and *in vivo* animal models (Bull et al., 2011). In retinal explants, the cellular architecture of the retina and the interactions between the cells are maintained; thus, the cells are studied *in situ*, not in isolation (Bull et al., 2011; Kretz et al., 2007). This method has been used to investigate cellular processes, such as development, outgrowth, neurodegeneration and survival, and techniques, such as cell transplantation, electrophysiology, genetic manipulation and proteomics (Bull et al., 2011; Cayouette and Raff, 2003; Donovan and Dyer, 2006; Hatakeyama and Kageyama, 2002; Kent et al., 2010; Koizumi et al., 2007; Kretz et al., 2007; Lin et al., 2009; McKernan et al., 2006; Sawamiphak et al., 2010; Walter et al., 1987; Wang et al., 2002; Xin et al., 2007). Thus, retinal explantation

* Corresponding author.
E-mail address: Patrice.Smith@carleton.ca (S.T. Hanea).

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represents a versatile and highly reproducible approach to studying a number of biological processes in an intact cellular system. In particular, the effects of different molecules on neuronal growth can be investigated (Böcker-Meffert et al., 2002).

1.2. Development of the protocol and comparison with other techniques

We describe an explant protocol which includes the optimized experimental conditions for embryonic mouse retinal samples. It is based on previous techniques particularly using embryonic chick retina (Fournier et al., 2000a, 2000b, 2001, 2002; Kent et al., 2010). The method used to isolate and assess growth in the embryonic chick retina has been adopted and optimized for rodent samples by incorporating our previously published work with adult retinal explants (Smith et al., 2009). As part of the optimization of the retinal explant technique, all elements of the procedure, such as dissection technique, reagents and time points were closely defined. The media composition was similar to those previously used for mouse explants (Bouchard et al., 2004; Smith et al., 2009). The retinal dissection technique was refined for embryonic tissue. Since this approach was focused on examining growth, the RGC layer was in contact with the adhesion substrate on the cover slip. This is unlike retinal explant techniques focused on studying neurodegeneration or cell transplantation, in which the RGC layer faces upwards, maintaining contact with the air above and media below (Bull et al., 2011). Generally, given the purpose for using retinal explants, modified methods are required. In particular, a model whereby RGCs face the substrate provides an efficient and effective method to study neuronal regeneration. This protocol involves the mounting of the intact tissue on a glass slide. Thus, the visualization of neuronal fibers can be achieved. In contrast, studies focused on analyzing cell dynamics, such as cell division in the retina, involve the sectioning of the retinal explant (Cayouette and Raff, 2003; Jolicoeur and Cayouette, 2014). Different immunostains are available for use on retinal explants. In this protocol, anti-βIII tubulin antibody, widely used to stain retinal explants, was employed (Bull et al., 2011; Fischer et al., 2004a, 2004b; Gaublomme et al., 2013; Müller et al., 2007; Smith et al., 2009). This particular visually clear stain, in combination with the precise placement of the retina – RGC layer downward – on the cover slip, permits the close examination of neurite growth and the impact of growth promoting or inhibitory factors. Overall, the protocol outlined herein has been refined to study embryonic mouse RGC neurite outgrowth and to limit any disturbances to the explants, while maintaining cell survival. In doing so, we have established a robust *ex vivo* system. Like in *in vitro* models, the experimental conditions are precisely controlled. However, unlike in dissociated cell cultures, the cellular connections are maintained. This *ex vivo* model, in which the integrity of the tissue is preserved, closely mimics the *in vivo* setting (Thangaraj et al., 2011). Thus, this retinal explant system allows for the close examination of neurite growth in a physiologically relevant model that maintains the interactions between the different cell types in the retina, reminiscent of the *in vivo* physiological setting.

1.3. Applications of the protocol

In this protocol, embryonic retinal tissue is used; specifically, embryonic day 18 retinas are explanted. By this developmental stage, most RGCs are born and they display high cell survival and inherent basal neurite growth (Cai et al., 2001; Cepko, 2014; Chen et al., 1995; Fawcett et al., 1989; Goldberg et al., 2002; Rapaport et al., 2004; Young, 1985). Overall, embryonic tissue particularly lends itself well to investigation, due in part to the better neuronal survival rates compared to older samples. This procedure could potentially be applied to postnatal or adult tissue and also to different species. In particular, our protocol is the culmination of a combined, optimized approach involving methodologies from investigations on embryonic chick and adult mouse retina (Fournier et al., 2000a, 2000b, 2001, 2002; Kent et al., 2010; Smith et al., 2009). Appropriate changes to media contents and dissection technique may need to be made to allow analyses of older retinal samples. Even so, studies on adult retinal explants are complicated by low cell survival and limited growth capability, and thus the possible need of a growth-conditioning lesion could affect research findings (Bähr et al., 1988; Cai et al., 2001; Chen et al., 1995; Fawcett et al., 1989; Goldberg et al., 2002; Kretz et al., 2007).

Only a small subset of adult RGCs survives in the long-term after optic nerve injury (Berkelaar et al., 1994). Recently, it has been suggested that alpha RGCs, a particular subtype of RGCs, show increased cell survival and axon regeneration after optic nerve injury, in comparison to other RGC subtypes (Duan et al., 2015). Using the outlined protocol, the growth of alpha RGCs could be examined. Specific markers, such as the anti-neurofilament H non-phosphorylated antibody (SMI-32), could potentially be used to identify these RGCs. Moreover, this explant approach can be used not only to examine neurite outgrowth but also growth cone dynamics. Importantly, in comparison to *in vitro* studies on isolated neurons which lack the required interactions among different cell types, *ex vivo* findings may translate much more closely to the *in vivo* setting. An explant approach coupled with *in vivo* manipulations on genetically modified mouse models could be used to delineate the molecular mechanisms underlying neuronal regeneration and further investigate the possible contribution of cellular interactions in this process.

Overall, the retinal explant procedure described here could be used to test the regenerative effects of potential therapeutic agents. The results from retinal explant investigations are applicable to other CNS regions and can help inform future preclinical studies (London et al., 2013). Ultimately, long-distance neuronal regeneration is critical in promoting functional recovery after neural injury. It is therefore imperative to identify potential growth promoting or inhibitory factors that could be targeted in order to facilitate regeneration and repair after CNS injury.

2. Materials and supplies

2.1. Animals

All experiments were performed on pregnant embryonic day 18 C57BL/6 mom mice and embryos in accordance with the corresponding guidelines and regulations.

2.2. Reagents

Alexa 488 anti-mouse IgG (Cell Signaling, cat. no. 4408S)

Anhydrous ethyl alcohol (Commercial Alcohols, cat. no. P016EAAN)

Anti-βIII tubulin antibody (Cell Signaling, cat. no. 4466S) and other RGC markers such as anti-neurofilament 165 (NF165) antibody (Developmental Studies Hybridoma Bank, cat. no. 2H3-c)

B-27 (Invitrogen, cat. no. 17504-044)

Bovine serum albumin (BSA; Sigma, cat. no. A9418)

Fetal bovine serum (FBS; Fisher)

Fluoromount G (Fisher, cat. no. OB100-01)

Hank's Balanced Salt Solution (HBSS; Invitrogen, cat. no. 14025-076)

Laminin (Fisher, cat. no. CB-40232)

L-glutamine (Invitrogen, cat. no. 25030-149)

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