Organotypic explant culture of adult rat retina for \textit{in vitro} investigations of neurodegeneration, neuroprotection and cell transplantation

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\textbf{ABSTRACT}

This protocol details a method for isolating retinal tissue from adult rats as an organotypic culture to study neurobiological processes in mature tissue. It combines the efficiency and control common to \textit{in vitro} techniques with close imitation of the \textit{in vivo} environment. Eyes from adult rats are enucleated and the neural retina is isolated. Tissue is cut into quarters, yielding eight retinal explants per animal, and cultured at a fluid/air interface on organotypic culture membranes. Explantation can be accomplished in thirty minutes per animal. Tissue is nourished by a serum-free medium and can be viably maintained for at least two weeks \textit{ex vivo}. Protocols are provided that describe histological processing, including techniques for whole-mount and cross-sectional immunohistochemical labelling. In addition, methods for simulating intraocular cell transplantation and pharmacological screening for neuroprotective therapies are also provided.
INTRODUCTION

Retinal explant culture

Organotypic culture involves the *ex vivo* maintenance of an organ or tissue, or parts thereof, as an *in vitro* model which recapitulates the *in vivo* cellular architecture of a tissue. Therefore, organotypic culture methods allow investigators to observe and manipulate the behaviour of complete tissues in a highly controlled *in vitro* setting while maintaining cells *in situ* with some semblance of physiological intercellular processes and communications. Beginning in the mid twentieth century with the work of Ames and colleagues, organotypic culture of the neural retina has played a central role in the history of neuroscience. Unlike other tissues, which often require slicing for organotypic culture (such as the hippocampus), the thin structure of the neural retina allows culture of the tissue intact, thereby facilitating study of generalizable neurobiological processes as well as those that are retinal-specific. Investigators have successfully cultured retinal tissue from a variety of species, but the most common source is the rodent.

Investigators have been employing various forms of organotypic retinal explant culture for decades in order to study a wide range of neurobiological processes including retinal development, CNS regeneration, cell death and neuroprotection, electrophysiological activity, and genetic modification. Due to the varying nature of such investigations, a remarkable diversity in retinal explant culture methods has arisen. This has contributed to great variability in expected experimental outcomes and tissue behaviour depending on the protocol being followed. For example, the culture of embryonic or neonatal retina provides very good tissue viability over a period of weeks *ex vivo*, during which time intrinsic developmental programs proceed. This feature has placed organotypic culture at the forefront of retinal developmental biology research. In contrast, culturing viable adult tissue for extended periods has proven problematic. Another highly variable aspect of retinal culture protocols is tissue orientation; while culturing retina with the ganglion cell layer opposed to glass is valuable for studying axon regeneration, it imposes extremely non-physiologic conditions that are not suited for studying pathological neurodegeneration, as occurs in human patients. Therefore, building upon numerous existing model systems, we sought to develop and characterise an organotypic retinal explant culture protocol that uses adult rodent tissue and that could be employed specifically to study cellular processes inherent to fully developed tissue, with a focus on pathology and treatment for retinal neurodegeneration.

Development of the current protocol

The current protocol utilizes neural retinal tissue from adult rats. During tissue isolation the optic nerve is transected to separate the retina from the posterior eye-cup, which results in axotomy of all retinal ganglion cells (RGCs). This triggers progressive degeneration of RGCs over time in culture, thus representing a model of inner retinal neurodegeneration. In addition, dissection separates the neural retina from the retinal pigment epithelium (RPE), leading to loss of photoreceptor outer segments and subsequent progressive photoreceptor degeneration. Therefore, this explant culture system may also model outer retinal neurodegeneration.

During development of the protocol, we extensively characterised the explant system to validate its ability to model certain aspects of *in vivo* retinal physiology accurately. This is an important benefit of the current protocol that is not consistently available.
for many other existing protocols, and which confers confidence that results obtained using the system as defined will roughly translate to in vivo physiology. First, we demonstrated that cultured tissue exhibited progressive, not immediate, neurodegeneration, and thereby reflecting an important facet of common neurodegenerative pathologies. Secondly, we demonstrated that, at least with respect to all interventions assessed thus far, the behaviour of retinal tissue in vitro mimicked that previously observed in vivo. Specifically, we observed gliosis in response to known reactive stimuli, and replicated neuroprotection by compounds previously reported to prolong RGC survival in vivo. If this model is employed for purposes not explicitly listed in this protocol, we recommend that control experiments be carried out to ensure faithful recapitulation of documented in vivo tissue behaviour. Lastly, we developed a defined, serum-free media formulation that maximised tissue viability while facilitating replicability, clean molecular analysis, and control over all exogenous substances. We now regularly use this established system to model neurodegeneration of adult tissue, and investigate novel neuroprotective treatments.

Advantages over other in vitro and in vivo experimental systems
Organotypic culture fills the gap between dissociated cell culture systems, which allow a high degree of experimental reproducibility, control, and efficiency, and in vivo animal models that (to varying degrees) reproduce the complexities of disease processes. Efficient methods for culturing dissociated retinal neurons exist and are widely used for the study of cell death, neuroprotection, and neuroregeneration. While these methods are able to generate thousands of cells for culture from a single retina, the behaviour of such cells grown in isolation is likely quite artificial. Moreover, the effects of experimental manipulation of molecular pathways may be different in isolated cells compared to that observed in whole tissues; dissociated cell culture is limited to investigation of only direct effects on cells of interest.

In contrast to dissociated cell culture, in vivo models of disease maintain intercellular relationships, thereby more realistically modelling human physiology and pathophysiology. The disadvantages of in vivo disease models, however, are clear. Animal models often require weeks to months for pathogenic processes to become apparent. Furthermore, a high degree of variability between animals often necessitates large sample sizes, which contribute to significant expense and time considerations. In addition, in vivo models often restrict precise control over experimental conditions at the cellular level.

Organotypic culture methods are neither as efficient as dissociated cell culture systems, nor as realistic as in vivo animal models. However, importantly, they provide a necessary compromise between the two systems. For example, organotypic cultures can be more efficient (in terms of time and resources) and controllable (in terms of microenvironment) than in vivo studies. They can also isolate direct tissue effects from off-target or systemic effects, and be used to explore the molecular interactions. Organotypic cultures also permit the study of cells in situ, rather than in isolation as in dissociated cell cultures. Therefore, they can provide a platform for confirming tissue effects of therapies identified using higher-throughput dissociated cell culture screens. Organotypic models can also contribute to replacing, reducing, and refining the use of animals in biomedical research.
**Disadvantages/considerations**
We have optimized a retinal explant culture system to model neurodegenerative disease in adult tissue. Like any experimental model system it has important limitations. While we have been able to demonstrate survival of retinal tissue to at least 17 days *ex vivo*, neuronal degeneration is progressive and affects multiple neuronal populations. We have defined the time course of RGC death in this model, but photoreceptor degeneration also occurs and it may not be possible to isolate the effects of these concurrent processes in this system. Moreover, while two and a half weeks is a relatively long time to maintain adult retinal tissue in culture, it may not be sufficient to model chronic processes. Furthermore, recapitulation of *in vivo* processes is not complete as our model is isolated from systemic processes, for example interactions with the immune system that contribute to some retinal pathologies.

While retinal explant experimental consistency has proven reliable after familiarisation with the protocol, we have experienced variability in RGC survival in cultures over time between laboratories and investigators (TVJ and NDB, unpublished observations). This could be a function of dissection technique, or subtle variability in reagent source/composition and experimental conditions. Therefore, we suggest that individual users characterize the behaviour of their cultures prior to experimentation in order to determine optimal time points for analysis.

**Comparison of our method and others**
Our organotypic retinal explant culture protocol represents one of many that have been described in the published literature, including four previously published in Nature Protocols. However, the current adaptation includes important modifications that make it valuable for a specific range of applications, including the study of neurodegeneration, neuroprotection, and cell transplantation - which we believe had been previously underserved by then-existing protocols.

Unlike numerous other protocols that employed retinal explant culture using tissue from embryonic or neonatal animals for similar purposes, the current method uses adult tissue, which is important when modelling age-related neurodegenerative disorders. Tissue from embryonic and neonatal animals is inherently more amenable to culture, and has been used extensively, however may not be applicable to human neurodegenerative disease, which occurs primarily in developmentally mature tissue.

Organotypic retinal culture methods also vary with regard to preparation and orientation of the tissue in culture. The current protocol maintains retinal tissue at a fluid/air interface supported by an organotypic culture membrane. Medium supplies nutrients from below and is drawn through the filter to nourish the tissue, thereby forming a thin film over the explant. This configuration allows for very good gas exchange and minimises tissue handling when maintaining cultures. Moreover, it maintains the entire neural retina in a morphologic configuration that approximates that found *in vivo*. In contrast, other explant protocols maintain tissue floating or submerged in medium, and/or mounted on a glass or plastic substrate. This is sometimes performed for the purposes of inducing axon extension from RGCs and is useful for studying neuronal regeneration, but is not applicable to studying pathogenic neurodegeneration or neuroprotection as it pertains to human disease.
Importantly, the protocol detailed here uses a serum-free, fully-defined medium. Many previous methods specify medium containing a high concentration of serum (most commonly horse or foetal bovine serum). The use of serum can increase experimental variability and does not permit identification of all biologically active components. The current protocol uses two commercially available serum-free neuronal supplements, N2 and B27, in combination with a basal media (Neurobasal-A) designed to support adult neurons in vitro. Details of the components included in both supplements are provided by Invitrogen Inc., however the exact concentration of B27 components is proprietary. It is important to note that while Invitrogen Inc. undertakes quality control testing for B27 supplement, we and others have observed batch variation with respect to culture viability. Therefore, we recommend batch testing B27 supplement, with subsequent acquisition of lots that produce optimal and consistent results.

Finally, using the current protocol, we have extensively characterized the behaviour of retinal tissue over time ex vivo to establish expected results and validate the method. While other published studies have been conducted utilizing retinal explant culture to investigate neuroprotection, neurodegeneration, and/or the effects of cell transplantation, a lack of evaluative data demonstrating reproducibility and comparison to in vivo behaviour limits their effective application prior to extensive characterization on the part of the end-user. In contrast, we have published numerous characterizations of this model and, to date, have used it to study retinal barriers to the integration of transplanted stem cells and examine novel neuroprotective therapies for RGCs. The published characterisation and validation of this system, along with this Protocol, should enable other investigators to confidently and reliably use this model in their laboratories.

**Experimental application of retinal explant cultures**
The current protocol was designed to model retinal neurodegeneration, in order to investigate potential neuroprotective and repair therapies. To this end, we have specifically characterized the expression of cell-selective marker proteins, RGC death, and morphological degeneration in cultured explants over time ex vivo. Furthermore, we have validated the use of this model for identifying therapies capable of alleviating RGC death, with the aim of using it as a screening tool for novel neuroprotective therapies prior to pre-clinical testing. In addition, we have found this model useful for studying the inner retinal integration of transplanted stem cells.

This explant system may also be potentially useful in a variety of experimental applications beyond those for which we have employed it thus far. For example, because the glial population remains viable and reactive in this system it may facilitate investigation of retinal glial biology, including reactive gliosis. A similar method has been utilized by other investigators for such a purpose. Furthermore, precise control of the tissue environment may lend the system to studying the effects of altering environmental conditions (e.g. light, atmospheric pressure, oxygen tension) on retinal neuronal survival. In addition, this system could be used to investigate non-neural processes (e.g. retinal neovascularization) that play a role in disease pathology.
**Considerations for experimental design**

The current Protocol describes organotypic culture of the adult retina in a flexible manner so that experiments may be tailored to a variety of neurobiological investigations. However, we will include some important considerations for successful experimental design that we have identified through our previous applications of this model.

As stated above, we have observed variability in explant viability over time *ex vivo* between individual users/labs. Therefore, it is advisable to characterise tissue behaviour (e.g. cell survival, rate of degeneration) before undertaking comparative experiments in order to verify viability and reproducibility, and identify appropriate time point(s) for analysis, to facilitate successful experimental design in each application.

It is also important to determine adequate sample sizes for each experimental group. When quantifying RGC loss, we have observed a level of variability that requires 6-8 explants per group in order to observe an approximately 30-50% difference in cell survival between treatment groups. During the course of this study we also observed that retinal explant viability could be affected by exogenous compounds included in drug formulations, such as drug vehicles and pharmaceutical preservatives. Therefore, we recommend that particular care be taken to control for all reagents present in drug preparations (e.g. DMSO, preservatives) when designing appropriate control groups for comparison.

There are several important considerations for quantification of neuronal survival in retinal explants that should be highlighted, specifically with regard to RGC analysis. We have found that immunohistochemical labelling of RGCs using cell-specific markers and microscopic quantification, in both transverse sections and whole-mounted tissue, can detect differences in cell survival across treatment groups. However, this type of analysis is not without limitations. Critically, antigen expression is known to vary after neuronal injury, which likely occurs prior to cell death and may influence accurate quantification of surviving cells. In addition, marker specificity must be considered given many antigens are expressed by multiple neuronal populations in the retina. Methods such as retrograde labelling of RGCs prior to explanation may improve quantification accuracy but would necessitate *in vivo* surgical procedures. Sourcing tissue from animals genetically engineered to stably express reporter genes in specific cell populations may also prove useful.

Finally, cell survival analysis may be undertaken in either transverse retinal sections or whole-mounted explants, with advantages and disadvantages inherent to both approaches (methods for both are provided below). Sectioning facilitates sampling throughout the whole explant across a number of slides (typically at least 10), thereby permitting analysis of dozens of antigens in each sample. We have found this approach provides a reliable estimate of cell survival in the tissue. However, explant whole-mount analysis can provide a larger sample size for cell survival quantification while also allowing visualization of dendritic arbours and/or axons, which may be useful for studying neurodegenerative processes.