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TITLE:

Characterization of a Novel Human Organotypic Retinal Culture Technique

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Human, Retina, Organotypic, Culture, Model development, Disease model

SUMMARY:

This study aims to develop a novel human organotypic retinal culture (HORC) model that prevents compromising retinal integrity during explant handling. This is achieved by culturing the retina with the overlying vitreous and the underlying retinal pigmented epithelium-choroid (RPE-choroid) and sclera.

ABSTRACT:

Previous human organotypic retinal culture (HORC) models have utilized detached retinas; however, without the structural support conferred by retinal pigmented epithelium-choroid (RPE-choroid) and sclera, the integrity of the fragile retina can easily be compromised. The aim of this study was to develop a novel HORC model that contains the retina, RPE-choroid and sclera to maintain retinal integrity when culturing retinal explants.

After cutting circumferentially along the limbus to remove iris and lens, four deep incisions were made to flatten the eyecup. In contrast to previous HORC protocols, a trephine was used to cut through not only the retina but also the RPE-choroid and sclera. The resultant triple-layered explants were cultured for 72 h. Hematoxylin and Eosin staining (H&E) was used to assess anatomical structures and retinal explants were further characterized by immunohistochemistry (IHC) for apoptosis, Müller cell integrity and retinal inflammation. To confirm the possibility of disease induction, explants were exposed to high glucose (HG) and pro-inflammatory cytokines (Cyt), to mimic diabetic retinopathy (DR). A magnetic assay (e.g., Luminex) was used to measure DR-related cytokine released into the culture medium.

H&E staining revealed distinct retinal lamellae and compact nuclei in retinal explants with the underlying RPE-choroid and sclera, while retinas without the underlying structures exhibited reduced thickness and severe nuclei loss. IHC results indicated absence of apoptosis and retinal inflammation as well as preserved Müller cell integrity. The magnetic assays showed significantly increased secretion of DR-associated pro-inflammatory cytokines in retinal explants exposed to HG + Cyt relative to baseline levels at 24 h.

We successfully developed and characterized a novel HORC protocol in which retinal integrity was preserved without apoptosis or retinal inflammation. Moreover, the induced secretion of DR-associated pro-inflammatory biomarkers when exposing retinal explants to HG + Cyt suggests that this model could be used for clinically translatable retinal disease studies.

INTRODUCTION:

The retina is a highly specialized ocular structure responsible for transforming incoming light energy to electric signals, which are then processed by the brain for visual perception. The human retina contains a dynamic range of cell types, highly organized in a unique lamellar structure consisting of two synaptic and three nuclei layers[1] (**Figure 1**). Retinal homeostasis is sustained by the intricate connections between neuroretinal cells, blood vessels, nerves, connective tissues and the RPE[1]. Due to the sophisticated retinal anatomy and physiology, mechanisms of many retinal diseases still remain poorly understood[2-5]. To better study retinal diseases, HORC models have been developed[6-9]. Compared to animal studies and in vitro cultures, HORC models are advantageous because they retain the dynamic cellular environment and complex neurovascular interactions in situ, providing a good model for clinical translation.

[Place Figure 1 here]

Previously characterized HORC protocols[6-9] have involved separating the retina from the underlying RPE-choroid and sclera using a surgical trephine. However, without the support provided by these underlying structures, the translucent retina becomes flimsy, difficult to handle and tools such as forceps can easily disrupt its integrity. Furthermore, isolating retina in culture without the RPE has been shown to cause ganglion cell apoptosis and photoreceptor degeneration[10-12]. Thus, an alternative HORC protocol that minimizes the loss of retinal integrity and better mimics the in vivo environment would be useful. This is particularly important when studying retinal disease mechanisms, as physical injury during explant handling could introduce artifacts. Therefore, the aim of this study was to develop a novel HORC model that includes the RPE-choroid and sclera in order to protect retinal integrity during explant handling and culture.

In order to achieve this aim, retinal explants “sandwiched” between the residual vitreous and the underlying RPE-choroid and sclera were extracted. In the sandwich explants, the vitreous weighs down the retina to prevent retinal detachment and folding, whereas the tough, fibrous sclera acts as both a scaffold for structural support and a contact point for forceps. Moreover, animal models have shown that retaining the RPE in culture can prevent retinal degeneration and glial proliferation, a response of Müller cells to danger signals such as hypoxia and inflammation[10-12].

To characterize the model, sandwich retinal explants were stained with Hematoxylin and Eosin (H&E) to assess anatomical structures and immunohistochemistry was performed, labeling explants with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, an apoptotic cell marker), glial fibrillary acidic protein (GFAP, a retinal inflammation and Müller cell activation marker), and vimentin, a marker of Müller cell integrity. To determine whether this model can be induced to develop molecular disease signs, the explants were exposed to high glucose (HG) with pro-inflammatory cytokines (Cyt), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), a culturing environment that has been shown to mimic diabetic retinopathy (DR) in both cell and animal disease models[13-15]. Magnetic assays were used in the DR model to measure Cyt released into the culture medium.

PROTOCOL:

Human donor eye cups were obtained from the New Zealand National Eye Bank following corneal excision for transplantation and as approved by the Northern B Health and Disability Ethics Committee (NTX/06/19/CPD/AM07).

NOTE: The culture should be done in a Class II biosafety cabinet to ensure sterile tissue culture conditions. The tissues must be cultured within 24 h post-mortem to avoid significant retinal integrity loss.

1. Extraction of the sandwich retinal explants from the eyecup

1.1. Remove the iris and lens.

1.1.1. Place the eye cup on a Petri dish, with the iris and lens facing upwards and the optic nerve head (ONH) contacting the Petri dish (**Figure 2A**).

1.1.2. Hold the eye cup steady at the limbus using forceps (**Figure 2B**).

1.1.3. Detach the iris and lens by making small cuts circumferentially along the outer edge of the limbus (**Figure 2A**).

1.1.4. Remove the iris and lens carefully. Avoid disturbing the retina during manipulation (**Figure 2C**).

NOTE: The lens will not fall into the eye cup as it is attached, via zonule fibers, to the ciliary body.

1.2. Flatten the eye cup.

1.2.1. With the eye cup still sitting upright, identify the ONH. This is easier with a bright white light source (**Figure 2D**).

1.2.2. Incise at the four quadrants towards the ONH (**Figure 2E**). The Petri dish can be rotated for easier handling. Do **NOT** cut the ONH.

1.2.3. Spread and flatten the eye cup carefully (**Figure 2E**).

1.2.4. Apply the forceps on the sclera instead of the retina to avoid disrupting retinal integrity.

NOTE: Peripheral retinal detachment and folding is unavoidable, as the weight of the overflowing vitreous will drag the retina. In these areas, remove the vitreous to prevent further retinal folding. Remember to leave residual vitreous to stabilize the retina on top of the RPE-choroid and sclera.

[Place Figure 2 here]

1.3. Collect sandwich retinal explants.

1.3.1. Place a surgical trephine on the retina in a region without retinal folds (**Figure 2F**).

1.3.2. Press hard to penetrate the sclera, which should generate a cracking sound.

1.3.3. Twist the trephine by 180° to ensure the sclera has been penetrated fully such that the retinal explant is now separated from the rest of the sample.

1.3.4. Apply the forceps at the sclera and transfer the sandwich retinal explant to the culture medium (**Figure 2G-I**).

1.3.5. Obtain 2-3 sandwich retinal explants from the peripheral retina of each quadrant.

NOTE: The sandwich retinal explant can sometimes be trapped in the opening of the trephine. Using forceps, gently tease out a small section of the base of the sclera. This occurs more often when the blade is blunt and can cause loss of the retina (**Figure 3A-C**).

1.3.6. Use a new trephine after cutting out 1-2 retinal explants as the blade becomes blunt easily (**Figure 3C**)

[Place Figure 3 here]

1.4. Culture the retinal explants.

1.4.1. Prepare the culture medium containing Dulbecco's Modified Eagle Medium nutrient mixture F-12 (DMEM-F12) and a 1x antibiotics and antimycotics mixture (AA, 100x stock)

1.4.2. Prior to explant extraction, place 500 µL of medium in a 24-well plate and equilibrate in the incubator. This is important as adding the medium afterwards can dislodge the retina.

1.4.3. Culture the sandwich retinal explants at 37 °C for up to 72 h in a humidified 5% CO_2 incubator in medium prepared in step 1.4.2.

1.4.4. To induce DR-like changes, culture retinal explants in medium containing DMEM-F12 with a combination of 32.5 mM HG with Cyt, TNF-α (10 ng/mL) and IL-1β (10 ng/mL).

2. Paraffin-embedding of the sandwich retinal explants

2.1. Fix the sandwich retinal explants.

2.1.1. Immerse the sandwich retinal explants in 10% formalin for at least 24 h.

2.1.2. Remove the formalin solution and transfer the sandwich retinal explants gently into tissue pads and cassettes.

2.1.3. Soak the sandwich retinal explants in 70% ethanol for at least 24 h.

2.1.4. Paraffin-embed retinal explants into blocks.

2.1.5. Cut paraffin-embedded retinal tissues into 5 µm thick sections using a microtome and mount onto glass slides. Store at room temperature until imaging.

2.2. Deparaffinize sections.

2.2.1. Immerse the sections in 70% xylene for 5 min.

2.2.2. Immerse the sections in 100% xylene for 5 min.

2.2.3. Rehydrate the sections in 70% ethanol for 5 min.

2.2.4. Rehydrate the sections in 100% ethanol for 5 min.

2.2.5. Wash the sections under running tap water for 10 min.

3. Characterization using H&E, IHC and a magnetic assay

3.1. H&E staining protocol

3.1.1. Deparaffinize the sections using step 2.2.

3.1.2. Hydrate the sections in tap water for 5 min.

3.1.3. Stain the sections in Gill's 2 hematoxylin solution for 5 min.

3.1.4. Wash the sections thoroughly under running tap water to remove excess stain.

3.1.5. Differentiate by dipping the sections twice in 1% acid alcohol.

3.1.6. Wash the sections quickly under running tap water.

3.1.7. Stain the sections blue by dipping six times in 1% lithium carbonate (10 mg/mL).

3.1.8. Wash the sections thoroughly under running tap water for 5 min to remove excess blue stain.

3.1.9. Dip the sections in 1% eosin 10 times.

3.1.10. Wash the sections quickly in tap water to remove excess stain.

3.1.11. Dehydrate the sections by dipping them 10 times in 100% ethanol. Do this twice.

3.1.12. Dip the sections in 70% xylene 10 times.

3.1.13. Dip the sections in 100% xylene 10 times.

3.1.14. Mount with a coverslip using dibutylphthalate polystyrene xylene (DPX) mounting medium.

3.1.15. Take images using a light microscope.

3.2. Immunohistochemistry

3.2.1. Deparaffinize the sections using step 2.2.

3.2.2. Place the slides into a solution containing 10 mM sodium citrate buffer with 0.05% Tween 20 at pH 6.0 and run an antigen retrieval in a pressure cooker automated at 121 °C for 2 min.

3.2.3. Wash sections in phosphate buffered saline (PBS) for 5 min. Do this 3 times.

3.2.4. Block the sections with PBS containing 0.1% Triton X-100 and 10% normal goat serum for 1 h at room temperature.

3.2.5. Incubate sections overnight at 4 °C with primary antibodies conjugated to secondary antibodies (Table of Materials).

3.2.6. Wash sections in PBS for 5 min. Do this 3 times.

3.2.7. Stain nuclei using 4',6-diamidino-2-phenylindole (DAPI) for 2 min.

3.2.8. Wash and mount sections using an anti-fade reagent.

3.2.9. Seal coverslips with nail polish.

3.2.10. Take images using a confocal laser scanning microscope.

3.3. Magnetic assay

3.3.1. Transfer 75 µL of media from each well to a 96-well-u-bottom plate at 72 h.

3.3.2. Analyze the cell supernatant for IL-18, IL-6, IL-8 and vascular endothelial growth factor (VEGF) after 24 and 72 h using a magnetic assay (e.g., Luminex). Follow the manufacturer's instructions to conduct the assay[16].

REPRESENTATIVE RESULTS:

Retinal integrity was preserved in this HORC model. Retinal integrity was preserved in the cultured sandwich retinal explants but was lost in the retina cultured without adjacent structures. H&E was conducted to examine the structural integrity of sectioned sandwich retinal explants after 72 h in culture. The sandwich retinal explants showed preserved integrity and a distinct lamellae structure from GCL to ONL with compact nuclei in INL and ONL (**Figure 4A**). However, disrupted retinal integrity was found at the edges of the same sample, where the retina had detached from the underlying RPE-choroid and sclera, showing reduced retinal thickness and sever loss of nuclei in INL and ONL (**Figure 4B**).

[Place Figure 4 here]

Characterization of retinal explants using IHC demonstrated well-preserved retinal integrity even after 72 h in culture. No TUNEL-positive cell nuclei, which mark cellular apoptosis, were found in retinal explants cultured in basal conditions, demonstrating sustained cellular vitality even after 72 h (**Figure 5A-C**). GFAP expression remained localized in GCL and IPL, as seen in normal retinal tissues[17,18]. There was no glial fibrosis, a sign of Müller cell activation and proliferation in response to retinal inflammation, which would otherwise be identified as upregulated GFAP expression that extends into the ONL[10-12](**Figure 5D-F**). Vimentin labelling (red) was highly expressed from GCL to ONL, showing preserved Müller cell integrity, as seen in normal retinal tissues (**Figure 5G-I**).

[Place Figure 5 here]

A DR model was developed by inducing sandwich retinal explants in HG and Cyt. HG and Cyt, IL-1β and TNF-α, increased the secretion of IL-18, VEGF, IL-6 and IL-8 relative to baseline levels. A magnetic assay was used to measure Cyt IL-18, VEGF, IL-6 and IL-8 secreted by retinal explants after culturing for 24 and 72 h under HG +Cyt conditions (**Figure 6**). To minimize inter-donor

differences, secreted cytokine levels were compared to baseline (indicated by the dotted red line), represented by cytokine levels secreted by the sandwich explants from the same donor but cultured in normal medium (prepared in step 1.4). After 24 h, secreted IL-18, VEGF, IL-6 and IL-8 levels increased significantly relative to baseline. After 72 h, only IL-18 and VEGF levels remained significantly elevated, whereas no significant difference was found in IL-6 and IL-8 levels compared to baseline.

[Place Figure 6 here]

FIGURE AND TABLE LEGENDS:

Figure 1: **Posterior ocular structures of the human eye.** Anterior to posterior, the retinal layers are: nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor inner segment (IS), and photoreceptor outer layer (OS). Cells within the retina include ganglion cells (blue), amacrine cells (yellow), bipolar cells (red), horizontal cells (purple), rod photoreceptors (pink) and cone photoreceptors (green). The vitreous is located anterior to the retina. The RPE, Bruch's membrane, choroid and sclera are located posterior to the retina. Note that the retinal image shown is only a schematic representation of the retina and the ratio of cells/retinal connectivity within each layer may not be indicative of the in vivo setting.

Figure 2: Images showing the procedure for collecting the sandwich retinal explants. For explant preparation, use dissecting scissors with one sharp tip and one blunt end, with the blunt end facing the inside of the globe to reduce tissue damage during incision. Also use forceps with blunt ends to avoid scratching to the intraocular tissues during handling. Using surgical scissors, with the blunt end facing the inside, cut along the limbus to remove the iris and lens (**A-C**). The ONH can be located if looking straight into the opened eyecup (**D**). Using forceps with blunt tips, hold the sclera to stabilize the globe (**E**). Divide the globe vertically into two halves by making two deep incisions towards the ONH but do not cut through the ONH. Repeat this along the horizontal meridian (**G**). Apply blunt forceps at the sclera and gently open the globe to a clover shape (**H-K**). Use forceps to carefully manipulate the vitreous to smoothen the folded retina, as the vitreous tugs onto the retina, but do not touch the retina directly (**I**). Remove the vitreous if it is causing retinal detachment or folding (not shown in **Figure 2** as the transparent vitreous is not well captured on photos). Locate areas where the retina is flat and extract retinal explants using a surgical trephine (**L**). Applying forceps at the sclera, carefully transfer the retinal explants into the pre-prepared culture medium (**M,N**). The entire sandwich explant should sink to the bottom of the well due to its weight, therefore each explant is submersed in the medium (**O**).

Figure 3: Troubleshooting. During the extraction process, the retinal explant may be trapped at the opening of the surgical trephine (**A**). Gently tease out the tissue from the base of the sclera without touching the retina (**B**). This can happen more often if the surgical trephine is used for extracting more than two retinal explants, as the blade can easily become blunt (**C**).

Figure 4: H&E images of retinal explants cultured in medium for 72 h. A) Structural integrity

maintained in areas attached to the underlying RPE-choroid and sclera, depicted by the distinct separation of each retinal layer from GCL to ONL and compact nuclei in INL and ONL. **B**) Severe loss in retinal integrity in regions detached from the RPE-choroid and sclera, as shown by the overall retinal thickness reduction and decreased numbers of nuclei in INL and ONL. Scale bar = 50 µm.

Figure 5: **IHC images of retinal explants after culturing in basal media for 72 h.** The lack of TUNEL-positive staining (green) suggests that no cellular apoptosis has occurred in the retinal layers from GCL to ONL (**A-C**). GFAP labelling (red) was restricted to GCL and IPL without glial fibrosis, which would be identified as extended expression of GFAP into the ONL (**D-F**). Vimentin (red) was highly expressed from GCL to ONL, locating the Müller cells that span through these retinal layers (**G-I**). Cell nuclei were stained with DAPI (blue). Scale bar = 50 µm.

Figure 6: **Cytokines release by the sandwich retinal explants cultured in HG and Cyt, IL-1β and TNF-α, after 24 and 72 h.** The secreted IL-18, VEGF, IL-6 and IL-8 levels increased significantly above baseline after 24 h ($p < 0.05$). After 72 h, IL-18 and VEGF levels remained significantly increased ($p < 0.05$), but no significant difference was found in IL-6 and IL-8 levels, compared to baseline. The dotted red line indicates the baseline level, which represents cytokine levels secreted by the explants from the same donor but cultured in normal medium.

DISCUSSION:

HORC is currently the most clinically translatable model in preclinical retinal research. Compared to in vitro cell culture models, HORC can better represent the anatomy of the human retina in situ, through retaining the dynamic retinal cell types and their connections with neurons, vasculatures and the extracellular environment[19]. Compared to animal models, HORC are more advantageous in studying the pathophysiology and designing pharmaceutical treatments for human retinal diseases due to inter-species variations, such as different retinal cell types as well as varying photoreceptor density and proportions, which could contribute to unsuccessful clinical trials despite positive results in animal models[19]. However, in previous HORC protocols, the retina was intentionally detached from the RPE-choroid and sclera. Without the tough sclera, the flimsy retina is difficult to handle and retinal integrity can be compromised if directly handled by forceps[6-9]. This is evidenced by the fact that previous studies using isolated retinas have reported increased Müller cell activation and TUNEL-positive nuclei by 72 h of culture. This is in contrast to our findings, suggesting that the sandwich method better preserves retinal integrity. Secondly, increased neuronal degeneration and glial proliferation were found in animal ORC models in retina cultured without RPE, compared to retina cultured with RPE[10-12].

To minimize the chances of compromising retinal integrity, sandwich retinal explants were utilized in this novel HORC protocol. In contrast to previous HORC protocols, the trephine cut through not only the retina, but also the RPE-vitreous and the sclera, such that the resultant explant is “sandwiched” and stabilized between the residual vitreous and the underlying structures. The residual vitreous weighs the retina down to prevent folding and detachment from the underlying structures while the sclera acts as a scaffold to support the retinal architecture to protect the retina from forceps-induced injury. Furthermore, culturing retina with RPE can

prevent retinal degeneration and glial proliferation in culture[10-12].

Remember the following points when extracting sandwich retinal explants. When cutting along the limbus, the lens will weigh the iris but will not fall inside the eye cup as it is attached, via zonule fibers, to the ciliary body. Apply the forceps on the sclera and avoid touching the retina to prevent disrupting retinal integrity. Fill the culturing wells with medium before placing the retinal explant inside the wells. Adding the medium in after placing the retinal explant inside the wells increases the risk of dislodging the retina off the RPE-choroid and sclera and flipping the entire explant upside down. When making deep incisions at the four quadrants, be mindful that the peripheral retina may detach due to the vitreous leaking out of the eyecup. To prevent detachment or folding of the retina, remove the vitreous in the area that is pulling on the retina. Although the previous point advises to remove the vitreous pulling on the retina, leave sufficient residual vitreous to weigh the retina on top of the RPE-choroid and sclera in order to increase the stability of the sandwich retinal explants. When cutting through the tough, fibrous sclera, the trephine can easily become blunt. As a result, excessive force may be required or the sclera may not be fully penetrated. Use a new trephine for every 1-2 extracted retinal explants to avoid incomplete penetrations through the sclera. Experience in careful handling is crucial to protect the retina; therefore, training with porcine eyes, which are similar in size and structure to the human eye, is advisable before using the limited donor scleral eye cups available.

Retinal integrity was preserved in the explants sandwiched between residual vitreous and the underlying RPE-choroid and sclera. H&E staining showed better structural integrity when the cultured retinal explant is attached to RPE-choroid and sclera, compared to culturing the retina alone. Supporting this finding, the lack of TUNEL-positive cell nuclei suggests an absence of apoptosis within all retinal layers, demonstrating preserved cellular viability even after 72 h. Furthermore, vimentin labelling confirmed Müller cell integrity and the restricted GFAP expression in GCL and INL, found in normal retina, again verified the lack of inflammatory or stress signals in the retina.

The HORC model characterized in this study resulted in preserved retinal structure and Müller cell integrity while preventing retinal inflammation and cellular apoptosis. Furthermore, it can be used to model retinal diseases, which is particularly useful to identify disease pathways and pharmaceutical mechanisms. Previous animal and in vitro studies have shown that DR inflammation can be induced when animal retina or cells are exposed to HG and Cyt[13-15]. In this novel HORC study, by applying HG and Cyt to the sandwich explants for 24 h in culture, secretion of IL-18, VEGF, IL-6 and IL-8 was significantly elevated above baseline. These cytokines are well known to increase in the vitreous and serum of patients with DR compared to non-diabetic controls, validating that this DR model is clinically translatable[20-25]. Although this study has only characterized DR using Luminex cytokine release assays, other techniques such as Western Blotting, IHC and polymerized chain reaction (PCR) can also be performed[26].

Despite the clear advantages outlined above, this novel HORC model also has some limitations. A major limitation of this protocol is the low supply of tissue. Donor eye cups, which have all ocular structures but the cornea generally removed for transplantation, are rarely available for

research due to the large demand of the sclera in surgical procedures such as glaucoma shunts, orbital implants, damage repair in retinal detachment and coverage of exposed sutures. In order to minimize this limitation, we suggest that all experimental groups are collected from the same donor. This removes the need to account for inter-donor-variabilities, such as age, duration of disease, baseline cytokine levels, and other factors that can contribute to confounding variables if comparing between different patients. Also, most donor tissues are from elderly people as it is harder to obtain samples from younger age groups, and even when available, the cause of death of young donors is often traumatic in nature leading to damaged tissues that are unsuitable for culture. Nonetheless, given that most chronic retinal diseases occur in aged patients, this model still retains usefulness in studying age-related chronic retinal diseases such as diabetic retinopathy and age-related macular degeneration.

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DISCLOSURES:

The authors have nothing to disclose.

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529

Figure 1

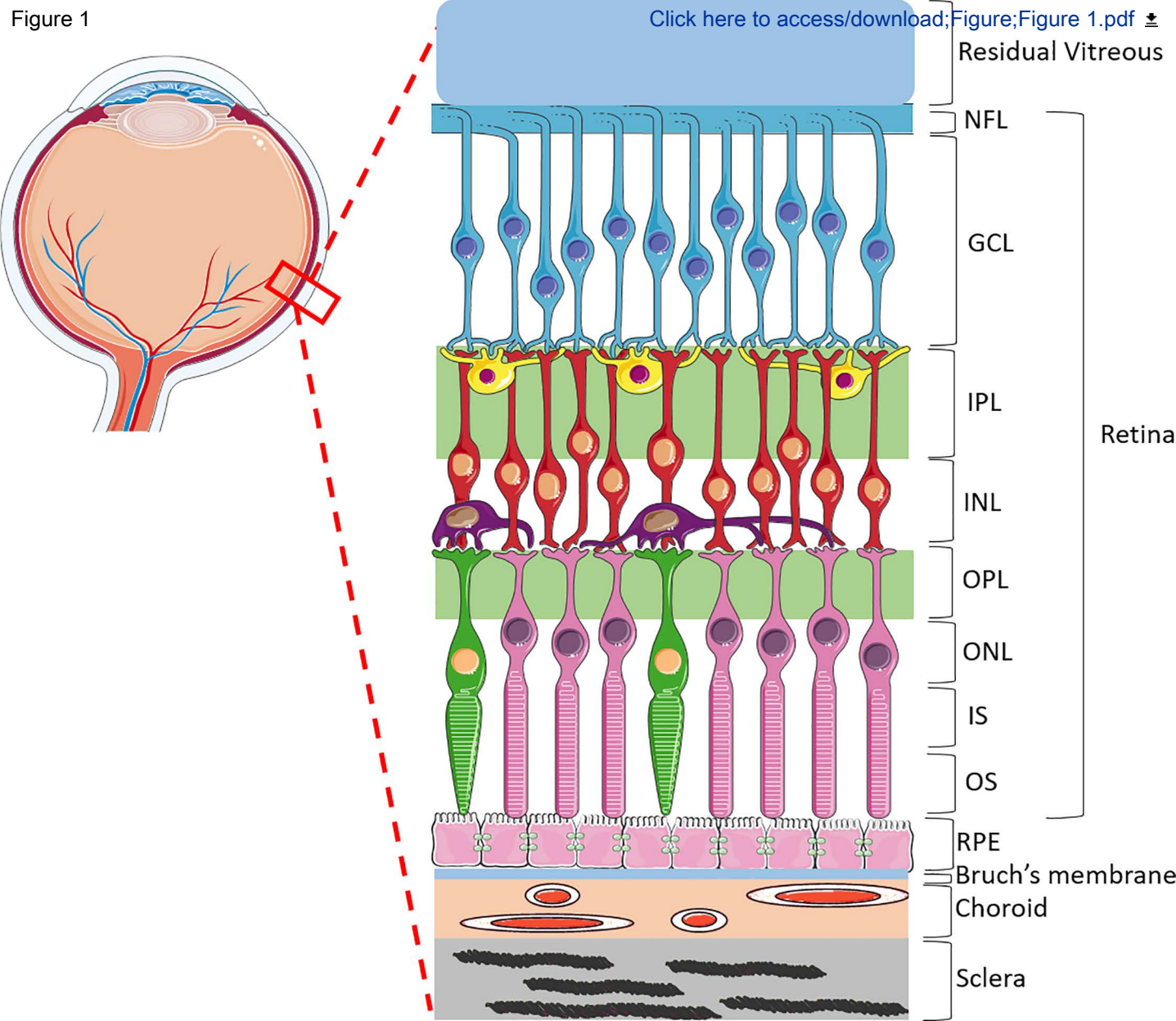


Figure 2

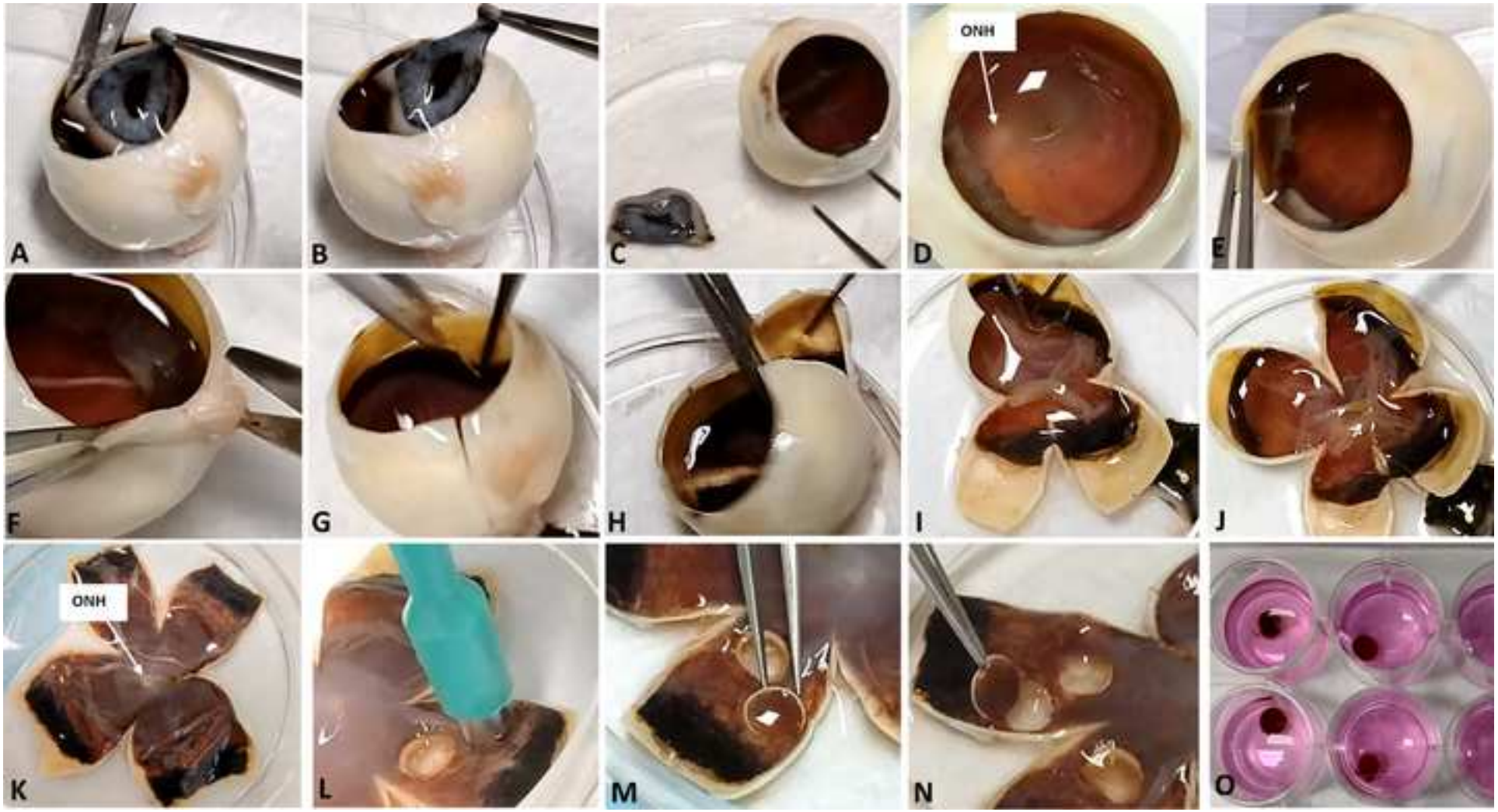
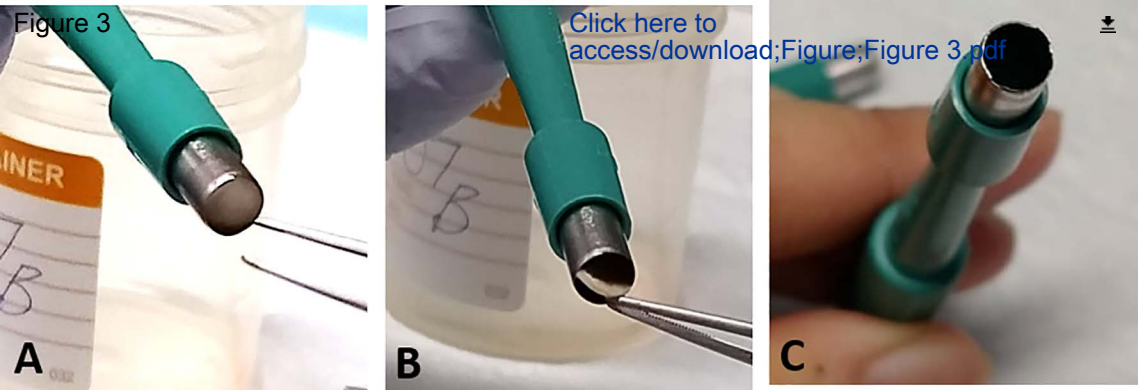
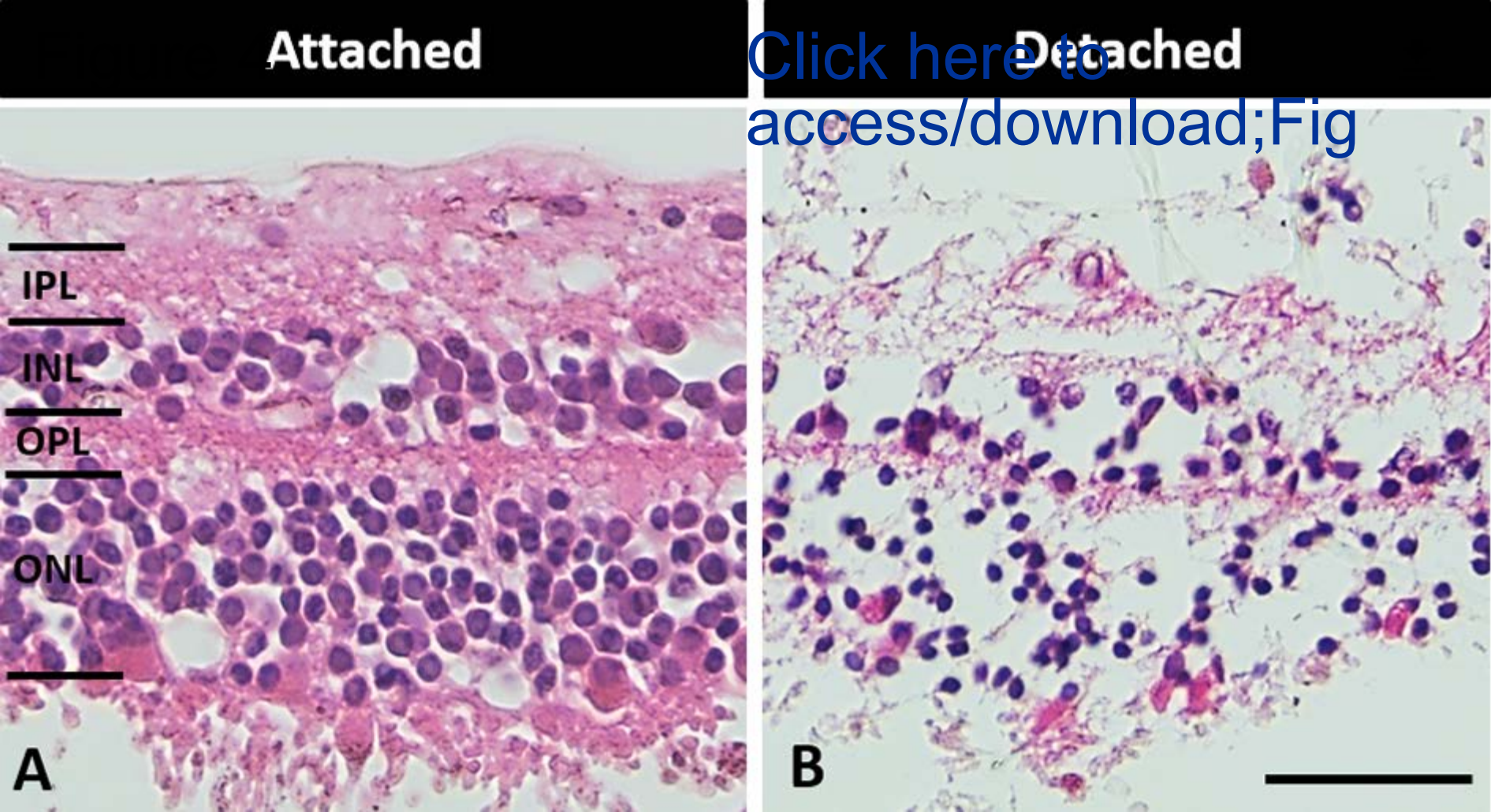


Figure 3





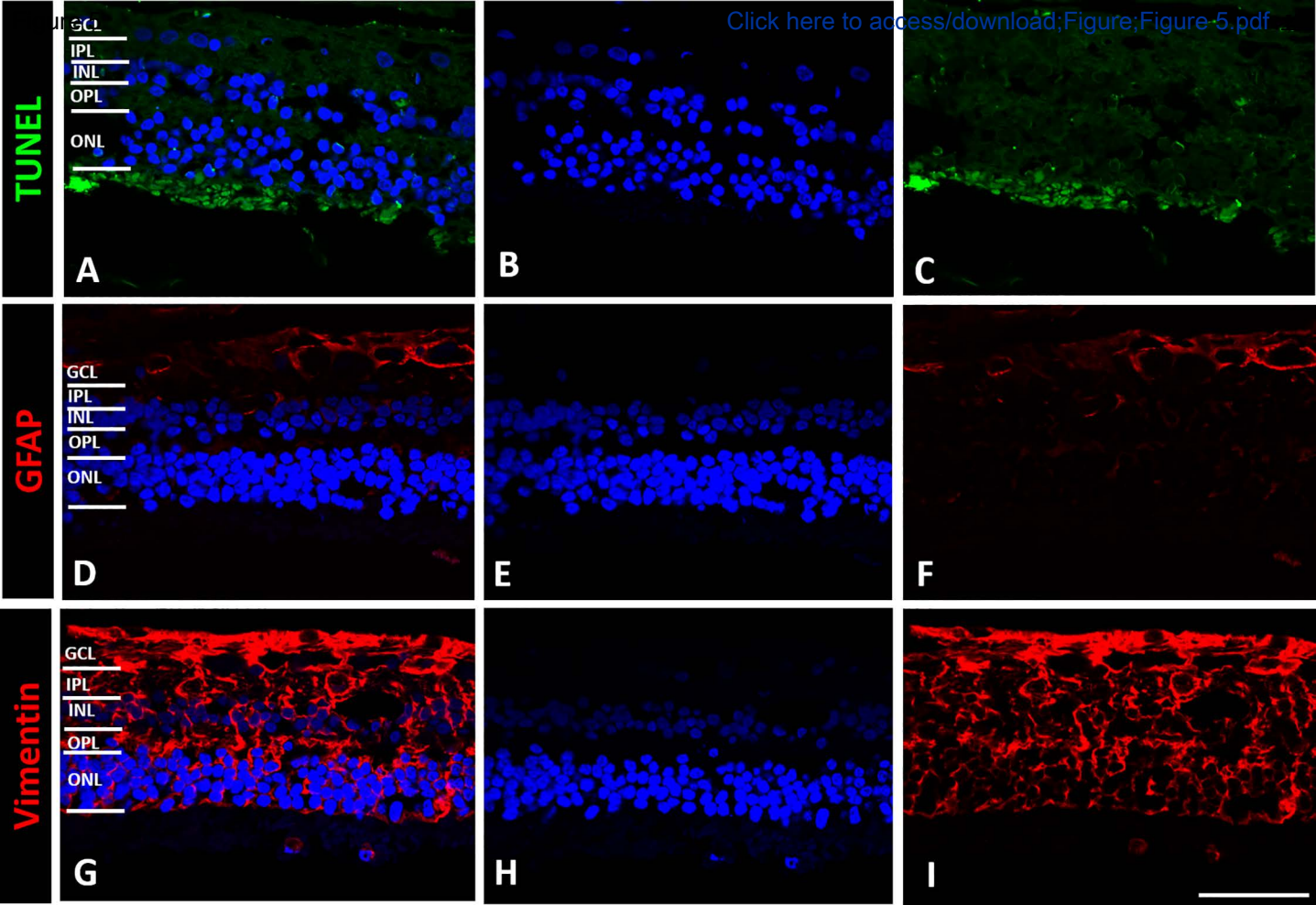
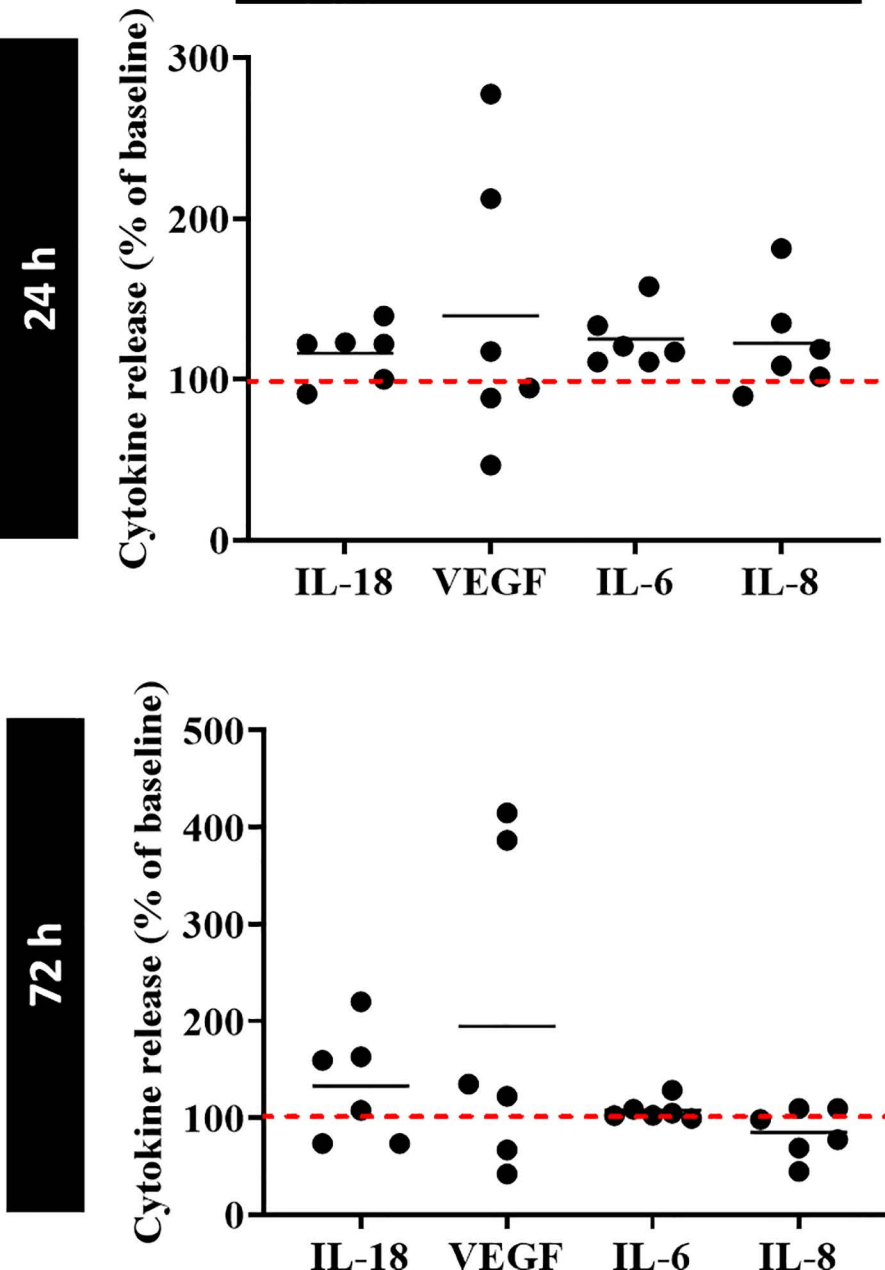


Figure 6



Name of equipment	Company	Catalog Number	Comments/Description
Disposable Biopsy Punches (5 mm)	Integra York PA Inc., USA	21909-142	Referred as surgical trephines in this article
Human Recombinant IL-1β	Peprotech, USA	200-01B	Working concentration: 10 ng/mL
Human Recombinant TNF-α	Peprotech, USA	300-01A	Working concentration: 10 ng/mL
DAPI (1 µg/mL)	Sigma Aldrich, Germany	#D9542	Nuclear stain. Working dilution 1:1000
DMEM/F-12, GlutaMAX supplement	Gibco, Thermofisher, Scientific Inc., USA	10565018	Dulbecco's Modified Eagle Medium nutrient mixture F-12 containing a 1× antibiotics and antimycotics mixture (AA, 100× stock)
Fine Scissors - Sharp-Blunt	Fine Science Tools (F.S.T)	14028-10	Tips: Sharp-Blunt, Cutting Edge: 27mm, Length: 10cm, Alloy/Material: Stainless Steel, Serrated: No, Tip Shape: Straight
Graefe Forceps	Fine Science Tools (F.S.T)	11050-10	Length:10cm, Tip shape: Straight, Tips: Serrated, Tip Width: 0.8mm, Tip Dimensions:0.8 x 0.7mm, Alloy/Materials: Stainless Steel
Mouse monoclonal GFAP-Cy3	Sigma Aldrich, Germany	#C9205	Primary antibody conjugated to Cy3. Working dilution 1:1000.
Mouse monoclonal Vimentin-Cy3	Sigma Aldrich, Germany	#C9080	Primary antibody conjugated to Cy3. Working dilution 1:50.
Rabbit polyclonal TUNEL (In Situ Cell Death Detection Kit, Fluorescein)	Sigma Aldrich, Germany	#11684795910	Primary antibody conjugated to Fluorescein-dUTP. Working dilution 1:10 with enzyme-buffer solution.

JoVe Rebuttal

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Thank you. We have gone through the manuscript stringently to ensure that all grammatical errors are fixed, and abbreviations are defined at first use.

2. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have now included more details in our methods section as suggested by the editor.

3. 1.3: As you have emphasized the delicate nature of the retina, please specify whether any surgical instrument is preferable for making these cuts and whether any particular care should be taken while making the cuts.

Details of the trephine, scissors and forceps used have now been included in the 'Table of Materials'

4. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. The title and a description of the table should appear after the Representative Results of the manuscript text. Please change the title from "Tables of Equipment" to "Table of Materials".

Thank you. This has now been changed accordingly.

5. Please capitalize the first letters of all words in the journal names in the reference list.

Thank you. This has now been changed accordingly.

6. Please add scale bars to legends for all micrographs.

Thank you. This has now been changed accordingly.

7. Please combine your Table of equipment and Table of materials into one Table of materials and sort everything alphabetically.

Thank you. We have combined the two tables as suggested by the editor.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Organotypic cultures of human retinal tissue usually use isolated retinas and do not include the retinal pigment epithelium (RPE) or choroid. The RPE and choroid are crucial in maintaining a healthy retina and thus developing a method which includes RPE and choroid is an important goal. The study provides a step by step protocol accompanied by useful illustrations. I suggest the following improvements.

Major Concerns:

1. The authors state that the tissues must be processed within 48h after death but no further details about the donor tissue are provided. More information is needed: such as what is the time delay between death and enucleation, how are the eyes being kept before the samples are taken and how does the time delay affect the quality of the tissue.

Thank you for your helpful suggestion. We have now included the donor information in a table under the methods section. Please note that the donor used for the images in Fig. 1 was the only one collected over 48 h as the globes were only used for showing the protocol but were not cultured. For culturing, donor eyes were always used within 24 h post-mortem. Previous studies using human donor explants have shown that there is no difference in tissue survival in HORCs cultured within 24 h post-mortem (PMID: 26432917). We have included the following sentences to clarify this within the manuscript:

The tissues must be cultured within 24 h post-mortem to avoid significant retinal integrity loss.

2. Figure 2 is very informative but the all-important step, i.e. how the incision of the eyecup in order to flatten it is made, is not shown or explained.

We agree with the reviewer that the incision part is a very important part of the protocol. As a result, we have included images of the incision process as suggested by the reviewer. Nonetheless, we would like to note that while the photo shows some indication of how the incision was conducted, the video will provide the level of detail and explanation required to replicate the process. We have also updated the figure legend for Fig. 2 as shown below:

Fig.2: Images showing the procedure for collecting the sandwich retinal explants. For explant preparation, use dissecting scissors with one sharp tip and one blunt end, with the blunt end facing the inside of the globe to reduce tissue damage during incision. Also use forceps with blunt ends to avoid scratching to the intraocular tissues during handling. Using surgical scissors, with the blunt end facing the inside, cut along the limbus to remove the iris and lens (A-C). The ONH can be located if looking straight into the opened eyecup (D). Using forceps with blunt tips, hold the sclera to stabilize the globe (E). Divide the globe vertically into two halves by making two deep incisions towards the ONH but do not cut through the ONH. Repeat this along the horizontal meridian (G). Apply blunt forceps at the sclera and gently open the globe to a clover shape (H-K). Use forceps to carefully manipulate the vitreous to smoothen the folded retina, as the vitreous tugs onto the retina, but do not touch the retina directly (I). Remove the vitreous if it is causing retinal detachment or folding (not shown in Fig.2 as the transparent vitreous is not well captured on photos). Locate areas where the retina is flat and extract retinal explants using a surgical trephine (L). Applying forceps at the sclera, carefully transfer the retinal explants into the pre-prepared culture medium (M,N). The entire sandwich explant should sink to the bottom of the well due to its weight, therefore each explant is submersed in the medium (O).

3. Table 1 is missing.

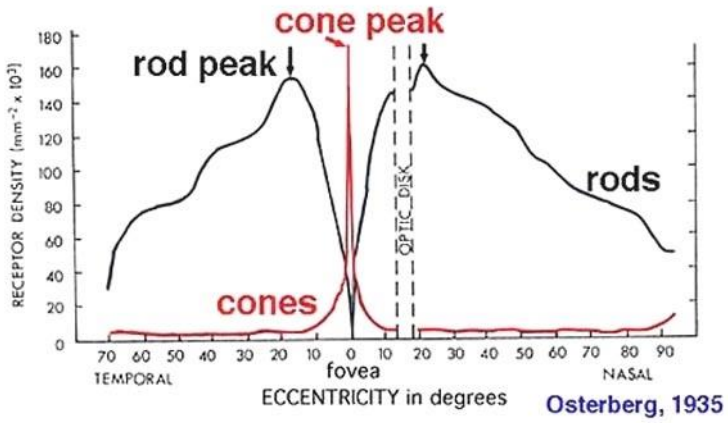
Apologies for the confusion. Table 1 is the 'Table of Materials' that was mistakenly placed at the end of the manuscript. We have now combined it with the instruments table and provided it as a separate file as required.

4. Table 2 lacks information on the dissection instruments, e.g. scissors, forceps etc.

Thank you for your observation. We have now included details on the forceps and scissors used in the Table of Materials. They are also emphasised in the legend of Figure 2 as shown in our response to your question 2 above.

5. Please explain why only peripheral retina is being used.

Previous studies (PMID: 21413409) have shown that there is a significant difference in the density of rod photoreceptors (Response figure 1). In our protocol, we used the peripheral retina as it allows the thickness and distribution of retinal cells to be more uniformly extracted, compared to the para-macular region and the fovea, where rod cells and cones cells peak, respectively.



Response figure 1: Graph showing the densities of rod and cone photoreceptors along the horizontal meridian. Cone cells peak at the fovea at a mean density of 161,900 cones/mm^2, decrease in density significantly outside the fovea and plateaus at approximately 10° away from the fovea. Rod cells are not present in the central 200 µm of the retina and the density of rod cells peak at 18° or 5mm away from the centre of fovea, as a ring of 160,000 rods/mm^2 around the fovea.

6. The quality of the retina shown in Figure 4B appears to be extremely bad. Did both images come from comparable retinas with respect to the post mortem fixation delay, donor age? A more quantitative comparison of the two treatment options should be provided. How does the quality of the explants vary within one retina? Other authors have obtained satisfactory result with respect to the quality of retina after culturing without RPE (e.g. Fernandez-Bueno et al. 2012,

Exp. Eye Res., Niyadurupola et al. 2010, BJO; Masri et al., 2019, TVST). These papers are cited but the fact that these authors achieved comparable tissue preservation to that shown in Fig. 4A is not discussed.

Both sections of retina in Figure 4A and 4B were from the same donor, fixed at the same time and utilised the same fixation protocol. In the process of collecting the explants, we observed that one area of the retina had lifted away from the underlying structures and was thus unsupported by the sclera/choroid, and this area was used for imaging Figure 4B. Given the poor tissue integrity of the detached retina (Figure 4B) as well as the fact that, as rightly pointed out by the reviewer, detached retinas have been extensively characterised in literature, we focussed our manuscript on the sandwich explant method instead. This is in keeping with the overall aim of our study which is to characterise a clinically translatable HORC model in which the retina is cultured along with its adjacent structures.

With regards to the disparity between our findings and previously published work, we believe that this maybe because these other studies utilised different culturing, fixing and storage protocols. Nonetheless, it is important to note that while Fernandez-Bueno et al. 2012 used tissues 3-4 hrs post-mortem, Müller cell activation shown by GFAP upregulation was present at day 3, which is not seen in our study. In Niyadurupola et al. 2011, 72 h culture resulted in a significant increase in TUNEL-positive cells across all retinal layers. In contrast, our study found no TUNEL-positive cells after 72 h culture with the sandwich culture method. In order to further clarify this in the manuscript, we have included the following sentences in the discussion section.

This is evidenced by the fact that previous studies using isolated retinas have reported increased Müller cell activation and TUNEL-positive nuclei by 72 h of culture. This is in contract to our findings, suggesting that the sandwich method better preserves retinal integrity.

7. Panels B,C, E,F and H, I in Fig. 5 are redundant. It would be much more useful to compare the two treatment options in this Figure.

We appreciate this feedback but would like to kindly point out that this is a methods manuscript as opposed to a traditional research paper. Therefore, the aim is to describe the protocol pertaining a new technique with no onus to compare this technique to an existing protocol. As a result, we did not compare our technique to the isolated retinas in terms of our immunohistochemical findings or following HG + Cyt treatment.

8. The authors exposed the explants to different solution in order to induce diseases, but the same treatment is not applied to isolated retina. Thus, it is not clear whether the sandwich method is required.

Thank you for your feedback. We expect that isolated retinas will also respond to treatment with HG and cytokines. We have not explored that in detail here because, as mentioned in our response to question 7 above, this is a methods paper as opposed to a traditional research paper. Therefore, the purpose of the manuscript is to describe the step-by-step protocol for a new technique as opposed to comparing the technique to existing ones. In particular, the purpose of our manuscript is to describe a protocol for a new sandwich method that preserves retinal integrity. As a result, the manuscript is not focussed on comparing the two culture methods but describing the protocol for our developed sandwich method.

Minor Concerns:

1. The schematic of the retina shown in Figure 1 could be reduced in size. Also, the diagram contains numerous inaccuracies in the retinal connectivity, so it might be better to make the layers more schematic.

Thank you for your observation. We have included a sentence in the figure legend to explain that we have used this image for schematic purposes only. The sentence included is:

Note that the retinal image shown is only a schematic representation of the retina and the ratio of cells/retinal connectivity within each layer may not be indicative of the *in vivo* setting.

2. Consider moving #4.4 further up.

Thank you for your suggestion. We have now moved #4.4 to #4.1.

3. The abbreviations HG and cyt are not defined

Thank you for your observation. We have now defined HG + Cyt at first mention in both the abstract and the introduction sections.

Reviewer #2:

Manuscript Summary:

Authors are presenting the novel, interesting protocol for human retinal explants culture. The protocol is understandable and clear, however there are several questions that should be addressed.

Major Concerns:

1. The authors claim that after 72 hours there is no apoptotic cells identified. I would say that this is not very impressive outcome, considering that rat explants for example are cultured for 7 or even 10 days. In my opinion it would be interesting to validate at what time-point the apoptosis in these explants starts to provide researchers with practical information how to utilize these explants in experiments.

We agree with the reviewer that rat explants can be cultured for extended periods of up to 10 days. However, previous studies using human donor explants (PMID: 21169273, PMID: 26432917, and PMID: 23022403) showed that culturing of donor retinas for more than three days results in significant GFAP upregulation (PMID: 23022403) and increase in TUNEL-positive cells (PMID: 21169273). In fact, Osbourne and colleagues (PMID: 26432917) found that TUNEL-positive/NeuN positive cells increased to up to 80% after one week of culturing their human retinal explants. It is in keeping with previous studies using human donor retinal cultures that we limited our experiment to three days. However, it is important to note that these previous studies used the isolated retina method. In contrast, our study using the sandwich retinal explant method resulted in no GFAP upregulation and no TUNEL-positive nuclear staining by 72 h. Therefore, our future studies will attempt to culture the explants for longer periods of time, similar to what can be achieved using rat retinas.

2. Statement that "HORC can preserve the anatomy and physiology of the human retina" is bit over for me. Considering that HORC are still denervated tissue and cut with trephine, it is risky to state that it preserves physiology of retina.

Thank you for your feedback. We have now changed the sentence to:

Compared to *in vitro* cell culture models, HORC can better represent the anatomy of the human retina *in situ*, through retaining the dynamic retinal cell types and their connections with neurons, vasculatures and the extracellular environment.

Minor Concerns:

1. It is not true that lens is attached to the iris, lens zonulas are originating from ciliary body, so should be more precised in the description.

Thank you, we have now updated this in the manuscript.

2. Im lacking some technical details. For example, if these explants are floating? Are they whole immersed in medium? What was volume of medium used? If they are floating it is trivial to state that vitreous is pressing retina if there is no solid base that explants rest on.

Thank you, we have updated the manuscript with the required details.

3. Im also lacking in discussion information abt penetration of supplements since the retina is sandwiched between sclera and vitreous. To long penetration of supplements may limit usefulness of this method.

Thank you for this important observation. We did not observe any issues relating to tissue perfusion such as loss of integrity in central parts of the explants. This is not surprising given that the tissue is only 5mm in diameter and wholly immersed in culture media.