Journal of Visualized Experiments

Long-term, serum-free cultivation of organotypic mouse retina explants with intact retinal pigment epithelium

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61868R2
Full Title:	Long-term, serum-free cultivation of organotypic mouse retina explants with intact retinal pigment epithelium
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Additional Information:	
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TITLE:

Long-Term, Serum-Free Cultivation of Organotypic Mouse Retina Explants with Intact Retinal Pigment Epithelium

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KEYWORDS:

neuroretina, RPE, organotypic explant cultures, serum-free medium, mouse, organ culture

SUMMARY:

The protocol describes organotypic explants of mouse neural retina, cultivated together with its retinal pigment epithelium (RPE), in R16 defined medium, free of serum and antibiotics. This method is relatively simple to perform, less expensive, and time-consuming when compared to in vivo experiments, and can be adapted to numerous experimental applications.

ABSTRACT:

In ophthalmic research, there is a strong need for in vitro models of the neuroretina. Here, we present a detailed protocol for organotypic culturing of the mouse neural retina with intact retinal pigment epithelium (RPE). Depending on the research question, retinas can be isolated from wild-type animals or from disease models, to study, for instance, diabetic retinopathy or hereditary retinal degeneration. Eyes from early postnatal day 2–9 animals are enucleated under aseptic conditions. They are partially digested in proteinase K to allow for a detachment of the choroid from the RPE. Under the stereoscope, a small incision is made in the cornea creating two edges from where the choroid and sclera can be gently peeled off from the RPE and neuroretina. The lens is then removed, and the eyecup is cut in four points to give it a four-wedged shape resembling a clover leaf. The tissue is finally transferred in a hanging drop into a cell culture insert holding a polycarbonate culturing membrane. The cultures are then maintained in R16 medium,

without serum or antibiotics, under entirely defined conditions, with a medium change every second day.

The procedure described enables the isolation of the retina and the preservation of its normal physiological and histotypic context for culturing periods of at least 2 weeks. These features make organotypic retinal explant cultures an excellent model with high predictive value, for studies into retinal development, disease mechanisms, and electrophysiology, while also enabling medium-throughput pharmacological screening.

INTRODUCTION:

In ophthalmic research, a variety of models are available to study the retina, including primary retinal cell cultures, retina-derived cell lines, retinal organoids, and in vivo animal models[1–5]. However, each of these models suffers from drawbacks. For instance, cells grow in isolation while the retina is a complex network with a multitude of cell-to-cell interactions. Thus, the behavior of isolated cell cultures is likely to be artificial compared to that observed in a whole tissue. This problem can in part be remedied using in vitro differentiated retinal organoids, which can be used to study development and basic biology[6]. Yet, as of today, retinal organoid generation still is time-consuming, labor-intensive, and suffers from reproducibility issues, requiring substantial further development work before organoids can be used for translational retinal research. Finally, studies on live animals, while arguably the model that comes closest to the requirements of ophthalmic research, are associated with strong ethical concerns. A good compromise between the efficiency of cell culture systems and the real-life situation of in vivo animal models are organotypic retinal explant cultures. Such cultures also reduce animal suffering since no in vivo interventions are performed.

Several methods have been described for culturing retinal explants from different species[5,7,8]. Our protocol describes a technique for the isolation of the mouse neural retina together with its retinal pigment epithelium (RPE). This technique will also be suitable for rat retinal cultures[9]. The culture of neural retina together with its RPE is of major importance for success. The RPE performs essential functions for the retina: transport of nutrients, ions, water, absorption of light and protection against photooxidation, re-isomerization of all-trans-retinal into 11-cis-retinal, which is crucial for the visual cycle, phagocytosis of shed photoreceptor membranes, and secretion of essential factors for the structural integrity of the retina[10]. Maintaining the RPE allows a successful development of photoreceptor outer and inner segments, keeping the retina viable for a longer time[11]. The procedure described below preserves the histotypic and physiological characteristics of the retina for at least two weeks[12]. Moreover, culturing the organotypic retinal explants in serum-free, antibiotic-free medium avoids the presence of unknown substances and enables a straightforward interpretation of the results[12].

Organotypic retinal explant cultures have been essential for improving our knowledge on retinal development and degeneration[7,13,14]. We show here that they are also a useful tool for medium-throughput pharmacological screening and that they can be employed to model a variety of retinal diseases, including diabetic retinopathy.

PROTOCOL:

Animal protocols compliant with §4 of the German law of animal protection were reviewed and approved by the Tübingen University committee on animal protection (*Einrichtung für Tierschutz, Tierärztlichen Dienst und Labortierkunde*; Registration No. AK02/19M). In this study, retinas were obtained from wild-type (WT) and *rd1* mice, the latter being a well characterized model for hereditary retinal degeneration[15]. Mice were housed under standard white cyclic lighting, had free access to food and water, and were used irrespective of gender.

1. Checklist

1.1. To ensure sterile conditions and avoid contaminations, clean, and disinfect the laminar air flow hood with 70% ethanol. Be sure to let the ethanol evaporate completely, to prevent intoxication of the retinal cultures.

1.2. Autoclave tools (e.g., scissors, forceps, and ophthalmic microscope scraping spoon) before use.

1.3. Prepare the following media in advance under a laminar-flow hood, under sterile conditions: Basal R16 medium (BM) (can be stored at 4 °C for 4 weeks), BM with 20% fetal calf serum (FCS) (same day use), BM with 0.12% proteinase K (44 mAnson U/mg) solution (same day use) and complete R16 medium with supplements (CM) as described by Romijn[16] (can be stored at 4 °C for 3 weeks) (see **Tables S1, S2,** and **S3**).

1.4. Preheat the proteinase K solution at 37 °C to activate it and use it in step 2.5.

2. Preparation

2.1. Sacrifice *rd1*/WT animal at post-natal day (P) 5 by decapitation. For animals older than P11, use CO_2 and/or cervical dislocation, as per the local animal protection regulations.

2.2. Depending on the age of the animal, prior to enucleation, if needed, open the eye lids using forceps and very carefully separate the eye lids, without touching or scratching the eye below.

2.3. Rapidly enucleate the eyes under a stereoscope using curved forceps.

2.4. Incubate the eyes in BM for 5 min at room temperature (RT).

2.5. Incubate the eyes in preheated BM, with 0.12% proteinase K at 37 °C for 15 min.

2.6. Perform the following steps inside a laminar air flow hood to ensure sterile conditions. To inactivate proteinase K, transfer eyes to BM containing 20% FCS and incubate for 5 min at RT.

2.7. Dissect the eyes under a stereoscope, aseptically, in a Petri dish containing fresh BM at RT. Initiate the dissection as soon as possible after the enucleation. The longer this time is, the harder

it is to dissect the retina, the eyes becoming very soft.

2.7.1. With forceps, hold the eye from the optic nerve. Using fine scissors, make a small incision in the cornea creating 2 edges from where the cornea, the choroid and the sclera can be gently peeled using 2 pairs of fine forceps (**Figure 1** steps 1–3). Alternatively, use a narrow-gauge cannula to make a first incision into the cornea and then insert one of the scissor blades into the opening.

2.7.2. Grasp the lens with fine forceps. Place a second pair of forceps perpendicularly to the first ones so that the first forceps are between the 2 shanks of the second one. Pull to extract the lens from the eye cup. If the vitreous and the ciliary body are still attached to the retina, remove them carefully (**Figure 1** step 4).

NOTE: Steps 2.7.1 and 2.7.2 need practice and ensure caution to not damage the retina.

2.7.3. Cut the retina perpendicular to its edges in four points, creating a four-leaf clover shape (**Figure 1** steps 5–6).

2.7.4. Using a pipette with broadly cut base of a 1 mL tip, hold the retina in a hanging drop of medium and transfer it to a culture dish filter insert placed in a 6-well culture plate. The RPE layer should face the membrane (**Figure 1** step 7).

2.7.5. Using a pipette, carefully remove the excess medium from the insert.

2.7.6. From the sides of the well, add 1 mL of CM per well and incubate in a sterile incubator at 37 °C with 5% CO_2. Do not submerge the retina in the medium as this will reduce oxygenation and cause tissue degeneration. The explant should remain at the interface between liquid and air, covered only by a thin film of liquid created by the surface tension of water.

2.8. Leave the retinal explant undisturbed for the first 48 h to facilitate recovery after the explantation procedure.

2.9. Change the medium every second day (48 h). Discard 700 µL of medium from each well and add 900 µL of fresh CM to the well. In this way, the amount of medium lost by evaporation is recovered and the retinal explant keeps some of the neuroprotective factors produced in the previous 48 h.

2.10. Incubate the removed medium in a separate and closed microcentrifuge tube along with the cultures to control and evaluate possible contamination (i.e., change in color of the medium).

NOTE: Retinal explants can be kept in culture for at least 2 weeks[12].

3 After culturing

NOTE: Explants can be used for different experimental applications (western blot, histology, whole mounts, genetic analysis, electrophysiology). Depending on the application, organotypic retinal explants can be snap frozen, lysed, or prepared for cryosectioning. The steps below describe histological preparation.

3.1. Perform a 45-min fixation with 4% paraformaldehyde (PFA), followed by gradual sucrose cryoprotection (10% sucrose for 10 min, 20% for 20 min and 30% for 2 h at room temperature (RT) or overnight (ON) at 4 °C). Add these buffers directly in the well.

3.2. Cut the membrane around the retinal explants.

3.3. Embed both the membrane and retinal tissue in the medium for frozen tissue.

[Place **Figure 1** here]

REPRESENTATIVE RESULTS:

After following the protocol, dissected and cultured retinal explants preserve their normal tissue architecture, with distinct layers, from the RPE to the ganglion cell layer (GCL), as shown in **Figure 2**. Outer nuclear layer (ONL) and inner nuclear layer (INL) size remained mostly stable for 2–3 weeks, with a slowly progressing cell loss and gradual thinning of these layers becoming more and more apparent if the culturing period is prolonged to 4 weeks and beyond. In the GCL, in contrast, because of the axotomy of the optic nerve, a marked thinning is usually observed within the first 4 days of culturing. Afterwards, the remaining cell population in the GCL (mostly displaced amacrine cells) will continue to be viable for another 3–4-weeks.

[Place **Figure 2** here]

Serum-free medium and the sustained in vitro environment allow to have full control over the experimental conditions. Here, we provide two examples for specific applications of this protocol. The first example illustrates the possibility to use retinal explants for drug testing or screening purposes. Organotypic retinal explant cultures were prepared from wild-type (WT) and *rd1* mouse models. The latter is a well characterized model for retinal degeneration[15]. In the *rd1* mouse retina, ONL degeneration is triggered by abnormally high levels of cGMP in rod photoreceptors[6,17]. Excessive cGMP causes increased activity of cyclic nucleotide gated ion channels (CNGCs) and cGMP-dependent protein kinase (PKG), leading to cell death[18]. The treatment of *rd1* mouse retinas with a structural analogue to cGMP (cyclic nucleotide #3; CN003), which targets both PKG and CNGC, was tested. After explantation at P5, the treatment paradigm described in **Figure 3A** was followed. Explant cultures were fixed with 4% PFA at P11 and prepared for cryosectioning (**Figure 3A**). To assess cell death of histological sections from treated, non-treated (NT), and WT specimens, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay[19] was performed. The analysis of TUNEL labeled cells showed a high percentage of dying cells in the ONL of the *rd1* untreated specimens, while CN003 protected *rd1* mouse photoreceptors when applied at a concentration of 50 µM[20] (**Figure 3B**).

A frequent complication of diabetes is diabetic retinopathy, a blinding disease which is difficult to faithfully reproduce in animal models[5]. The second example highlights the use of organotypic retinal explant cultures to characterize retinal cell viability under conditions emulating type-2 diabetes mellitus (T2DM)[21]. Here, we used 20 mM of the glycolysis inhibitor 2-deoxy-glucose (2-DG)[22] and administered it to the culture medium for 24 h from P10 to P11. We show that subjecting WT retinal explants to such in vitro simulated diabetic conditions leads to extensive neuronal cell death of the retina (**Figure 3C**). This paradigm in turn may then be used, for instance, to study degenerative mechanisms or to test retinoprotective treatments in a diabetes context.

[Place **Figure 3** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Step-by-step procedure for the preparation of organotypic retinal explant cultures. (**A**) Mouse eyes are enucleated and transferred to a solution of proteinase K to allow separation of sclera and choroid from the retina and RPE. A small cut in the sclera/choroid layer is introduced. (**B**) Two forceps are used to peel the sclera/choroid layer. (**C**) The black choroid layer can be seen during the peeling. The underlining dark retinal pigment epithelium (RPE) remains attached to the eyeball. The sclera and choroid are removed along with the optic nerve. (**D**) The forceps are used to carefully remove the lens and vitreous humor by peeling around the limbus. (**E**) The retina retains a bowl-like shape. (**F**) To flatten the retina for culturing in a dish, 4 cuts in equal distance around the retina are made with a scissor, giving it a clover-like shape. The retina culture is transferred to a membrane culture insert in a 6-well plate with the use of a cut 1 mL pipette tip. The retina still retains some of the bowl-shape. However, upon removal of the excess liquid surrounding the retina, it will unfold to a planar structure. (**G**) In the culture membrane setup, the retina culture is resting on a porous polycarbonate (PC) membrane on top of a solution of complete R16 medium. To ensure viability, the culture must be kept in a humidified sterile incubator at 37 °C with 5% CO_2, and the medium should be replaced every 48 h.

Figure 2: Cell types found in retinal explant culture. Retinal explant culture at P11 derived from *rd1* mutant (**A**) and WT animals (**B**) showing nuclear staining with DAPI (left, blue), rod photoreceptors (center, red) and Müller cells (right, green). Nuclear staining highlights all the major cellular layers of the retina such as, retinal pigment epithelium (RPE), outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL). Specific cell types in the nuclear layers, such as rods and Müller cells, are immunolabeled with alpha arrestin[23] and glutamine synthetase[24] antibodies, respectively. (**C**) Shows full length section of a whole *rd1* mouse retina, with DAPI staining highlighting the consistency, integration, and development of the retina. These retinas were cultured for 6 days. Procedure description: Retina and RPE derived from *rd1* or WT animals were isolated at P5 and cultured as described in the protocol, until P11 with a medium change every 48 h. Cultures were fixed with 4% PFA at P11 and cryosectioned.

Figure 3: Two examples for applications of organotypic retinal explant cultures. (**A**) Procedure description: Retina and RPE derived from *rd1* or WT animals were isolated at post-natal day (P) 5 and cultured as described above, with a medium change every 48 h. At P7 and P9, the spent

medium was discarded and fresh CM containing active compound at a concentration of 50 µM was added to the plate. Cultures were fixed with 4% PFA at P11 and cryosectioned. (**B**) Compound testing on *rd1* retina. Sections were obtained from WT, treated (003), and non-treated (NT) organotypic retinal explant cultures. TUNEL assay (red) was used as a marker for cell death. Nuclear staining with DAPI (blue). The quantification shown in bar graph illustrates the percentages of dying cells in WT, *rd1* NT, and *rd1* 003 condition. Treatment with compound 003 significantly reduced *rd1* photoreceptor cell death. (**C**) Simulation of type-2 diabetes mellitus (T2DM) on WT retina using 2-deoxy-glucose (2-DG) treatment. TUNEL assay was performed on NT and T2DM specimens. The quantification indicates a highly significant increase in cell death.

DISCUSSION:

The protocol presented describes organotypic explant cultures of mouse retina with intact RPE in defined R16 medium, free of serum and antibiotics. This protocol was originally developed starting in the late 1980s[7,25] and since then it has been continuously refined[6,11,12]. Notable applications include studies into the mechanisms of hereditary retinal degeneration and the identification of retinoprotective drugs[20,26,27].

For a successful experiment, some important considerations need to be taken into account. Here are some important troubleshooting points to help enhance the quality of cultures. First, the retinal cultures may display excessive folding and/or rosette formation[28]. This can be caused by touching the retina with a forceps during the explantation procedure. Moreover, the ciliary body must be completely removed from the explant, as this can increase retinal folding during culture.

Second, during the transfer of the retina to the well plate in a hanging drop, if the retina faces the membrane the wrong side down, keep it in the drop hanging from the pipette tip and very gently push the medium in and out of the tip (without detaching the hanging drop) to flip the retina around. Finally, if the RPE remains attached to the sclera and detaches from the retina, it is most likely caused by an insufficient predigestion of the sclera. This problem could be especially important when working with eyes from older animals or non-rodent species (e.g., pigs) and may be resolved by increasing the proteinase K concentration.

Conducting organotypic retinal explant cultures is a complex procedure that requires adequate training and experience. Lack of training can lead to variability in the quality of the retinal explants. For these reasons, it is important to monitor and verify viability and reproducibility, characterizing, for instance, the rate of cell death with the TUNEL assay. The use of an antibiotic-free medium makes the retinal explants vulnerable to contamination by bacteria and fungi. To minimize this risk, we recommend that particular care is taken to work under truly aseptic conditions. Another limitation of in vitro retinal culturing are differences in physiochemical environment when compared to the in vivo retina (e.g., choroidal and retinal blood supply, oxygen and glucose levels, intraocular pressure, composition of the vitreous). A continuous perfusion system, perhaps embedded into a dedicated bioreactor[29] could make this model closer to the in vivo condition. Furthermore, the axotomy of the optic nerve during retinal dissection will lead to ganglion cell death, that can induce stress responses[8]. Therefore, it is recommendable that the explant be left to adapt to culturing conditions for at least 2 days in vitro before it is

subjected to a specific manipulation or treatment.

The described method is usually performed on immature retinal tissues, which may survive well for 4 weeks in vitro[7,30]. However, the procedure is tailorable to a variety of applications, including culturing of adult retina. Although different published approaches describe the isolation of the adult retina without its RPE[31,32], the incubation with papain solution for up to 1 h at 37 °C before dissection allows the RPE to stay attached to the retina even when derived from an adult mouse[33].

The serum-free medium and the chemically defined in vitro environment provide for an entirely defined and reproducible manipulation of the experimental conditions. Therefore, organotypic retinal explant cultures are valuable tools in the field of ophthalmology and neuroscience, and have been used for studying retinal diseases[34], retina development[35,36], retinal stem cell therapy[37], genetic modifications[38], and pharmacological screening. As a specific example of drug testing, here we used retinal explant cultures to test a cGMP analogue (CN003), known to reduce photoreceptor cell death in animal models for inherited retinal disease[20] (**Figure 3B**). Another possible application of the technique is described in **Figure 3C**, which illustrates how the precise control of the tissue environment can be exploited to emulate diabetic conditions[21]. Because of the preservation of tissue architecture over the entire culturing period, organotypic retinal explant cultures are also suitable for electrophysiological studies. Neuronal functionality on retinal explants have been investigated using patch-clamp recording[39] and multi-electrode-array (MEA) recording[30,40]. The latter allows recording of electrical activity of neuronal populations at the same time and has been exploited to characterize photoreceptor and ganglion cell functionality in culture conditions. In a broader perspective, the organotypic explant culture systems can also be applied in other fields such as prion research, where organotypic slice cultures have been used for ex vivo prion replication[41], or clinical research, where explant cultures were used to test the therapeutic efficacy of hypothermia[42].

The organotypic explant culturing technique is relatively simple to perform and, when compared to corresponding in vivo experiments, is less expensive and time-consuming, and avoids the ethical concerns related to live animal studies. The precise control over experimental conditions and the preservation of RPE and tissue complexity make the method a valuable tool to improve our knowledge on retinal physiology and pathophysiology and enable numerous experimental applications.

ACKNOWLEDGMENTS:
This research work received financial support from the European Union (*trans*M*ed*; H2020-MSCA-765441), the German research council (DFG; PA1751/8-1, 10-1) and the China scholarship council.

DISCLOSURES:
The authors have nothing to disclose.

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41. Falsig, J., Aguzzi, A. The prion organotypic slice culture assay--POSCA. *Nature Protocols*. **3** (4), 555–562 (2008).
42. Klemm, P. et al. Hypothermia protects retinal ganglion cells against hypoxia-induced cell death in a retina organ culture model. *Clinical and Experimental Ophthalmology*. **47** (8), 1043–1054 (2019).

Figure 1

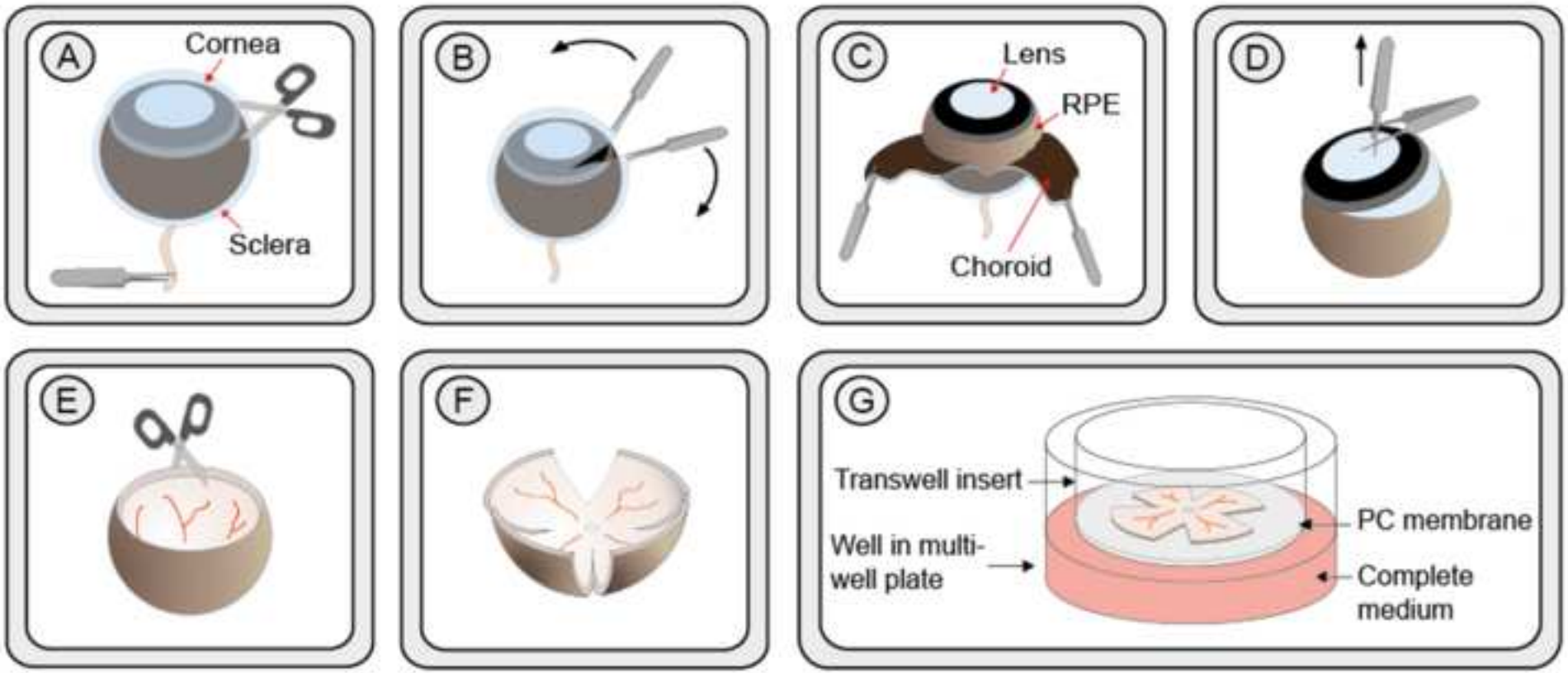


Figure 2

Click here to access/download;Figure;Figure2_v2.png

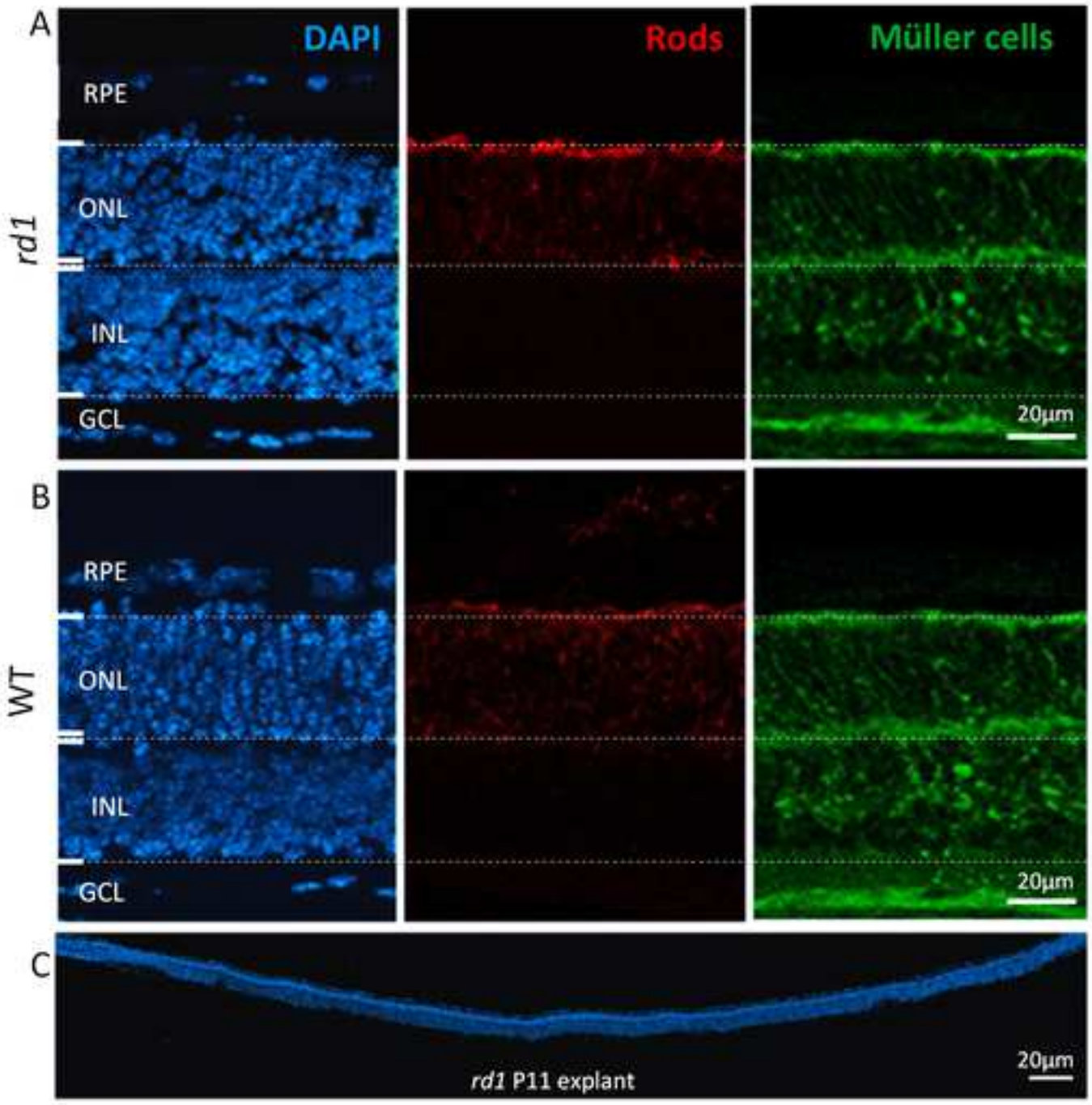
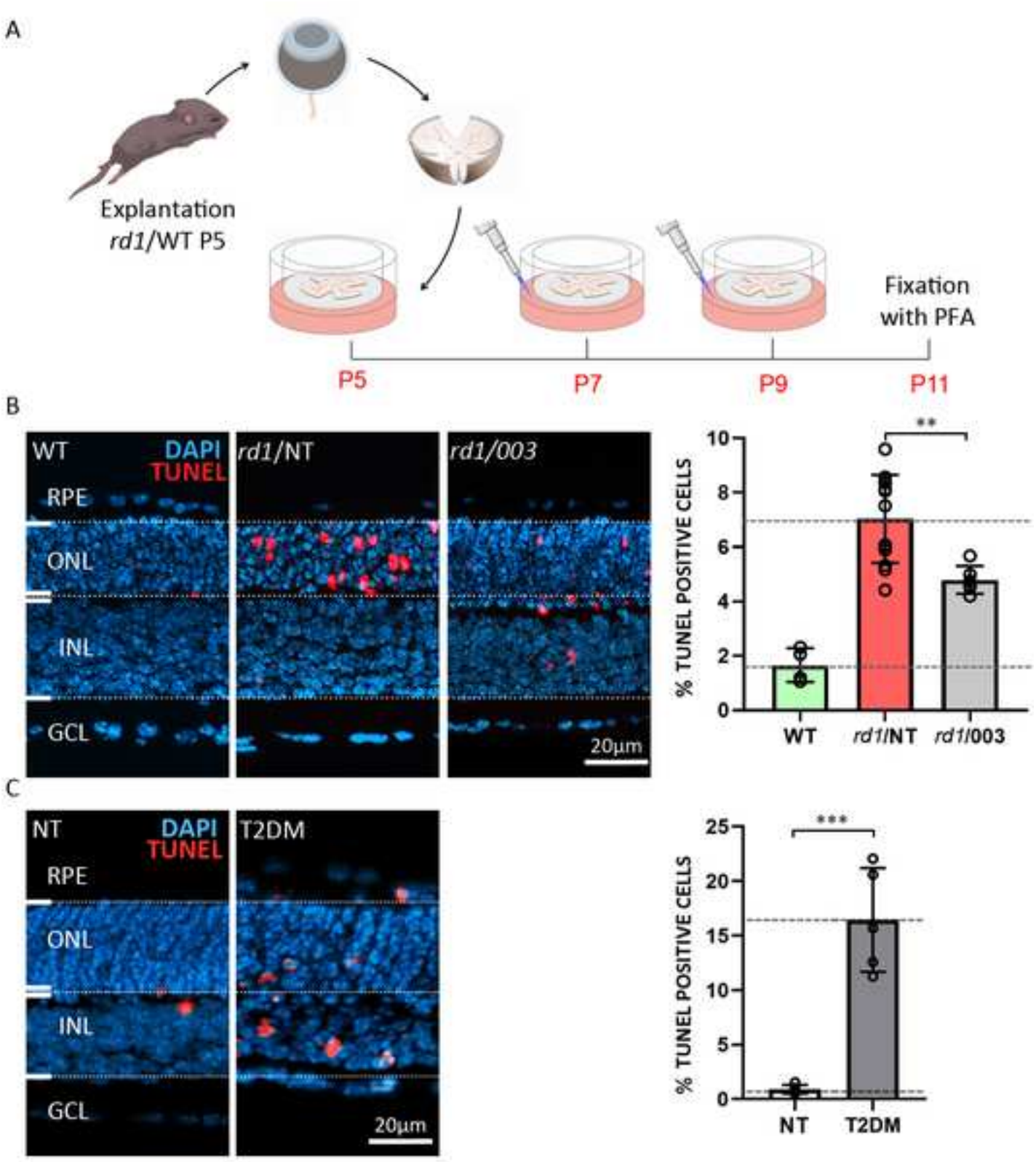


Figure 3



Name of Material/Equipment	Company	Catalog Number	Comments/Description
Biotin	Sigma	B4639	
(+/-)-α-LipoicAcid (=Thiotic acid)	Sigma	T1395	
BSA	Sigma	B4639	
CDP-Choline-Na	Sigma	30290	
Corticosterone	Sigma	C2505	
$CuSO_4 \times 5H_2O$	Sigma	C8027	
DL-Tocopherol	Sigma	T1539	
Ethanolamine	Sigma	E0135	
FCS	Sigma	F7524	
Filtropur BT100, 1L, 0.2µm	SARSTEDT	83.3942.101	for Basal Medium
Forceps	F.S.T	15003-08	
Glutamine	Sigma	G8540	
Glutathione	Sigma	G6013	
Insulin	Sigma	I6634	
L-CysteineHCl	Sigma	C7477	
Linoleic Acid	Sigma	L1012	
Linolenic Acid	Sigma	L2376	
$MnCl_2 \times 4H_2O$	Sigma	M5005	
Na-pyruvate	Sigma	P3662	
$NaSeO_3 \times 5H_2O$	Sigma	S5261	
Ophthalmic microscope scaping spoon	F.S.T.	10360-13	
Progesteron	Sigma	P8783	
Proteinase K	MP Biomedicals	21935025	44 mAnson U/mg
R16	Gibco	07491252A	
Retinol	Sigma	R7632	
Retinyl acetate	Sigma	R7882	
Scissors	F.S.T	15004-08	
Sterile filter 0.22µm	MILLEX GP	SLGP033RS	for supplements
T3	Sigma	T6397	
Tocopherylacetate	Sigma	T1157	

Transferrin	Sigma	T1283
Transwell permeable supports	Corning	3412
Vitamin B1	Sigma	T1270
Vitamin B12	Sigma	V6629
Vitamin C	Sigma	A4034

Editorial comments:

1) Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues (1.3.2, please use American English, e.g., fetal, not foetal) and to check spacing between numbers and units (e.g., no space between numbers and %).

Reply: We apologize for the mistakes and have corrected this in the revised manuscript.

2) Please provide enough detail to allow viewers/readers to replicate your protocol. For example, 2.1, please specify the most optimal method to sacrifice the animals and the age and strain of the mice used in this study. Especially because you have discussed different scenarios with retinal tissue from mice of different ages.

Reply: We thank the Editor for this comment. Regarding 2.1, we have now specified the most optimal method to sacrifice the animals as well as the age and strain of the mice used in this study in the revised manuscript (lines 86-87, 105-106).

3) For 1.2, please specify all the tools to be used, and avoid the use of "etc" in academic writing.

Reply: We have now changed this in the revised manuscript.

4) Please label and cite all figures in order, Figure 1 legend corresponds to Figure 2's description in the text.

Reply: We apologize for the mistake and have corrected this in the revised manuscript.

5) Please consider moving some descriptions from the legends to the protocol or representative results, citing those figures.

Reply: We thank the Editor for this comment and we made some changes accordingly.

6) Please include experimental details that would accompany Figures 2 (immunolabelling) and 3 (treatment with 003, TUNEL, 2-deoxyglucose) so that the reader/viewer can see how these steps result in the representative results shown in these figures. As it is not the focus of the protocol, a citation would also suffice.

Reply: Thank you for pointing this out. We have now provided citations to include experimental details for the immunolabelling in Figure 2 and treatment with 003, TUNEL, 2-deoxyglucose in Figure 3.

7) Please discuss limitations of this protocol in the discussion.

Reply: We thank the Editor for this comment. Organotypic retinal explant culture protocol is a complex procedure and one factor that could limits a successful experiment is lack of adequate training (lines 265-268). Moreover, the use of an antibiotic-free medium makes the retinal explants vulnerable to contamination by bacteria and fungi (lines 268-271). Other limitations of *in vitro* retinal culturing are differences in physiochemical environment when compared to the *in vivo* retina (*e.g.* choroidal and retinal blood supply, oxygen and glucose levels, intraocular pressure, composition of the vitreous and the axotomy of the optic nerve) and are described in lines 271-275. As another possible limitation we added the lack of perfusion. The inclusion of a perfusion system might match the *in vivo* condition even more closely

8) Please spell out the complete journal titles in the references.

Reply: We thank the Editor for this comment. We have now revised this accordingly.

Reviewer 1:

In the manuscript titled "Long-term, serum-free cultivation of organotypic mouse retina explants with intact retinal pigment epithelium", the authors described a straight-forward protocol to prepare and culture retina explants from mouse eyes and presented two examples to demonstrate the applications of this method. The protocol is well written and should prove useful for readers learning how to carry out the procedure. However, there are some changes and additions suggested to improve the quality and clarity of the manuscript:

Major Concerns:

1) The authors claimed that retina explant cultures are viable for at least four weeks. They should provide data (e.g. histology analysis and cell death analysis) to show the viability of retina explants at different time points, for example after 1, 2, 3, and 4 weeks of culture.

> Reply: We thank the reviewer for this comment. Since we do not show the viability of retina explants at different time points in this article, we rephrased this to “at least two weeks” in the revised manuscript (lines 73, 147) and provided citations for retinal explant culturing longer than 2 weeks (line 73, 147, 280).

2) In figure 2, the DAPI staining of the WT retina explant section indicates diffused ONL, INL, and RPE layers. It seems that these layers were mixed up without obvious boundaries. Does it mean that this retina explant was losing its structure?

> Reply: We thank the reviewer for this comment. The picture showing DAPI staining of the WT retinal explant section may not have been adequately focused and therefore it may have been difficult to distinguish the boundaries between layers, despite the presence of the dotted line. We have changed the picture to illustrate retinal layering better.

3) In figure 2 legend, they should mention how long these explants were kept in culture and the age of the animals used for this experiment.

> Reply: The cultures shown in Figure 2 had been cultured from P5 to P11, *i.e.* for a culture period of 6 days *in vitro*. We have now revised Figure 2 legend accordingly.

4) The retina explant cultures must be viable for functional studies for example electrophysiology as this manuscript mentioned. The authors should also include this data in the manuscript.

> Reply: We thank the reviewer for this comment. Retinal explants are indeed adaptable to many experimental applications, including electrophysiology. However, our laboratory does not conduct experiments in electrophysiology and therefore cannot provide results. However, we now provide additional references in the text that refer to the use of retinal explants for electrophysiological studies (line 293-299).

Minor Concerns:

1) In section 2.7, the corresponding figure for the text should be figure 1 not figure 2.

> Reply: We apologize for this mistake and have corrected this in the revised manuscript.

2) In section 2.7. 1, the authors didn't mention how to remove the cornea.

> Reply: We apologize for this mistake and have corrected this in the revised manuscript (line 119)

3) In picture 3 and 4 of figure 1, the lens looks more like a cornea. The authors need to modify the cartoon.

Reply: We thank the reviewer for this comment and modified the cartoon accordingly.

4) From section 2.1-2.5, please specify where these steps should be processed (in a laminar airflow hood?) and how to keep eyes aseptic.

Reply: We thank the reviewer for this suggestion. We specify when steps should be processed under a laminar airflow hood (line 113). If not mentioned, the step can be processed in non-sterile conditions.

5) Please also indicate the key point(s)/step(s) to make a successful RPE/neural retina explant culture in the manuscript.

Reply: We thank the reviewer for this comment. The steps to make a successful RPE/neural retina explant culture are the incubation of the eye with proteinase K (line 112), which allows the detachment of the sclera and choroid from the RPE (line 119-121) as well as adequate training and experience in order to obtain a successful outcome (lines 265-268).

Reviewer 2:

The manuscript by Belhadj et al., describes a very useful method for organotypic culture of mouse retinal tissue in conjunction with the supporting retinal pigment epithelium. The protocol is well-described, detailed and easy to follow. The culture method will be useful for studies on retinal development, pathophysiology of retinal diseases as well as for small-scale drug screening.

Major Concerns:

1) 1.2 There should be a list of all the tools needed and/or they should all be listed in 1.2.

Reply: All the tools needed were indeed not specifically mentioned. We thank the reviewer for this suggestion. We modified the list accordingly. Please also note that a table of materials was submitted together with the manuscript (line 93).

2) 1.4 Do the author mean that proteinase K should be preheated before being added to the medium described in 1.3.3? Or at which step is pure proteinase K used?

Reply: The 0,12% proteinase K solution in BM should be prepared in advance (line 99). The pure proteinase K is a powder and shouldn't be preheated. At the day of explantation, an aliquot of the 0,12% proteinase K solution in BM should be preheated at 37°C to activate the enzyme (line 102) and to then use it in step 2.5. We have now added this information to 1.4.

3) 2.4 What is the temperature of the BM?
4) 2.7 What is the temperature of the BM?

Reply: Thank you for pointing this out. The basal medium is at room temperature in these steps. This information has been added to the manuscript (lines 111 and 116-117).

5) 2.7.6 Are the retinal explants cultured in water-air interface? According to the method described, no medium is covering the retina. Won't the retinal explants dry out?

Reply: Yes, the retinal explants are cultured at the water-air interface, a thin liquid film created by the surface tension of water is covering them. This has now been explained in the protocol (step 2.7.6).

6) How are the retinas lifted from the polycarbonate culturing membrane for downstream applications? This step could be included.

Reply: Thank you for this suggestion, the manuscript was indeed not mentioning this part. As we added it in 3.1, the fixation and cryoprotection buffers can be added directly in the well. Then, the membrane around the retinal explant is cut and both the membrane and retinal tissue are embedded in medium for frozen tissue (lines 167-172). Therefore, histological section from retinal explants usually also show the (sectioned) culturing membrane.

7) Figure 2C. TUNEL staining is not visible. What does P11 denote? This should be clearly described. Is it the age of the mouse or days of culture? Days of culture should be indicated.

Reply: We thank the reviewer for the comment. We have now removed the TUNEL staining from Figure 2 as it is also shown in Figure 3. P11 stands for post-natal day 11 which is the age of the mouse. We clarified this in the revised manuscript (line 214-216).

8) Lines 175-180. Representative figures should be included to show with a time course along the culture period.

Reply: We thank the reviewer for this suggestion. Such time-courses, showing representative images of retinal explant cultures for culture periods ranging from 1-4 weeks, were in fact done already in previous studies (Caffe et al., 1989; 2001). While it might be of interest to repeat such a time-course again, perhaps using a more modern representation, we feel that to repeat this for the current article would be out of scope of a video article that focusses on the retinal explantation technique.

In the revised manuscript, we now refer more clearly to the older studies showing time-courses for different retinal explant culturing periods (Lines 198-203)

9) How many days of culture for the retinas shown in Figure 2?

Reply: The retinal explants were cultured for 6 days, from P5 to P11. We clarified this in the revised manuscript (line 214).

10) The *rd1* mouse model should be described at the first mention; the same applies to TUNEL.

Reply: A short description of the *rd1* model was added, as well as a citation of the paper characterizing it, in the beginning of the protocol (line 86-87).

11) How long time between the enucleation and the initiation of the organotypic culture? Is there a maximum time before the retinal tissue is affected? Has this been tested?

Reply: This has not been tested. However, we recommend to initiate the dissection as soon as possible after the enucleation. We indeed noticed that the longer this time is, the harder it is to dissect the retina, the eyes becoming very soft. We added this information (lines 174-175).

12) Figure 3C. The images of NT and T2DM do not look to be of same magnification.

Reply: We thank the reviewer for the observation. We changed the image of figure 3C accordingly.

13) Any troubleshooting steps could be described.

Reply: We thank the reviewer for this suggestion. We have added a “Troubleshouting” section to the manuscript (lines 149-161), highlighting possible problems and how to address them. Moreover, as mentioned in the manuscript (lines 265-268) sufficient practice on retinal dissection and work under aseptic conditions is essential.

Minor Concerns:

1) DAPI and TUNEL apply to all panels in Figure 3B and C so it should be indicated accordingly.

Reply: The figure has been changed accordingly, thank you for the suggestion.

Table 1
Basal R16 Medium: Preparation

Substance	Catalogue-Nr.	MW	Amount	dissolve in	conz. Stock-lsg	conz. compl. Medium	stability	storage (R202)
$NaSeO_3$ x $5H_2O$ $MnCl_2$ x $4H_2O$ $CuSO_4$ x $5H_2O$	S5261 M5005 C8027	262,94 197,91 249,68	7,9 mg 1,0 mg 2,5 mg	·dissolve both together in 100ml ddH_2O ·filter	300µM 50 µM 100 µM	30/60nM 5nM 10/20nM	Unlimited	4°C/-20°C
Biotin (4°C)	B4639	244,31	10 mg	·dissolve in 9,8ml ddH_2O ·add 0,1ml 1N NaOH (pH 10-12) ·heat to 35°C (~10min) ·neutralise with 0,1ml 1N HCl ·put therefrom 1ml in 9ml ddH_2O ·filter	0,1mg/ml	0,1µg/ml	6 Months	4°C
Ethanolamine (4°C)	E0135	61,08	10 µl	·add to10ml ddH_2O ·filter	1mg/ml	1 µg/ml		4°C
CDP-choline-Na (4°C)	30290	510,31	25,6 mg	·dissolve in 10 ml ddH_2O ·filter	2,56 mg/ml	2,56 µg/ml		4°C

· dissolve 1 vial R16 Medium (powder) in 500ml ddH_2O
· add following supplements: 2,73g $NaHCO_3$ (Merck:106 329, 500g) (32,5mM in compl. Medium)
0,1ml $NaSeO_3$/$MnCl_2$/$CuSO_4$ (Stock)
1,0ml Biotin (Stock)
1,0ml Ethanolamine (Stock)
1,0ml CDP-choline-Na (Stock)
· fill up to 800ml with ddH_2O
· filter
· **stability: 3 months**

NOTE: filtering is required for sterilization, use a filter with a sterile filter with 0.2 µm size or similar.

Table 2
Supplements: Preparation

Substance	Catalog-Nr.	MW	Amount	dissolve in	Conc. in Stock-solution	Conc. compl. Medium	Stability	Storage
BSA (4°C)	B4639	~65kDa	4 g	·dissolve in 100ml ddH_2O ·filter (stir slowly!)	4%	0,2%	2 years	4°C
Transferrin (4°C)	T1283	~80kDa	25 mg	·dissolve in 2,5ml ddH_2O ·filter	10mg/ml	10µg/ml	6 months	-20°C
Progesteron (RT)	P8783	314,47	2,52 mg 25mg/ 40ml	·dissolve in 4ml 100%EtOH ·filter ·put therefrom 0,1ml in 9,9ml sterile 0,1% BSA	6,3µg/ml	0,0063µg/ml	6 months	-20°C
Insulin (-20°C)	I6634	~6kDa	20 mg	·dissolve in10ml 10mM HCl ·filter	2mg/ml	2µg/ml	6 months	-20°C
T3 (-20°C)	T6397	651,01	2 mg 20mg/ 99ml	·dissolve in 9,9ml 0,01%BSA ·+0,1ml 1N NaOH ·filter ·put therefrom 0,1ml in 9,9ml sterile 0,01% BSA	2µg/ml	0,002µg/ml	6 months	-20°C
Corticosterone (RT)	C2505	346,47	8 mg 40mg/ 20ml	·dissolve in 4ml 100% EtOH ·filter ·put therefrom 0,1ml in 9,9ml sterile 0,1% BSA	20µg/ml	0,020µg/ml	6 months	-20°C
ThiaminHCl	T1270	337,27	27,7 mg	·dissolve in 10ml ddH_2O	2,77mg/ml	2,77µg/ml	6 months	-20°C

(Vitamin B1) (4°C)				·filter				
Vitamin B12 (4°C)	V6629	1355,42	3,1 mg 31mg	·dissolve in 10ml ddH_2O ·filter	0,31mg/ml	0,31µg/ml	6 months	-20°C
(+/-)-α-LipoicAcid (=Thiotic Acid)(RT)	T1395	206,32	45 mg	·dissolve in 10ml 100%EtOH ·filter ·put therefrom 0,1ml in 9,9ml sterile 1% BSA	45µg/ml	0,045µg/ml	6 months	-20°C
Retinol / Retinyl acetate (-20°C)	R7632 R7882	286,5 328,5	20 mg 20 mg	·dissolve both together in 2ml 100%EtOH ·filter ·put therefrom 0,05ml in 9,95ml sterile 0,1% BSA	0,05mg/ml 0,05mg/ml	0,1 µg/ml 0,1 µg/ml	6 months	-20°C
DL-Tocopherol / Tocopherylacetat (4°C)	T1539 T1157	430,7 472,73	200 mg 200 mg	·dissolve both together in 2ml 100%EtOH ·filter ·put therefrom 0,1ml in 9,9ml sterile 10% BSA (vortex→stay milky)	0,5mg/ml 0,5mg/ml	1 µg/ml 1 µg/ml	6 months	4°C
Linoleic Acid/ Linolenic Acid (-20°C)	L1012 L2376	280,4 278,4	111 µl 108 µl	·dissolve both together in1,781ml 100%EtOH ·filter ·put therefrom 0,1ml in 9,9ml sterile 10% BSA (vortex→stay milky)	0,5mg/ml 0,5mg/ml	1 µg/ml 1 µg/ml	6 months	-20°C
L-CysteineHCl (RT)	C7477	157,63	70,9 mg	·dissolve in 10ml ddH_2O ·filter	7,07mg/ml	7,09 µg/ml	6 months	-20°C
Glutathione (4°C)	G6013	307,32	10 mg 20mg	·dissolve in 10ml ddH_2O ·filter	1mg/ml	1µg/ml	6 months	-20°C
Na-pyruvate	P3662	110,04	500 mg	·dissolve in 10ml ddH_2O	50mg/ml	50 µg/ml	6 months	4°C

(4°C) R202				·filter				
Glutamine/ Vitamin C (4°C)	G8540 A4034	146,15 176,13	25 mg 100 mg	·dissolve both together in 10ml ddH_2O ·filter	2,5mg/ml 10mg/ml	25 µg/ml 100 µg/ml	6 months	-20°C

· aliquot all the supplement

NOTE: filtering is required for sterilization, use a sterile filter with 0.22 µm size or similar.

Amount of product advised to be weighted easily

Table 3
Complete Medium

Supplement	Amount for 100ml complete Medium/ Amount for 50ml	
Basal R16 Medium	80,0ml	40,0ml
BSA	5,0ml	2,5ml
Transferrin	100µl	50µl
Progesterone	100µl	50µl
Insulin	100µl	50µl
T3	100µl	50µl
Corticosterone	100µl	50µl
Thiamine HCl	100µl	50µl
Vitamin B12	100µl	50µl
(+/-)-α-Lipoic Acid (=Thiotic Acid)	200µl	100µl
Retinol/Retinylacetate	200µl	100µl
DL-Tocopherol/-acetate	200µl	100µl
Linolic Acid/Linolenic Acid	200µl	100µl
L-Cysteine HCl	100µl	50µl
Glutathione	100µl	50µl
Na-pyruvate	100µl	50µl
Glutamine/Vitamine C	1,0ml	0,5ml
ddH_2O	12,3ml	6,15ml
Volume	100ml	50ml

· stable for 3 weeks