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.