Point by point answer to the reviewer's comments

Reviewer #1:
Scientific Quality: Grade B (Very good)
Language Quality: Grade B (Minor language polishing)
Conclusion: Minor revision
Specific Comments to Authors: Comments to the authors The manuscript by Freude KK et al., demonstrates that it is possible to generate RGCs and Müller glial cells from retinal organoids obtained from human induced pluripotent stem cells, by following protocols previously established. The expression of specific markers for these cells in their retinal organoids, was confirmed by RT-PCR and immunocytochemical determinations. The use of magnetic-activated cell sorting (MACS) method, a useful top-notch technique, allowed the authors to isolate and expand both cell types from the organoids. In general, the manuscript represents a valuable piece of information which potentially could be useful for treating retinal diseases compromising survival of RGC such as Glaucoma .

We thank the reviewers for their overall positive evaluation.

Minor points 1) The abstract is not very well written, and confusing and should be rewritten. The phrase the authors wrote in the "aim" of the manuscript is much more precise; I would suggest expanding the phrase the authors wrote in the aim and use it as an abstract.

This was a helpful comment and we have changed the abstract accordingly.

2) Relevant contributions of other authors to obtain retinal organoids (i.e: V. Canto Soler´s group) and about the crosstalk between MGC and retinal neurons should be included in the references.

We have incorporated the reference and suggestion. Page 6 line 176

Reviewer #2:
Scientific Quality: Grade A (Excellent)
Language Quality: Grade A (Priority publishing)
Conclusion: Accept (High priority)
Specific Comments to Authors: The manuscript entitled "Retinal organoids derived from human induced pluripotent stem cells - possibilities and limitations for studying retinal neurodegeneration" by Freude et al., addresses very interesting issue of the pluripotent stem cells application to model diseases in 2D and 3D tissue formats. Manuscripts describes 3D retinal organoid culture and very simple and robust approach for Muller glia and

retinal progenitors isolation using magnetic activated cell sorting. The paper is well and clearly written, title corresponds to the topic of the manuscript. All Figures have a very good quality supporting the accuracy of the data presented. However, I did not find what Pax6 antibody Authors used in their experiments.

We thank the author for his positive evaluation and we have added the Pax6 antibody information to the materials section Page 10 lines 300-301.

Reviewer #3:
Scientific Quality: Grade D (Fair)
Language Quality: Grade B (Minor language polishing)
Conclusion: Major revision
Specific Comments to Authors: This study reports the generation of retinal organoids from human iPSC by adapting a method reported earlier in 2014. The protocol seem to work well and generates authentic appearing retinal organoids within 56 days, for downstream applications. This can be appreciated in Fig 1. However, there are many concerns with respect to the characterizations done, which needs more rigor. Specific comments: For organoids: Fig 2 1. IHCs are sub-optimal, as stated by the authors themselves. Apart from trying tissue clearing, it would have been simpler to take sections for IHCs. This doesn't require any special chemical treatments. Even muller processes across the retina can be clearly visualized.

We agree with the reviewer that sectioning and subsequent ICC could have been an option. In our hands cryopreservation and sectioning was suboptimal to whole mounts ICC, which was mainly problematic due to the collapse of the optic cups in the process. Therefore, we focused on wholemount ICC, which gives in general a better overview of cell contributions as intended in the manuscript. When we discuss the wholemount procedure being suboptimal we referred to the commonly used procedure of fructose glycerol clearance. We also implemented Ethyl cinnamate based clearance, which gave superior results and that is what we recommend in the paper as the best wholemount clearance procedure for optic cups. See methods page 10 lines 315/316 and results page 13 lines 407-412.

Fig 2F shows a beautiful optic cup with RPE margins, as confirmed by TEM. It will be useful to give the experimental replicates assessed.

Thank you for pointing this out we have added the assessed replicates to the materials section page 8 line 249.

For MACS enriched cells: Fig 3 1. ICC with multiple markers will be required. Also, it can be clubbed with RT-PCR assay.

The reviewer has a point here and that is what we indented to do, but due Corona lab access restriction we were not able to complete this assessment. Due to the fact that the optic cup protocol takes 56 days and the subsequent culturing of cells after FACS takes 14 days, plus the ICC and RT-PCR assays this is unfortunately out of the scope at the moment. We have added in the discussion that such rigorous analyses are crucial and discussed the markers for future studies. Please see page 15 staring line 484 until page 16 line 493.

2. Enrichment efficiency need to be checked by FACS or by comparing the positive and negative cell pools.

We have added the numbersr of retinal organoids applied in the MACS experiment (Methods Page 9 Line 287/288). Prediction of efficiency numbers is a little more difficult with the MACS approach since it is based on columns and magnetic bead interaction and not on countable fluorescence signals. Optic cups contain on day 56 on average 50.000 cells, but there is natural variation of at least 5.000 – 10.000 cells due to different sizes and composition of the retinal organoids. The successfully sorted cell numbers were low with on average 200 to 300 cells per sort. See results page 14/15 lines 452-457.

3. Chx10 can't be cytosolic and overlap with Nestin. Infact they are exclusive. Anti-Chx10 staining may be just the background.

We agree with the reviewer that the ICC should be mainly nuclear, but some publications show indeed faint cytosolic expression pattern (DOI: 10.1016/j.biomaterials.2017.10.052) and we observe both nuclear and cytosolic signals in our retinal organoids. The ICC results after MACS are puzzling and we are grateful that the reviewer points this out. We have commented on it and underlined the need for more thorough follow up in the discussion to validate cell identity. Results page 15 lines 467-469

4. Was the sorting efficiency 100% that all the cells in the field are positive for all the markers tested? Either the antibodies have non-specificity or the markers chosen are not-specific to RGC & Muller glia.

The efficiency was very high at around 90-95% of the sorted cells, but the number of cells pulled down was very small. The antibodies gave specific signals, since the MGs after MACS were negative for the RGC markers and the RGCs were negative for the MG markers, which were used as controls for each other. Obviously, MACS sorting with one cell surface marker will not

exclusively pull down only one cell population and extended 2D cultures are needed to address the purity of the lineages. The cells analyzed here are progenitors and even though we believe this approach has great potential we are aware and have discussed that future studies should address increased efficiency of MACS, maintenance of lineage identity in 2D, maturation in 2D and validation through marker analyses (added to the discussion page 16 lines 492-502)

Overall, it is an useful study, but requires rigorous characterization of retinal organoids and isolated RGCs/Muller glia.
We agree and have incorporated this into the discussion (page 16 page 16 lines 492-502), but it is out of scope for this submission.

General comments: 1. Title is not appropriate and should be revised.

The title is revised to better match the study.

Retinal neurodegeneration is diverse, while the authors are focusing on RGC pathology in this MS. Retinal organoid protocol development, their characterization and an RGC isolation method has formed the main body of the manuscript. It doesn't explain any possibilities or limitations.

We have added possibilities and limitations to the discussion (Page 17 lines 545-554)

2. Why referencing Fig 2A later than 2C. Better rearrange the figure order otherwise.

This has been addressed.