Re: Authors' response to the reviewers' comments for Assetta et al., "Generation of Human Neurons and Oligodendrocytes from Pluripotent Stem Cells for Modeling Neuron-oligodendrocyte Interactions"

We would like to thank the reviewers and editors for their careful assessment of our manuscript and really appreciate their insightful comments and constructive suggestions. We have addressed every single point raised by the reviewers, major and minor, with new data and further clarification. Specifically, we have performed additional experiments to validate the purity and proper development of our induced cell populations in single and mixed cultures (Fig. 2D and 3D). We have also elaborated and referenced the mentioned parts of our manuscript to adequately inform the readers of the utility and caveats residing in our methods. The text revision is extensive throughout the manuscript. While these changes are made at this turbulent time when our productivity is markedly compromised owing to the pandemic-related delays, they do significantly strengthen our work, which, in our opinion, now is better aligned with the research scope of the *JoVE*.

We hope this rebuttal will meet the editors' and reviewers' full approval and move forward the publication of our article. Below, we cite the editorial and reviewers' comments in full in *italic* typeface and provide our response in **bold** typeface.

Editorial Comments:

• *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.*

We have followed this top suggestion and proofread our entire package to our best.

• *Protocol Language: Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.*
1) All steps of your protocol need to be re-written in the imperative voice. For example, "HEK293T cells plated in T75 flasks are transfected with plasmids expressing Ngn2" should be "Plate HEK293T cells in T75 flasks and transfect them with plasmids expressing Ngn2".

We have changed the text in the protocol section exactly as suggested.

• *Protocol Detail: 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 specific details (e.g. button clicks for software actions, numerical values for settings, etc) to all 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. Some examples:*
1) 1.1.1: Describe cell plating, cell density. How is transfection performed? Mention culture environmental conditions.

2) 1.1.2: How is collection done? What is the centrifugation speed (in g) and duration.
3) 1.2.1: how is cell culture performed?
4) 1.2.2: describe the detachment steps. How much accutase? How long is it incubated?
5) Please use the above comments as a guide for your entire protocol and add all specific details.

All these mentioned details are incorporated into our manuscript.

• Protocol Highlight: Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

This critical step of Protocol Highlight has been executed and all the listed points have been completely taken into account.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The Discussion section is now reorganized and expanded from three to five paragraphs, with all the listed points underlined.

• Figure/Table Legends: Define all error bars.

All the error bars are now clearly defined in the figure legends.

• References: Please spell out journal names.

The original list of references was generated by using the EndNote with the JoVE style downloaded from the website. Now the journal full names are all spelt out as suggested.

• Commercial Language:JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding

language in your manuscript are WiCell Research Resources (Wicell, Matrigel, PEI, mTeSR, accutase, etc.

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We have carried out this editing procedure as suggested.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

We do not include any previously published figures in this manuscript; we cite and summarize our prior results to inform the readers.

Reviewers' Comments:

Reviewer #1:
Manuscript Summary:
In the manuscript, Assetta et al. described a new method in inducing neurons and oligodendrocyte lineage cells from iPS cells. The authors also provided a detailed protocol in co-culturing iNeurons and iOligodendrocytes and successfully characterized cell-specific markers for both cell types. The protocol is well-written and the method described in the manuscript will be of great interests for researchers who want to use the iPS cells to study neuron-oligodendrocyte interactions in various neurological diseases, including Alzheimer's disease.

We wholeheartedly appreciate the reviewer's comprehensive comments.

Major Concerns:
None

Minor Concerns:
1. As the protocol describes a co-culture harboring induced neurons and oligodendrocytes, the authors should comment on the purity of the culture - are there any astrocytes (GFAP+) or microglia (Iba1+) present in the co-culture? If so, what are the percentages for these cell types? Since both of these cell types interact with neurons and oligodendrocytes and are involved in

AD, it will be helpful to clarify if they are present in the co-culture system which is intended to model AD.

We are grateful for the reviewer's comment that points to the overall strength of the modern stem cell-based model system, which allows generation of a homogenous, well-characterized population of desired brain cell type. For our co-cultures, we induced the human neurons and OPCs separately along their individual, fate-determining developmental courses before we carefully mixed them to grow together. Microglia and astrocytes thus are not expected to be present in our preparations. To ensure the absence of these two glial cell types, we examine the expression of several marker genes in our neuron-OPC co-cultures and compared that to validated pure human microglia and astrocytes (Fig 3D). We cannot detect any qPCR signal for all the tested microglial markers, *TMEM119*, *TREM2* and *CD33*, and only notice minimal levels of astroglial genes *GFAP* and *ALDHL1*. We did not choose the widely accepted microglial marker IBA1 (AIF1) as this gene is indeed expressed at a low level in OPCs, according to independently published RNA-Seq datasets.[1-3] We hope this additional set of data will further corroborate the purity of our co-culture system. The text changes are at Line 378-380 and 428-431.

2. As AD is a neurodegenerative disease, the authors should discuss the relevance between the length of co-culture and the nature of this disease.

This point indeed constitutes a major limitation for our methodology and we agree with the reviewer that this should be discussed in the manuscript. We have expanded the DICUSSION to include a new paragraph about the limitations of our methods, and addressed this point there (Line 467).

3. In figure 1, "day 6" image is missing.

Our apologies for this error. Due to an unforeseen issue of software compatibility, the image did not appear on all platforms. The error has now been fixed.

Reviewer #2:

Manuscript Summary:

The manuscript by Assetta et al presents a straight-forward and efficient protocol for deriving neurons and oligodendrocyte lineage cells from human pluripotent stem cells for single culture or co-culture. The authors have significantly reduced the time to generate these cells from possibly months in traditional protocols to just a few weeks thus greatly increasing the tractability of this in vitro culture system. However, a satisfactory characterization of the generated cells is lacking, which brings to question its effectiveness. This and other concerns are outlined in more detail below.

We thank the reviewer for his/her concise comments.

Major Concerns:
1) The authors describe the generation of human induced neurons from pluripotent stem cells, but it is not clear what types of neurons are generated. Are they excitatory? Inhibitory? What other characteristics do they exhibit?

We thank the reviewer for this comment which is indeed worthwhile to be addressed in the manuscript. In brief, the induced neuron protocol described here is an improvement of the widely employed method based on the single transcription factor Neurogenin 2 (Ngn2), which has been documented to generate functional excitatory cortical neurons, highly resembling the layer 2/3 counterparts *in vivo*.[4] Our specific method is further enhanced by the unique feature of being glia-free, and has been used in our prior work in which we demonstrated the resultant human neurons exhibit a typical pyramidal morphology and electrophysiological characteristics, quantitatively reproducible and independent of the cell line of origin.[5,6] We have revised the INTRODUCTION (the third paragraph, Line 118) accordingly to provide such information .

2) In figure 1C, the image shown for the "DAY 4" is washed out and the image for "DAY 6" is missing.

Our apologies for this error, which resulted from a software compatibility issue for the image display. This now is fixed and corrected.

3) In figure 2B, only a small subset of the total cells appear to be Olig2 positive. How pure are these OPC cultures?

The reviewer again raised a good point, which is the general limitation for identifying the specific cell type or development stage by using a single marker. Olig2 is a traditional marker for all oligodendrocyte lineage cell types, and its expression level fluctuates along the oligodendroglial differentiation and is not necessarily high at the early OPC stage.[1,2] In fact, as revealed in Fig. 2E, *OLIG2* expression increases with maturation and is the lowest in OPCs. Therefore, researchers in the field of oligodendrocyte biology, particularly those who develop and use stem cell-derived oligodendroglia, prefer the classical surface marker O4 which correlates closely with the functionality.[7-10] Consistent with these notions, we can detect Olig2 immunoreactivity in roughly 25% of our OPCs (Fig. 2B, right bottom panel) and ~95% of the population positive for O4 (Fig. 2B, right top panel). We are thus confident in the efficiency of our protocol and have added the aforementioned quantification as the exemplary information in the figure legends (Line 399).

4) In figure 2D, the authors nicely show expression of OPC and OL markers in the induced human cultures. Can the authors show a corresponding absence of NPC markers such as PAX6 and nestin?

We performed the exact experiment as the reviewer suggested and are able to show that the expression of NPC marker PAX6 diminishes dramatically in our induced OPCs and

further decreases to nearly background in differentiated oligodendrocytes. We agree with the reviewer that this piece of data is informative and have incorporated it as our new Fig. 2D with a detailed description in the corresponding figure legend (Line 401).

5) The authors state that myelin wrapping is observed in the induced co-cultures of iNs and iOLs, however the MBP immunostaining shown in figure 2B and figure 3B is not convincing. The gold standard for showing myelination is using TEM. Without TEM images of myelin the most the authors can state with the images they present is that MBP+ OL processes ensheath axons.

We appreciate that the reviewer indicates this inaccurate part in our original manuscript. We have modified our text and figure legends accordingly to avoid any confusion and overstatement (Line 131, 316 and 374).

6) In figure 3C, the authors show a clear increase in synaptic marker staining along neuron cell bodies and dendrites upon co-culture with induced oligodendrocyte lineage cells. Is the increase due to interactions with iOPCs or iOLs? It is not clear.

We are grateful for reviewer's comment on this important issue which was not explained adequately in our previous submission. As demonstrated in the literature[7,9,10] and shown in our time course examination Fig. 2E, the stem cell-derived OPCs, once stimulated by myelinogenic molecules like triiodothyronine (T3), can further differentiate and transition through the intermediate developmental stages toward becoming mature myelinating oligodendrocytes – by an accelerated course. Such stimulated cultures then become heterogenous in population and comprise self-renewing OPCs, pre-oligodendrocyte, pre-myelinating oligodendrocytes and myelinating oligodendrocytes.[11] These subpopulations in continuum would account for different portions at different time points. In this manuscript, we start the co-cultures by mixing the induced neurons and OPCs together and then allow OPCs to mature in the described co-culture medium. The imaging was performed at D21 of co-culturing, at the time when pre-myelinating oligodendrocytes are expected to dominate the oligodendroglial population (Fig. 2E). We have thus revised our description already to clarify this (Line 350-357).

How do early-stage oligodendrocyte lineage cells interact with neurons and influence the synapse formation independent of myelination? This question is currently under active investigation at the Huang lab, by using this particular co-culture system. Our preliminary data suggest OPCs are able to secrete neurotrophins to enhance the neuronal excitability, potentially through promoting synapse formation. We believe the protocol described here will be encouraging and helpful for future studies tackling the neuron-oligodendroglia synaptic interactions.

Minor Concerns:
7) It's not clear what "up to six weeks" (line 117) is referring to in the last paragraph of the introduction

By writing "up to six weeks" we meant that we are able to maintain the co-culture with a proper morphology and gene expression profile for at least six weeks. We have further

clarified by removing this phrase from line 117 and explaining in the REPRESENTATIVE RESULTS section (Line 377).

8) In the protocol the authors state that lentivirus preparation takes ~6 days, but they only describe ~4 days of work.

Our apologies for the confusion. This difference mainly reflects the flexibility in the actual production procedure. We typically have a 5-day procedure: plating (1 day), transfecting (1 day), media change (1 day) and collection (1, but up to 3 consecutive days), ultracentrifugation and suspension (1 day). We have made the changes to make our description consistent (Line 142).

Reviewer #3:
Manuscript Summary:
In Alzheimer's disease (AD) the role of oligodendrocytes is yet to be determined, with a number of evidence to suggest a strong contribution of this cell type to the ongoing neurodegenerative processes in this disease. Authors here describe protocols to generate functional neurons and oligodendrocytes from human pluripotent stem cells, including a co-culture system to model the neuron-oligodendrocyte connections. More specifically, they describe methods for generation induced oligodendrocyte precursor cells (iOPCs) and induced oligodendrocytes (iOLCs) in a relatively short period of time.

We are grateful for the reviewer's concise and positive evaluation.

Major Concerns:
1. In line 172-172 authors say that detached neurons can be frozen without significant cells death. There is always some cell death after thawing, in CS10 can be even up to 20% for neurons, therefore it is beneficial for the reader to specify the expected % of cells death. Please indicate that number based on your experience, especially as the number of neurons to be seeded in the co-culture system is advised to be different if seeding fresh or thawed neurons (protocol 3.2.1, line 252).

We thank the reviewer for drawing attention to this technical point and agree that such information should be included. We do normally notice about 15-20% cell death when we thaw neurons frozen in CS10, and consider this level of loss reasonable for storage and future retrieval. We have added this specific piece of information into the PROTOCOL section and discussed this for the utility (Line 195).

2. Image in Figure 1C day 6 is missing, whereas the day 4 image has poor contrast settings. Please improve these images for better representation for your data.

Our apologies for this error, which resulted from a software compatibility issue for the image display. This now is fixed and corrected.

3. What is specifically meant by "initial seeding density" in line 196?

We have now clarified this by adding extra words to reference this to the same seeding density mentioned in line 187, on day -1 of the protocol.

4. Catalog number is missing for the NPC freezing media (line 205).

This has now been added in the Table of Materials.

5. Add references to all figures in the Protocol section.

We have incorporated the figure references into our PROTOCOL section.

6. As authors are comparing different differentiation conditions to maturation markers, Abeta levels, or synapsin puncta (Figure 3 and 4), it would be most appropriate to present statistical analysis on this data.

Our apologies that the information regarding the statistical significance did not go through in our previous manuscript. We have that presented appropriately in the figures and legends.

7. The data looks promising, but what is missing is the quantification of the percentage on NPCs, OPCs and OLCs obtained compared to the total cellular make up of the differentiated culture. Authors describe the use of IHC differentiation markers, but limited data in presented to give a feel of the efficiency of these differentiation protocols.

We thank the reviewer for again raising a great point. We first validated that the NPCs we generated based on the established approach of SMAD inhibition[12] are homogenous and well-characterized, with more than 95% of cells positive for markers including Nestin, Pax6 and Sox1 but negative for the pluripotential marker Oct4. We have performed additional experiment (Fig. 2D) to show that our protocol efficiently differentiates NPCs into OPCs, with the expression of NPC marker Pax6 considerably reduced in OPCs (>90%) and further diminished in oligodendrocytes (>99%). Hence, we are confident that NPC account for only a minimal fraction of the total cellular make-up in our co-cultures. Moreover, our IHC staining for the standard OPC marker O4 indicated a satisfactory level of purity, with more than 95% of our resultant OPCs being positive (Fig. 2B).

However, the maturation triggered by the myelinogenic compounds (including T3 triiodothyronine and Clemastine) in our described maturation medium diversifies the cell population. Consistent with previous reports on generating functional oligodendrocytes,[7,9,10] we showed in our time course study (Fig. 2E) that our OPCs, upon myelinogenic stimulation for maturation (by T3 triiodothyronine and/or Clemastine), differentiate and transition through intermediate stages (like pro-oligodendrocyte and pre-myelinating oligodendrocytes) toward myelinating oligodendrocytes.[11] Such oligodendroglial subpopulations in continuum constitutes varying percentages of the total cells along the maturation process, with more mature cells dominating at a later

time point and vice versa. We have revised our REPRESENTATIVE RESULTS section to mention this caveat (Line 350-357). Overall, in our opinion, the purity and efficiency of our protocol in generating human OPCs are evident.

8. How long are the authors capable to keep the neuronal oligodendrocyte co-culture healthy in culture? Are there any changes observed on the neuronal activity in this co-culture system? The possibility of a successful long term neuronal co-culture and increased support for neuronal activity through the co-culture system is a very interesting aspect while studding neuronal degeneration, and therefore these concepts should be discussed here as attributes of these protocols.

We completely agree with the reviewer on this critical aspect of the utility of our model system. In one of our attempts we managed to grow the co-cultures for five weeks with a reasonably healthy morphology and appropriate gene expression pattern. We have not yet systemically examined the neuronal activity in our long-term co-cultures, but plan to do so in our follow-up research, in which we intend to study the oligodendroglial influences on synaptic functions, potentially independent of myelination. We have revised our REPRESENTATIVE RESULTS and DISCUSSION as suggested to elaborate this interesting concept for future application (Line 377 and 467-480).

Minor Concerns:
1. In steps 1.2.2 and 2.1.2 please add the % confluence of ES cells at this stage prior to accutase treatment.

We have provided this detail as suggested.

2. In protocol 1.2 and 2.1 please give the concentration of Matrigel and GFR Matrigel used for all iPSC, neurons and OPCs.

We again thank the reviewer for his/her careful assessment. Such information has been added in.

REFERENCES

1 Marques, S. *et al.* Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system. *Science.* **352** (6291), 1326-1329, (2016).
2 Hrvatin, S. *et al.* Single-cell analysis of experience-dependent transcriptomic states in the mouse visual cortex. *Nat Neurosci.* **21** (1), 120-129, (2018).
3 Zhang, Y. *et al.* Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. *Neuron.* **89** (1), 37-53, (2016).
4 Zhang, Y. *et al.* Rapid single-step induction of functional neurons from human pluripotent stem cells. *Neuron.* **78** (5), 785-798, (2013).

5 Huang, Y. A., Zhou, B., Nabet, A. M., Wernig, M. & Sudhof, T. C. Differential Signaling Mediated by ApoE2, ApoE3, and ApoE4 in Human Neurons Parallels Alzheimer's Disease Risk. *J Neurosci.* 10.1523/jneurosci.2994-18.2019, (2019).

6 Huang, Y. A., Zhou, B., Wernig, M. & Sudhof, T. C. ApoE2, ApoE3, and ApoE4 Differentially Stimulate APP Transcription and Abeta Secretion. *Cell.* **168** (3), 427-441.e421, (2017).

7 Douvaras, P. & Fossati, V. Generation and isolation of oligodendrocyte progenitor cells from human pluripotent stem cells. *Nat Protoc.* **10** (8), 1143-1154, (2015).

8 Garcia-Leon, J. A. *et al.* Generation of a human induced pluripotent stem cell-based model for tauopathies combining three microtubule-associated protein TAU mutations which displays several phenotypes linked to neurodegeneration. *Alzheimers Dement.* **14** (10), 1261-1280, (2018).

9 Yamashita, T. *et al.* Differentiation of oligodendrocyte progenitor cells from dissociated monolayer and feeder-free cultured pluripotent stem cells. *PLoS One.* **12** (2), e0171947, (2017).

10 Chanoumidou, K., Mozafari, S., Baron-Van Evercooren, A. & Kuhlmann, T. Stem cell derived oligodendrocytes to study myelin diseases. *Glia.* **68** (4), 705-720, (2020).

11 Goldman, S. A. & Kuypers, N. J. How to make an oligodendrocyte. *Development.* **142** (23), 3983-3995, (2015).

12 Chambers, S. M. *et al.* Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol.* **27** (3), 275-280, (2009).