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TITLE:

Coculture of Axotomized Rat Retinal Ganglion Neurons with Olfactory Ensheathing Glia, as an In Vitro Model of Adult Axonal Regeneration.

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KEYWORDS:

olfactory ensheathing glia (OEG), adult axonal regeneration, in vitro assay, retinal ganglion neurons (RGN), coculture, axotomy

SUMMARY:

We present an in vitro model to assess olfactory ensheathing glia (OEG) neuroregenerative capacity, after neural injury. It is based on a coculture of axotomized adult retinal ganglion neurons (RGN) on OEG monolayers and subsequent study of axonal regeneration, by analyzing RGN axonal and somatodendritic markers.

ABSTRACT:

Olfactory ensheathing glia (OEG) cells are localized all the way from the olfactory mucosa to and into the olfactory nerve layer (ONL) of the olfactory bulb. Throughout adult life, they are key for axonal growing of newly generated olfactory neurons, from the lamina propria to the ONL. Due to their pro-regenerative properties, these cells have been used to foster axonal regeneration in spinal cord or optic nerve injury models.

We present an in vitro model to assay and measure OEG neuroregenerative capacity after neural

injury. In this model, reversibly immortalized human OEG (ihOEG) is cultured as a monolayer, retinas are extracted from adult rats and retinal ganglion neurons (RGN) are cocultured onto the OEG monolayer. After 96 h, axonal and somatodendritic markers in RGNs are analyzed by immunofluorescence and the number of RGNs with axon and the mean axonal length/neuron are quantified.

This protocol has the advantage over other in vitro assays that rely on embryonic or postnatal neurons, that it evaluates OEG neuroregenerative properties in adult tissue. Also, it is not only useful for assessing the neuroregenerative potential of ihOEG but can be extended to different sources of OEG or other glial cells.

INTRODUCTION:

Adult central nervous system (CNS) neurons have limited regenerative capacity after injury or disease. A common strategy to promote CNS regeneration is transplantation, at the injury site, of cell types that induce axonal or neuronal growth such as stem cells, Schwann cells, astrocytes or olfactory ensheathing glia (OEG) cells[1–5].

OEG derives from the neural crest[6] and locates in the olfactory mucosa and in the olfactory bulb. In the adult, olfactory sensory neurons die regularly as the result of environmental exposure and they are replaced by newly differentiated neurons. OEG surrounds and guides these new olfactory axons to enter the olfactory bulb and to establish new synapses with their targets in the CNS[7]. Due to these physiological attributes, OEG has been used in models of CNS injury such as spinal cord or optic nerve injury and its neuroregenerative and neuroprotective properties become proven[8–11]. Several factors have been identified as responsible of the pro-regenerative characteristics of these cells, including extracellular matrix proteases production or secretion of neurotrophic and axonal growth factors[12–14].

Given the technical limitations to expand primary OEG cells, we previously established and characterized reversible immortalized human OEG (ihOEG) clonal lines, which provide an unlimited supply of homogeneous OEG. These ihOEG cells derive from primary cultures, prepared from olfactory bulbs obtained in autopsies. They were immortalized by transduction of the telomerase catalytic subunit (TERT) and the oncogene Bmi-1 and modified with the SV40 virus large T antigen[15–18]. Two of these ihOEG cell lines are Ts14, which maintains the regenerative capacity of the original cultures and Ts12, a low regenerative line that is used as a low regeneration control in these experiments[18].

To assess OEG capacity to foster axonal regeneration after neural injury, several in vitro models have been implemented. In these models, OEG is applied to cultures of different neuronal origin and neurite formation and elongation—in response to glial coculture—are assayed. Examples of such neuronal sources are neonatal rat cortical neurons[19], scratch wounds performed on rat embryonic neurons from cortical tissue[20], rat retinal explants[21], rat hypothalamic or hippocampal postnatal neurons[22,23], postnatal rat dorsal root ganglion neurons[24], postnatal mouse corticospinal tract neurons[25], human NT2 neurons[26], or postnatal cerebral cortical neurons on reactive astrocyte scar-like cultures[27].

In these models, however, the regeneration assay relies on embryonic or postnatal neurons, which have an intrinsic plasticity that is absent in injured adult neurons. To overcome this drawback, we present a model of adult axonal regeneration in cocultures of OEG lines with adult retinal ganglion neurons (RGNs), based on the one originally developed by Wigley et al.[28–31] and modified and used by our group[12–18,32,33]. Briefly, retinal tissue is extracted from adult rats and digested with papain. Retinal cell suspension is then plated on either polylysine-treated coverslips or onto Ts14 and Ts12 monolayers. Cultures are maintained for 96 h before they are fixed and then immunofluorescence for axonal (MAP1B and NF-H proteins)[34] and somatodendritic (MAP2A and B)[35] markers is performed. Axonal regeneration is quantified as a percentage of neurons with axon, with respect to the total population of RGNs and axonal regeneration index is calculated as the mean axonal length per neuron. This protocol is not only useful for assessing the neuroregenerative potential of ihOEG but can be extended to different sources of OEG or other glial cells.

PROTOCOL:

NOTE: Animal experimentation was approved by national and institutional bioethics committees.

1. ihOEG (Ts12 and Ts14) culture

NOTE: This procedure is done under sterile conditions in a tissue culture biosafety cabinet.

1.1. Prepare 50 mL ME10 OEG culture medium as provided in **Table 1**.

1.2. Prepare 5 mL of DMEM/F12-FBS, as provided in **Table 1**, in a 15 mL conical tube.

1.3. Warm both media at 37 °C in a clean water bath, for 15 min.

1.4. Thaw Ts12 and Ts14 cells vials at 37 °C in a clean water bath.

1.5. Resuspend and add cells to the DMEM/F12-FBS culture medium prepared in step 2.

1.6. Centrifuge for 5 min at 300 x *g*.

1.7. Aspire the supernatant.

1.8. Add 500 µL of ME10 medium and resuspend the pellet.

1.9. Prepare a p60 cell culture dish with 3 mL of ME10 and add the cellular suspension, dropwise.

1.10. Move to distribute the cells uniformly across the plate.

1.11. Culture cells at 37 °C in 5% CO_2.

NOTE: After reaching confluence, at least another passage must be done to optimize cells for coculture. 90% confluence is needed before seeding them on the coverslips for coculture. A confluent p-60 has a mean cell number of 7 x 10^5 for Ts14 and 2.5 x 10^6 for Ts12 cell lines. Ts12 and Ts14 cell lines should be passaged every 2–3 days.

2. Preparation of ihOEG (Ts12 and Ts14) for the assay

NOTE: This step must be done 24 h before RGN dissection and coculture.

2.1. Treat 12 mm Ø coverslips with 10 µg/mL poly-L-lysine (PLL) for 1 h.

NOTE: The coverslips can be left overnight in PLL solution.

2.2. Wash the coverslips with 1x phosphate buffer saline (PBS), three times.

2.3. Detach Ts12 and Ts14 ihOEG cells from p60 cell culture dish.

2.3.1. Add 4 mL of DMEM/F12-FBS culture medium (**Table 1**) to a 15 mL conical tube. Warm at 37 °C in a clean water bath.

2.3.2. Remove the medium from plates and wash cells with 1 mL of 1x PBS-EDTA, once.

2.3.3. Add 1 mL of trypsin-EDTA to the OEG cells and incubate for 3–5 min at 37 °C, 5% CO_2.

2.3.4. Collect cells with a p1000 pipette and transfer them to medium prepared in step 3.1.

2.3.5. Centrifuge for 5 min at 200 x *g*.

2.3.6. Aspire the supernatant.

2.3.7. Add 1 mL of ME10 medium and resuspend the pellet.

2.3.8. Count the cell number in a hemocytometer.

2.4. Seed 80,000 Ts14 cells or 100,000 Ts12 cells onto the coverslips in 24-well plates in 500 µL of ME10 medium.

2.5. Culture cells at 37 °C in 5% CO_2, for 24 h.

3. Retinal tissue dissection

NOTE: 2-month old male Wistar rats are used as RGN source. Two retinas (one rat) for 20 wells

of a 24-well cell dish. Autoclave surgical material before use. Papain dissociation kit is commercially purchased (**Table of Materials**). Follow the provider´s instructions for reconstitution. Reconstitute D,L-2-amino-5-phosphonovaleric acid (APV) in 5 mM stock and prepare the aliquots.

3.1. On the day of the assay, prepare the following media.

3.1.1. Prepare a p60 cell culture dish with 5 mL of cold EBSS (vial 1 of the papain dissociation kit).

3.1.2. Prepare a p60 cell culture dish with reconstituted vial 2 (papain) of the papain dissociation kit plus 50 µL of APV, 250 µL of vial 3 (DNase) plus 5 µL of APV.

3.1.3. In a sterile tube mix 2.7 mL of vial 1 with 300 µL of vial 4 (albumin-ovomucoid protease inhibitor). Add 150 µL of vial 3 (DNase) plus 30 µL of APV.

3.1.4. Prepare 20 mL of Neurobasal-B27 medium (NB-B27) as provided in **Table 1**.

3.2. Sacrifice a rat by asphyxiation with CO_2.

3.3. Remove the head by decapitation with guillotine; place it in a 100 mm Petri dish and spray the head with ethanol 70% before placing it in a laminar flow hood.

3.4. Cut the rat´s whiskers with scissors so they do not interfere with the eye manipulation.

3.5. Grip the optic nerve with forceps to pull out the eyeball enough to be able to make an incision across the eye with a scalpel.

3.6. Remove the lens and vitreous humor and pull out the retina (orange-like tissue), while the remaining layers of the eye stay inside (including the pigment epithelial layer).

3.7. Place the retina in the p60 cell culture dish prepared in step 3.1.1.

3.8. Transfer the retina to the p60 cell culture dish prepared in step 3.1.2 and cut it with the scalpel in small pieces of an approximate size < 1 mm.

3.9. Transfer to a 15 mL plastic tube.

3.10. Incubate the tissue for 30 min, in a humidified incubator at 37 °C under 5% CO_2, with agitation every 10 min.

3.11. Dissociate cell clumps by pipetting up and down with a glass Pasteur pipette.

3.12. Centrifuge the cell suspension at 200 x *g* for 5 min.

3.13. Discard supernatant and to inactivate papain, resuspend the cell pellet in the solution prepared in step 3.1.3. (1.5 mL for 2 eyes).

3.14. Carefully pipet this cell suspension into 5 mL of reconstituted vial 4.

3.15. Centrifuge at 200 x *g* for 5 min.

3.16. While centrifuging, completely remove the ME-10 medium from the OEG 24 well cell plate (previously prepared in step 2) and replace it with 500 µL of NB-B27 medium per well.

3.17. Discard the supernatant and resuspend the cells in 2 mL of NB-B27 medium.

3.18. Plate 100 µL of retinal cell suspension, per well of the m24 plate, onto PLL-treated or OEG monolayers-coverslips.

3.19. Maintain cultures at 37 °C with 5% CO_2 for 96 h in NB-B27 medium.

4. Immunostaining

4.1. After 96 h, fix the cells for 10 min by adding the same volume of 4% paraformaldehyde (PFA) in 1x PBS to the culture medium (600 µL) (PFA final concentration 2%).

4.2. Remove the media and PFA from the 24-multiwell plate and once again add 500 µL of 4% paraformaldehyde (PFA) in 1x PBS. Incubate for 10 min.

4.3. Discard the fixer and wash 3 times with 1x PBS for 5 min.

4.4. Block with 0.1% Triton X-100/1% FBS in PBS (PBS-TS) for 30–40 min.

4.5. Prepare the primary antibodies in PBS-TS buffer as follows: SMI31 (against MAP1B and NF-H proteins) monoclonal antibody (1:500). 514 (recognizes MAP2A and B proteins) rabbit polyclonal antiserum (1:400).

4.6. Add primary antibodies to cocultures and incubate overnight at 4 °C.

4.7. Next day, discard the antibodies and wash the coverslips with 1x PBS, 3 times, for 5 min.

4.8. Prepare the secondary antibodies in PBS-TS buffer as follows: For SMI-31, anti-mouse Alexa Fluor 488 (1:500). For 514, anti-rabbit Alexa-594 (1:500).

4.9. Incubate cells with the corresponding fluorescent secondary antibodies for 1 h, at RT, in the dark.

4.10. Wash the coverslips with 1x PBS, 3 times, for 5 min, in the dark.

4.11. Finally, mount coverslips with mounting medium (**Table of Materials**) and keep at 4 °C.

NOTE: Whenever necessary, fluorescent nuclei staining with DAPI (4,6-diamidino-2-phenylindole) may be performed. Before mounting, incubate the cells for 10 min in the dark with DAPI (10 µg/mL in 1x PBS). Wash the coverslips 3 times with 1x PBS and finally, mount the coverslips with the mounting medium.

5. Axonal regeneration quantification

NOTE: Samples are quantified under the 40x objective of an epifluorescence microscope. A minimum of 30 pictures should be taken on random fields, with at least 200 neurons, to be quantified for each treatment. Each experiment should be repeated a minimum of three times.

5.1. Quantify the percentage of neurons with axon (SMI31 positive neurite) relative to the total population of RGNs (identified with MAP2A/B 514 positive immunostaining of neuronal body and dendrites).

5.2. Quantify the axonal regeneration index or mean axonal length (µm/neuron). This parameter is defined as the sum of the lengths (in µm) of all identified axons, divided by the total number of counted neurons, whether they presented an axon or not. Axonal length is determined using the plugin NeuronJ of the image software ImageJ (NIH-USA).

5.3. Calculate the mean, standard deviation, and statistical significance using the appropriate software.

REPRESENTATIVE RESULTS:

In this protocol, we present an in vitro model to assay OEG neuroregenerative capacity after neuronal injury. As shown in **Figure 1**, the OEG source is a reversible immortalized human OEG clonal cell line -Ts14 and Ts12-, which derives from primary cultures, prepared from olfactory bulbs obtained in autopsies[15,17,18]. Retinal tissue is extracted from adult rats, digested, and retinal ganglion neurons (RGN) suspension is plated on either PLL-treated coverslips or onto ihOEG monolayers, Ts14 or Ts12. Cultures are maintained for 96 h before they are fixed. Axonal and somatodendritic markers are analyzed by immunofluorescence and axonal regeneration is quantified.

Ts14 OEG identity is assessed by immunostaining with markers described to be expressed in ensheathing glia (**Figure 2**), such as S100 β (2A) and vimentin (2B); GFAP expression was also analyzed to discard astrocyte contamination (2C). As shown, Ts14 expressed S100 β and vimentin but not GFAP.

In the axonal regeneration assay, Ts14 regenerative capacity is compared to Ts12 in RGN-OEG cocultures, using PLL substrate as a negative control (**Figure 3**). Both the percentage of cells with

axons as well as the average length of the regenerated axons were significantly higher in neurons cocultured on Ts14 monolayers, compared to neurons plated on either Ts12 cells or PLL (**Figures 3D,E**). Representative images show a lack of capacity of RGN to regenerate their axons over PLL or Ts12 cells (**Figures 3A,B**), while Ts14 stimulates the outgrowth of axons in RGN (3C).

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of rat retinal ganglion neurons with olfactory ensheathing glia cells coculture, as a model of adult axonal regeneration. Immortalized human OEG (ihOEG) clonal cell lines - Ts12 and Ts14- derived from primary cultures from olfactory bulbs. Retinal ganglion neurons from adult rats are plated on either PLL-treated coverslips (negative control) or onto Ts14 or Ts12 monolayers. Cultures are maintained for 96 h before they are fixed and axonal and somatodendritic markers are analyzed by immunofluorescence. Percentage of neurons with axon and mean axonal length/neuron are quantified to assay RGN axonal regeneration.

Figure 2: Identity of ihOEG cell line Ts14. Immunofluorescence images of Ts14 in culture, labeled with anti-S100 β (panel A, green) and vimentin (panel B, red). GFAP expression (panel C, red) was also analyzed to discard astrocyte contamination. Nuclei are stained with DAPI (blue).

Figure 3: Assay for axonal regeneration in cocultures of OEG lines with adult retinal ganglion neurons (RGNs). (**A–C**) Immunofluorescence images showing somatodendritic labelling with 514 antibodies, which recognizes microtubule-associated protein MAP2A and B, in red, and with axon-specific SMI31 antibody in green, against MAP1B and NF-H proteins. Green arrows indicate RGN axons (SMI31-positive: green) and yellow arrows indicate neuronal bodies and dendrites (514 positive: red and yellow). (**D,E**) Graphs show mean and standard deviation of the percentage of neurons exhibiting axons and the axonal regeneration index, a parameter reflecting the mean axonal length (µM) of axons per neuron. A minimum of 30 pictures (40x) were taken on random fields and quantified for each cell sample. Experiments were performed in triplicate, from three different rats (N = 3), retinal tissue pooled from both eyes, with duplicates for each experimental condition (each glia population tested). Asterisks indicate the statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS: non significance (ANOVA and post hoc Tukey test comparisons between parameters quantified for Ts14 vs Ts12, Ts14 vs PLL, and Ts12 vs PLL).

DISCUSSION:

OEG transplantation at CNS injury sites is considered a promising therapy for CNS injury due to its constitutive pro-neuroregenerative properties[7–9]. However, depending on the tissue source—olfactory mucosa (OM-OEG) versus olfactory bulb (OB-OEG)—or the age of the donor, considerable variation exists in such capacity[26,31,33,36]. Therefore, it is of importance to have an easy and reproducible in vitro model to assay the neuroregenerative capacity of a given OEG sample, before initiating in vivo studies. In this protocol, adult rats' axotomized RGN are cocultured onto a monolayer of the OEG to assay. Subsequent analysis of RGN axonal and somatodendritic markers by immunofluorescence is performed to assess RGN axonal regeneration.

An initial difficulty of the assay is the source of OEG. In this work, we use reversible immortalized human OEG (ihOEG) clonal lines, previously established and characterized by our group[15–18], which provide an unlimited supply of homogeneous OEG. Two of these ihOEG cell lines are Ts14, which maintains the regenerative capacity of the original cultures and Ts12, a low regenerative line that is used as a low regeneration control in these experiments[18] Nevertheless, although technical limitations exist to expand human primary OEG cells, they can also be obtained from nasal endoscopic biopsies—OM—or, in case of OB-OEG, from cadaver donors.

Preparation of monolayer OEG cultures is a crucial procedure, as too many cells could cause the coculture to detach from the plate. Therefore, prior to OEG preparation for the assay, it is recommended that the user determines the optimal number of cells to be plated, depending on their size and division rate.

Another critical issue is the retinal tissue dissociation, after retina dissection. It is necessary to break up the tissue fragments, following incubation in the dissociation mix. If done too vigorously, the cells will be destroyed, but tissue fragments will be left intact if done too weakly. In order to obtain a homogeneous cell suspension, we suggest filling and emptying a Pasteur pipette 10–15 times, with a tip of intermediate diameter, while avoiding bubbling. Pasteur pipettes with wide tips can be narrowed using a Bunsen burner.

To assess the capacity of different glial populations to foster adult neurons' axonal regeneration, we have determined that 96 h is the time interval that best suits the aim because: 1) it is the longest time to maintain the culture alive without disturbing the OEG monolayer; and 2) it is the time needed for neurons to grow axons long enough to reveal differences between the regenerative capacities of different OEG populations or other non-regenerative cells (i.e., fibroblasts[12–18,32,33]). It would certainly be interesting to determine the time course of the regeneration process, as it could provide information about the differential regenerative properties of different glial populations, at shorter times of the co-culture. In our hands, for regenerative glia, the time course between 72–96 h is quite similar for all the cell lines, although axons are shorter at 72 h (unpublished data). Also, 96 h of co-culture, permits to study OEG-dependent mechanisms of adult axonal regeneration[12,14].

During axonal regeneration quantification, it is important to take a minimum of 30 pictures at 400 augments (40x objective), at different random areas of the coverslip, but following the complete axons of the photographed neurons. Therefore, the experimenter must take serial pictures in the chosen areas to measure the real axonal lengths.

Other in vitro approaches have also been developed to evaluate OEG regenerative functions. In these models, OEG is applied to cultures of different neuronal origin and, in response to glial coculture, neurite formation and elongation are assayed[19–27]. However, the regeneration assay relies on embryonic or postnatal neurons, which have an intrinsic plasticity absent from injured adult neurons. This model consisting of adult axonal regeneration in cocultures of OEG lines with adult retinal ganglion neurons (RGNs) overcomes this drawback. In addition, we are dissecting adult retinas, and because we cut optic nerve and axons retract in the process of dissection, we

obtain neuronal bodies clean of myelin, to perform the coculture. This is the difference with other parts of the adult CNS, where myelin can hinder very much with the dissection to obtain clean neurons for the coculture.

Based on the one originally developed by Wigley et al.[28–31], we highlight the following improvements in the protocol. First, the use of neurobasal medium supplemented with B27 as OEG-RGN coculture medium, which allows growth of neuronal cells and positively affects the reproducibility of the experiment. Second, we characterize and quantify axonal regeneration by using a specific marker of the axonal compartment; and third, we use an additional direct parameter, the mean axonal length/neuron, that assesses the axonal growth regenerative potential of OEG.

In summary, we consider that this is a simple, reproducible, time saving, and medium-cost assay, not only useful for assessing the neuroregenerative potential of ihOEG, but also because it can be extended to different sources of OEG or other glial cells. Moreover, it could be used as a valuable proof of concept of the neuroregenerative potential of an OEG or glial sample, before translation to in vivo or clinical studies.

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DISCLOSURES:

The authors have nothing to disclose.

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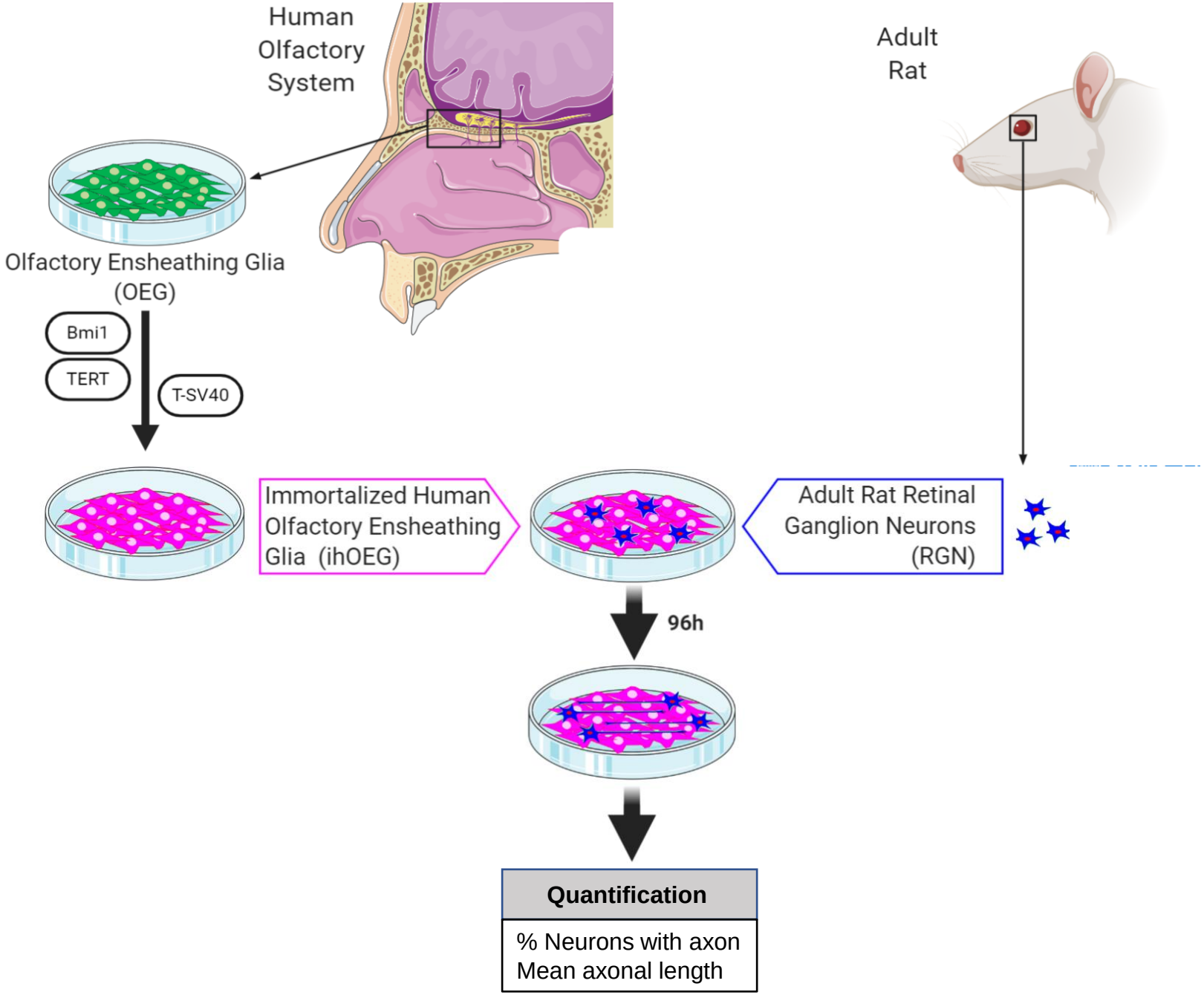
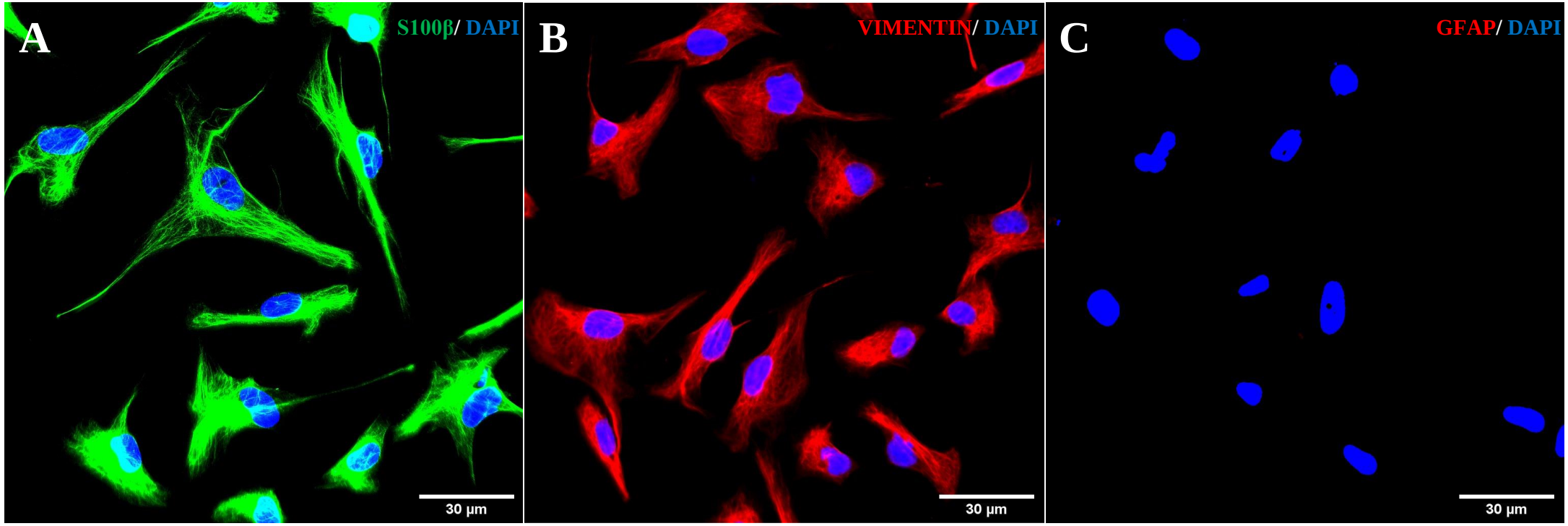
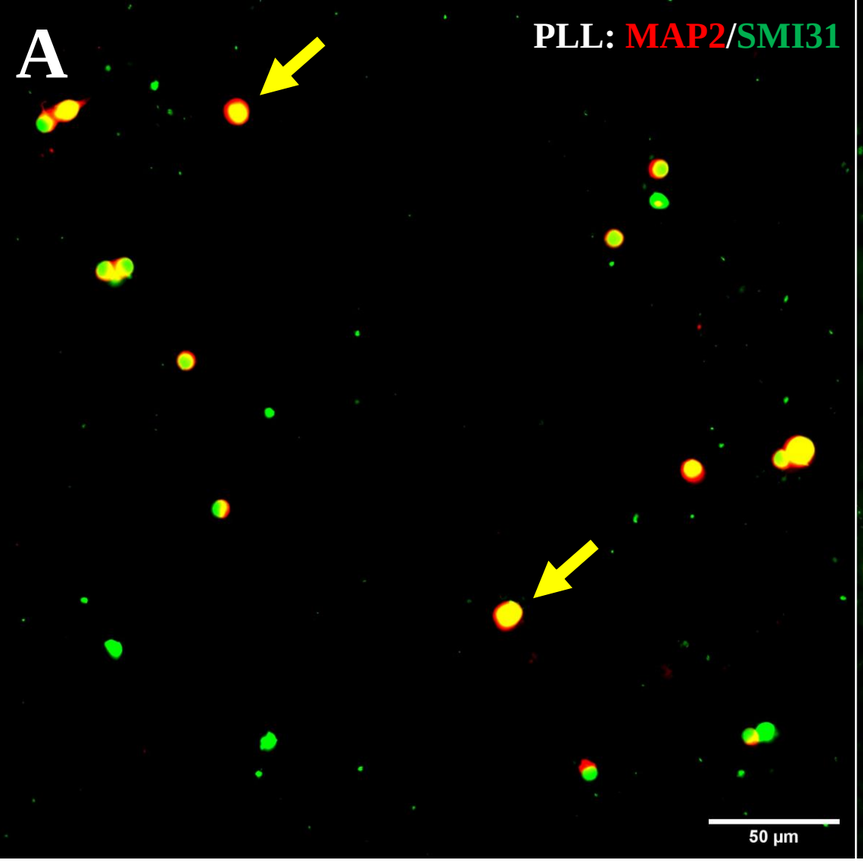
Figure

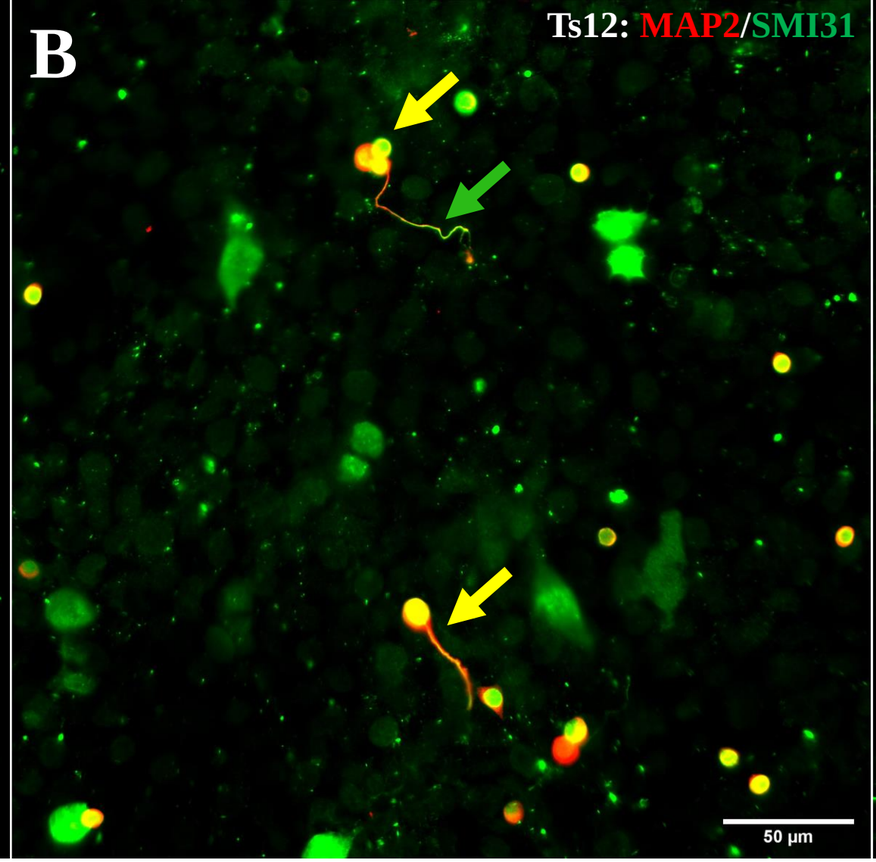


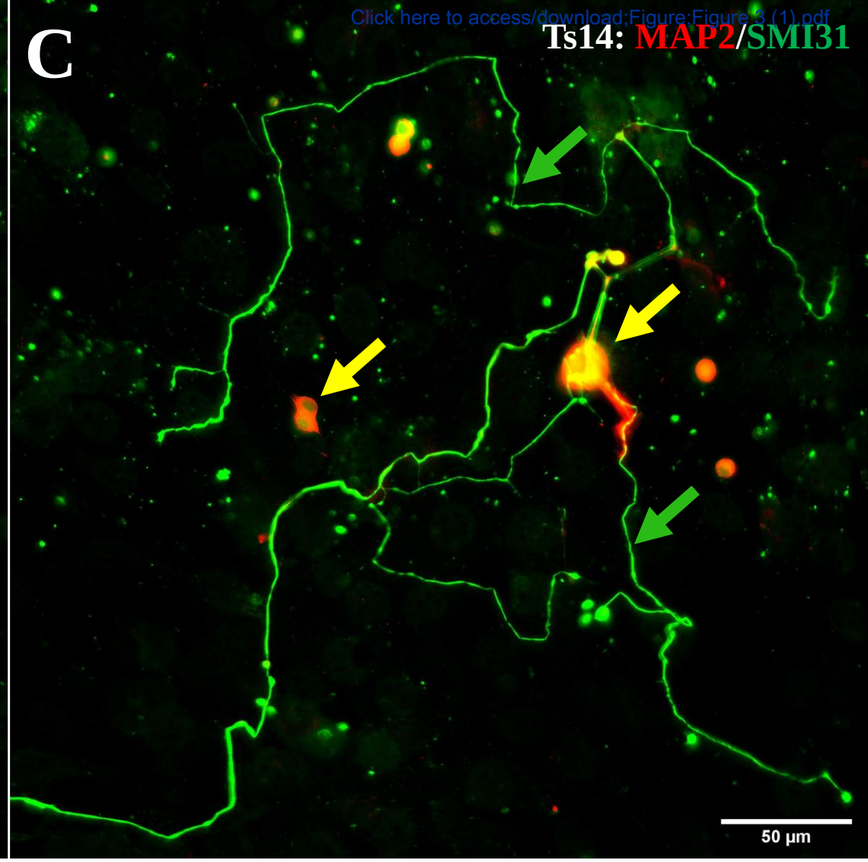
Figure

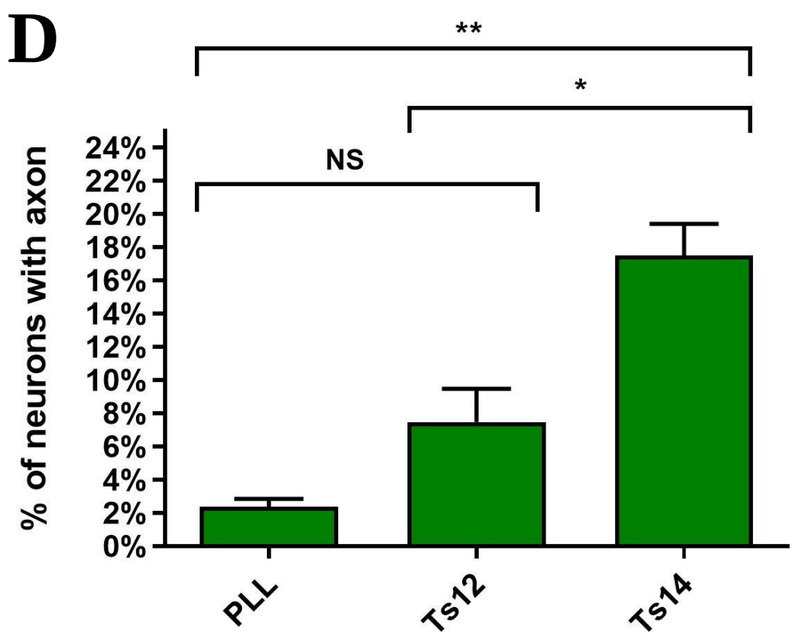
Click here to access/download;Figure;Figure 2 (2).pdf

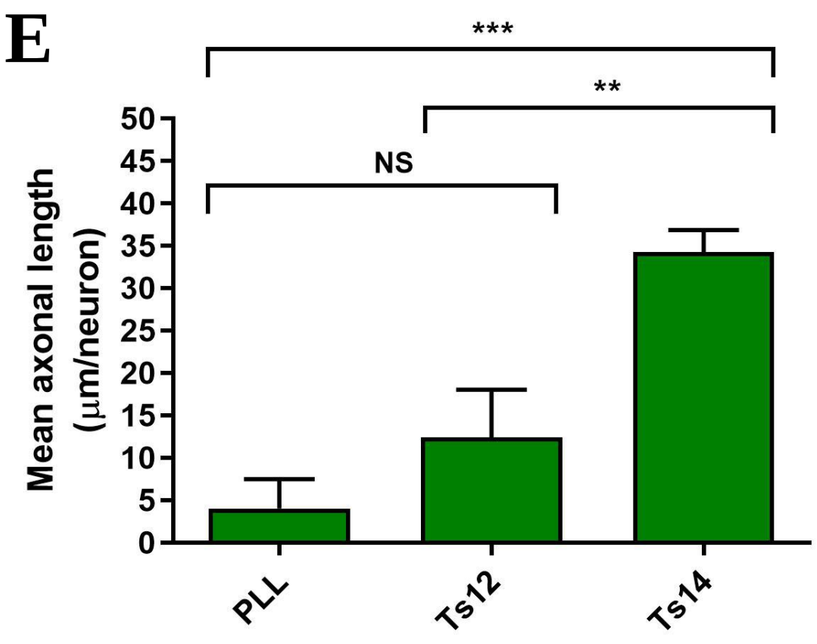












Table

	Final concentration
ME10 medium	
DMEM-F12	
Fetal Bovine Serum (FBS)-Hyclone	10%
Glutamine	2 mM
Pituitary extract	20 µg/mL
Forskolin	2 µM
Penicillin/Streptomycin/Amphotericin B	1X
DMEM-F12-FBS medium	
DMEM-F12	
Fetal bovine serum (FBS)	10%
Neurobasal-B27 (NB-B27) medium	
Neurobasal medium	
B-27 supplement	1X
Glutamine	2 mM
Penicillin/Streptomycin/Amphotericin B	1X

Name of Material/Equipment	Company	Catalog Number	Comments/Description
antibody 514	Reference 34		Rabbit polyclonal antiserum, which recognizes MAP2A and
antibody SMI-31	BioLegend	801601	Monoclonal antibody against MAP1B and NF-H proteins
anti-mouse Alexa Fluor 488 antibody	ThermoFisher	A-21202	
anti-rabbit Alexa Fluor 594 antibody	ThermoFisher	A-21207	
B-27 Supplement	Gibco	17504044	
D,L-2-amino-5-phosphonovaleric acid	Sigma	283967	NMDA receptor inhibitor
DAPI	Sigma	D9542	Nuclei fluorescent stain
DMEM-F12	Gibco	11320033	Cell culture medium
FBS	Gibco	11573397	Fetal bovine serum
FBS-Hyclone	Fisher Scientific	16291082	Fetal bovine serum
Fluoromount	Southern Biotech	0100-01	Mounting medium
ImageJ	National Institutes of Health (NIH-USA)		Image software
L-Glutamine	Lonza	BE17-605F	
Neurobasal Medium	Gibco	21103049	Neuronal cells culture medium
Papain Dissociation System	Worthington Biochemical Corporation	LK003150	For use in neural cell isolation
PBS	Home made		
PBS-EDTA	Lonza	H3BE02-017F	
Penicillin/Streptomycin/Amphotericin B	Lonza	17-745E	Bacteriostatic and bactericidal
Pituitary extract	Gibco	13028014	Bovine pituitary extract
Poly -L- lysine (PLL)	Sigma	A-003-M	

l B.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Here in this protocol paper, the authors describe an interesting method for (1) harvesting and culturing sensory neurons from the ganglionic cell layer of the rat retina, and (2) plating them on top of a mono-layer of immortalized human OEG cell line to assess and quantify the regenerative role of the OEG cells. The method is well described, is easy to understand, easy to follow, and also potentially easy to adapt to evaluate regenerative potential of any other cells that can be grown as a monolayer in 2D culture. The biggest advantage is that the neurons gathered here are fully differentiated, adult cells; this means that they should not be able amplify or grow on their own without explicit aid from the co-cultured cell in the mono-layer.

Major Concerns:

My only major concern is that the description in step 3 (lines 178-204) needs more specific descriptive details at multiple places.

- After cutting the whiskers (in step 3.4), the authors state "Pick the optic nerve with forceps and, with a scalpel, make an incision across the eye". Do they mean to keep the eyeball in the orbital fossa and insert the forceps behind the eye to grip the optic nerve, or to take the eyeball out first and then proceed? Some details should be added between these steps to clarify the dissection process a little more.
We thank the reviewer for this suggestion. We have specified to grip the optic nerve to pull out the eyeball enough to be able to do the procedure, instead of "pick", that can be misinterpreted with taking the eyeball completely out.

- In step 3.8, the instruction is to cut the retina into small pieces with scalpel. Here, the authors should specify how big or small these pieces should be. Is the size significant? As they discuss in line 318, the tissue dissociation is an important step - one can then conclude that the size of the retinal cut-ups might have a significant bearing on the overall yield of the neurons.
Thank you for the comment. We have now added to text that pieces have to be of an approximate size < 1 mm.

- In this same step, the authors may also want to describe if the removal of retinal pigment epithelial layer needs to be performed, and if so, how thoroughly should it be performed.
Thank you to the reviewer for the comment. When using this method (as it will be shown in the video) the whole retina comes out and is isolated from the rest of the eyes' layers. We have now added to the text: "…pull out the retina (orange-like tissue), while the remaining layers of the eye stay inside (including the pigment epithelial layer)."

- In step 3.16 (line 200), it should be specified that the entire content of ME-10 should be replaced by NB-B27, instead of stating "aspire ME-10 medium from the OEG 24 well cell dish" These are the main examples. However, I encourage the authors to give more specific details at other places as well, if deemed appropriate.
We now specify to completely remove the ME-10 medium and to replace it with the NB-B27 medium.

Minor Concerns:

- In line 51 in the abstract, the word "neurogenerative" is not appropriate, since the OEGs do not generate neurons, but rather they help the axons regrow. Hence, I recommend it should be amended to "neuroREgenerative"or "reparative" capacity.
Thank you to the reviewer for warning us of the writing mistake. We have now corrected the word to "neuroregenerative".

- In step 5.1 and 5.2 (lines 238-245) the authors describe the method for quantification of axon regrowth by measuring (1) % of cell bodies with axons, and (2) mean axonal length at a single timepoint of 96 hours after co-culture.
I think if the authors can provide a timeline of sorts here, for example, at 2 hours (enough to allow the cells to settle in the new culture conditions), 2 days (48 hours) and 4 days (96 hours) after plating the co-culture, the results can elucidate the regenerative potential of the test cell population more clearly. I understand that this may require additional lab work, which is why, I request the authors to at least address this point in their discussion. For the validity of the described protocol, this point is not critically important (hence, I have put this as a minor concern).
Thank you to the reviewer for his/her interesting considerations with respect to the time course of the experiment. To assess the capacity of different glial populations to foster adult neurons axonal regeneration, we have determined that 96h is the time interval that best suits our aim, because: 1) it is the longest time to maintain the culture without disturbing the OEG monolayer; 2) it is the time needed for neurons to grow axons long enough to reveal differences between the regenerative capacities of different OEG populations or other non-regenerative cells, i.e. fibroblasts (see references 12-18,32,33). It would certainly be interesting to determine the time course of the regeneration process, as it could provide information about the differential regenerative properties of the different glial populations, at shorter times of the co-culture. In our hands, for regenerative glia, the time course between 72-96 hours is quite similar for all the cell lines, although axons are shorter at 72 hours (unpublished data). Also, 96 hours of co-culture, permits to study OEG-dependent mechanisms of adult axonal regeneration (see references 12,14).
We have now added these considerations to the discussion.

- In lines 290-291, the statement "Experiments were performed in triplicate with duplicated samples for each experimental condition" is confusing. Did the authors do 3 technical replicates AND 2 biological replicates (meaning N = 6) or just 3 technical replicates with the same sample for each replicate? Please rephrase to clarify this. Also clearly state the N value here.
We thank the reviewer for highlighting this ambiguity. We have modified the sentence to clarify the type of replicates that were made, so it now states: "Experiments were performed in triplicate, from three different rats (N=3), retinal tissue pooled from both eyes, with duplicates for each experimental condition (each glia population tested)".

- In the line 326-327, the authors raised a very good point "Therefore, the experimenter must take serial pictures in the chosen areas, to measure the real axonal lengths". I recommend that the authors clarify here, with regards to "the minimum of 30 fields" mentioned in line 289. If the user takes 2 serial pictures at each random point, does the reader then only do 15 random points in this way (15x2 = 30)? Or do the authors recommend that the user should image 30 random Regions of interest (ROI), where each ROI may contain serial pictures of completely imaged axons?
We understand the need to clarify this point. As the reviewer says, we choose random points; if there is in an axon in the chosen field, we take the necessary pictures to follow the axon. In total we take 30 pictures corresponding to, approximately, 10 random regions of interest.
We clarify this point in the manuscript in Step 5 of the Protocol, Figure 3 legend and in the Discussion.

Reviewer #2:

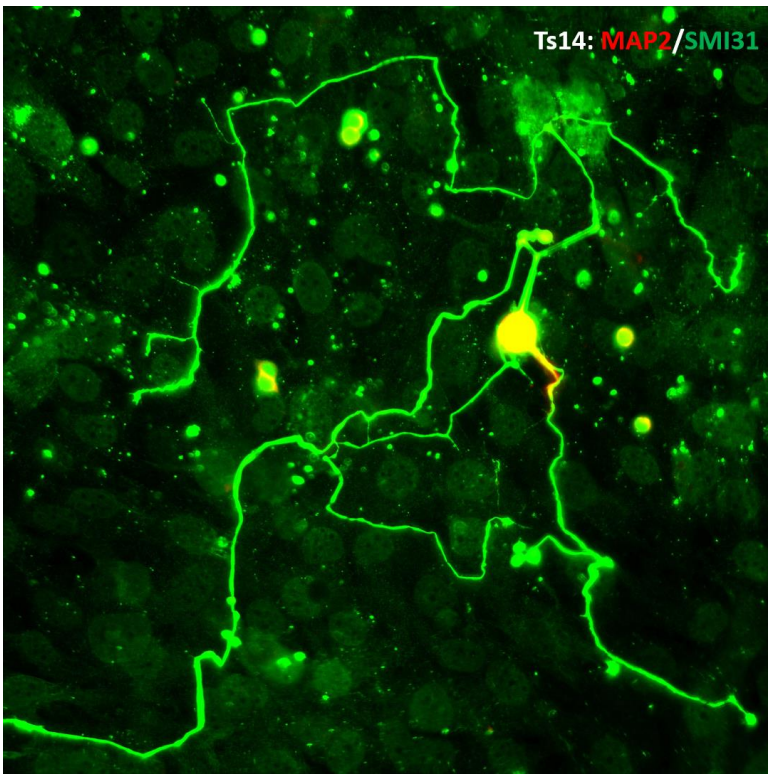
Manuscript Summary:

In this manuscript the authors present an in vitro model system to assay and measure OEG neurogenerative capacity after neural injury. For this, retinas, extracted from adult rats and retinal ganglion neurons (RGN) are cocultured onto the reversibly immortalized human OEG (ihOEG) monolayer. Axonal and somatodendritic markers in RGNs are analyzed after 96 hrs by immunofluorescence and the number of RGNs with axon and the mean axonal length/neuron are quantified. This protocol evaluates the OEG neuroregenerative properties in adult tissue when compared to other existing protocols which rely on embryonic or postnatal neurons with intrinsic plasticity. Axonal regeneration has been quantified as percentage of neurons with axon, respect to total population of RGNs and axonal regeneration index is calculated as mean axonal length per neuron. The authors mention that this protocol is useful for assessing the neuroregenerative potential of ihOEG of different origins. Though the study is presented with some minor modification from previously existing protocols, the authors could introduce readily available reversibly immortalized adult human OEG cells in the protocol. That adds some novelty to this in vitro model system.

Major Concerns:

A major concern is, after co-culture experiment no differential labelling has been done to show that the RGNs are grown on the OEG layer. It could have been good if the authors can explain which are the other contexts in which this protocol can be used as a model of adult axonal regeneration as this is just a short period demonstration of 96 hours.

Thank you to reviewer 2 for the suggestion. We consider that we have a differential labelling because, the markers that are shown, are neuronal markers and they are not present in glial cells. The reviewer may refer to the fact that we do not present a co-labelling, showing glia and neurons in the same pictures. Normally, we just focus in RGN markers to study neuronal regeneration, assessing the existence of a monolayer by contrast of phase microscopy during all the experiment. Below these lines, we have included a picture in which we have increased the intensity and contrast of the images, for the reviewer to be able to perceive the monolayer below the RGN, as the SMI31 marker also labels the nuclei in the sample.



To measure the regenerative property of a cell line, the timing of 96h stablished in this protocol is enough for neurons to be able to regenerate axons, if they are in the correct environment. Nevertheless, the time window could be set up to a shorter or longer period if needed, but 96 hours is ideal to permit regeneration of long axons, without disturbing the OEG monolayer.

As for the contexts of the assay of the neuro-regenerative capacity of OEG, regeneration only occurs in the presence of a monolayer of a regenerative OEG population, not taking place over PLL or over a fibroblast monolayer (please check our references in the manuscript 12-18, 32 and 33).

We added a paragraph in the discussion considering these facts.

Other specific concerns are listed below.

1. Line 122, The approximate quantity of cells to be plated can be mentioned (eg: 3x106 cells/ml).
We understand the suggestion of the reviewer. The number of cells to be plated depends on the size of the cell line, as we mention in lines 316-317, and in the confluence of the cells when they were frozen. Normally, a confluent p-60 has a mean cell number of 7 x 10^5 for Ts14 and 2,5 x 10^6 for Ts12 cell lines.

2. Line 127, the approximate time for each passage can be mentioned though it depends on cell growth.
As reviewer 2 says, each cell line growth rate is different. That is why we assume that the specification of amplifying the cells when they are at 90% confluence, is informative enough for the reader to know when to proceed to the passage of cells. Nevertheless, we have now specified the time for each passage for our lines Ts14 and Ts12 (every 2-3 days).

3. Line 203, A cell strainer with appropriate pore size can be used in this step to remove undigested clumps and debris as those will interfere further culture.
Thank you for this suggestion, but we have verified that it is not necessary the use of the cell strainer if the retina is well processed. Also, the passage of the cell suspension through vial 4 (ovomucoid component of the papain kit) and its posterior centrifugation, avoids the interference of debris with further cell culture.

4. Line 203, it is important to mention, where exactly have to plate the resuspended cells. is this the coculture stage? please clarify
We have now specified this step in the protocol, stating that we add 100 µL of retinal cell suspension, per well of the m24 plate, onto PLL-treated or OEG monolayers-coverslips.

5. Line 213, Is it 0.1% Trypsin? please correct appropriately.
The blocking solution is composed by 0.1% Triton X-100 (to generate pores in the cell membrane) and 1% FBS (to block unspecific attachment of antibodies) in PBS. Trypsin is used to detach the cells.

6. If Ts14 stimulates the outgrowth of axons in RGN, the rate of regeneration can be assessed by keeping Day 1, axons as a control. The axonal regeneration in 24 hr,48hr,72hr and 96 hr can be recorded to confirm that the results are actual axonal regeneration and not the pre-existing axons.
To measure the regenerative property of Ts14 cell line, the timing of 96h stablished in this protocol is enough for neurons to be able to regenerate axons, if they are in the correct environment. Nevertheless, a shorter or longer time window could be set up if needed, but 96 hours is ideal to permit regeneration of long axons, without disturbing the OEG monolayer.
Misquantification of pre-existing axons is ruled out by means of the PLL negative control. At 96 h, RGN over PLL practically do not extend axons, compared to RGN over an OEG cell layer. Even more, the presence of pre-existing axons would be difficult after the process of retinae extraction and papain digestion.

We have now added these considerations to the discussion.

7. Will the papain treatment simulate neuronal injury? Please add a statement about this.
We thank the reviewer for raising this possible concern. When the retina is extracted from the eye, the RGNs are axotomized and, consequently, injured. The papain is used to disaggregate the retina tissue and then obtain the RGN population. In fact, it is a soft treatment.

8. line 204, is there a medium change necessary as the directly plated digested tissue pellet will contain lot of cell debris which negatively affect the survival of the attached cells? This step is usually part all the tissue digestion protocols for cell culture.
The kit eliminates the debris, specifically the vial 4 centrifugation (steps 3.14 and 3.15) and accordingly, the retinal cell suspension is clean and ready to be directly plated in the corresponding wells.

9. How it will be ruled out that the retinal digest contain only RGN, clarify
When the retinal extraction is performed, the retinal tissue is easily recognizable because of its orange-like color). Thus, the remaining layers stay inside the eye after cutting it and extracting lens and vitreous humor. Although other neuronal cell types (amacrine, horizontal or bipolar neurons) may be present in the retinal digest, regenerative RGN are easily distinguished by their characteristic morphology.

10. Figures and Figure legends are not numbered and titled properly, please make appropriate changes
After revision, we have not seen any mistakes in the figures or figure legends.

11. Only Ts14 OEG identity not Ts12 is assessed by immunostaining, explain
The aim of Ts14 characterization in this paper is to show the readers the characteristic OEG markers. Ts12 and Ts14 cell line's identities are already published by our group (references 17 and 18 in the manuscript) so we do not consider it is necessary to assess the identity of both lines in this work.

12. Why no nuclear staining has done for neuronal cells to make sure they are cells itself, explain
Antibody 514 positive cells are neuron cells because this marker labels the somatodendritic compartment, (see Figure 3). Precisely we use this labelling to identify and count neurons, and then we use SMI31 to detect their axons.

<u>Minor Concerns:</u>

It is desirable to change the title as "Coculture of axotomized rat retinal ganglion neurons with olfactory ensheathing glia, as an invitro model of adult axonal regeneration"
We thank the reviewer for the suggestion. We have now specified <u>in vitro</u> in the title.

Reviewer #3:

Manuscript Summary:

The Methods Article by Portela-Lomba and co-authors presents an in vitro model to assess olfactory ensheathing glia (OEG) neuro-regenerative capacity, which is based on a co-culture of axotomized adult retinal ganglion neurons (RGN) on OEG monolayer and subsequent study of axonal growth. This manuscript described the protocol in which the immortalized human OEG (ihOEG) was cultured as a monolayer, and retinal ganglion neurons (RGN) were co-cultured on the OEG monolayer. While the manuscript is well-written, the following points should be considered to improve the accuracy of the protocol.

Major Concerns:

1. In the Summary and Abstract, and other sections, the authors stated that this model could measure OEG neuro-generative capacity after neural injury. However, this protocol were not involved in the neural injury. Actually, the author only measured the neural growth on the OEG monolayer.
We thank the reviewer for raising this possible misunderstanding. Nevertheless, when the retina is extracted from the eye, the RGNs are axotomized and, consequently, injured. In our negative control PLL we demonstrate that adult RGN cannot regenerate by itself, and it is the OEG monolayer which provides the appropriate environment for RGN to be able to extend axons.

2. The authors only showed the identity of ihOEG cell line Ts14 in Figure 2. The identity of ihOEG cell line Ts12 should also be provided.
As answered previously to reviewer 2, the aim of Ts14 characterization in this paper is to show the readers the characteristic OEG markers. Ts12 and Ts14 cell line's identities are already published by our group (references 17 and 18 in the manuscript) so we do not consider it is necessary to assess the identity of both lines in this work.

3. The step-by-step labels of the procedure are imperfect and should be re-labeled. e.g., Line 188 and 189, Step 3.1.1 and Step 3.1.2 are not found. So, the '1. On the day of the assay, prepare the following media:' might be labeled as 3.1, and the '1.1 A p60 cell culture dish with 5mL of cold EBSS' might be 3.1.1.
We have followed the Instructions for Authors from the journal editors. The protocol must be a numbered list: step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol.
Below we provide a screen capture of a recent JOVE paper showing the protocol structure, accepted by the journal editors (Fujiki R, Lee JY, Jurgens JA, Whitman MC, Engle EC. Isolation and Culture of Oculomotor, Trochlear, and Spinal Motor Neurons from Prenatal Islmn:GFP Transgenic Mice. J Vis Exp. 2019;(153):10.3791/60440. doi:10.3791/60440):

Protocol

All experiments utilizing laboratory animals were performed in accordance with NIH guidelines for the care and use of laboratory animals and with the approval of the Animal Care and Use Committee of Boston Children's Hospital.

1. Setting Up Timed Matings Prior to the Dissection

1. To generate prenatal embryonic mice for motor neuron harvest, weigh each female mouse and set up timed mating between adult *Isl*^{MN}*:GFP* transgenic mice 11.5 days prior to the day of neuron isolation. For the purpose of developing this protocol, 129S1/C57BL/6J *Isl*^{MN}*:GFP* mice, aged 2−9 months, were used and timed mating was set up in the evening.
2. Examine female mice for vaginal plugs the following morning. Consider the date on which the plug is identified as embryonic day (E) 0.5.
3. Weigh female mice and examine for pups using ultrasound (see **Table of Materials**) between E8.5−11. Check for the signs of successful mating.
    1. Confirm the successful mating by detecting weight gain in female mice (usually >1.5 g on E9.5 if there are more than 5−6 embryos).
    2. Visually confirm embryos under ultrasound. Embryos are easily detectable by ultrasound after E9.5. Ultrasounds are conducted only on females that have gained weight because they are more often pregnant than those that do not.
    NOTE: Female mice can gain weight for reasons other than pregnancy, so weight gain alone is not a reliable indicator of pregnancy. Ultrasound confirmation prevents unnecessary sacrifice of females that are not pregnant but is not crucial if unavailable.

Minor Concerns:

1. Overall, this manuscript needs English expression and grammar checked. e.g., Line 76 growth signals might be "growth factors", dish might be plate or vice versa. In addition, Line 151, 80.000 Ts14 cells should be 80,000 Ts14 cells; Line 171, mix 2,7 mL vial 1 might be mix 2.7 mL vial 1; and so on.

We have now changed the words "growth signals" in line 76 to growth factors. Regarding to the synonyms plate and dish, both are correct and usable.

We have now unified the format of the decimal signs.

2. The Legal Unit of Measurement should be unified. e.g., minutes and min, hours and h; 200 g and 200 x g; ml and mL, and so on.

We have now unified the format of the Units of Measurement.

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Author(s): María Portela-Lomba, Diana Simón, Cristina Russo, Javier Sierra, María Teresa Moreno-Flores

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