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Retinal organoid induction system for derivation of 3D retinal tissues from human pluripotent stem cells

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TITLE:

Retinal Organoid Induction System for Derivation of 3D Retinal Tissues from Human Pluripotent Stem Cells

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SUMMARY:

Here we describe an optimized retinal organoid induction system, which is suitable for various human pluripotent stem cell lines to generate retinal tissues with high reproducibility and efficiency.

ABSTRACT:

Retinal degenerative diseases are the main causes of irreversible blindness without effective treatment. Pluripotent stem cells that have the potential to differentiate into all types of retinal cells, even mini-retinal tissues, hold huge promises for patients with these diseases and many opportunities in disease modeling and drug screening. However, the induction process from hPSCs to retinal cells is complicated and time-consuming. Here, we describe an optimized retinal induction protocol to generate retinal tissues with high reproducibility and efficiency, suitable for various human pluripotent stem cells. This protocol is performed without the addition of retinoic acid, which benefits the enrichment of cone photoreceptors. The advantage of this protocol is the quantification of EB size and plating density to significantly enhance the efficiency and repeatability of retinal induction. With this method, all major retinal cells sequentially appear and recapitulate the main steps of retinal development. It will facilitate the downstream applications, such as disease modeling and cell therapy.

INTRODUCTION:

Retinal degenerative diseases (RDs), such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are characterized by the dysfunction and death of photoreceptor cells, typically lead to irreversible vision loss without effective ways to cure[1].The mechanism underlying these diseases is largely unknown partially due to lack of human disease models[2]. Over the past

decades, significant advances have been accomplished in regenerative medicine through stem cell technology. Many researches, including ourselves, have shown that human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), can differentiate into all types of retinal cells, even mini-retinal tissues through various differentiation approaches[3-11], providing a huge promise in disease modeling and cell therapy[12,13,14].

However, the induction process from hPSCs to retinal cells is highly complicated, time-consuming, and of low repeatability, which requires researchers with rich experience and high skills. During the complex and dynamic induction process, a number of factors will impact yield of retinal tissues[15-17]. Also, different induction methods often vary considerably in timing and robust expression of retinal markers, which might confound the sample collection and data interpretation[3]. Therefore, a straightforward protocol of retinal differentiation from hPSCs with step-by-step guidance would be in demand.

Here, based on our published studies[18-21], an optimized retinal induction protocol to generate retinal organoids (ROs) with rich cone photoreceptors from hPSCs is described, which does not require the supplement of retinoic acid (RA). This protocol focuses on the description of the multi-step method to generate neural retina and RPE. EBs formation is the essential part of the early induction stage. Both size and plating density of EBs are quantitatively optimized, which scientifically enhances the yield of retinal tissues and promotes the repeatability. In the second part of the induction, Optic vesicles (OVs) self-organize in the adherence culture and ROs form in the suspension culture, the time courses and efficiencies of this part vary considerably in different hPSC lines. The maturation and specification of retinal cells in ROs mainly occur in the late stage of induction. Without the addition of RA, mature photoreceptors with both rich cone and rod can be produced.

The purpose of this protocol is to quantitatively describe and detail each step for inexperienced researcher to repeat. Various hPSC lines have been successfully induced into ROs by this protocol with robust yield of cone-rich retinal tissues and high repeatability. HPSCs-derived ROs with this protocol can recapitulate the main steps of retinal development *in vivo*, and survive for long-term, which facilitate the downstream applications, such as disease modeling, drug screening, and cell therapy.

PROTOCOL:

1. Culture and expansion of hPSCs

1.1 HPSC culture

1.1.1 Coat two wells of a 6-well plate with extracellular matrix (ECM, hESC-qualified Matrix). Prepare 50 mL of an ECM solution containing 8–12 μg/mL of ECM in Dulbecco's Modified Eagle's Medium (DMEM). In 50 mL of DMEM, add 1 mL of the thawed ECM stock solution (50x). Add 1 mL of the ECM solution to each well of a 6-well plate. Incubate it for 1 h in an incubator at 37 °C

and 5% CO_2.

1.1.2 Prepare hPSC maintenance medium (MM) according to the manufacturer's instruction.

1.1.3 Pre-warm MM at room temperature (RT) for 30 min.

1.1.4 Thaw a cryogenic vial of hPSCs (hiPSCs or hESCs) (about 1 x 10^6) from a liquid nitrogen tank by incubation in a water bath at 37 °C for 30 s.

1.1.5 Take out the vial, carefully disinfect it using a 75% disinfection alcohol spray. Put it in a biosafety cabinet.

1.1.6 Transfer the cell suspension from the vial to a 15 mL tube, add 5 mL of pre-warmed MM drop by drop to the tube using a 5 mL pipette. Meanwhile, gently shake the tube to blend the hPSCs .

1.1.7 Centrifuge the tube at 170 x *g* for 5 min. Remove most of the supernatant using a 1 mL pipette carefully and leave behind about 50 µL supernatant to avoid losing the cells.

1.1.8 Add 1 mL MM to the tube, resuspend the pellet by gently pipetting up and down once or twice with a 1 mL pipette.

NOTE: Single cells of hPSCs are difficult to survive. Small cell clumps with 3–5 cells are preferred to keep hPSCs growing in colonies.

1.1.9 Remove ECM from the pre-coated wells (step 1.1.1), add 1.5 mL MM to each well, and then distribute 0.5 mL cell suspension per well.

1.1.10 Gently shake the plate to distribute the hPSCs uniformly, put the plate in an incubator at 37 °C and 5% CO_2. Do not move the plate for at least 24 h to promote the cell adherence.

1.1.11 Change MM every other day and passage the hPSCs when the confluence has reached about 80%.

1.2 Passaging of hPSCs

NOTE: The maintenance of the undifferentiated state in hPSCs is quite critical for further applications. Under the adherent conditions, hPSCs grow in colonies with well-defined border. The cells should be passaged when the confluence of hPSCs reaches about 80%.

1.2.1 Observe the cells under a microscope, the clearly visible differentiated cells (<5%) should be marked and mechanically removed before passaging.

1.2.2 Prepare the ECM-coated plate as described in step 1.1.1.

1.2.3 Pre-warm MM and 1x phosphate buffer saline (PBS) without Ca^{2+} and Mg^{2+} at RT.

1.2.4 Pre-warm 0.5 mM EDTA (in 1x PBS) solution in a water bath at 37 °C.

1.2.5 Remove the medium from the culture plate using a vacuum-aspiration system, add 1mL 1x PBS in each well to wash the cells using a 1 mL pipette and repeat twice.

1.2.6 Add 1 mL EDTA solution per well to dissociate the hPSCs in a cell culture incubator at 37 °C and 5% CO_2 for 5 min. Do not exceed the recommended incubation time in order to avoid dissociation to single cells.

1.2.7 Take out the plate and check for the detachment of cells under the microscope. The confluent hPSCs loosen up and each cell border can be seen, but the cells cannot easily come off by gently shaking the cell plate.

1.2.8 Remove the EDTA solution with a 1 mL pipette, add 1 mL of MM to stop the dissociation. Gently pipette the hPSCs once or twice with a 1 mL pipette to resuspend the cells. (Don't need centrifuge to collect cells.)

NOTE: If most of cells come off from the plate after incubation of EDTA, cells can be collected by centrifuge.

1.2.9 Remove ECM from the pre-coated wells (step 1.2.2), add 1.5 mL MM per well.

1.2.10 Transfer 150–200 µL of cell clumps to each well. Generally, hPSCs can be passaged at a ratio of 1:6. For example, cells from one well of a 6-well plate can be distributed to six new wells.

1.2.11 Gently shake the plate to distribute the hPSCs uniformly and culture the hPSCs in the incubator at 37 °C and 5% CO_2 for at least 24 h without touching the plate.

1.2.12 Change MM every other day as described in step 1.1.

2 Retinal differentiation from hPSCs

NOTE: When the colonies reach ~80% confluence (**Figure 1B**), they can be guided to differentiate into retinal organoids following the protocol schematized in **Figure 1A**. To ensure the hPSCs have high quality and good yield, regularly evaluate the pluripotency with molecular markers such as OCT4 or NANOG using IFC or QPCR. HPSCs should be discarded if differentiate cells account for more than 5% of the total cells. Check for mycoplasma contamination with a mycoplasma detection kit according to the manufacturer's instructions. Use only mycoplasma-free hPSCs as mycoplasma can alter the differentiation capability of hPSCs.

2.1 Prepare media and reagents

2.1.1 Prepare neural induction medium (NIM) by mixing the following: 500 mL Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, 1:1), 5 mL 1% N2 supplement, 0.5 mL 0.1% heparin (2 mg/mL in 1x PBS), and 5 mL 1% MEM Non-Essential Amino Acids (NEAA).

2.1.2 Prepare retinal differentiation medium (RDM) containing 300 mL DMEM/F-12, 200 mL DMEM basic, 10 mL 2% B27 supplement, 5 mL 1% Antibiotic Antimycotic, and 5 mL 1% MEM NEAA.

NOTE: Both NIM and RDM are not filtered but the sterility test is performed. Take out 1 mL medium and add it into a 35 mm dish, culture for 3–7 days in an incubator at 37 °C and 5% CO_2. The media can be stored at 4 °C and should be used within 2 weeks to ensure the activity of the components.

2.1.3 Prepare 10 mM Blebbistatin (1,000x) in DMSO. Add 1,710 µL DMSO to dissolve 5 mg Blebbistatin to obtain 10 mM stock solution (1,000x), aliquot at 10 µL/tube, and store at -20 °C.

NOTE: All media and reagents should be warmed at RT for 30 min before use, unless otherwise mentioned.

2.2 Embryoid body (EB) formation

2.2.1 On day 0 (D0), initiate the differentiation. Take out one well of hPSCs in a 6-well plate, which grows to an ~80% confluence. Collect the cells with EDTA dissociation solution as described in steps 1.2.1 to 1.2.6.

2.2.2 Remove the EDTA solution, add 1 mL MM containing 10 µM Blebbistatin to stop cell dissociation, collect the cells with a 1 mL pipette. The size of cell clumps is one of the key factors impacting the yield of EBs. Approximately, five cells per clumps are preferred to produce the right size of EBs on D5 to D7.

NOTE: This is a key step. Do not pipette the cells too many times since single cells of hPSCs are hard to form EB-like aggregates.

2.2.3 Transfer the cell suspension (about 2 x 10^6 cells) to a 100 mm ultralow-attached petri dish and add 9 mL MM containing 10 µM Blebbistatin to the dish.

2.2.4 Gently shake the dish twice to distribute the cells uniformly, put the dish in the incubator at 37 °C and 5% CO_2.

2.2.5 On D1, after the cells are cultured for at least 24 h, take out the dish and observe it under the microscope. A large number of the small cell aggregates will be formed spontaneously by this time (**Figure 1C**).

2.2.6 Prepare 12 mL of mixture with MM and NIM at 3:1 ratio (9 mL MM and 3 mL NIM) in a 15 mL tube.

2.2.7 Transfer the cell cultures to a 15 mL centrifuge tube by a 10 mL pipette perpendicularly, add 10 mL of the pre-warmed mixture to the dish.

2.2.8 Centrifuge the tube at 60 x *g* for 3 min to collect the aggregates, remove the supernatant using a 5 mL pipette and leave behind about 500 µL to avoid losing cells.

2.2.9 Add 2 mL of the mixture to the tube, transfer the suspension to the same dish (step 2.2.7).

2.2.10 Gently shake the dish to make the cell aggregates uniformly distributed, put back the dish in the incubator.

2.2.11 On D2, prepare 12 mL of a new mixture with MM and NIM at 1:1 ratio (6 mL MM and 6 mL NIM) in a 15 mL tube. Change cell medium with the fresh prepared mixture by repeating the steps from 2.2.5 to 2.2.10.

2.2.12 On D3, change cell medium with 15 mL NIM as described above. Culture the cells for at least 5 days under the suspension conditions.

NOTE: During D1 to D3, the medium should be changed each day, providing enough nutrition. Since D3, NIM can be changed every other day. Also, EBs can be divided into several dishes to provide abundant nutrition.

2.3 Seed the EBs

NOTE: On D5 to D7, choose an appropriate time point to plate the EBs on the ECM-coated dishes according to the size of EBs. Approximate 200 µM in diameter of EBs is appropriate for the retinal differentiation. In general, one well of hPSCs in a 6-well plate can produce about 300 to 1,000 EBs. The variation of EB yielding is varied by the hPSC lines.

2.3.1 On D4, prepare ECM-coated dishes for EBs adherent culture. Add 5 mL ECM to each 100 mm tissue culture dish (surface treated), put them in the incubator overnight.

2.3.2 On D5, remove ECM from the pre-coated dishes, add 10 mL pre-warmed NIM to each dish.

2.3.3 Take out the dish containing EBs. Check the quality of EBs under the microscope and ensure that they are quite bright and round in shape. The size of the EBs is approximate 200 µM in diameter. Collect all EBs in a 15 mL tube. Transfer the EBs from the dishes to a 15 mL tube by a 5 ml pipette. Let the EBs settle down for 5 min. Remove the most supernatant, leaving behind about 2 mL medium.

2.3.4 Distribute the EBs into the coated dishes containing 10 mL NIM drop by drop with a 1 mL

pipette. Seed the EBs at the density of approximate 2–3 EBs per cm^2. For example, add about 120–180 EBs into a 100 mm dish. To roughly judge the EB number, take out one drop of EBs suspension onto a coverslip, count the number of EBs under the microscope.

NOTE: Plating density of EBs is one of key factor impacting the efficiency of retinal induction. The density can be also adjusted by each hPSC line.

2.3.5 Gently shake the dishes to distribute the EBs uniformly. Put them in the incubator at 37°C and 5% CO_2. Don't move the dishes for at least 24 h to enhance the adherence of EBs (Caution).

2.4 Induction of optic vesicles (OVs) and retinal pigment epithelium (RPE) in adherent conditions

NOTE: After EBs seeded on the ECM coated surface, hPSCs can develop OV-like structures consisting of neural retinal domain surrounded by the RPE domain, which can be observed as early as D20 after differentiation. In this protocol, specific growth factors or signaling molecules are not required to guide the hPSCs into the retinal fate except the addition of the supplements of N2 and B27 in the media.

2.4.1 On D8–D9, take out the dishes and observe the EBs under the microscope. All EBs will be attached and spread out on the dishes (**Figure 1D**). Add 10 mL fresh NIM to each 100 mm dish containing 10 mL old medium. Put them back in the incubator.

NOTE: Do not remove the old medium.

2.4.2 On D12, change half of the medium with NIM using a 10 mL pipette. Keep the culture in the incubator.

2.4.3 On D16, remove all NIM from the dishes using a vacuum-aspiration system. Add 20 mL RDM to each dish. Keep culturing in RDM and change half of the medium every other day.

2.4.4 During D10–D30, observe the morphological changes of the cells twice a week under a microscope and evaluate the efficiency of retinal differentiation.

NOTE: Since D10, eye field (EF) domains are self-organized in the peripheral zones of adherent EBs. The OV-like structures appear between D20 to D25, gradually protrude from the dish, and self-form an optic cup, which is surrounded by the pigmented RPE (**Figure 1E**). The OVs can be easily recognized with the bright, refractive, and thick NR ring.

2.5 Detach and culture OVs and RPE in suspension to obtain retinal organoids (ROs)

2.5.1 On D28–D35, most of OVs appear in the dishes. Use a Tungsten needle or a needle with 1 mL syringe to mechanically detach the morphologically identifiable OVs along with the adjacent RPE. Culture them in suspension.

NOTE: The appearance and yield of OVs and RPE vary widely in different hPSC lines. So, the time point of detaching OV and RPE is flexible. Obvious OVs with the adjacent RPE can be detached, and then moved to a low adhesive culture dish containing RDM. Keep culturing the rest of the cells until all OVs and RPEs are lifted up.

2.5.2 Put 50–60 OVs into each 100 mm low attachment culture dish containing 15 mL RDM for the ROs formation (**Figure 1F**).

2.5.3 Change RDM every 2–3 days until D42, when the ROs are well round-shaped.

3 Retinal development and maturation

NOTE: In this protocol, serum is required to keep the ROs grow and mature for long-term culture.

3.1 Retinal lamination and specification in ROs

3.1.1 Prepare 10 mL of 100 mM taurine (1,000x) in 1x PBS. Weigh 125 mg taurine, dissolve in 10 mL of 1x PBS. Filter the solution with a 0.22 μm syringe filter. Aliquot at 500 μL/tube, and store at -20 °C.

3.1.2 Prepare retinal culture medium 1 (RC1). Mix the following components: 300 mL DMEM/F-12, 200 mL DMEM basic, 50 mL fetal bovine serum,10 mL 2% B27 supplement, 5 mL 1% Antibiotic Antimycotic, 5 mL 1% MEM NEAA, 0.5 mL 100 μM taurine, and 5 mL 2 mM L-alanyl-L-glutamine.

3.1.3 Prepare retinal culture medium 2 (RC2) containing 450 mL DMEM/F-12, 50 mL fetal bovine serum, 5 mL 1% N2 supplement, 5 mL 1% Antibiotic Antimycotic, 0.5 mL 100 μM taurine, and 5 mL MEM NEAA.

NOTE: The RC1 and RC2 are not filtered. Take out 1 mL medium, add it into a 35 mm dish, culture it for 3–7 days in the incubator at 37 °C and 5% CO_2, to ensure the sterility before use. The medium can be stored at 4 °C and should be used within 2 weeks to ensure the activity of the components. All media and reagents should be pre-warmed at RT for 30 min before use.

3.1.4 On D42, switch the culture medium from RDM to RC1.

3.1.5 Tilt the dishes at about 30˚ and settle the ROs down for 30 s. Remove the old RDM with a 10 mL pipette leaving behind about 1 mL medium to avoid losing ROs. Add 15 mL fresh RC1 to each dish.

3.1.6 Gently shake the dishes to distribute the ROs uniformly. Put the dishes back in the incubator. Change the whole medium twice a week thereafter.

3.1.7 During D50–D90, select out high quality of ROs for long-term culture, which were round-

shaped with thick and bright NR. Place 30–40 ROs in a 100 mm low attachment dish with 20 mL RC1, change the whole medium twice a week.

3.1.8. For the long-term suspension culture of ROs, pipette the ROs to avoid RO-RO reattaching using a pipette. Transfer ROs to new culture dishes once a month to avoid ROs sticking to the surface of the dishes.

NOTE: Under the suspension culture conditions, ROs are round-shaped, comprising a bright and thick NR ring attached with more or less RPE at one side. Laminated neural retina develop and retinal cell subtypes sequentially appear with retinal ganglion cells first generated, followed by photoreceptor cells, amacrine cells, and bipolar cells.

3.2 Human photoreceptor maturation with enrichment of cones in ROs

3.2.1 After D90, switch the medium from RC1 to RC2, which is suitable for photoreceptor maturation.

3.2.2 Change the medium as described in steps 3.1.4–3.1.5.

NOTE: Under this culture condition, ROs can grow for long-term (**Figure 1G**), up to D300 tested. Retinal cells in ROs become mature, and all cell subtypes of neural retina, including muller glial cells, rods and cones are also acquired. Without any addition of RA, cone photoreceptors are also rich in ROs.

REPRESENTATIVE RESULTS:

The retinal induction process in this protocol mimics the development of human fetal retina. To initiate the retinal differentiation, hPSCs were dissociated into small clumps and cultured in suspension to induce the formation of EBs. On D1, the uniformed cell aggregates or EBs formed (**Figure 1C**). The culture medium was gradually transitioned into NIM. On D5, EBs were plated onto the ECM-coated culture dishes. Cells gradually migrated out of the EBs (**Figure 1D**). From D10, eye fields self-organized in the peripheral zone of adherent EBs. On D16, the induction medium was replaced by RDM. Afterwards, the NR domains gradually formed, protruded from the dish, and self-formed OV-like structures surrounded by the RPE cells (**Figure 1E**). During D28–D35, OVs along with the adjacent RPE were lifted up with a sharp needle and cultured in suspension. Under the suspension culture conditions, ROs self-formed comprising neural retina (NR) attached with more or less RPE sphere at one side (**Figure 1F**) and could survive and mature overtime as long as FBS were added to the medium.

As retinal differentiation and specification progressed, hPSCs produced all major retinal cell subtypes sequentially. The subtypes of neural retina gradually lined up in layers, mimicking the architecture features of native human retina (**Figure 2A–G**). Retinal ganglion cells (RGCs) were first generated from retinal progenitors and accumulated in the basal side of NRs. Photoreceptor cells located in the apical side, while amacrine cells, horizontal cells, bipolar cells, and muller glial cells all located in the intermediate layer of NRs.

With this protocol, ROs developed into the highly mature photoreceptors with both rods and cones (**Figure 2G–I**). Photoreceptors increased rapidly in the developing out nuclear layer (**Figure 2G**) after week 8, and gradually matured from week 17 onward. From week 21, all subtypes of photoreceptors including rods, red/green cones, and blue cones can be detected in ROs. Both rich rods and cones can be obtained in this induction protocol without any addition of RA throughout the whole differentiation process.

FIGURE AND TABLE LEGENDS:

Figure 1: Induction and morphological features of retinal organoids from hPSCs. (**A**) Schematics of retinal induction from hPSCs. (**B**) A typical colony of hPSCs (10x). (**C**) EBs on D1 (4x). (**D**) On D7, plated EBs were attached and spread out on the dishes (4x). (**E**) On D25, the optic vesicle like structures (OVs) formed and protruded from the dish (indicated by the red circle), surrounded by pigmented RPE (4x). (**F**) Retinal organoids self-formed after OVs were lifted up and cultured in suspension conditions (the arrows pointed NR and RPE (4x). (**G**) A retinal organoid comprising NR (red arrow) and RPE (black arrow) on D180 (4x). Scale bars = 200 μm.

Figure 2: Subtypes of retinal cells were sequentially detected in three-dimensional retinal tissues. Example images of major retinal cell types expressing specific markers by immunofluorescent staining. (**A–B**) Retinal progenitor cells expressed Ki67 (**A**) and VSX2 (**B**). (**C**) Islet1 positive retinal ganglion cells located in the basal side of the neural retina. (**D**) Amacrine cells positive for AP2α. (**E**) Muller glial cells positive for SOX9. (**F**) PKCα positive bipolar cells. (**G**) Recoverin positive photoreceptor cells. (**H**) Rhodopsin positive rod photoreceptors. (**I**) L/M-opsin positive cone photoreceptors. Scale bar = 50 μm.

DISCUSSION:

In this multi-step retinal induction protocol, hPSCs were guided step by step to gain the retinal fate, and self-organized into retinal organoids containing laminated NR and RPE. During the differentiation, hPSCs recapitulated all major steps of human retinal development *in vivo*, from EF, OV, and RPE, to retinal lamination, generating all subtypes of retinal cells, including retinal ganglion cells, amacrine cells, bipolar cells, rod, and cone photoreceptors, and muller glial cells in a spatial and temporal order. The recapitulation of retinal development would benefit the downstream applications, such as retinal disease modeling.

A couple of protocols have been established to generate retinal organoids from hPSCs[3–10,13–20]. According to the culture conditions, the protocols can be classified into 2D, 3D, and the combination of 2D and 3D approaches[9,13]. The 2D approaches[6,10,22] mean all the induction process occurs in the adherent culture conditions, generating retinal cells without architecture from hPSCs. In contrast, the 3D approaches[7,11,23] mean all the induction process is under the suspension culture conditions, yielding organized retinal tissues. For example, Sasai, Y. et al.[7,24] reported a SFEBq method (serum-free floating culture of embryoid-body-like aggregates with quick re-aggregation) to guide ESCs to differentiate into optic cups in suspension culture. Using the multi-steps 3D ap-

proaches[8,11,18,20,25] including this protocol, hPSCs have been induced toward retinal fates and organoids under both adherent and suspension culture conditions.

To induce hPSCs to neural retinal fate, a series of exogenous factors have been added to the media in many protocols. For example, Lamba, et al.[26] added a combination of noggin (an inhibitor of the BMP pathway) and Dickkopf-1 (dkk1, an antagonist of the Wnt/β-catenin signaling pathway) and insulin-like growth factor-1 (IGF-1) to direct ESCs to an anterior neural fate. Osakada, et al.[6] added DAPT (a Notch signaling pathways inhibitor) and Left-Right Determination Factor A (a WNT signaling pathways inhibitor) to obtain rod and cone photoreceptor precursors. Kuwahara, et al.[27] and Capowski, et al.[3] added BMP4 for brief, early exposure of hPSCs culture to improve OV production. By contrast, this optimized retinal induction protocol is simple and low cost without requiring extrinsic signaling modulators except the basic supplement of N2 and B27.

Retinoic acid (RA) plays an important role in retinal development and photoreceptor determination[28-30]. Most of the protocols were developed with the supplement of RA (0.5-1 μM) in certain periods. Our studies have demonstrated that two high concentration of RA or too long period of RA treatment result in rod-rich photoreceptors but inhibit cone differentiation[8,18]. However, in this optimized protocol, RA is not added to the culture media throughout the whole differentiation process[18], promoting the production of cone photoreceptors, which is responsible of human day-time vision and color vision and required for cell replacement of RD treatment. Although some studies reveal thyroid hormone signaling directs cone subtypes in mice and human retina[31,32], the regulator for cone commitment is still unclear[33]. In Kim, et al.[34] and Lowe, A., et al.[35], the long-term culture also without any exogenous retinoic acid generated cone-rich retinal organoids, which is consistent with this optimized protocol.

The key point of this protocol to grasp is to make high quality of EBs and to seed EBs appropriately. Cells grow fast during early EBs suspension culture. Medium should be changed every day and be enough to provide abundant nutrition. The size of EBs, approximate 200 um in diameter, is appropriate for the retinal differentiation. The planting density of EBs at 2–3 EBs per cm^2 is suitable for most of hPSC lines. The most advantage of this optimized protocol is the quantification of EB size and plating density to significantly enhance the efficiency and repeatability of retinal induction. We have clearly described all the steps in detail, which largely helps the inexperienced researchers to learn and repeat the retinal induction.

In addition, retinal induction efficiency largely depends on the quality and differentiation potency of the hPSCs[36,37]. Different hPSCs have different efficiencies. Some hPSC lines indeed are under poor efficiency, which might be due to the reprogramming methods, somatic cells, and so on. This protocol has been confirmed suitable for various hPSCs to obtain 3D retinal organoids and the RPE, including various hESCs and hiPSCs reprogrammed from fibroblasts, blood, and urine cells[18,20,21]. In general, with this protocol described above, one well of hPSCs (about 80% confluence) in a 6-well plate can generate about 1,000 EBs, yielding roughly 200 ROs. Therefore, this protocol with high efficiency is suitable for large-scale production of retinal organoids and benefits the downstream applications including basic and translation study.

In summary, the optimized retinal induction protocol is simple and low cost with high repeatability and efficiency, offers promising personalized models of retinal diseases and provides abundant cell source for cell therapy, drug screening, and gene therapy test.

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DISCLOSURES:

Xiufeng Zhong is the patent inventor related to the generation of retinal cells from human pluripotent stem cells.

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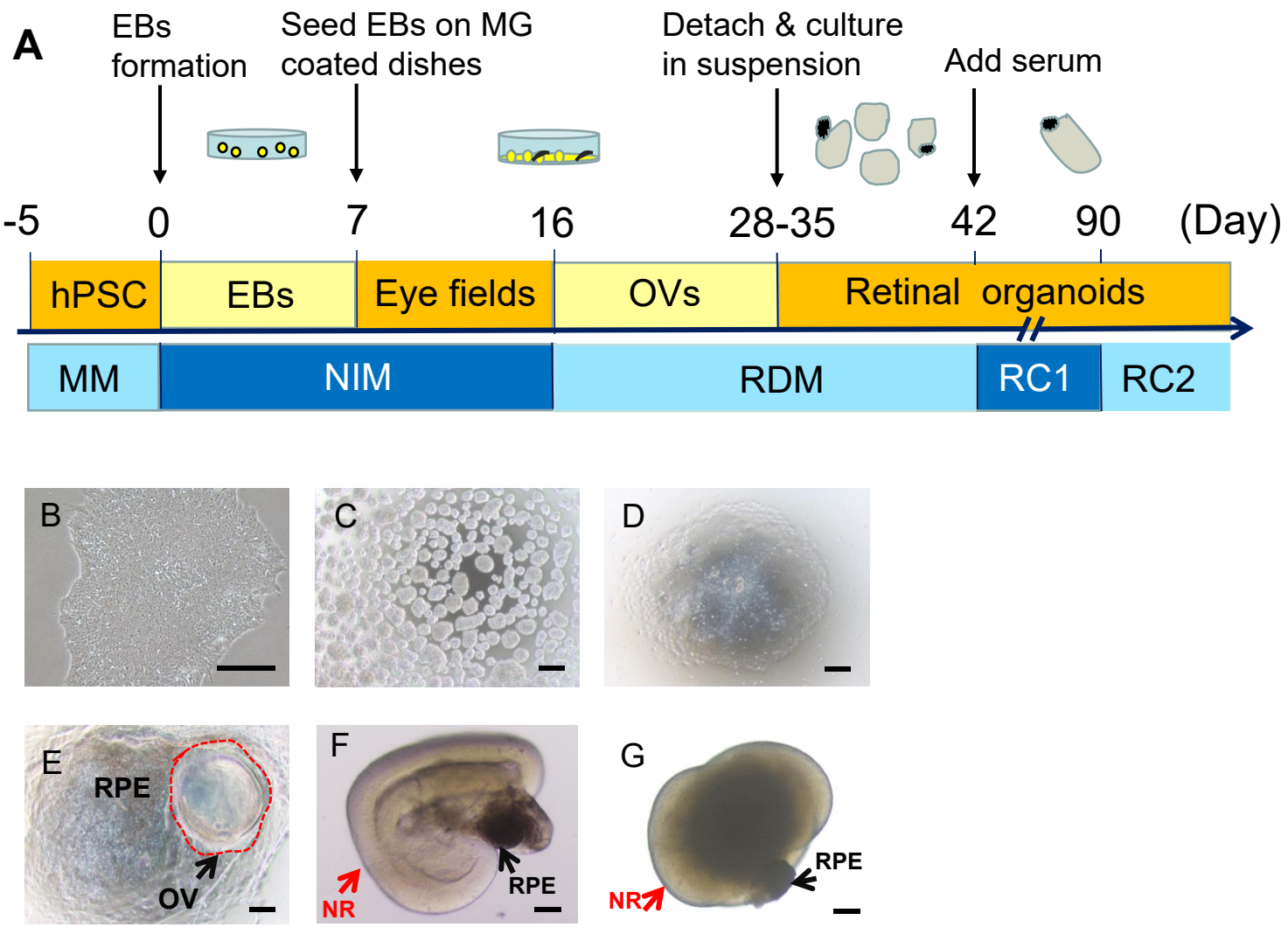
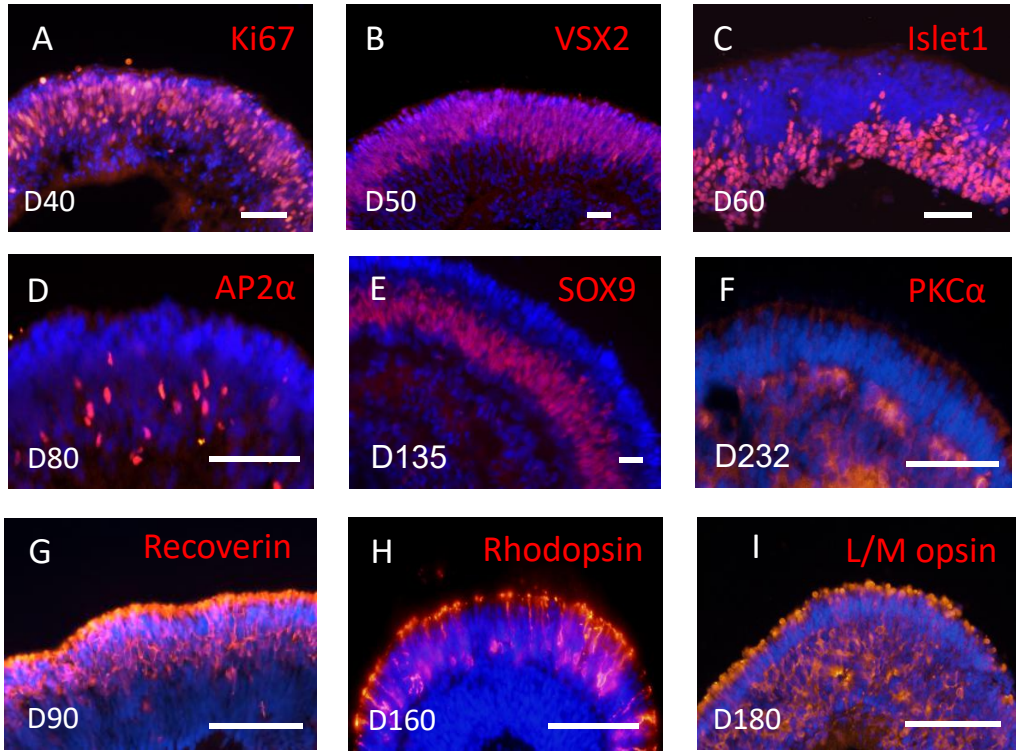
Figure

Click here to access/download;Figure;JOVE protocol Figure 1-2.25.pdf



Figure



Materials

Name	Company	Catalog Number
(−)-Blebbistatin	Sigma	B0560-5mg
1 ml tips	Kirgen	KG1313
10 ml pipette	Sorfa	3141001
100 mm Tissue culture	BIOFIL	TCD000100
100 mm Tissue culture	Falcon	353003
15 ml Centrifuge tubes	BIOFIL	CFT011150
35 mm Tissue culture dishes	Falcon	353001
5 ml pipette	Sorfa	313000
50 ml Centrifuge tubes	BIOFIL	CFT011500
6 wells tissue culture plates	Costar	3516
Anti-AP2α Antibody	DSHB	3b5
ANTIBIOTIC ANTIMYCOTIC 100X	Gibco	15240062
Anti-ISL1 Antibody	Boster	BM4446
Anti-Ki67 Antibody	Abcam	ab15580
Anti-L/M opsin Antibody	gift from Dr. jeremy	/

Anti-PAX6 Antibody	DSHB	pax6
Anti-rabbit 555	Invitrogen	A31572
Anti-Recoverin Antibody	Millipore	ab5585
Anti-Rhodopsin Antibody	Abcam	ab5417
Anti-sheep 555	Invitrogen	A21436
Anti-SOX9 Antibody	Abclonal	A19710
Anti-VSX2 Antibody	Millipore	ab9016
B-27 supplement W/O VIT A（50X）	Gibco	12587010
Cryotube vial	Thermo scientific-NUNC	375418
DAPI	DOJINDO	D532
Dimethyl sulphoxide(DMSO) Hybri-max	Sigma	D2650-100ML
DMEM	Gibco	C11995500BT
DMEM /F12	Gibco	C11330500BT
EDTA	Invitrogen	15575-020
FBS	NATOCOR	SFBE
Filter	Millipore	SLGP033RB

GlutaMax, 100X	Gibco	35050061
Heparin	Sigma	H3149
Matrigel， 100x	Corning	354277
MEM Non-Essential Amino Acids Solution (100X)	Gibco	11140050
mTeSR1	STEM CELL	85850
N2 supplement	Gibco	17502048
Phosphate-buffered saline (PBS) buffer	GNM	GNM10010
Taurine	Sigma	T0625
Ultra-low attachment culture dishes 100mm petri dish, low-attachment	Corning	CLS3262-20EA

Comments
ROCK-inhibitor
1 ml
Pipette
100 mm Petri dish
100 mm Petri dish
Centrifuge tubes
35 mm Petri dish
Pipette
Centrifuge tubes
Culture plates
Primary antibody
Antibiotic-Antimycotic
Primary antibody
Primary antibody
Primary antibody

Primary antibody
Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor
Primary antibody
Primary antibody
Donkey anti-Sheep IgG (H+L) Secondary Antibody, Alexa Fluor
Primary antibody
Primary antibody
Supplement
1.8 ml
4',6-Diamidino-2-phenylindole dihydrochloride; multiple suppliers
Multiple suppliers
Medium
Medium
0.5 M PH 8.0
Serum
0.22μm，sterile Millex filter

L-alanyl-L-glutamine
2 mg/ml in PBS to use
Extracellular matrix (ECM)
MEM NEAA
hPSCs maintenance medium (MM)
Supplement
Without Ca+、Mg+,PH7.2±0.1 0.1M
Supplement
Petri dish

Response to Editor and Reviewers

Dear editor and reviewers:

We would like to thank you and your colleagues for considering our manuscript entitled "**An optimized retinal organoid induction system for derivation of 3D retinal tissues from human pluripotent stem cells**". We thank the editor and reviewers for the thoughtful comments and constructive advice which have dramatically improved the manuscript. We have revised the whole manuscript carefully and hope the issues mentioned by the reviewer have been clarified. All revisions have been clearly marked in the revised manuscript with change tracking. A point to point response is attached to the editor's and reviewers' comments.

Please fine blow the point-to-point response to editor's and reviewers' comments.

Editor：

Editorial comments 1: *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

Response: We thank the editor for the suggestion and have thoroughly proofread the manuscript along with stylistic improvements.

Editorial comments 2: *Please revise the following lines to avoid previously published work: 55-56, 65-66, 112-113, 243 -244.*

Response: We thank the editor for pointing out these sentences had similarity with previously published work and we have revised them to avoid previously published work.

Editorial comments 3: *Please revise the text to avoid the use of any personal*

pronouns (e.g., "we", "you", "our" etc.).

Response: We thank the editor for the suggestion. We have checked the original manuscript and revised them unless we think it is necessary to keep.

Editorial comments 4: *JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.*
For example: Matrigel, mTeSR1, GlutaMAX, etc.

Response: We thank the editor for the suggestion. We have replaced all the commercial language to the generic terms and reference all the commercial products in the Table of Materials.

Editorial comment 5: *Expand the Introduction to include all the citations.*

a) *A clear statement of the overall goal of this method*

b) *The rationale behind the development and/or use of this technique*

c) *The advantages over alternative techniques with applicable references to previous studies*

d) *A description of the context of the technique in the wider body of literature*

e) *Information to help readers to determine whether the method is appropriate for their application*

Response: We thank the editor for the suggestion. We have expanded the introduction including all the citations.

For "a) A clear statement of the overall goal of this method", we have added this

part in Line 68-70, “The purpose of this protocol is to quantitatively describe and detail each steps for inexperienced researcher to repeat. Various hPSC lines have been successfully induced into ROs by this protocol with robust yield of cone-rich retinal tissues and high repeatability.”

For “b) The rationale behind the development and/or use of this technique”, we have explained in Line 41-46, “Over the past decades ..... providing a huge promise in disease modeling and cell therapy”.

For “c) The advantages over alternative techniques with applicable references to previous studies”,we have clearly summarized in Line 56-66, “Here, based on our published studies ..... Without the addition of RA, mature photoreceptor of with both rich cone and rod can be produced.”.

For “d) A description of the context of the technique in the wider body of literature”, we have added more literatures to described the background in the first paragraph of the introduction (Reference 1-14).

For “e) Information to help readers to determine whether the method is appropriate for their application”, we have explained the mainly three parts of the protocol, the purpose and the downstream application in paragraph 3-4 of the introduction. It will help readers to understand the protocol and determine whether the protocol is suitable for them.

Editorial comment 6: *Please define the confluency at which the cells are passaged.*

Response: We apologize for the confusion. We have revised the description into “The cells should be passaged when the confluence of hPSCs reaches about 80%.”.

Editorial comment 7: *Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be*

visualized to tell the most cohesive story of the Protocol.

Response: We thank the editor for the suggestion. We have revised the protocol steps to a one-line space between each steps and highlight the protocol steps for video in yellow.

Editorial comment 8: *Please revise the Discussion to include:*

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

Response: We thank the editors for the suggestion. We have expanded the discussion to include all the parts.

For "a) Critical steps within the protocol", we have adequately discussed in paragraph 5 of the discussion.

For "b) Any modifications and troubleshooting of the technique", we have described in paragraph 4, 5 of the discussion.

For "c) Any limitations of the technique", we have mentioned and discussed in paragraph 6 of the discussion.

Editorial comment 9: *Please title case and italicize journal titles and book titles in the Reference section. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.*

Response: We thank the editor for the suggestion. We have revised the references in the order by using Endnote in JOVE style.

Editorial comment 10: *Figure 1A: Please remove the commercial names from the*

figure and replace them by generic names (e.g., mTeSR1).

Response: We thank the editor for the suggestion. We have deleted the commercial name: mTeSR1, and change it to the generic name: MM (hPSCs Maintenance medium).

Editorial comment 11: *Figure 1B-1G: Please include the details of the magnification in the Figure Legends.*

Response: We thank the editor for the suggestion. We have added the details of the magnification for each figure legends.

Editorial comment 12: *Please sort the Table of Materials in alphabetical order.*

Response: We thank the editor for the suggestion. We have revised the Table of Materials in alphabetical order.

Reviewer 1

Manuscript Summary.

Lack of evidence of improvement: *The authors in this manuscript develop a modified protocol to generate human retinal organoids with a combination of 2D and 3D method. While the protocol is interesting, there is no comparison data presented to show if it is a better protocol than the previously, but the authors call it an optimized protocol, it is inconclusive. There is no evidence for the generation of retinal ganglion cells, amacrine cells, horizontal cells, etc. Probably, it is in the previously publishes. However, on the day 180 (1G), we did not see any out segments, therefore, it is even not an improved method.*

Response: We thank the reviewer for the suggestion. Since the journal focused on the protocols. We mainly described the detail steps of the protocol for the readers to repeat. We have published detail comparison data in the previous original papers [18-20].In the protocol, we described the yield of EBs and ROs as "One well of hPSCs (about 80% confluence) in a six-well plate can generate about 1000 EBs, yielding roughly 200 ROs". This protocol can generate retinal tissues with high efficiency.

Also we have added more results to show the retinal subtype cells in Figure 2.

Comment 1: *The authors always allege this protocol is low-cost, but there are still expensive N-2 and B-27 supplements in the medium and there is also no comparison information.*

Response: We thank the reviewer for the suggestion. In our original manuscript, we used the word "low-cost" caused this optimized protocol is simple and low cost without requiring extrinsic signaling modulators, such as noggin, Dickkopf-1, IGF-1, DAPT and so on. But N-2 and B-27 supplements are the main and important basic supplements for neural development. They are also necessary in our protocol. We have revised and reduced the use of "low-cost" to avoid confusing.

Comment 2: *Which steps are the main steps in the retinal development? The protocol just provides quite limited data of the early stage and inferior late stage.*

Response: We thank the reviewer for the suggestion. In our protocol, the main steps in the retinal development is in step 3, retinal development and maturation. During the suspension culture of ROs, the retinal subtype cells gradually formed, retinal specification and maturation.

After the modification, we added many NOTEs to help the reader to understand the main steps of this protocol. We added more results of retinal development in Figure

2.

Comment 3: *what is the 1x Matrigel in 1.1.1?*

Response: We thank the reviewer for the suggestion. We have replaced Matrigel to ECM to avoid the use of the commercial language, revised the preparation of ECM clearly. We described it as “Prepare 50 mL of an ECM solution containing 8-12 μg/mL of ECM in Dulbecco's Modified Eagle's Medium (DMEM). In 50 mL of DMEM, add 1 mL of the thawed ECM stock solution (50x).” .

Comment 4: *hPSCs are more than iPSCs and ESCs in 1.1.3*

Response: We thank the reviewer for the careful comment, we have corrected it to “hPSCs (hiPSCs or hESCs)”.

Comment 5:*1000g is not suitable for cells in 1.1.6*

Response: We thank the reviewer for the suggestion. We are sorry for the mistake we made. We have corrected the centrifugal force from “1000 x g” to “170 x g” and also corrected another same mistake in step 2.2.8, to “60 x g”.

Comment 6: *how much is the most, using vacuum-aspiration system is easy to lose all the cells, what is the volume that should be left and how to deal with the left medium in 1.1.6?*

Response: We thank the reviewer for the suggestion. We have changed it to “Remove most of the supernatant by a 1 mL pipette carefully and leave behind about 50 μL supernatant to avoid losing the cells.”

Comment 7: *There are so many repeats "watch the cells under the microscope" in*

1.2.6, 1.2.7, 2.1.4, 2.1.7, 2.1.8, 2.1.11, 2.2.3. Besides, it is better to replace "watch" to "detect", "observe", or "examine"

Response: We thank the reviewer for the comments for the improvements of wording, we have carefully checked and revised them.

Comment 8: *why the mediums should be prepared for a week before use in 2.1.1, it should be explained?*

Response: We thank the reviewer for the suggestion. We have corrected it and added the detail of preparation of the medium in step 2.1.1-2.1.2 as "NOTE: Both NIM and RDM are not filtrated, but performed the sterility test. Take out 1 mL medium, add it into a 35 mm dish, culture for 3-7 days in an incubator at 37 °C and 5% CO2. The media can be stored at 4 °C and should be used within 2 weeks to ensure the activity of the components.".

Comment 9: *2.1.7 is a totally repeat*

Response: We thank the reviewer for the suggestion. We have revised to avoid repeating.

Comment 10: *Point 3.1.2, how to define the high quality?*

Response: We thank the reviewer for the suggestion. We have added the description of the ROs to define the high quality as "select out high quality of ROs for long-term culture, which were round-shaped with thick and bright NR."

Comment 11:*There is no evidence, even no reference for Laminated neural retina develop and retinal cell subtypes sequentially appear with retinal ganglion cells first generated, followed by photoreceptor cells, amacrine cells and horizontal cells." in*

3.1.2 ;

Response: We thank the reviewer for the suggestion. We have added more results to show the generation of the retinal subtype cells and the laminated neural retina in Figure 2.

Comment 12: *In point 3.2.2, figure 1G is not a good example for the "well-laminated"*

Response: We thank the reviewer for the suggestion. We have revised and added suitable results in Figure 2 to show the laminated NR.

Comment 13: *More evidence is required for the generations of different retinal cells, for the laminated organoids.*

Response: Please refer to our response to ***Comment 11***.

Reviewer 2

Comment 1: *On line 88, why is it recommended to prepare the media 1 week in advance, when some of the components, like B27, are only stable for 1 week at 4°C after supplemented?*

Response: We thank the reviewer for the suggestion. We have corrected it and added the detail of preparation of the medium in step 2.1.1-2.1.2 as “NOTE: Both NIM and RDM are not filtrated, but performed the sterility test. Take out 1 mL medium, add it into a 35 mm dish, culture for 3-7 days in an incubator at 37 °C and 5% CO2. The media can be stored at 4 °C and should be used within 2 weeks to ensure the activity of the components.” .

Comment 2: *The decision of not adding retinoic acid is understandable to obtain a higher cone-enrich retina, but a citation to Kim et al 2019 (PMID: 31072937) would be a good addition to support this. In addition, it would be good to give the readers the option of adding retinoic acid to the culture media to generate more rod photoreceptors. The authors could also discuss the work of Kaya et al 2018 (PMID: 31814692) which suggests 9-cis-retinal can accelerate photoreceptor differentiation.*

Response: We thank the reviewer for the suggestion. The reference Kim et al 2019 (PMID: 31072937) that reviewer provided to us is a good addition to support the opinion of the influence of RA for retinal development. We have learned the relevant literatures and gained a lot. We have added this part in the paragraph 4 of the discussion.

We focused on our retinal induction protocol and discussed the relevant study in this manuscript, so we haven't discussed the supplement of RA to generate more rod photoreceptor.

Comment 3: *Authors discuss that no external factors need to be added to achieve high numbers of retinal organoids. While some factors are mentioned, bone morphogenetic protein 4 (BMP4) has been shown to improve the morphology and number of neural retina formation (Capowski et al 2018, PMID: 30567931). It would be good to also comment on this factor.*

Response: We thank the reviewer for the suggestion. The reference that reviewer provided to us is a good addition to discuss the different signaling modulators for retinal development, we have added this part to benefit the discussion in the paragraph 3.

Comment 4: *Finally, the protocol would benefit with an additional figure including staining of the differentiated retinal cell types: ganglion cells, amacrine cells, horizontal cells, bipolar cells, Müller glia cells, rod and cone photoreceptors, as the*

authors do in Figure 5 of Luo et al 2018 (PMID: 29999566).

Response: We thank the reviewer for the suggestion. We have added more results to show the retinal cell subtypes in Figure 2.