Journal of Visualized Experiments

Directed Induction of Retinal Organoids from Human Pluripotent Stem Cells

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62298R2
Full Title:	Directed Induction of Retinal Organoids from Human Pluripotent Stem Cells
Corresponding Author:	Zi-Bing Jin Beijing Institute of Ophthalmology Beijing, Beijing CHINA
Corresponding Author's Institution:	Beijing Institute of Ophthalmology
Corresponding Author E-Mail:	jinzb502@ccmu.edu.cn
Order of Authors:	Xiao Zhang
	Zi-Bing Jin
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please specify the section of the submitted manuscript.	Medicine
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Beijing,China
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)

TITLE:

Directed Induction of Retinal Organoids from Human Pluripotent Stem Cells

AUTHORS AND AFFILIATIONS:

Xiao Zhang[1], Zi-Bing Jin[1*]

[1] Beijing Institute of Ophthalmology, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing Ophthalmology & Visual Science Key Laboratory, Beijing, China.

*Corresponding author: Zi-Bing Jin (jinzb502@ccmu.edu.cn)

Email of Co-author:

Xiao Zhang (zhang_xiao26@foxmail.com)

SUMMARY:

Using a self-organizing method, we develop a protocol with the addition of COCO that could significantly increase the generation of photoreceptors.

ABSTRACT:

Retinal cell transplantation is a promising therapeutic approach, which could restore the retinal architecture and stabilize or even improve the visual capabilities to the degenerated retina. Nevertheless, progress in cell replacement therapy presently faces the challenges of requiring an off-the-shelf source of high quality and standardized human retinas. Therefore, an easy and stable protocol is needed for the experiments. Here, we develop an optimized protocol, based on a self-organizing method with the use of exogenous molecules and reagent A as well as manual excision to generate the three-dimensional human retina organoids (RO). The human Pluripotent Stem Cells (PSCs)-derived RO expresses specific markers for photoreceptors. With the addition of COCO, a multifunctional antagonist, the differentiation efficiency of photoreceptor precursors and cones is significantly increased. The efficient use of this system, which has the benefits of cell lines and primary cells, and without the sourcing issues associated with the latter, could produce confluent retinal cells, especially photoreceptors. Thus, the differentiation of PSCs to RO provides an optimal and biorelevant platform for disease modelling, drug screening and cell transplantation.

INTRODUCTION:

Pluripotent stem cells (PSCs) are characterized by their self-renewal and ability to differentiate into all kinds of somatic cells. Thus, organoids derived from PSCs have become an important resource in regenerative medicine research. Retinal degeneration is characterized by the loss of photoreceptors (rods and cones) and retinal pigment epithelium. Retinal cell replacement could be an encouraging treatment for this disease. However, it is not feasible to obtain human retinas for disease research and therapy. Therefore, retinal organoids (ROs) derived from PSCs, which effectively and successfully recapitulate multi-layered native retinal cells, are beneficial for basic and translational research[1-3]. Our research focuses on RO differentiation to provide sufficient and quality cells for studying retinal degeneration[4].

Methods for differentiating ROs are continuously emerging, with three-dimensional (3D) suspension differentiation pioneered by the Sasai laboratory in 2012[5]. We introduced the CRX-tdTomato tag in the human embryonic stem cells (hESCs) to specifically track the photoreceptor precursor cells and modified the method with the addition of COCO, a multifunctional antagonist of the Wnt, TGF-β, and BMP pathways[6]. COCO has been shown to efficiently improve the differentiation efficiency of photoreceptor precursors and cones[6,7].

Altogether, by modifying the classical differentiation method, we have developed an accessible protocol to harvest abundant photoreceptor precursors and cones from human ROs for analyzing the retinal disease associated with the photoreceptors through laboratory investigations and for further clinical application/transplantation.

PROTOCOL:

This study was approved by the institutional Ethics Committee of Beijing Tongren Hospital, Capital Medical University. H9 hESCs were obtained from the WiCell Research Institute and genetically engineered to tdTomato-tagged cell line.

1. Generation of human ROs

1.1. Culture the hESCs under feeder-free conditions.

1.1.1. Coat one well of a 6-well plate with 1 mL of 0.1 mg/mL reagent A (**Table of Materials**) at 37 °C for at least half an hour following the manufacturer's instructions. Thaw an aliquot of $1x10^6$ hESCs.

NOTE: Prepare 3 mL of pre-warmed reagent B (**Table of Materials**) and transfer the cryopreserved cells (1 mL) into 3 mL of fresh medium. Do not pipette the hESCs to single cells.

1.1.2. Centrifuge at 200 x *g* for 5 min and remove the supernatant.

1.1.3. Seed the cells into the reagent A-coated plate with 2 mL of reagent B and change 2 mL of reagent B daily. Passage cells when reaching approximately 80% confluency (usually around 4 days).

1.2. Day 0

1.2.1. Dissociate hESCs to a single-cell suspension using medium I (**Table 1**). Prepare medium I by mixing the following: 20% (v/v) KnockOut serum replacement (KSR), 0.1 mM MEM non-essential amino acids solution (NEAA), 1 mM pyruvate, 3 µM IWR-1-endo (IWR1e), 30 µM COCO, 100 U/mL penicillin, 100 µg/mL streptomycin (PS), 0.1 mM β-mercaptoethanol, and Glasgow's Eagle's minimal essential medium (GMEM).

NOTE: Before the start of the dissociation, prepare medium I and transfer 12 mL of medium I with 20 µM Y-27632 into a 10 cm Petri dish, 500 µL of reagent C (**Table of Materials**) containing 0.05 mg/mL of reagent D (**Table of Materials**) and 20 µM Y-27632 in a 1.5 mL tube. Perform the above steps in the dark, as component IWR1e in medium I is light-sensitive.

1.2.2. Wash the hESCs with pre-warmed 1x Dulbecco's phosphate-buffered saline (DPBS) buffer.

1.2.3. Add the prepared 500 µL reagent (in the 1.5 mL) tube and incubate the hESCs for 3.5 min at 37 °C and 5% CO_2.

1.2.4. Detach the cells by flicking the side and bottom of the plate for a few seconds and add 500 µL of prepared medium I from the Petri dish into the hESC plate.

NOTE: Prepare a 1.5 mL tube with 900 µL of 1x DPBS and use a hemocytometer for cell counting.

1.2.5. Harvest the cells in a new 1.5 mL tube and pipette the cell suspension up and down and then take out 100 µL from the tube and then add into the tube with 900 µL of DPBS for cell counting.

1.2.6. Disperse the left 900 µL cell suspension and then dilute the cells with prepared medium I in the Petri dish to 9 x 10^4 cells/mL.

NOTE: The whole volume of the medium is 12 mL; 1.08 x 10^6 cells are needed in total.

1.2.7. Add 100 µL of cell suspension to each well of a non-adherent, V-bottom, 96-well plate (**Table of Materials**).

NOTE: Use a multichannel pipette to shorten the time and ensure that each well contains an equivalent cell number. Shake the Petri dish each time before removing 100 µL portions. It is important that the cells are uniformly distributed.

1.2.8. Lightly spin down the 96-well plate in a low-speed shaker for 5 min and then incubate at 37 °C and 5% CO_2.

NOTE: Keep the plate in dark. Set the day as day 0.

1.3. Day 2

1.3.1. Add 1% reagent A (**Table of Materials**). Prepare reagent A (protein concentration of 10 mg/mL) by adding 133.4 µL of reagent A into 2 mL of medium I.

NOTE: Maintain reagent A at 4 °C overnight before use to achieve complete and uniform melting. Please note the product information and search the catalog number the and the lot number in the official website of the company to have the protein concentration of reagent A as each bottle

of reagent A is at a different protein concentration. If the protein concentration is low, an increased volume of reagent A would be helpful.

1.3.2. Add 20 µL of prepared reagent A to each well and pipette twice in the center to scatter the dead cells.

NOTE: Maintain the reagent A and the 96-well plate under cool conditions and complete all the steps on ice. Place the plate in dark.

1.4. Day 2-12

1.4.1. Clean the bottom of the 96-well plate and incubate at 37 °C and 5% CO_2 until day 6. At day 6, change half of the medium by removing 58 µL of the medium from each well and adding 60 µL of medium I.

NOTE: Use a multichannel pipette to complete the half medium change. Conduct this step gently to ensure that no cell pellets are removed from the well. Aspirate the medium into a clean 10 cm Petri dish. If there are cell pellets inside, add them back to the 96-well plate.

1.5. Day 12- 18

1.5.1. Change the medium to medium II (**Table 1**)at day 12. Prepare medium II using 1% (v/v) reagent A by mixing the following: 10% fetal bovine serum (FBS), 0.1 mM NEAA, 1 mM pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mM β-mercaptoethanol, 100 nM SAG dihydrochloride and GMEM. Store it under cool and dark conditions.

1.5.2. Harvest the cell pellets in a 15 mL conical tube from the 96-well plate and allow the pellets to settle naturally for 5 min at room temperature.

NOTE: Remove the medium and pellets gently under the surface to avoid bubbles.

1.5.3. Remove the supernatant while taking care of the organoids. Transfer the cell aggregates to a 10 cm suspension dish containing 18 mL of prepared medium II with reagent A.

NOTE: Do not add the prepared medium II into the 10 cm dish in advance, as the reagent A should be at 4 °C.

1.5.4. Incubate at 37 °C and 5% CO_2 until day 18.

1.6. From day 18 onwards

1.6.1. Change the medium to medium III (**Table 1**). Prepare medium III under dark conditions by mixing the following: 10% FBS, 1x supplement 1, 0.5 µM retinoic acid (RA), 100 µM taurine, 100

U/mL penicillin, 100 µg/mL streptomycin and 1:1 mixture of Dulbecco's modified eagle medium (DMEM)/nutrient mixture F-12.

1.6.2. On day 18, when a semitransparent optic vesicle is generated, cut the organoids using a microsurgical knife.

NOTE: Cleave the organoid, usually into four pieces, in a Petri dish with medium II. Each piece should grow into an intact optic cup in the following weeks.

1.6.3. Aggregate all the pellets to the center of the dish. Harvest the cells into a 15 mL conical falcon tube and allow natural settling. Then gently remove the supernatant.

NOTE: Due to their size, organoids can be aggregated at the center by rotating the Petri dish in one direction for 90° on a horizontal plane a few times.

1.6.4. Suspend and disperse the pellets in two 10 cm Petri dishes, with 18 mL of medium III per dish, and gently transfer the dishes to the incubator to avoid cell aggregation. Continue culturing in medium III at 37 °C and 5% CO_2 and change the medium weekly. The CRX expressed after day 45 and until day 120, we could detect the out-segment of the photoreceptor.

2. Analyzing human ROs

2.1. FACS analysis

2.1.1. Assemble three CRX-tdTomato and three H9 ES-derived organoids from the dishes by using the cut 1 mL tips. Wash the organoids with 1 mL of pre-warmed (room temperature) DPBS.

2.1.2. Prepare the digestion buffer by mixing 0.25% trypsin-EDTA Solution with 0.05 mg/mL of reagent D.

2.1.3. Dissociate the organoids into single cells by using the prepared digestion buffer for 8 min at 37 °C. Then add the same volume of DPBS with 10% FBS and 0.05 mg/mL of reagent D to inactivate the reaction.

2.1.4. Lightly suspend and scatter the cells and then filter using a 100 µm cell strainer and use a Fluorescence-Activated Cell Sorting (FACS) system at 561 nm excitation laser line and 780/60 filter to analyze the CRX-tdTomato positive signals.

NOTE: CRX initially expresses at day 45 and increases with the maturation of organoids. Ten thousand cells are used for each test, for each timepoint, at least three repeats are completed.

2.2. Fluorescence intensity quantification of ROs

2.2.1. Prepare the viable organoids randomly for imaging.

NOTE: Set the parameters and use the same filters and parameters for all the fluorescence intensity monitors.

2.2.2. Capture images and analyze the mean fluorescence intensity using suitable software (e.g., ImageJ).

2.2.3. Import the images and then convert to 8-bit using **Image | Type**.

2.2.4. Adjust the threshold by using the default parameters by **Image | Adjust | Threshold.**

2.2.5. Determine the measured area and then evaluate the gray value by using **Analyze | Measure**.

NOTE: The mean values in the result panel are the mean fluorescence intensity of the measured area.

REPRESENTATIVE RESULTS:

The schematic illustration depicts the differentiation protocol to improve precursor cells with COCO (**Figure 1**). From PSC to ROs, numerous details could cause result variations. It is recommended to record every step and even the catalog number and lot number of every medium to track the entire procedure.

Herein, we provide bright field images for days 6, 12, 18, and 45 (**Figure 2**). On day 6, the organoids are usually around 600 µm in diameter in a 96-well plate, with dense connections inside and bright rim (**Figure 2A**). On day 12, the optic vesicle-like structures initially generate (**Figure 2B**). From day 12 to day 18, the presence of optic vesicle structures is clear, and they continued to grow after day 18. The organoids without the vesicle-like architecture are discarded (**Figure 2C**). By day 30, the vesicle-like architecture is more obvious, and it is easier to distinguish the superior ROs from the inferior ones (**Figure 2D-E**). The organoids that lose the translucent structure (asterisks in **Figure 2D-E**), should be removed in the following days.

The organoids express the CRX, which is a marker of photoreceptor precursors, from day 45 onwards (**Figure 2F,2I**). Other photoreceptor precursor markers, such as RCVRN and OTX2, were also positively detected at day 45 (**Figure 2G-H**). The addition of COCO promotes the generation of photoreceptor precursors.

FIGURE AND TABLE LEGENDS:

Figure 1. Timetable for stepwise treatment for RO differentiation from hESC.

Figure 2. Human retinal organoid generation. (**A-B**) Early-stage optic vesicle-like structure formed in a 96-well plate. The black arrows indicate the optic vesicle-like structures. (**C**) The first day of suspension culture in a Petri dish on day 18. (**D-E**) Optic cup structures are observed at this stage. The stars show the inferior organoids (**F**) The bright field image on day 45. (**G-H**)

Immunostaining results of RCVRN (**G**) and OTX2 (**H**) on day 45. (**I**) TdTomato-positive signals indicate the expression of CRX on day 45 in (**F**). Scale bars = 400 nm.

Table 1: Medium I, II, and II recipes

DISCUSSION:

Retinal organoid differentiation is a desirable method for the generation of ample functional retinal cells. The RO is a composite of different retinal cells, such as ganglion cells, bipolar cells, and photoreceptors, generated by pluripotent stems cells toward the neural retina[4,5,8,9]. Although confluent ROs could be harvested, it is time-consuming, which may require long culturing periods (up to 180 days). However, for photoreceptor transplantation, or studying cone-rod or rod-cone dystrophy, it is advantageous to obtain a relatively high percentage of photoreceptors in the 3D culturing system[10].

It is also challenging to monitor the organoids' development without interrupting the normal developmental processes. Therefore, we used CRX, a cone-rod homeobox protein, predominantly expressed in photoreceptor precursors, as a target gene to trace photoreceptor precursor cells during their 3D differentiation. With the tdTomato system, CRX-expressing cells can be spatio-temporally tracked by the red fluorescence without interrupting retinalization during their 3D differentiation. The utilization of CRX-tdTomato system could accelerate the process of drug screening for photoreceptor precursor differentiation in the ROs.

Using our method, around 70% organoids could develop into retinal organoids, which display the vesicle-like structures. Importantly, with the incision of superior organoids, we usually could harvest around 100 retinal organoids from a 96-well plate. Additionally, abundant photoreceptor precursors are generated in the early stage of RO maturation with the COCO culture, which helps progression toward direct differentiation of certain cells through pathway regulation[11,12]. The protein concentration of reagent A is crucial for the differentiation. Altogether, sufficient reagent A with the aggregates in the early days as well as the cutting of the organoids on day 18 are important to harvest abundant ROs with high quality. This method also promotes the development of directional differentiation of photoreceptor cells in 3D organoids and contributes to the transplantation of photoreceptor cells.

ACKNOWLEDGMENTS:

We thank members of 502 laboratory for their technical supports and helpful comments regarding the manuscript. This work was partly supported by the Beijing Municipal Natural Science Foundation (Z200014) and National Key R&D Program of China (2017YFA0105300).

REFERENCES:

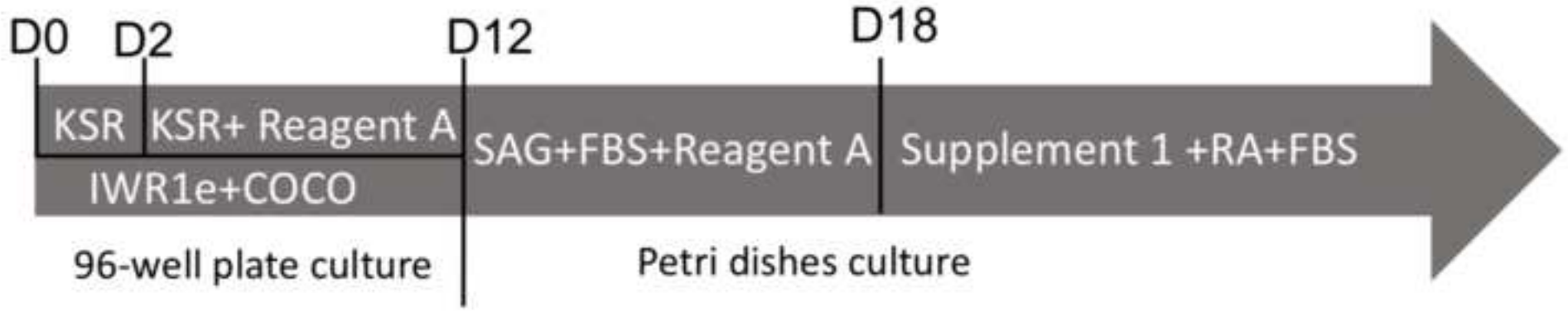
1 Xie, H. et al. Chromatin accessibility analysis reveals regulatory dynamics of developing human retina and hiPSC-derived retinal organoids. *Science Advances* **6** (6), eaay5247 (2020).

2 Lu, Y. F. et al. Single-Cell Analysis of Human Retina Identifies Evolutionarily Conserved and Species-Specific Mechanisms Controlling Development. *Developmental Cell.* **53** (4), 473-491 (2020).

3 Cowan, C. S. et al. Cell Types of the Human Retina and Its Organoids at Single-Cell Resolution. *Cell.* **182** (6), 1623- 1640 (2020).
4 Jin, Z. B. et al. Stemming retinal regeneration with pluripotent stem cells. *Progress in Retinal and Eye Research.* **69,** 38-56 (2019).
5 Nakano, T. et al. Self-formation of optic cups and storable stratified neural retina from human ESCs. *Cell Stem Cell.* **10** (6), 771-785 (2012).
6 Pan, D. et al. COCO enhances the efficiency of photoreceptor precursor differentiation in early human embryonic stem cell-derived retinal organoids. *Stem Cell Research and Therapy.* **11** (1), 366 (2020).
7 Zhou, S. et al. Differentiation of human embryonic stem cells into cone photoreceptors through simultaneous inhibition of BMP, TGFbeta and Wnt signaling. *Development.* **142** (19), 3294-3306 (2015).
8 Deng, W. L. et al. Gene Correction Reverses Ciliopathy and Photoreceptor Loss in iPSC-Derived Retinal Organoids from Retinitis Pigmentosa Patients. *Stem Cell Reports.* **10** (4), 1267-1281 (2018).
9 Gao, M. L. et al. Patient-Specific Retinal Organoids Recapitulate Disease Features of Late-Onset Retinitis Pigmentosa. *Frontiers in Cell and Developmental Biology.* **8,** 128 (2020).
10 Zhang, C. J., Ma, Y., Jin, Z. B. The road to restore vision with photoreceptor regeneration. *Experimental Eye Research.* **202**, 108283 (2020).
11 Reichman, S. et al. From confluent human iPS cells to self-forming neural retina and retinal pigmented epithelium. *Proceedings of the National Academy of Sciences of the U. S. A.* **111** (23), 8518-8523 (2014).
12 Kuwahara, A. et al. Generation of a ciliary margin-like stem cell niche from self-organizing human retinal tissue. *Nature Communications.* **6,** 6286 (2015).

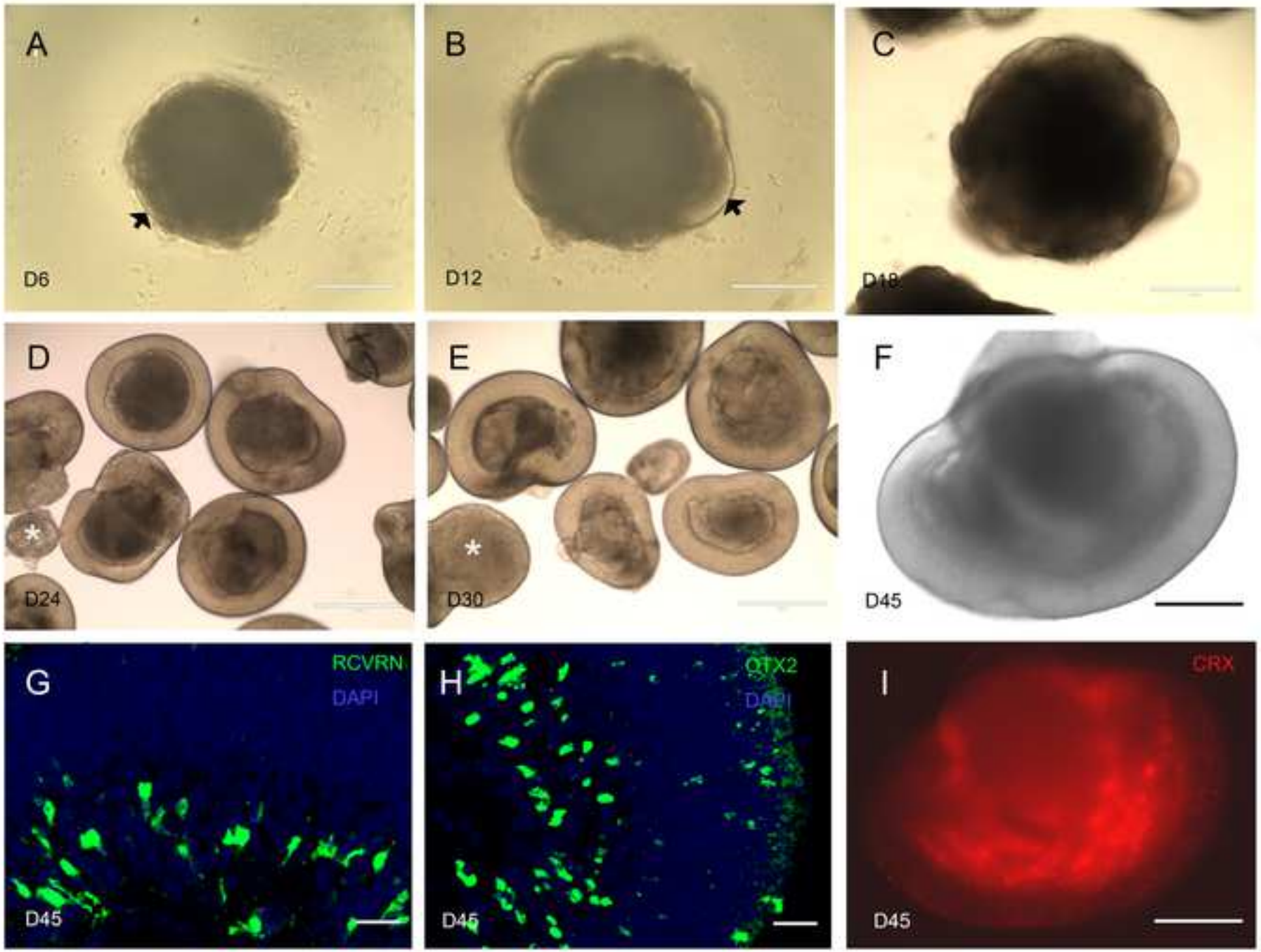
Figure

Click here to access/download;Figure;Figure 1.png



Figure

Click here to access/download;Figure;Figure2.jpg



Medium Tables

Medium I (50 mL)

	KSR	G-MEM	NEAA	Pyruvate	b-ME	IWR1e	COCO	PS
Percentage% OR final concentration	20	78	0.1mM	1mM	0.1mM	3 µM	30 µM	1
Volume	10 mL	39 mL	0.5 mL	0.5 mL	90.9 µL	5 µL	10 µL	0.5mL

The store concentration of IWR1e is 30 mM, COCO is 0.15 M

Medium II (50 mL)

	FBS	G-MEM	NEAA	Pyruvate	b-ME	SAG	PS
Percentage% OR final concentration	10	88	0.1 mM	1 mM	0.1 mM	100 nM	1
Volume	5 mL	44 mL	0.5 mL	0.5 mL	90.9 µL	2.5 µL	0.5mL

The store concentration of SAG is 2 mM

Medium III (50 mL)

	FBS	DMEM/F12-Glutamax	Supplement 1	RA	Taurine	PS
Percentage% OR final concentration	10	88	1	0.5 µM	100 µM	1
Volume	5 mL	44 mL	0.5 mL	5 µL	50 µL	0.5mL

The store concentration of RA is 5 mM, Taurine is 100 mM

Name of Material/Equipment	Company	Catalog Number	Comments/Description
2-mercaptoethanol	Life Technologies	21985-023	
COCO	R&D Systems	3047-CC-050	DAN Domain family of BMP antagonists
DMEM/F-12	Gibco	10565-042	
DMSO	Sigma	D2650	
DPBS	Gibco	C141905005BT	
EDTA	Thermo	15575020	
Fetal Bovine Serum (FBS), Qualified for Human Embryonic Stem Cells	Biological Industry	04-002-1A	
GMEM	Gibco	11710-035	
KnockOut Serum Replacement-Multi-Species	Gibco	A3181502	
MEM Non-essential Amino Acid Solution （100X）	sigma	M7145	
Pen Strep	Gibco	15140-122	
Primesurface 96 V-plate	Sbio	MS9096SZ	Cell aggregation in 1.2.7
Pyruvate	Sigma	S8636	
Reagent A	BD	356231	Matrigel in 1.1.1
Reagent B	StemCell	5990	mTeSR- E8 ， PSCs basal medium in 1.1.2
Reagent C	Gibco	12563-011	TrypLE Express in 1.2
Reagent D	Roche	11284932001	DNase I , in 1.2
Retinoic acid	Sigma	R2625-100MG	
SAG	Enzo Life Science	ALX-270-426-M001	
Supplement 1	Life Technologies	17502-048	N-2 Supplement (100X), Liquid, supplemet in medum III
Taurine	Sigma	T-8691-25G	

Trypsin-EDTA (0.25%), phenol red	Gibco	25200056	organoids dissociation in 2.1.3
Wnt Antagonist I, IWR-1-endo - Calbiochem	Sigma	681669	Wnt inhibitor
Y-27632 2HCl	Selleck	S1049	

Dear Editor and reviewers,

Thank you very much for the comments. The words in red are the reply from us.

Editorial comments:

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

The protocol has been checked from cover to cover.

Reviewers' comments:

Reviewer #2:

Manuscript Summary:

The authors have sufficiently addressed the reviewers' concerns; and edited the text. They have also added a table with media formulations.

Major Concerns:

N.A.

Minor Concerns:

N.A.

Reviewer #4:

Manuscript Summary:

In this study the authors present a differentiation protocol to generate retinal organoids (ROs) from pluripotent stem cells. This is a protocol based on the original protocols described by Sasai's lab with slight modification and addition of factors to promote differentiation. Overall, the manuscript is informative however, a few points need to be addressed for clarity.

Major Concerns:

The authors should also revise grammar and writing throughout the manuscript as sometimes the message can be a bit confusing.

The use of a CRX-td Tomato line is advantageous but similar lines have been described in a number of studies. The authors do not present the percentage of CRX positive cells obtained by FACs and do not mention the overall efficiency of the protocol. I. e. from 96 embryoid bodies (EBs) formed at the beginning of the protocol how many will form retinal vesicles.
The authors claim this is an efficient protocol to generate ROs, but they do not mention if the protocol has been replicated with multiple cell lines. They should add further explanation on that and name the lines that were used to date.

The expression of CRX is growing with time and reaches to around 50% at day 120. (Pan, D. et al.,2020). There are another three lines are differentiated into ROs using this method in our publish (Gao, M. L. et al., 2020).

“around 70% organoids could develop into retinal organoids, which display the vesicle-like structures. Importantly, with the incision of superior organoids, we usually could harvest around 100 retinal organoids from a 96-well plate.” Has been added to the discussion part from line 284-286

Minor Concerns:
Abstract:
Please clarify what they mean by 'The efficient use of this system could produce confluent retinal cells, which has the benefits of cell lines and primary cells, and without the sourcing issues associated with the latter.' The sentence is somehow convoluted.

It means the ROs is better than the cell lines and primary cells, as the ROs harbor the advantage of the prolificacy for cell lines and the reality for primary cells, meanwhile they avoid the well-known immortalization for cell line and the scarcity for primary cells.

Last line of the abstract should say 'cell transplantation' and not 'cell transplanting'.

It has been updated at line 33

Introduction:
Authors should mention which hESC line they use and provide a bit more of information on CRX so that lay readers clearly understand their choice of reporter lines. This is only provided later in the Discussion.

"H9 hESCs were obtained from the WiCell Research Institute and genetically engineered to tdTomato-tagged cell line." It has been mentioned in the protocol line 59-61

Protocol:

1) It would be easier to start each heading with the day of the Differentiation, for example in 1.2 say 'Day 0- Detach hESCs and treat with a single-cell suspension using medium I'.
Also this should read: 'Detach hESC into single cells and add to differentiation medium I.' It is not very clear what they mean by 'treat with a single cell-suspension'

The "day 0", "day2", "day2-12", "day 12-18", "from day 18 onwards" has been added.

"Dissociate hESCs to a single-cell suspension using medium I." it has been updated at line 81

2) In 1.1.3 it says "Seed the cells into the Matrigel coated plate and change the medium daily and change the passage of cells when the cells reach approximately 80% confluency…' This is not clear. Do they mean change the media daily until the cells are ready to passage at when they reach 80% confluency? Again, writing should be checked throughout.

It has been changed at line 76-78

3) Please give more detail:
For example in 1.2.2 the text says, ' add 500ul of TrypLE Express to the 6 well plate well containing cells'.

"Add the prepared 500 µL reagent (in the 1.5 mL tube) and incubate the hESCs for 3.5 min at 37 °C and 5% CO_2." It has been updated at line 94-95

In 1.2.5 it says, 'harvest cells in a 1.5 ml Eppendorf tube.' This is important because cells are usually collected in larger tubes in other protocols.

We believe the 1.5 ml tube is easier to handle. There is no centrifuge for those cells.

In 1.2.8 it says, 'Lightly spin down the 96-well plate in a low-speed shaker for 5 minutes and then incubate at 37 °C and 5% CO2.

NOTE: Keep the plate in dark. Set the day as day 0.'
Can the authors clarify what they mean by a low-speed shaker? Do they mean centrifuge? Please, also specify the speed.

It is not centrifuge; the speed is less than 50 rpm. The role of the low-speed shaker is to help blending and settling the cells. It is optional, not necessary.

4) It is not clear when they add the Matrigel. Is it at Day 1 or day 2?

It is from day 2 to day 18, which is clear in figure 1.

5) To instruct the reader the authors should state the morphology of the cells or organoids at each stage and refer to appropriate figure. For example, when media is changed at Day 12 the authors state that cell pellets should be harvested. Surely these are not cell pellets anymore, but they EBs or organoids already?

Figure 2 A-F evidently show the morphology of six important timepoints.

6) In 1.6.2 On day 18, a semitransparent optic vesicle is generated, and organoids should be cut using a microsurgical knife.
NOTE: Cleave the organoid, usually into four pieces, in a Petri dish with medium II. Each piece should grow into an intact optic cup in the following week.
The word optic cup here is not appropriate as it implies that the cut vesicle will undergo invagination and form both RPE and neural retina. These should still be called vesicles. Nomenclature for the various stages should be revised.

It is vesicle on day 18, but it will grow into optic cup in the following weeks.

7) Please provide a bit more instruction on cutting the vesicles.
I think it would be shown in the video.

Reviewer #5:

Manuscript Summary:

The manuscript describes a protocol for differentiating retinal organoids with an increased yield of photoreceptor precursors. The authors adapt an established organoid protocol by adding a multi-target antagonist, claiming to promote photoreceptor precursor differentiation.

Major concerns:

1. This protocol is essentially describing the organoid differentiation method published in Pan, D. et al 2020. The authors cite the paper in lines 53, and 54. However, they make claims based on the data from that paper in the abstract (lines 29-31), introduction (lines 56-57), results (lines 251-252), and discussion (lines 273-279, 281-283). The authors occasionally use nearly identical language in the results/discussion sections of the two manuscripts (e.g. line 276). The authors also responded to multiple reviewer comments by saying the missing or requested data was in the other paper. This manuscript is not that paper- it should stand on its own and be distinct in its claims and purpose. The authors either need to include more citations and refer to specific images from that paper (as per many of the reviewer comments), or present new data or images to substantiate their claims about the method in this manuscript.

It has been revised. There is no describe for the bipolar, RGCs or other retinal cells, except the general description of the compose of RO in the discussion.

2. The protocol is lacking in cohesiveness. The analysis steps of the protocol are deficient in detail (particularly the imaging portion), and it is not clear why they are included as none of the text or figures really correspond to them. Nothing in the title, summary, or abstract suggests that the protocol includes any type of analysis, and nowhere is it explicitly explained why one would be following those steps. It would be helpful for the authors to clearly state in the manuscript the purpose of the analysis steps, and describe the nature of the data a user may obtain from them. The analysis steps also seem to only be applicable if the user is growing organoids with a CRX reporter line. If that is the actual intention of the protocol, then the title/manuscript could be reframed, such as "Differentiation and analysis of photoreceptors from retinal organoids using a CRX reporter line" or something more appropriate. If the intention is for more general differentiation, then the analysis steps do not necessarily belong in this protocol.

“around 70% organoids could develop into retinal organoids, which display the vesicle-like structures. Importantly, with the incision of superior organoids, we usually could harvest around 100 retinal organoids from a 96-well plate.” The expression of CRX is growing with time

and reaches to around 50% at day 120. (Pan, D. et al.,2020). There are another three lines are differentiated into ROs using this method in our publish (Gao, M. L. et al., 2020). Clearly, the method could be used to other lines and the efficiency of this protocol has been added to the discussion at line 284-286

3. Retinal organoids are a knowingly long differentiation process. The authors discuss the use of this method for generating photoreceptors and claims that the differentiation efficiency of cones is "significantly increased", but they don't give any indication of when photoreceptors are actually developed, and don't show any images past day 45. The protocol stops at day 18 and just says to change medium weekly, but provides no guidance past that point. Figure 2 shows tdTomato expression at D45, which is presumably showing photoreceptor precursor cells, but how long does the user need to differentiate to get mature photoreceptors? Opsin expression? This could be as far out as 200+ days of differentiation.

The further information has been added at line 193-194 “The CRX expressed after day 45 and until day 120, we could detect the out-segment of the photoreceptor.”

Minor concerns:
Overall:
There are still language, syntax, and grammar issues that make parts of the manuscript confusing and should be edited.
Summary/Abstract:
1. The wording of the summary (as well as the abstract) makes it sound like the method is serum-free, when it seems the authors are trying to say that the method is built off of a serum-free method? The syntax or wording should be changed in both places to make it clearer, because this particular protocol is not serum-free and the current wording is misleading.

It has changed to self-organizing method in the summary and abstract.

2. The summary and abstract state on lines 17 and 25, "we develop an optimized protocol…" However, this manuscript is not describing the development of the protocol. The word "develop" should be changed to "describe" or something similar.

The addition of the COCO is the development.
Introduction:

1. Using a word in its own definition- in line 38, saying that pluripotent stem cells are characterized by their pluripotency is redundant and not helpful to the reader.

The word “pluripotency” has been deleted.

Protocol:

1.1.1 What is the solvent for reagent A? I believe where "do not pipette to single cell" was changed to "do not dissociate to single cell", what was really meant was "do not over-triturate cells" as there is no dissociation in this step.

It has been changed at line 72

1.2 The first sentence is unclear. Maybe change to "Dissociate hESCs to a single-cell suspension using medium I." Just reference the media table rather than spelling it out (here and in subsequent steps). The wording of this note is very unclear and needs editing.

It has been changed to “Dissociate hESCs to a single-cell suspension using medium I.” at line 81

1.2.2 Which reagent?

It is the prepared mixture in 1.2 in a 1.5 mL tube.

1.2.4 Where is the 100 ul being taken out of, and where is it being added to? The wording is unclear.

“Harvest the cells in a new 1.5 mL tube and pipette the cell suspension up and down and then take out 100 µL from the tube and then add into the tube with 900 µL of DPBS for cell counting.” It has been modified at step 1.2.4

1.2.5 What does disperse mean? Please include the number of cells that are being seeded per well or per organoid.

“Disperse the left 900 µL cell suspension” has been updated.

“ 9 x 10^4 cells/mL” and “100 µL per well”, It is quite clear there are 9000 cells per well.

1.2.7 Please include the speed of the shaker.

It is not centrifuge; the speed is less than 50 rpm.

Discussion:

1. Line 266-267: retinal pigmented epithelium is not part of the neural retina.

It has been deleted.

2. Line 267: unclear what "confluent Ros" means.
It means the number of the ROs is considerable.

3. Lines 277-279: how does the utilization of CRX-tdT help the direct differentiation of photoreceptors? Either something is misworded here and needs clarification, or this claim needs to be deleted.
It has been deleted.

Figure 2:
1. Please update the figure legend to indicate what the arrows and asterixis mean.
Summary needs rewording. It claims possibility of photoreceptor increase here when this paper doesn't show it.
The figure legend has been updated and the increased photoreceptor is described in our publish. (Pan, D. et al.,2020)

Medium Tables:
1. All medium tables show the working/final percentage or concentration, but the volume is assumably of a stock/starting concentration. Please include relevant concentrations for the volumes of reagents being added (e.g. the starting concentration for IWR1e?)
It has been added in Table 1.
With appropriate further editing, and with good videos, this "paper" could be a useful addition to the retinal research community.