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We present an optimized protocol for guided differentiation of retinal pigment epithelium (RPE) cells from human-induced pluripotent stem cells (iPSC). De novo-generated RPE cells are mature, polarized, and mimic the cellular and molecular profile of primary RPE; they are also suitable for in vivo cell transplantation studies. The protocol includes an enrichment step, making it useful for large-scale GMP manufacturing. RPE cells produced following this protocol are appropriate for cell replacement therapy for macular degeneration and disease modeling.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

An improved protocol for generation and characterization of human-induced pluripotent stem cell-derived retinal pigment epithelium cells

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SUMMARY

We present an optimized protocol for guided differentiation of retinal pigment epithelium (RPE) cells from human-induced pluripotent stem cells (iPSC). De novo-generated RPE cells are mature, polarized, and mimic the cellular and molecular profile of primary RPE; they are also suitable for in vivo cell transplantation studies. The protocol includes an enrichment step, making it useful for large-scale GMP manufacturing. RPE cells produced following this protocol are appropriate for cell replacement therapy for macular degeneration and disease modeling.

For complete details on the use and execution of this protocol, please refer to Surendran et al. (2021).

BEFORE YOU BEGIN

Culturing, maintenance, and differentiation of iPSC lines to Retinal Pigmented Epithelium (RPE) required standard cell culture practices like aseptic handling of cultures, wearing personal protective gear along with appropriate equipments like biosafety cabinets, CO2 incubators, centrifuge, water-bath and microscope. Culturing of cells in plates coated with extracellular matrices (ECM) required humidified incubators at 37°C with 5% CO2 levels. Before differentiation, iPSCs were characterized to check for expression of pluripotent markers, chromosomal aberrations by karyotyping, and sterility tests to determine if the cultures are contamination-free (bacterial and mycoplasma).

In our previously published paper (Surendran et al., 2019), we reported rosette selection on day 20–22 of differentiation - for enrichment of RPE cells. The current protocol provides a detailed procedure for the generation of RPE through a combination of chemical and manual selection processes for enrichment of RPE at early and late stages of the differentiation process. In principle, iPSCs are coaxed into neuroectodermal fate by suppressing pluripotency factors like FGF2 using dual SMAD and WNT inhibition. This is achieved by the use of small molecules like SB431542 and LDN193189. SB431542 inhibited Activin/TGF-β pathways and LDN193189 acted as a BMP4/7 inhibitor while IWR1 silenced the canonical WNT signaling pathway. The developmental stages that iPSCs go through are the formation of anterior neural plate (ANP), eye field specification, and bilayered optic cup.
To facilitate retinal cell proliferation, the embryoid bodies are cultured in low serum media supplemented with N1, thus forming retinal progenitors which are enriched to produce matured RPE. The matured RPE represent tightly packed hexagonal cells with tight junctions, and brown to black pigmentation with apical-basal polarity. This protocol generated pigmented and ciliated RPE exhibiting functional characteristics such as polarized secretion of cytokines – PEDF and VEGF. Characterization methods to determine their authenticity and purity are also laid down along with this protocol.

Institutional permissions
The study was approved by the Institutional Committee for Stem Cell Research (IC-SCR) registered with the National Apex Committee for Stem Cell Research and Therapy, Indian Council of Medical Research (ICMR), New Delhi, India.

Note: Acquire all critical reagents mentioned in the key resources table.

Acquire/thaw iPSCs and maintain them as undifferentiated cultures in mTeSR-based medium. Prepare media according to the recipes mentioned in the protocol.

Note: The iPSCs maintenance and differentiation media can be used for up to 2 weeks when stored at 4°C. Thus, it is not advisable to prepare all media at once, rather it is better to prepare it when needed at the specific step of the procedure.

△ CRITICAL: All procedures are performed in a BSL-2 certified laboratory biosafety cabinet with standard aseptic techniques. Cultures are grown and maintained in a humidified incubator at 37°C with 5% CO₂.

△ CRITICAL: Plan to test the cultures regularly for sterility and karyotyping.

General experimental preparations

◎ Timing: 1 h for step 1

◎ Timing: 6–10 days for step 2

◎ Timing: 1 h for step 3

A detailed schematic of human-induced pluripotent stem cell (iPSC) differentiation through neuroectodermal induction and retinal progenitors to RPE cells is shown in the graphical abstract. The reproducibility of this RPE differentiation protocol has been tested in multiple iPSC lines, including TC-1133 (RUCDR/NIH; Baghbaderani et al., 2015), ERPl001-A, ERPl002-A, and ERPl003-A (Knala et al., 2020). Therefore, we expect similar differentiation results in other iPSC lines following the detailed protocol as described here.

Note: The entire protocol must be performed in a sterile environment, such as a biosafety cabinet.

1. Preparation of Cell Therapy Systems-Vitronectin (CTS™-VTN) for iPSC expansion.
   a. Thaw CTS-VTN (0.9 mg/mL) at room temperature (20°C–25°C) for 5–10 min, then place on ice.

   Note: CTS-VTN can be divided into usage-size aliquots in polypropylene tubes and stored at −60°C to −80°C.
b. Room temperature storage and/or shaking might result in an appearance of light turbidity. This does not impact product performance.

**Note:** To coat the wells of a 6-well plate, add 60 μL of CTS-VTN into a 15 mL tube containing 6 mL of sterile CTS™ DPBS (1 x) (-Ca²⁺/-Mg²⁺) at room temperature.

c. Gently resuspend by pipetting the CTS-VTN solution up and down.

**Note:** This results in a working concentration of 9 μg/mL (i.e., a 1:100 dilution). Diluted CTS-VTN solution can be stored at 4°C for not more than 7 days. Add 1 mL of diluted CTS-VTN solution to each well of a 6 well plate. Swirl and/or rock the plate to ensure even coating.

d. When used to coat a 6 well plate (10 cm²/well) at 1 mL/well, the final concentration will be 0.9 μg/cm².

e. Incubate the coated plates at 37°C for 1 h.

**Note:** The culture plate can now be used or stored at 2°C–8°C, wrapped in laboratory film, for up to one week. Make sure wells do not dry out. A volume of basal media may be added to the well(s) 1 h after coating to ensure that the wells do not dry out. If a portion of a well does dry out, this well cannot be used. Prior to use, pre-warm the culture plate to room temperature (20°C–25°C).

f. Aspirate the CTS™-VTN solution and discard it immediately prior to use. It is not necessary to rinse off the culture plate after the removal of CTS™-VTN. Cells can be seeded directly onto the CTS™-VTN-coated culture plates.

2. iPSC culture and maintenance. **Troubleshooting 1.**

**Note:** You must have a prepared CTS-VTN coated plate before starting this protocol. If you are using a CTS-VTN coated plate that has been stored at 4°C, the plate must be allowed to equilibrate to room temperature for 1 h prior to starting.

**Note:** 1 cryovial packed with 1 million cells should be thawed into 1 well of a 6 well plate (10 cm² surface area).

a. Add 5 mL of cold mTeSR plus medium to a sterile 15 mL tube.

**Note:** A 1:5 ratio is recommended to effectively dilute the Cryostor CS10 (1 mL of cells and 5 mL of mTeSR plus).

b. Remove cells from the liquid nitrogen storage tank.

c. Thaw cells quickly in a 37°C water bath using a “Figure 8” motion until you see a pea-sized ball of ice.

⚠️ **CRITICAL:** To avoid cell death, do not thaw the cells completely in the water bath.

d. Using a 2 mL pipette, slowly add mTeSR plus to the cells drop by drop and collect in a 15 mL tube.

e. Cap the 15 mL tube and gently invert the tube 4–5 times to mix the CryoStor CS10 and mTeSR plus.

f. Centrifuge at 200 g, 3 min at 25°C ± 5°C.

g. While cells are spinning, aspirate CTS-VTN from the plate.
h. Gently aspirate the supernatant from the cell pellet and re-suspend cells in 2 mL of fresh mTeSR plus containing 10 μM ROCK inhibitor (Y-27632).

**Note:** The use of ROCK inhibitor Y-27632 increases cell survival and cell health. Reconstitute and store Y-27632 as per manufacturer’s instructions. [https://www.tocris.com/products/y-27632-dihydrochloride_1254](https://www.tocris.com/products/y-27632-dihydrochloride_1254).

**CRITICAL:** Complete removal of DMSO present in storage media by washing is very important due to its known toxicity. The operation needs to be gentle and quick.

i. Plate the cells onto the CTS-VTN coated well.

j. Place culture plate in the incubator; gently rock the plate in a plus direction for homogeneous distribution of the cells.

k. The next day, replace media with 2 mL of fresh mTeSR plus to remove Y-27632.

**CRITICAL:** For media changes, always add/remove medium carefully close to the wall of the wells to avoid detaching the cells.

l. Colonies should appear within 2–3 days.

m. Change medium every day and when the colonies cover 70%–80% of the plate they are ready to be passaged.

3. iPSC passaging.
   a. Prepare CTS-VTN-coated plates as described previously and pre-warm mTeSR plus.
   b. Aspirate the medium and wash the well with (1 mL/well of 6 wells) 1×PBS.
   c. Add ReLeSR to the culture plate and aspirate ReLeSR within 1 min, such that colonies are exposed only to a thin film of the liquid.
   d. Incubate the plate at 37°C for 4 min ± 30 s.
   e. Gently rinse the plate(s) or well(s) with 1 mL of mTeSR plus for complete detachment of cells.
   f. Detach the colonies by holding the plate with one hand and using the other hand to firmly tap the side of the plate for approximately 30–60 s.
   g. Transfer the detached cell aggregates to a 15 mL tube using a 5 mL serological pipette.
   h. Cell aggregates should be appropriately sized for plating (mean aggregate size of approximately 50–200 μm).
   i. Centrifuge the sterile 15 mL or 50 mL serological tube for 3 min at 200 g by keeping acceleration and deceleration at a maximum of 9 for the centrifuge.
   j. Upon completion of centrifugation, remove the supernatant, gently tap the pellet and add appropriate volume of mTeSR plus along with 10 μM Y-27632, depending on the split ratio, to a 15 mL or 50 mL tube.
   k. Plate the cell colonies onto the CTS-VTN coated dishes.
   l. Rock the plate in a plus direction to ensure even distribution of colonies in the wells.
   m. Place the plate in a 37°C incubator with 5% CO₂. Do not move the plate for 24 h.
   n. After 24 h, observe the plate under the microscope to confirm that the colonies are attached to the plate. The next day, replace media with 2 mL of fresh mTeSR plus to remove Y-27632.
   o. Change medium every day until plate(s) reaches 70% confluency in less than 7 days.

**Note:** Discard any plate(s) that does not become 70% confluent within 7 days. Before every passage, the supernatant is pooled in from all plate(s) into a single sterile 15 mL tube and stored at −80°C until it is tested for sterility.

4. Matrigel aliquoting and coating.
Matrigel is aliquoted and stored at −20°C. It is important that any material that will come in contact with the Matrigel be ice-cold. Matrigel will solidify and adhere to any item that is above 10°C. Please avoid multiple freeze/thaw cycles.

a. Before beginning place your frozen serological pipettes, pipet tips, 15 mL tubes and matrigel aliquots on ice in the bio-safety cabinet (BSC). Be sure to spray the ice bucket down thoroughly with 70% ethanol before placing it in the BSC.

b. Dilute matrigel at 1:100 in cold DMEM-F12 medium. For example, add 100 μL matrigel in 10 mL DMEM-F12 media.

**CRITICAL:** Take care to keep fingertips above the matrigel level. The warmth from your fingertips will cause the matrigel to solidify. Change to a new cold pipet tip frequently.

**Note:** This 1:100 dilution will make 1% matrigel solution ready to use for coating culture plates.

c. Using the cold serological pipette, transfer 1% matrigel solution to the culture plate at 1 mL/ well of 6 well plate.

d. Swirl and/or rock the plate to ensure even coating.

e. Incubate at 37°C for at least 1 h.

f. Alternatively, if you are not using matrigel coated plates on the same day as coating, wrap plates in parafilm, and store in 4°C fridge for up to 1 week. Make sure wells do not dry out.

**Note:** A volume of basal media may be added to the well(s) 1 h after coating to ensure that the wells do not dry out. If a portion of a well does dry out, this well cannot be used.

Prior to use, remove matrigel solution. Avoid air dry of matrigel-coated wells before seeding cells.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-Melanoma gp100 antibody (EP4863(2)) (1:100) | Abcam | ab137078 |
| Anti-ZO-1 antibody (1:250) | Life Technologies | 61-7300 |
| Anti RPE65 antibody (401.8B11.3D9) (1:150) | Abcam | ab13826 |
| Mouse monoclonal [T311] to Tyrosinase (1:250) | Abcam | ab738 |
| MiTF Recombinant Rabbit Monoclonal Antibody (JF100-01) (1:150) | Novus Biologicals | MAS-32554 |
| TYRP1 Antibody (TA99) (1:150) | Abcam | NB52-32906 |
| Anti-Oct4 antibody (1:250) | Abcam | ab19857 |
| Anti-cone arrestin (1:300) | Kind gift from Dr. W. Clay Smith, University of Florida | N/A |
| Fluoromount-GTM mounting medium | Invitrogen | 00-4959-02 |
| Chemicals, peptides, and recombinant proteins | | |
| Fetal Bovine Serum (FBS) | HyClone | SH30071.03 |
| MEM alpha modified medium | Sigma-Aldrich | M4526 |
| N1 Supplement (100X) | Sigma-Aldrich | N6530 |
| ReLeSR | STEMCELL Technologies | 05872 |
| CryoStor CS10 | STEMCELL Technologies | 07930 |
| mTeSRTM plus basal media and 100X supplement | STEMCELL Technologies | 100-0274/ 100-0275 |
| Gentle cell dissociation reagent (GCDR) | STEMCELL Technologies | 100-0485 |
| StemproTM AccutaseTM | Thermo Fisher Scientific | A1110501 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| CTS DMEM/F12        | Thermo Fisher Scientific | A1370801  |
| Revita Cell supplement | Thermo Fisher Scientific | A2644501  |
| Dulbecco’s phosphate buffered saline (DPBS) | Thermo Fisher Scientific | A1285601  |
| Knockout Serum (KOSR) | Thermo Fisher Scientific | A1099201  |
| Sodium Pyruvate      | Thermo Fisher Scientific | 11360-070  |
| Sodium bi-carbonate   | Thermo Fisher Scientific | 25080-094  |
| HEPES buffer         | Thermo Fisher Scientific | 15630-080  |
| MEM-Non-essential Amino Acids (NEAA) | Thermo Fisher Scientific | 11140-050  |
| Glutamax             | Thermo Fisher Scientific | A1286001  |
| Matrigel             | Corning | 354277     |
| IWR-1                | Sigma-Aldrich | I0161      |
| Taurine              | Sigma-Aldrich | T0625      |
| Hydrocortisone solution (50 μM) | Sigma-Aldrich | H6909      |
| T3ido-Thyronine       | Sigma-Aldrich | TSS16      |
| CTS-Vitronectin, truncated recombinant human (hVTN-N) | Thermo Fisher Scientific | CTS279S3  |
| IGF-1                | Thermo Scientific | PHG0078    |
| Rock Inhibitor (Y27632) | Tocris | 1254       |
| SB431542             | Tocris | 1614       |
| LDN193189            | Tocris | 6053       |
| Cyclosporine A       | AbbVie | NDC 0074-7269-50 |
| Dexamethasone        | Somerset Therapeutics | NDC 70609-021-01 |
| Tropicamide          | Akorn | NDC 17478-102-12 |
| Phenylephrine        | Akorn | NDC 17478-201-15 |
| Goniovisc            | Akorn | NDC 17478-064-12 |
| Erythromycin         | Akorn | NDC 17478-070-35 |
| Proparacaine         | Akorn | NDC 17478-263-12 |

Critical commercial assays

| Human Serpin F1/PEDF DuoSet ELISA kit | R&D Systems | DY1177 |
| PowerUp™ SYBR™ Green Master Mix | Applied Biosystems | A25742 |
| TaqMan™ Universal PCR Master Mix | Applied Biosystems | 4304437 |
| RNeasy® mini kit | Qiagen | 74104 |
| Verso cDNA synthesis kit | Thermo Fisher Scientific | AB1453B |

Deposited data

| Sequencing of iPSC-derived retinal cells | Eyestem Research, Surendran et al., 2021. PMID: 33468244. | GSE140545 |

Experimental models: Cell lines

| Human induced pluripotent stem cell line TC-1133 | Baghbaderani et al., 2015. PMID: 26411904 | RUCDR Infinite Biologics (RUCDR/NIH) |
| ERPL001-A, ERPLi002-A, and ERPLi003-A | Konala et al., 2020. PMID: 32278301 | In-house generated iPSC lines |

Oligonucleotides

| MITF F | Sigma-Aldrich | CCAGGCATGAAACACACATTTC |
| MITF R | Sigma-Aldrich | TCCATCAAACCAAGATTTC |
| RPE65 F | Sigma-Aldrich | GATCTGAGTCTGAAAAAGG |
| RPE65 R | Sigma-Aldrich | TGGGGAGCTGACTCAATTC |
| BESTROPHIN F | Sigma-Aldrich | GGCAGAACAAAGCAGGTTTG |
| BESTROPHIN R | Sigma-Aldrich | ACCGAAGGTTGTCATCTCAG |
| TYROSINASE F | Sigma-Aldrich | ACCCATGGACATACCCGGG |
| TYROSINASE R | Sigma-Aldrich | AGAGATCTGAGTCTGAAAGTT |
| TYRP1-F | Sigma-Aldrich | CCGGAAACACCAGTGGAGGG |
| TYRP1-R | Sigma-Aldrich | TCTGTTGAAAGTGTCAGAG |
| RLBP1-F | Sigma-Aldrich | AGCTGAGTAGAATGGAG |
| RLBP1-R | Sigma-Aldrich | CAAGAAGGCTGACCACAT |
| PMEL17-F | Sigma-Aldrich | CCTTCTGAGTCTGCAGCTC |
| PMEL17-R | Sigma-Aldrich | TCCAAAGTCACAGGTAGAG |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| B-ACTIN-F           | Sigma-Aldrich | TCACCCACACTGTGCCCATCTACGA |
| B-ACTIN-R           | Sigma-Aldrich | CAGGGGAACCGGCTATTGGCCAATGG |
| AFP                 | Thermo Fisher Scientific | HS00173490_m1 |
| HAND2               | Thermo Fisher Scientific | HS00232769_m1 |
| TH                  | Thermo Fisher Scientific | HS01002188_g1 |
| OCT4                | Thermo Fisher Scientific | HS03005111_g1 |
| GAPDH               | Thermo Fisher Scientific | HS02758991_g1 |
| AFP                 | Thermo Fisher Scientific | HS00173490_m1 |

Software and algorithms

GraphPad Prism | Commercial proprietary software | https://www.graphpad.com |
ImageJ | National Institutes of Health | https://imagej.nih.gov/ij/ |
FlowJo | Becton, Dickinson & Company | https://www.flowjo.com |

Other

- 6 well tissue culture treated plates: Nunc | 140675 |
- 6 Well low attachment suspension culture plates: Corning | CLS3471 |
- Cryovials: Nunc | 368632 |
- Cell Scraper: Nunc | 179693 |
- Coming Transwell 12 well 3.0 micron insert: Corning | CLS3402 |
- QuantStudio™ 3 Real-Time PCR System: Thermo Scientific | N/A |
- BD LSRRfortessa™ Cell Analyzer: BD Biosciences | N/A |
- Centrifuge 5810R: Eppendorf | 022625101 |
- Forma™ Steri-Cycle™ i160 CO2 165 L Incubator: Thermo Scientific | S1030301 |
- Microscope IX73: Olympus | N/A |
- Minispin® Plus personal microcentrifuge: Eppendorf | N/A |
- Paraformaldehyde: Sigma | 158127 |
- Triton X 100: Thermo Fisher Scientific | 85111 |
- VarioskanTM Lux multimode microplate reader: Thermo Fisher Scientific | VL0000D0 |
- Aquaguard-1: PromoKine | PK-CC01-867-1B |

STEP-BY-STEP METHOD DETAILS

Differentiation of iPSC into retinal pigmented epithelial (RPE) cells

- © Timing: Day 0–2 for step 1
- © Timing: Day 3–8 for step 2–7
- © Timing: 4 h–2 days for step 8 a
- © Timing: 3 h for step 8 b
- © Timing: 4–5 h for step 8 c
- © Timing: 4–5 h for step 8 d
- © Timing: 2 months for step 9
- © Timing: 1 h for step 10
- © Timing: 1 day for step 11
- © Timing: 1 day for step 12
1. Formation of Embryoid Bodies (EBs) by forced aggregation of iPSCs. **Troubleshooting 2.**

   a. Pre-warm the mTeSR plus, CTS™ DPBS (1 x) (-Ca²⁺/-Mg²⁺) to 37°C.

   **Note:** One 6-well plate (at 80% confluence, about 1 million cells per well) is sufficient to initiate the RPE differentiation through EB formation (Figure 1A inset).

   b. The cells from 6 wells will be going into 6 wells of ultra-low attachment plates (in order to avoid any form of attachment) for the formation of EBs.

   c. Prepare ultra-low attachment plates by adding 1 mL of mTeSR plus medium to each well.

   d. Aspirate the spent medium from the plate containing iPSCs, and rinse the plate once with CTS™ DPBS (1 x) (-Ca²⁺/-Mg²⁺) (Refer Table 1 for volume).

   e. Aspirate CTS™ DPBS (1 x) (-Ca²⁺/-Mg²⁺) and add ReLeSR Solution to the plate containing iPSCs. Adjust the volume of ReLeSR Solution for various plate sizes (Refer Table 1 for volume).
f. Add 1 mL/well of ReLeSR™ and aspirate ReLeSR™ within 1 min, such that colonies are exposed to a thin film of liquid.
g. Incubate at 37°C for 4 min ± 30 s.
h. Add an appropriate amount of mTeSR plus to each plate (Refer Table 1 for volume) to stop the dissociation reaction.
i. Gently pipet the dissociated cells up and down sufficiently to disperse the colonies into a single-cell suspension.
j. Make sure to pipet gently to minimize the formation of bubbles.
k. Transfer the iPSC suspension from each well into a separate 15 mL tube and centrifuge the tube(s) at 200 g for 3 min to pellet the cells.
l. Carefully aspirate the supernatant(s) from the iPSC pellet(s).
m. Resuspend the pellet(s) with an appropriate amount (approximately 6 mL) of mTeSR plus medium.
n. Add resuspended cells to each 6 well ultra-low attachment plate containing 6 mL mTeSR plus medium (1 mL/well) with 10 μM ROCK inhibitor.
o. This day is considered Day 0. Incubate the cells for 24 h in a 37°C, 5% CO2 incubator to allow them to form EBs. Replace media with 2 mL of fresh mTeSR plus to remove Y-27632.
p. Day 1 and Day 2: Gently swirl the EB plate and bring the EBs to centre of the dish. Remove 1 mL of medium from well corner and add 1 mL of fresh mTeSR plus medium (Figure 1A).

2. Differentiation induction and plating of EBs.
   a. Prepare DIM as per Table 2.

Note: Reconstitute and store growth factors as per manufacturer’s instructions.

https://www.sigmaaldrich.com/LN/en/product/sigma/i0161.
https://www.tocris.com/products/sb-431542_1614.
https://www.tocris.com/products/ldn-193189-dihydrochloride_6053.
https://www.stemcell.com/products/human-recombinant-igf-i.html.

| Table 1. Correlation of media volume to surface area of culture plate |
|---------------------------------------------------------------|
| Culture vessel | Surface area | DPBS | ReLeSR | Culture medium |
|----------------|--------------|------|--------|---------------|
| 6-well plate   | 10 cm²/well  | 2 mL/well | 1 mL/well | 2 mL/well     |
| 12-well plate  | 4 cm²/well   | 1 mL/well | 0.5 mL/well | 1 mL/well    |
| 24-well plate  | 2 cm²/well   | 0.5 mL/well | 0.3 mL/well | 0.5 mL/well |
| 35-mm dish     | 10 cm²       | 2 mL  | 1 mL   | 2 mL          |
| 60-mm dish     | 20 cm²       | 4 mL  | 2 mL   | 4 mL          |
| 100-mm dish    | 60 cm²       | 12 mL | 5 mL   | 12 mL         |

| Table 2. Differentiation induction medium (DIM) |
|-----------------------------------------------|
| Component                                      | For 500 mL | Final concentration |
| DMEM/F12                                       | 425 mL     |                   |
| Fetal Bovine Serum (FBS)                       | 50 mL      | 10%                |
| Sodium pyruvate (100 mM)                       | 5 mL       | 1 mM               |
| Sodium bicarbonate (7.5% solution)             | 5 mL       | 8.92 mM            |
| HEPES buffer (1 M)                             | 5 mL       | 10 mM              |
| Non-essential amino acids (100x)               | 5 mL       | 1x                 |
| N1 media supplement (100x)                     | 5 mL       | 1x                 |
| IWRI (2 mM)                                    | 500 μL     | 2 μM               |
| SB431542 (10 mM)                               | 500 μL     | 10 μM              |
| LDN 193189 (1 mM)                              | 50 μL      | 100 nM             |
| IGF-1 (100 μg/mL)                              | 50 μL      | 10 ng/mL           |
b. Gradually shift the medium of the suspension cultures with EBs from mTeSR plus to differentiation induction media (DIM).

c. Day 3: Replace one part of mTeSR plus media with DIM (3:1) (Example: For 1 mL media, 750 μL mTeSR plus media, and 250 μL DIM).

d. Day 4: Replace one part of mTeSR plus media with DIM (1:1) (Example: For 1 mL media, 500 μL mTeSR plus media, and 500 μL DIM).

e. Day 5: Give complete media change (100%) with DIM.

f. Day 6: Matrigel coating and EB plating. Troubleshooting 3.

   i. Gently swirl the EB plate and transfer EBs from 3 wells of a 6 well plate into a 15 mL tube.

   ii. Allow the EBs to settle to the bottom of the tube at room temperature (20°C–25°C) inside the bio-safety cabinet (it will take about 3 min). Do not centrifuge the EBs. Remove supernatant (retain 1 mL of media), add 2 mL of DIM and then add 500 μL of resuspended media in a drop-wise manner using 1 mL pipette tip such that the EBs are distributed uniformly across the plate.

   iii. Gently shake the culture plate to aid the uniform distribution of the aggregates and carefully place it back in the incubator. Do not disturb for 24 h.

   Note: We used AggreWell™800 Microwell Culture Plates (Stem Cell Technologies) for EB formation for the same RPE differentiation protocol. We did observe uniformity in terms of size of EBs generated using the multi-well plates. However, we did not find significant advantage with respect to final RPE numbers, processing time or cost incurred. Therefore, we continued using ultra low attachment dish to form EBs.

g. Day 7–8:

   i. After 24 h, observe the plates for EB attachment. The majority of the EB’s will attach and start exhibiting outgrowth from the periphery.

   Note: Floating EB’s can be discarded at this point while changing medium.

   ii. Do complete media change with 100% DIM (Refer Table 1 for volume).

3. Introducing Differentiation Propagation Media (DPM).

   a. Prepare DPM as shown in Table 3.

   Note: Add N1 supplement to filtered media.

Media can be stored at 4°C for 2 weeks.

b. Day 9: Gradually shift the medium from DIM to differentiation propagation media (DPM). Replace one part of DIM media with DPM (1:1) (Example: For 1 mL media, 500 μL DIM and 500 μL DPM).

Table 3. Differentiation propagation media (DPM)

| Components                        | For 500 mL | Final concentration |
|-----------------------------------|------------|---------------------|
| DMEM/F12                          | 465 mL     |                     |
| Fetal Bovine Serum                | 10 mL      | 2%                  |
| Sodium pyruvate (100 mM)          | 5 mL       | 1 mM                |
| Sodium bicarbonate (7.5% solution)| 5 mL       | 8.92 mM             |
| HEPES buffer (1 M)                | 5 mL       | 10 mM               |
| Non-essential amino acids (100x)  | 5 mL       | 1x                  |
| N1 media supplement (100x)        | 5 mL       | 1x                  |
c. Day 10: Give complete media change (100%) with DPM.

d. Day 11–22: Feed cultures with DPM on alternate days until day 22 (Refer Table 1 for volume) (Figures 1B and 1C).

4. Introducing Retinal Pigment Epithelium Maturation Media (RPEMM).

a. Prepare RPEMM as shown in Table 4.

Note: Add growth factors & supplement to filtered media. Reconstitute and store growth factors as per manufacturer’s instructions.

https://www.sigmaaldrich.com/IN/en/product/sigma/t0625.
https://www.sigmaaldrich.com/IN/en/product/sigma/h6909.
https://www.sigmaaldrich.com/IN/en/product/sigma/t5516.

Media can be stored at 4°C for 2 weeks.

b. Gradually switch the medium from DPM to Retinal Pigment Epithelium Maturation Media (RPEMM).

c. Day 23: Replace one part of DPM media with RPEMM (1:1) (Example: For 1 mL media, 500 μL DIM and 500 μL RPEMM).

d. Day 24: On day 24 switch to complete RPEMM (Figure 1D).

e. Day 25–45: Feed the cultures with RPEMM on alternate days until day 40 (refer Table 1 for volume).

5. Day 40 (+5): Enrichment step by passaging of RPE.

RPE cells can be passaged between day 35 to day 45 after the onset of pigmentation and this step helps in enriching the pigmenting cells (Figure 1E). Troubleshooting 4.

a. Matrigel coating-prepare matrigel coated plates as previously stated (Refer step 4 under preparations).

b. For 1 × 6 well plate, aliquot 3 mL of gentle cell dissociation reagent (GCDR) and 3 mL StemPro Accutase in a 15 mL tube and pre-warm in incubator at 37°C for 30 min.

c. Aspirate the culture medium and gently wash once with CTS™ DPBS (1×) (-Ca²⁺/-Mg²⁺) 1 mL/well.

d. Incubate the cells with 1:1 ratio of pre-warmed GCDR and Accutase at 37°C for 11–12 min.

Note: If required incubate for an additional 2 min at step 4 to ensure complete dissociation into single cells.

e. Pipette gently to dissociate into single cells or aggregates of 5–10 cells and dilute with RPEMM media.

f. Collect cell suspension in a 15 mL tube and centrifuge at 200 g for 3 min. Remove supernatant.

g. Aspirate matrigel just before seeding cells and add RPEMM media in each well (Refer Table 1 for volume).

h. Resuspend cell pellet in RPEMM and seed cells 1:2 or 1:3 in matrigel coated plates.

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**Table 4. Retinal pigment epithelium maturation media (RPEMM)**

| Components                      | For 500 mL | Final concentration |
|---------------------------------|------------|---------------------|
| MEM-x modified                  | 462 mL     |                     |
| Fetal Bovine Serum              | 25 mL      | 5%                  |
| GlutaMAX (100x)                 | 5 mL       | 1×                   |
| Taurine (50 mg/mL)              | 2.5 mL     | 0.25 mg/mL          |
| Hydrocortisone (20 mg/mL)       | 550 μL     | 10 μg/mL            |
| Tri-iodo-thyronine (2 mg/mL)    | 15 μL      | 0.0065 μg/mL        |
| N1 media supplement (100x)      | 5 mL       | 1×                   |

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Figure 2. Characterization of RPE

(A–E) Immunostaining of iPSC derived RPE cultures for the marker expression during stages of retinal differentiation. Images showing the expression of RPE commitment marker MITF (A), melanocyte protein- PMEL17 (B), RPE maturation markers TYRP1, RPE65 and TYR (C–E) and tight junction marker ZO-1 (D and E).

(F) Flow cytometry analysis of iPSC derived RPE cells for positive (MITF, RPE65, TYRP1) and negative (OCT4) markers.
i. Allow cells to attach without disturbing for 24–48 h.

j. After proper attachment of cells, resume media change.

6. Day 47–75 or later.
   a. Feed the cultures with RPEMM on alternate days until day 75–80.
   b. OPTIONAL STEP: Day 60 (±5): Passaging RPE.

RPE cells could be passaged again around day 60 (±5) by following the steps mentioned in Enrichment step by passaging of RPE (Refer step 5 in step-by-step method details) and maintain up to day 90–100 by changing medium on alternate days. This step can be implemented for further scale-up if there is a need to generate larger numbers of RPE cells for pre-clinical safety and toxicological studies.

7. Day 75 or later: Selection and cryopreservation.
RPE cells show cuboidal morphology at day 75 with visible deposits of melanin pigment (Figure 1F). Cultures are frozen using commercially available cryoprotectant - CryoStor® CS10 Freeze Media - BioLife Solutions in 1.8 mL NUNC cryotubes.
   a. Wash the cells twice with CTS™ DPBS (1 x) (−Ca²⁺/−Mg²⁺).
   b. Add pre-warmed GCDR and Accutase at 1:1 ratio and incubate for 10–12 min at 37°C.

**Note:** If enrichment step by passaging the RPE cells is not performed at day 40/45 then follow step c, else directly move to step d.

   c. Manually remove the visibly non-pigmented cluster areas if any (non-black) using 20–200 μL pipette tips and discard them. Gently flush the pigmenting patches out from the wells using 1 mL pipette tips.

**Note:** Repeat the flushing carefully in presence of RPEMM until all the visibly pigmented patches have been successfully detached.

   d. Collect the cells and mix with equal volumes of RPEMM. Centrifuge at 200 g for 2 min at room temperature (20°C–25°C).

**CRITICAL:** After counting the cells, determine the number of cryovials required to freeze approximately 1 million cells per vial in 1 mL of cold freezing medium. Label accordingly with cell line name, cell type, initials and date on each cryovial.

   e. To the RPE cell pellet add Cryostor CS10 freezing medium and gently mix the RPE cell suspension.
   f. Cryopreserve the cells (in suspension) in cryovials at 1 million cells per vial.
   g. Place the cryovials in Mr. Frosty freezing container and store at −80°C for 24 h.
   h. After 24 h, transfer the cells to the liquid nitrogen storage tank and update cryotank binder.

8. Characterization of RPE in vitro.
   a. Protein expression by Immunocytochemistry.
   Immunocytochemistry of differentiation cultures help visualize the expression level and localization of stage-specific retinal markers and authenticates successful differentiation of iPSC to RPE (Figures 2A–2E; Table 5).
Day 1.

i. Aspirate the culture medium and gently wash thrice with CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺) with fixing solution and incubate for 15–20 min at room temperature (20°C–25°C).

ii. Remove fixing solution and gently wash thrice with CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺).

iii. Permeabilize and block by adding blocking solution for 30 min at room temperature (20°C–25°C).

iv. Gently wash thrice with CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺).

v. Add primary antibody diluted in antibody diluent and incubate overnight (16–20 h) at 4°C.

Day 2.

vi. Remove primary antibody and wash thrice with CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺).

vii. Add secondary antibody diluted in antibody diluent and incubate in dark for 60 min at room temperature (20°C–25°C).

viii. Remove secondary antibody solution and wash thrice with CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺).

ix. Counterstain with 1 mg/mL concentration of DAPI and incubate in dark for 10 min at room temperature (20°C–25°C).

x. Remove DAPI solution and wash once with CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺).

xi. Add CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺) (300 µL for 1 well of a 4 well plate) and visualize under a fluorescent microscope.

Pause point: Fixed cells from step (ii) can be stored in CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺) at 4°C for 1 week before proceeding to staining.

b. Immunophenotyping by Flow cytometry.

Flow cytometry analysis of RPE cells helps to quantify the levels of protein expression providing reconfirmation of successful differentiation of iPSC to RPE (Figure 2F).

Enzymatic dissociation of RPE into single cells.

i. Aspirate culture media and wash cells with CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺).

ii. Harvest cells enzymatically (GCDR and Accutase at 1:1 ratio for 10–12 min; (Refer Section 7, steps a-d under step-by-step method details) and gently pipet to make a single cell suspension.

iii. Centrifuge at 200 g for 3 min and discard the supernatant.

iv. Wash the pellet by resuspending in CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺) and centrifuge at 200 g for 3 min.

v. Fix the cells by adding 1 mL of 4% PFA for 10 min at room temperature (20°C–25°C).

vi. Wash the cells with CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺) and centrifuge at 200 g for 3 min and completely remove CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺).

vii. Add 1 mL of ice-cold methanol to the pellet and incubate at 4°C for 20 min.

viii. Wash cells with FACS buffer (Refer Table 6) and centrifuge at 200 g for 3 min.

ix. Stain the cells with primary antibody at an optimized concentration in 100 µL FACS buffer and incubate for 30 min at room temperature (20°C–25°C).

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### Table 5. Immunocytochemistry (ICC) reagents

| Reagents            | Final concentration | Amount (for 10 mL) |
|---------------------|---------------------|--------------------|
| Fixing solution     |                     |                    |
| Paraformaldehyde (PFA) | 400 mg             |                    |
| CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺) | 1 x | 10 mL             |
| Blocking solution   |                     |                    |
| CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺) | 1 x | 10 mL             |
| Triton-X-100 (10%)  | 0.1%                | 100 µL             |
| FBS                 | 4%                  | 400 µL             |
| Antibody diluent    |                     |                    |
| CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺) | 1 x | 10 mL             |
| FBS                 | 2%                  | 200 µL             |
x. Repeat wash with FACS buffer and centrifuge at 200 g for 3 min.
xi. Add secondary antibody in 100 μL FACS buffer to the pellet at optimized concentration and incubate in dark for 30 min.
-xii. Vortex the vials every 10 min to ensure mixing.
-xiii. Wash with CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺) and resuspend in FACS buffer.
-xiv. Transfer the cells to flow tubes for flow cytometry analysis.

Pause point: Fixed cells from step (vi) can be stored in CTS-DPBS (1 x) (-Ca²⁺/-Mg²⁺) at 4°C for 48–72 h before proceeding to permeabilization and blocking.

Note: Permeabilization, blocking, and immunolabelling are done simultaneously to avoid cell loss during washing steps.

c. Gene expression analysis by quantitative real-time PCR.
Gene expression profiling helps to understand the molecular signature of these de novo generated RPE cells in comparison to undifferentiated iPSC.

Purification of total RNA.

Pellet preparation:
-i. Collect the cells in a sterile 1.5 mL tube (Refer Section 7, steps a-d under step-by-step method details) and centrifuge for one min at 200 g to pellet down the cells.
-ii. Remove the supernatant carefully and snap freeze the pellet using liquid nitrogen. Store the snap-frozen cells at −80°C until RNA isolation.

RNA isolation:
-iii. For RNA isolation, use the RNeasy Mini kit (Qiagen) and perform all the steps at room temperature (20°C–25°C).
-iv. Add 500 μL RLT buffer to the Cell pellet and add equal volume of 70% ethanol. Add the mixture into the spin column and spin at 8,000 g for 1 min.
v. Wash the column once with buffer RW1 and twice with buffer RPE. Perform all the washing steps at 8,000 g for 1 min.
vi. For the final elution, add 20 μL of RNase free water directly to the spin column, incubate for a minute, and spin at 8,000 g for 1 min to elute the RNA.
-vii. Keep the RNA immediately on ice and quantify using a nanodrop.

cDNA synthesis:
-viii. cDNA transcription is carried out using a Verso cDNA synthesis kit.

△ CRITICAL: Perform all the reaction setup steps on ice.

ix. Verso cDNA kit protocol recommends template RNA concentrations ranging from 1 pg to 1 μg. Prepare the cDNA synthesis reaction mix on ice by using the volumes mentioned in Table 7.
x. Incubate at 42°C for 45 min for cDNA synthesis to occur and 95°C for 2 min for enzyme inactivation. The synthesized cDNA can be used for qPCR analyses.

Note: RT enhancer used from the kit degrades dsDNA during the transcription of RNA and it gets inactivated at 95°C in the second step. This eliminates the need for DNase treatment.

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Table 6: FACS buffer

| Reagents            | Final Conc | Amount (for 10 mL) |
|---------------------|------------|--------------------|
| PBS                 | 1 x        | 10 mL              |
| FBS                 | 2%         | 200 μL             |
| Triton-X-100 (10%)  | 0.25%      | 250 μL             |
RT-qPCR.
Analyzing the expression of various genes by RT-qPCR. RT-qPCRs were done using QuantStudio3 Real-Time PCR machine and the results were analyzed using the 2-\(\Delta\Delta C_t\) method. Perform RT-qPCR experiment preferably with minimal light.

xi. qPCR using SYBR green: Prepare 20 μL reaction mixture containing 10 μL of SYBR green mix (2×), 1 μL each of forward and reverse primer (stock 10 μM), 2 μL of cDNA (50 ng), and 6 μL of water (Figure 2G).

xii. Run the qPCR reaction as mentioned in Table 8.

xiii. qPCR using Taqman probes: Prepare a 20 μL reaction mixture containing 10 μL of Taqman master mix (2×), 1 μL of the gene-specific probe (stock 20×), 2 μL of cDNA (50 ng), and 7 μL of water (Figure 2H).

xiv. Run the qPCR reaction as mentioned in Table 9.

xv. Quantification of the gene expression: Here, the expression of test genes is normalized against the expression level of Beta-ACTIN/GAPDH as housekeeping genes. Calculate the fold change is by normalizing the expression of the samples (RPE) against a control (iPSC).

d. Measurement of extracellular secretion of protein by Enzyme-linked immunosorbent assay (ELISA).

Secretion of Pigment-Epithelium Derived growth Factor (PEDF) is an important criterion to assess the polarity of \textit{in vitro} generated RPE (Figure 2I). Quantification of PEDF is done enzymatically using a commercially available kit (Human Serpin F1/ PEDF Duoset ELISA kit).

i. Coat the ELISA plate with 100 μL of capture antibody and incubate overnight at room temperature (20°C–25°C).

ii. Wash the coated wells with wash buffer 3 times. Perform all the steps at room temperature (20°C–25°C).

iii. Add 300 μL of reagent diluent as a blocking solution and incubate for 1 h. Repeat washing 3 times with wash buffer.

iv. Add 100 μL of the sample (the day on which the cell supernatant is harvested) and standards diluted in reagent dilution to the coated wells and incubate for 2 h. Carefully wash thrice with wash buffer.

v. Add 100 μL of detection antibody diluted in reagent diluent and incubate for 2 min.

vi. Carefully wash thrice with wash buffer.

vii. Add 100 μL of Streptavidin-HRP to each well. Cover the plate and incubate for 20 min in dark.

viii. Carefully wash thrice with wash buffer.

| Table 7. cDNA synthesis reaction | Components | Volume |
|----------------------------------|------------|--------|
| 5x cDNA synthesis buffer         | 4 μL       |
| dNTP mix                         | 2 μL       |
| Oligo dT                         | 1 μL       |
| RT enhancer                      | 1 μL       |
| Verso enzyme mix                 | 1 μL       |
| RNA                              | 1 μg       |
| Nuclease free water              | Upto 20 μL |

| Table 8. qPCR cycle parameters- SYBR green | Steps       | Temperature | Time | Cycles |
|-------------------------------------------|-------------|-------------|------|--------|
| UNG incubation                            | 50°C        | 2 min       | 1    |
| Polymerase activation                      | 95°C        | 2 min       | 1    |
| Denaturation                               | 95°C        | 15 s        | 40   |
| Anneal/Extension                           | 60°C        | 1 min       |      |
ix. Add 100 μL of substrate solution and incubate for 20 min. Avoid exposing the plates to direct light.

x. Add 50 μL of Stop Solution to each well and mix the plate gently.

xi. Determine the optical density of each well immediately at 450 nm and 540 nm for background subtraction using Varioskan LUX microplate reader.

xii. The concentration of PEDF from the samples is analyzed by plotting the standard values as a graph.

9. Preclinical safety and efficacy studies in animal model.
Sub-retinal transplantation of freeze-thawed RPE cells in animal model (rat) helps to evaluate the efficacy and safety of these cells in vivo, thereby providing important IND enabling pre-clinical data.

a. Animal maintenance.
   i. Breed a colony of Pigmented RCS (RCS-p+/Lav) rats (NIH Rat Resource and Research Center, #315).
   ii. Feed animals a standard laboratory chow and maintain animals on a 12 h dark/light cycle.
   iii. Wean animals at postnatal day (PD) 21 and administer oral cyclosporine A (210 mg/L; Gengraf, North Chicago, IL) in drinking water. Animals should be kept on cyclosporine A until sacrifice.

10. Cell preparation.
Preparing the cells prior to injection under aspetic conditions following this protocol is extremely important for best performance of the cells post transplantation into animals.

   a. Remove cells from liquid nitrogen storage and thaw in a 37°C water bath for 2 min.
   b. Add 1 mL of pre-warmed DMEM/F12 media dropwise to the vial.
   c. Transfer cells to a 15 mL tube containing 5 mL of pre-warmed DMEM/F12 media.
   d. Rinse cryotube with 1 mL of DMEM/F12 media and add to 15 mL tube.
   e. Centrifuge cells at 150 g for 5 min at room temperature (20°C–25°C).
   f. Aspirate supernatant and re-suspend cells in 1 mL of pre-warmed DMEM/F12 media.
   g. Carefully pipette up and down to re-suspend the cells.
   h. Remove 10 μL of cell suspension and add to 10 μL of 0.08% Trypan blue solution.
   i. Pipette up and down and load hemacytometer (10 μL each side).
   j. Count viable cells.
   k. Centrifuge cell suspension again at 150 g for 5 min.
   l. Resuspend cells in appropriate volume to achieve desired concentration for injection.

11. Subretinal injections (Wilson et al., 2017).
We tested cryopreserved RPE frozen at day 75–80 for subretinal injections and we strongly believe that at this stage iPSC-RPE are suitable for application in transplantation studies. Transplantation of cells at earlier stages of differentiation is possible but progenitor cell behavior is not well known in vivo.

   a. One hour before injections, dilate pupils with 1% tropicamide and 2.5% phenylephrine.
   b. Anesthetize eyes with topical 0.5% proparacaine HCl.
   c. Sedate animals with intraperitoneal ketamine/xylazine (100/10 mg/kg).
   d. Cut the corner of the eyelid to allow for visualization of the posterior retina. Use a hemostat to reduce blood accumulation.
e. Make a small scleral/choroidal incision (~1 mm) 2-mm posterior to the limbus in the dorso-temporal region using 27 gauge needle tip.

f. Make a small lateral corneal puncture using a 30-G needle to limit increases of intraocular pressure and reduce efflux of cells following injection.

g. Deliver two microliters of cell suspension containing the total cell dose or control media into the subretinal space using a fine glass pipette (internal diameter, 75–150 μm) inserted into the subretinal space. The glass pipette is connected to a 10 μL Hamilton syringe (Hamilton, Reno, NV) with small-bore (400 μL total volume) microtubing.

h. After delivery, using a glass coverslip, look at the retina under the microscope and score the subretinal bleb based on size and injection related issues, for example: bleeding, bubbles, cells in the vitreous.

i. Place 0.5% erythromycin ointment on eyes and let animals recover from anesthesia.

j. On surgery day, administer intraperitoneal dexamethasone (1.0 mg/kg). Administer dexamethasone to animals every other day for 2 weeks post-cell transplantation to minimize a potential inflammatory response.

Note: Injections should occur between PD22 and PD25. Animals need to be placed on cyclosporine A one day prior to injections.

12. Optokinetic Tracking (OKT).
Optokinetic thresholds should be assessed using a virtual optomotor system (VOS; CerebralMechanics) comprising of four computer monitors arranged in a square, displays facing inwards. On the monitors, a virtual cylinder displays sine-wave gratings that can be rotated either clockwise or counter clockwise permitting evaluation of both the left and right eyes independently; the left eye responds to clockwise, and the right eye to counterclockwise movement (Figure 4C).

a. Place the animal on the pedestal in the center of the four computer monitors. Allow the animals 5 min to acclimate to the pedestal.

b. Using the Cerebral Mechanics software, set up the simple staircase examination for both eyes, keeping the contrast set to 100%.

c. The program will begin with a low spatial frequency, the cylinder will rotate and if the grating is resolved, the animal will respond with a reflexive head and neck movement tracking the grating. If the animal responds click ‘yes’, if the animal doesn’t respond click ‘no’. Based on this input, the spatial frequency of the grating will then incrementally increase until the animals no longer track the stimulus, resulting in a maximal spatial frequency threshold.

d. Once the exam is finished, take the animal off the pedestal and return them to their home cage.

Note: The RCS rat has a near normal OKT through PD60. To determine the functional rescue resulting from the cell transplantation, this exam is most optimal no earlier than PD90.

13. Fundus photography.
A fundus camera or retinal camera is designed to observe the inner regions of eye, mainly the retina. Fundus images can be obtained at any time post-injection to verify successful transplantation of cells and the length of cell survival (Figure 4A).

a. Dilate pupils with 1% tropicamide and 2.5% phenylephrine.

b. Anesthetize eyes with topical 0.5% proparacaine HCl.

c. Sedate animals with intraperitoneal ketamine/xylazine (100/10 mg/kg).

d. Keep eyes lubricated with 2.5% Goniovisc Hypromellose.

e. Using the Micron IV (Phoenix-Micron, Inc.) imaging system use the white light to capture bright field images of the retina.

f. When imaging, use the optic nerve head as a guide, but then position the animal so that the peripheral/temporal region of the retina, where the injection took place, is in view.

g. Place 0.5% erythromycin ointment on eyes and let animals recover from anesthesia.
14. Tissue preparation.

Tissue samples must be appropriately harvested and prepared for histology and immunohistochemistry studies. Sample preparation include processes such as fixation, dehydration, embedding and sectioning.

a. After euthanasia following IACUC guidelines, enucleate the eye as rapidly as possible.
b. Place the enucleated eye into cold 4% paraformaldehyde (PFA). Try to maintain a 10–20× fixative to tissue ratio.
c. After 10–60 min of fixation, remove the anterior chamber of the eye including the lens and replace the remaining eye cup into 4% PFA. Place samples in PFA on ice.
d. Place the eyes in 2°C–8°C for 24–48 h.
e. After 24–48 h, remove the PFA solution and add an equal volume of cold 10% sucrose solution in 1×PBS.
f. Place the eyes in 2°C–8°C for 24 h.
g. Remove the 10% sucrose solution and replace the solution with an equal volume of cold 20% sucrose solution.
h. Place the eyes in 2°C–8°C for 24 h.
i. Remove the 20% sucrose solution and replace the solution with an equal volume of cold 30% sucrose solution.
j. Place the eyes in 2°C–8°C for 24 h.
k. Remove the eye cup from the 30% sucrose solution and dab dry on a piece of filter paper.
l. Place the eye cup cut side down on filter paper to allow the vitreous to drain out of the eye cup. Repeat until the filter paper remains dry. See Figure 3A to view complete vitreous removal on filter paper (black represents vitreous imprint on the filter paper).
m. Place the eye cup into a labeled cryomold and fill with OCT media.

Figure 3. Tissue preparation for histopathology

(A–C) Diagramatic representation of vitreous removal on filter paper (A), orientation of eye cup (B) and freezing of cryomold (C) for histopathology.
n. Orient the eye cup toward the front of the cryomold and orient the injection site at about 2’oclock for OS (oculus sinister left) eyes and 10’oclock for OD (oculus dextrus right) eyes (Figure 3B).

o. Freeze the cryomold, containing the eye, in a liquid nitrogen bath. Ensure that the liquid nitrogen does not directly touch the OCT media, but only contacts the walls of the cryomold (Figure 3C). Direct contact of liquid nitrogen to OCT media creates air bubbles, ruining sample integrity.

p. Once the media no longer has a glossy shine, remove it from the liquid nitrogen bath.

q. Tightly wrap the cryomold in aluminum foil to try to limit the block’s exposure to air.

r. Place wrapped blocks in a -20°C freezer.

15. Histology / Immunohistochemistry. Hematoxylin and eosin (H&E) and immunohistochemical staining are valuable tools for detecting histopathological changes and specific antigens in tissues.
a. Section eye blocks at 12 μm.

b. Obtain approximately 40 slides containing 4 sections per slide from each eye.

c. Collect sections in a 5-slide series to provide a representative section every 60 μm on each slide throughout the eye-cup.

d. Stain the first slide of each series with cresyl violet or hematoxylin and eosin and examine retinal damage/toxicity and evidence of photoreceptor rescue (Figure 4B).

e. For immunohistochemistry, dry slides for at least 30 min after removing from freezer.

f. Block for 45 min with 4% horse serum, 1% bovine serum albumin (BSA), 0.5% triton x in phosphate buffered saline (PBS).

g. Add primary antibodies (anti-cone arrestin, generously provided by W. Clay Smith, PhD, University of Florida) to blocking buffer and incubate overnight (minimum 15 h) at 4°C.

h. The next day, wash 3×5 min with 1×PBS.

i. Add secondary antibodies (1:300) in blocking buffer and incubate for 45 min in the dark at room temperature (20°C–25°C).

j. Wash 3×5 min with 1×PBS.

k. Incubate with DAPI in the dark for 10 min.

l. Wash 2×5 min with 1×PBS.

m. Coverslip the slides with 100 μL of Fluoromount G.

16. Quantification of photoreceptors.
The outer nuclear layer thickness (ONL) was measured with the help of immunohistochemical staining as the primary indicator of photoreceptor rescue (Figures 4D–4F).

a. Use a scanning laser confocal microscope (Leica SP5 using LAS AF software; Leica) to image stained slides. Keep laser intensity settings (gain) consistent for each emission wavelength.

b. Collect Z-stack images at ×10 and ×20 magnification at 1024×1024-resolution with 1-μm step size.

c. Keep color channels separate as each color channel z-stack is flattened. Save each color channel image and the color merged image as TIFF files.

d. Count ONL cell bodies within the rows of the ONL in both temporal (injected) and nasal (non-injected) regions to obtain retinal thickness – nuclei values.

e. Count cone arrestin positive cells in both temporal (injected) and nasal (non-injected) regions to obtain cones per image values.

f. Have three observers blinded to the dosage and age group generate counts.

g. Using data collected in steps d-f, average ONL counts and photoreceptor counts per group and present data from both temporal (injected) and nasal (non-injected) regions.

EXPECTED OUTCOMES

Human iPSC lines are created using various reprogramming methods and from different tissue types like fibroblasts, peripheral blood etc.). As a result, it shows variation in propensity of differentiation into specific cell/tissue types, including RPE. We can produce 20–24 million RPE with a starting population of 1 million iPSCs. The yield being >20 times the starting population with minimal manipulation makes the protocol scalable and robust. RPE generated following this protocol were frozen or expanded further for another doubling at a more mature stage around day 60 or later.

From the perspective of developmental biology, there are four major steps in the protocol:

Induction stage leading to commitment towards retinal lineage resulting in eye field specification. We expect over 90% expression of Paired box 6 (PAX6) and Retinal homeobox (RAX) protein with a concomitant decrease of the pluripotency marker OCT-4 at around differentiation day 20.
Propagation stage for expansion of cells that undergoes optic vesicle patterning expresses high levels of visual system homeobox 2 (VSX2/CHX10) and the melanocyte inducing transcription factor (MITF) from day 20 onwards.

RPE specification stage marks a drastic change of cellular morphology and onset of pigmentation. Brown to black spots indicating melanin production appears in the cultures and continue to develop between day 30–40. At this stage, Pre-melanosome marker PMEL17 strongly co-expresses with MITF (Figures 2A and 2B). Tight junction boundaries around hexagonal cells can be visualized by Zone Occludase-1 (ZO-1) staining.

RPE maturation and ciliation stage, wherein visual cycle protein RPE65 and melanocyte protein PMEL17 expressed abundantly coupled with tyrosinase (TYR) and tyrosinase-related protein 1 (TYRP1), key enzymes involved in melanin production (Figures 2C–2E).

20%–30% pigmented RPE at day 40–45 were harvested and cultured on transwell chambers with 0.4 μm polyester membrane. Upon transitioning these cultures to the air-liquid interface (ALI) in transwell chambers, RPE cells underwent polarization and produced high levels of pigment epithelium-derived factor (PEDF) in the apical chamber exposed to air than the basal layer containing the medium (Figure 2I). Confocal imaging of RPE grown in transwell chambers using Z-stack analysis clearly demonstrated appropriate localization of primary cilia markers, ARL13B and acetylated-tubulin (data not shown).

LIMITATIONS
This protocol starts with neuroectoderm induction involving combined dual SMAD and WNT inhibition. Notably, most of the existing protocols for neural differentiation from iPSCs also depend on combined dual SMAD and BMP inhibition. Moreover, early specification of the anterior neuroectoderm and formation of forebrain (cortical neurons) requires WNT inhibition. Therefore, it is of utmost importance to eliminate the undesired neural (or neural stem cell) population from the cultures as early as possible. Though further steps in this protocol do not support the growth and expansion of neural cell (non-retinal) types, the selection step at day 40–45 or later helps significantly in enriching the RPE population thus facilitating a better yield of functional RPE.

In some batches, the onset of pigmentation may slow down (after day 30–35) due to various reasons involving the initials steps of differentiation induction. One of the main reasons is the improper EB size and plating density resulting in delayed retinal induction and RPE specification. Although cluster selection and making uniform EB’s has been optimized, the process is still not ready for automation. Nevertheless, once the brown/black spots appear and if the pigmentation is progressing steadily, the expected outcomes will be met within the anticipated time frame. Nevertheless, despite these caveats we feel that even without automation we have achieved a process that meets the scale needs of manufacturing RPE economically. The current protocol is particularly suitable for in vitro disease modeling, drug screening and regenerative medicine.

TROUBLESHOOTING
Problem 1
Human iPSC quality – Refer “iPSC culture and maintenance section” under Preparations, step 2.

Potential solution
It is very important to maintain iPSC cultures devoid of any spontaneously differentiated cells. A mechanical colony cutting technique can be adopted to get rid of unwanted cells before the next passage. Please refer to the iPSC passing section using ReLeSR which selectively choses iPSCs leaving the differentiated cells on the plate.
Problem 2
Irregular sized EBs with improper boundary – Refer “Formation of Embryoid bodies (EBs) by forced aggregation of iPSC” section, step 1.

Potential solution
In case the iPSC cultures are impure and or unhealthy, it leads to formation of irregular EBs with blebbing. So, it is paramount to maintain good quality iPSC without differentiated cells. The addition of a Rho-kinase inhibitor is also essential for proper EB formation.

Problem 3
Size and density of EBs to be plated – Refer “Differentiation induction and plating of EBs” section, step 2.f.

Potential solution
It is very important to maintain medium sized EBs. Bigger EBs could potentially lead to overcrowding of cells which could impact differentiation. Huge EB cores could deter crosstalk between cells leading to minimal pigmentation and non-epithelial morphology of cultures.

Problem 4
Cell morphology and percentage of pigmentation affects RPE passaging – Refer ”Day 40 (+5): Enrichment step by passaging of RPE” section, step 5.

Potential solution
Ensure that majority of cells show RPE- (cuboidal to hexagonal) like morphology and that a minimum of 20% of cells show pigmentation before attempting to passage. Since epithelial cells require cell to cell contact, ensure a large seeding ratio.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rajarshi Pal (rajarshi.pal@eyestem.com).

Materials availability
Human normal donor iPSC lines TC-1133 (RUCDR/NIH), and the disease lines ERPLi001-A, ERPLi002-A, and ERPLi003-A (Konala et al., 2020) are available from the Eyestem Biobank and can be found at https://eyestem.com/collaborations/research-alliance/.

Data and code availability
The published article PMID: 33468244 includes mRNA sequencing dataset generated and analyzed during RPE differentiation. Sequencing data is deposited in the NCBI GEO datasets accessible at GSE140545 (Surendran et al., 2021).

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AUTHOR CONTRIBUTIONS
H.S. and L.S. developed the RPE differentiation protocol and wrote the manuscript. H.S., L.S., V.B.R.K., and J.S. performed differentiation and characterization experiments. J.S., W.T., and R.R. performed animal experiments and did the analysis. R.R. and R.P. revised and proof-read the manuscript.
DECLARATION OF INTERESTS
The authors declare no competing interests.

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