Generation of Retinal Ganglion Cells from Reprogrammed Keratocytes of Non-Glaucoma and Glaucoma Donors

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Human induced pluripotent stem cell (hiPSC)-based disease modeling can be successfully recapitulated to mimic disease characteristics across various human pathologies. Glaucoma, a progressive optic neuropathy, primarily affects the retinal ganglion cells (RGCs). While multiple groups have successfully generated RGCs from non-diseased hiPSCs, producing RGCs from glaucomatous human samples holds significant promise for understanding disease pathology by revealing patient-specific disease signatures. Given that keratocytes originate from the neural crest and previous reports suggest that ocular fibroblasts from glaucomatous donors carry pathogenic signatures, it is highly plausible that these signatures imprinted within the keratocytes will also be present in the derived RGCs. Thus, we aimed to generate RGCs from both glaucomatous and non-glaucomatous donor keratocytes and validate disease-specific signatures in 3D retinal organoids and in isolated RGCs. Our protocol describes the generation of iPSCs from keratocytes of both glaucomatous and non-glaucomatous donors, followed by their differentiation into retinal organoids. Subsequent isolation and culturing of RGCs were performed. Disease signatures in the RGCs were validated in both 3D retinal organoids (ROs) and 2D RGC cultures, and glaucomatous RGCs in 3D and 2D cultures demonstrated increased cleaved CASP3 and significant RGC loss, indicating disease imprints in the hiPSC-derived RGCs. This model offers a venue and high throughput platform for studying glaucomatous disease pathology and holds significant potential for drug discovery using RGCs derived from human donors. © 2025 The Author(s). Current Protocols published by Wiley Periodicals LLC.

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Keywords: glaucoma • induced pluripotent stem cells • keratocytes • retinal ganglion cells • retinal organoids

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INTRODUCTION

Induced pluripotent stem cell-based disease modeling holds significant promise for studying complex and intricate disease mechanisms in neurodegenerative diseases, including optic neuropathies. Glaucomatous optic neuropathy, a global predominant cause of visual impairment, affects the retinal ganglion cells (RGCs), which are the terminal visual neurons. Although promising *in vivo* model systems are currently available to address the pathology of glaucomatous neurodegeneration, human induced pluripotent stem cells (hiPSC)-based RGC generation stands alone by mimicking disease pathology specific to humans and discerning species-specific variations (Shi et al., 2017). Moreover, the iPSC-derived adult RGC can be maintained in culture for a long time, enabling us the opportunity to understand disease-specific physiological and pathological changes occurring in the mature retina and enhance our understanding of developing precision medicine therapeutics, future transplantation and drug screening studies (Romano & Hicks, 2007). In vitro studies for glaucoma disease modeling have mainly employed RGCs sourced from adult retina (Park et al., 2019; Boal et al., 2023) or RGCs derived from embryonic stem cells (ESCs) (Wu et al., 2021). However, a significant focus of recent studies has been on RGCs generated from hiPSCs. As a reliable and ethically approved source, hiPSCs are regarded as an invaluable resource for disease modeling. Methods published over the years have used the approaches of deriving RGCs using iPSCs sourced from various parent somatic cells (Ji & Tang, 2019) such as blood cells, keratinocytes (Gudiseva et al., 2021; Yokoi et al., 2017) dermal fibroblasts (Rabesandratana et al., 2020), urine cells (G. Li & Luo, 2024), and IMR-90 lines (Agarwal et al., 2023; K. Li et al., 2017). Efforts have also been made to derive diseased RGCs, including the use of CRISPR/Cas9 gene editing to introduce the OPTN (E50K) mutation into hiPSCs (VanderWall et al., 2020) and deriving iPSC RGCs from patient blood cells carrying the *SIX6* risk allele (Teotia et al., 2017). Moreover, prior reports have shown the successful establishment of hiPSC lines from glaucoma patients with CYP1B1 mutations (Bolinches-Amoros et al., 2018) and from a patient with congenital glaucoma (Zhang et al., 2018). Nevertheless, the downstream generation of RGCs and their phenotypic and functional characteristics have yet to be explored.

While iPSC-derived RGCs offer a valuable tool for understanding disease phenotypes, a crucial aspect lies in the source of the somatic cells used to derive these iPSCs. For instance, disease modeling in iPSCs is strongly influenced by the parent somatic cells, largely due to the epigenetic memory these cells retain (Poetsch et al., 2022). The patient's derived iPSCs exhibit significantly greater transcriptomic variability compared to controls, underscoring their potential for studying disease phenotypes (Daniszewski et al., 2022). Evidence from previous studies, such as variations in somatic mutation profiles across tissues related to specific cellular functions and distinct transcriptional and epigenetic patterns in iPSCs derived from different parental somatic cells, highlights the significance of selecting parental somatic cells when studying disease mechanisms (Garcia-Nieto et al., 2019; Polo et al., 2010). Moreover, another study demonstrated differences in the potential of iPSCs to form dopaminergic neurons as an influence of parental somatic cells (Chlebanowska et al., 2020). This evidence suggests that ocular sources could provide a potentially valuable tool to study glaucomatous RGC phenotypes. Evidence

from genomewide association studies showed that ocular fibroblasts from glaucomatous donors carry pathogenic signatures (Roodnat et al., 2024). Keratocytes are derived from the neural crest cells during embryonic development (Naylor et al., 2016). Thus, it is highly plausible that the genetic and epigenetic signatures imprinted within the keratocytes will also be present in the derived RGCs and may enhance our understanding of the disease phenotype effectively. Although previous studies have made limited efforts to generate iPSCs from keratocytes (Bikkuzin et al., 2019), to the best of our knowledge, no studies have successfully generated iPSCs from keratocytes of glaucoma donors and derived RGCs from them.

Moreover, none of the studies have investigated whether RGC phenotypes from keratocyte-derived iPSCs differ between glaucomatous and non-glaucomatous sources.

Thus, we have established the protocol for generating hiPSCs and RGCs downstream by using keratocytes from glaucomatous and non-glaucomatous donor eyes. We have characterized the iPSCs and karyotyped them to evaluate chromosomal loss during reprogramming, and RGCs have been generated downstream. These *de novo* RGCs were then characterized by cellular markers, and most importantly, we evaluated apoptotic marker expression between glaucomatous and non-glaucomatous ROs and their derived RGCs, which depicted a significant apoptotic signature in glaucomatous RGCs compared to non-glaucomatous RGCs.

CAUTION: Precautions must be taken while handling the human donor tissues, including wearing personal protective equipment such as lab coats and face masks and implementing double gloving for all procedures. All tissue handling and cell culture procedures need to be performed under a sterile laminar hood. All tips, viral media, and containers used for reprogramming must be autoclaved and disposed of in biosafety containers.

METHODOLOGY

Human donor tissue collection and demographics

The study utilized de-identified human specimens in accordance with institutional IRB approval, which exempts the need for informed consent and approved IRB. The study and the methods performed adhered to Indiana University School of Medicine's approved guidelines for Not Human Subject Research (NHSR) and to the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) for handling de-identified human specimens. The human non-glaucomatous and glaucomatous donor corneas were collected from the Lions World Vision Institute (Tampa, FL) and Lions Gift of Sight (Minnesota) eye banks and processed according to the Declaration of Helsinki. All the donors were Caucasian, and no significant differences were noted between the ages of the non-glaucomatous and glaucomatous donors. The detailed demographics of all donors have been provided in Supplementary Table 1. Donors with corneal infection, ocular pathology other than glaucoma, or other progressive central nervous system diseases were excluded. Experiments were performed as three independent repeats. All experiments with human donor tissues were performed in accordance with the Indiana University Institutional Biosafety Committee approval (Proposal #IBC1318).

NOTE: Appropriate informed consent is necessary for obtaining and use of human study material.

CULTURING OF KERATOCYTES FROM HUMAN CADAVERIC DONORS

BASIC PROTOCOL 1

Somatic cells can be reprogrammed into iPSCs; however, it has been shown that the parent somatic cells can influence iPSC phenotypes largely due to residual epigenetic memory (Poetsch et al., 2022; Raab et al., 2014). Thus, keratocytes, derived from the human cornea, can be an effective resource for studying ocular pathologies, particularly

Table 1 Reagents and Consumables for Basic Protocol 1

Reagents		
Item	Company	Cat. no.
1× Phosphate-buffered saline (PBS)	Corning	21-040-CV
Type II Collagenase	Gibco	17101-015
Dulbecco's Modified Eagle Medium (DMEM)	Corning	15-017-CV
Fetal bovine serum (FBS)	ATLAS BIOLOGICALS	F-0500-D
Penicillin-streptomycin	Gibco	15140-122
TryPLE	Gibco	12605-010
Consumables		
Item	Company	Cat. no.
Laminar flow hood	Thermo Fisher Scientific	1300 SERIES A2
Sterile dish	Corning	430167
15-mL centrifuge tube	Corning	430766
Centrifuge	Thermo Fisher Scientific	Sorvall ST 8
6-well cell culture dishes	Fisherbrand	FB012927
CO_2 incubator	Thermo Fisher Scientific	HERACELL VIOS 160i
Fluorescent microscope	Nikon	ECLIPSE Ti2

in diseases like glaucoma, where adult RGCs are not available for downstream analysis. The procedure detailed here can be used to isolate and culture primary keratocytes from human donor corneas. The source tissues were either dissected corneas or whole globes. If whole globes were used, the corneas were dissected in a sterile hood, as previously published (Hameed et al., 2024). The keratocytes were cultured following the protocols published previously (Binte et al., 2022; McKay et al., 2020) with slight modifications. The below protocol also details the steps involved in passaging the keratocytes for downstream experiments.

Materials

Required reagents and consumables for Basic Protocol 1 are listed in Table 1.

Isolation and culturing of keratocytes

1. Process all human donor tissues in a sterile laminar hood.
2. Transfer the non-glaucomatous and glaucomatous corneas to a sterile dish and rinse the corneas with 1× phosphate-buffered saline (PBS).
3. Carefully remove any remaining scleral tissues using microfine-point scissors while holding the corneas with forceps.
4. Using a sterile scalpel blade, remove the corneal epithelium and endothelium from the top and bottom of the tissue, respectively.
5. Wash the tissue with 1× PBS to remove the remnants of epithelium and endothelium.
6. Cut the tissue into 1–2 mm pieces and transfer to a 15-mL centrifuge tube.

7. Add collagenase type II (30 mg/mL in 1× PBS) to the tissue pieces to dislodge the cells, and incubate at 37°C for 3 hr.
8. Remove the enzyme by brief centrifugation at 70 × *g* and room temperature for 5 min
9. Collect the cells and resuspend them in DMEM containing 10% FBS and 1× Pen Strep.
10. Culture the cells in a 6-well culture dish in a CO_2 incubator under standard cell culture conditions.
11. Change the medium after 3 days of incubation, then exchange the medium every 2 days until the keratocytes reach 70% to 80% confluency.

Passaging of keratocytes

12. Remove medium from the confluent keratocyte culture and wash once with 1× PBS.
13. Add 1 mL TryPLE express and incubate at 37°C for 3 min.
14. Neutralize the TryPLE by adding 1 mL complete DMEM. Collect the dislodged cells and transfer to a 15-mL centrifuge tube.
15. Centrifuge at 70 × *g* and room temperature for 5 min and remove the supernatant carefully.
16. Resuspend in complete DMEM and seed the cells for downstream experiments.

REPROGRAMMING DONOR KERATOCYTES INTO iPSCs

BASIC PROTOCOL 2

Previous studies have reprogrammed keratocytes into iPSC using integrating and non-integrating viral vector systems including retroviral systems such as FUW-SOKM-harboring lentiviruses (LvSOKM) (Joseph et al., 2016), pMXs vectors (Chien et al., 2012), and Epi5 reprogramming vectors (Bikkuzin et al., 2019) to simulate different disease etiologies or explore therapeutic strategies. The current protocol employed the CytoTune^{TM}-iPSC 2.0 Sendai reprogramming kit, which is based on three non-integrative Sendai viral vectors containing hKOS, hc-Myc, and hKlf4 for iPSC generation. These vectors collectively express the four Yamanaka factors: Oct3/4, Sox2, Klf4, and c-Myc. These viral vectors are necessary for reprogramming and transitioning the cells to the pluripotent stem cell state. The vectors Oct3/4 and Sox2 regulate the genes critical for maintaining pluripotency, Klf4 controls cell proliferation, differentiation, and survival, while c-Myc promotes cell proliferation and metabolism during reprogramming (Liu et al., 2008). The keratocytes cultured from both glaucomatous and non-glaucomatous donor corneas at passage three, following characterization (Supporting Protocol 1), were used for iPSC generation. The detailed protocol for iPSC generation, clonal propagation, and passaging is given below.

Materials

Required reagents and consumables for Basic Protocol 2 are listed in Table 2.

NOTE: All iPSC cells should be seeded in rh-laminin 521-coated culture dishes.

NOTE: Ensure that clones are selected without cross-contaminating them with other clones in the plate.

Keratocytes reprogramming into iPSCs

1. Coat a 6-well plate with rh-laminin 521 at a final concentration of 10 µg/mL in Hanks' Balanced Salt Solution (HBSS) and incubate the plates overnight at 4°C.

Table 2 Reagents and Consumables for Basic Protocol 2

Reagents		
Item	Company	Cat. no.
Rh Laminin 521	Corning	354221
Hank's Balanced Salt Solution (HBSS)	Corning	21-023-CV
MEM-α	Thermo Fisher Scientific	12571063
KnockOut serum replacement	Thermo Fisher Scientific	10828010
Primocin	Invivogen	ant-pm-1
Glutamax	Gibco	35050-061
10 ng/mL bFGF	R&D System	GMP rhFGF-basic
The CytoTuneTM-iPSC 2.0 Sendai reprogramming kit	Thermo Fisher Scientific	A16517, Lot no. L2190071
TryPLE	Gibco	12605-010
Versene	Thermo Fisher Scientific	15040066
ROCK inhibitor (ROCKi)	Millipore Sigma	6880001-1MG
Essential 8 (E8) flex medium	Thermo Fisher Scientific	A2858501
Consumables		
Item	Company	Cat. no.
Laminar flow hood	Thermo Fisher Scientific	1300 SERIES A2
Centrifuge	Thermo Fisher Scientific	Sorvall ST 8
6-well cell culture dishes	Fisherbrand	FB012927
12-well cell culture dishes	CytoOne	CC7682-7506
CO_2 incubator	Thermo Fisher Scientific	HERACELL VIOS 160i
Inverted phase contrast microscope	Nikon	ECLIPSE Ts2

2. Trypsinize the keratocytes and seed at a density of 1×10^5 cells/well in a rh-laminin 521-coated dish.
3. Feed the cells with complete DMEM until they reach 60% to 70% confluency at the time of transduction.
4. Add the viral vectors according to the kit's transduction volumes (13.5 µl CytoTune™ 2.0 hKOS, 12.5 µl CytoTune™ 2.0 hc-Myc, and 8 µl CytoTune™ 2.0 hKlf4) into 1 mL biopsy medium (400 mL MEM-α, 50 mL KnockOut serum replacement, 1 mL Primocin, 5 mL Glutamax, and 10 ng/mL bFGF).
5. Remove the virus-positive medium 12–16 hr post-transduction and add 2 mL fresh biopsy medium every 24 hrs until day 5.
6. Passage the cells to a second rh-laminin 521-coated dish on day 5 using TryPLE express at a seeding density of 2×10^5 cells/well.
7. Feed the cells with 2 mL biopsy medium containing 50 µM ROCKi and 10 ng/mL bFGF.
8. On day 6, feed the cells an equal volume of biopsy medium containing 50 µM ROCKi, 10 ng/mL bFGF, and essential E8 flex medium.

9. From day 7 to day 30, feed the cells with E8 flex medium with 10 ng/mL FGF and 50 µM ROCKi every day.
10. Manually pick 1–2 mm iPSC colonies under a 10× objective on an inverted phase contrast microscope between days 17 and 30 for clonal propagation. The quality control and identity of selected clones after clonal expansion are confirmed by karyotyping (Basic Protocol 3) and characterization (Support Protocol 1).

Clonal propagation

11. Pick at least 12 colonies from each donor and transfer each colony into individual wells of a rh-laminin 521-coated 12-well plate without cross-contamination.
12. Gently dissociate the colonies with a 1-mL pipette tip and feed with E8 flex medium containing 10 ng/mL FGF and 50 µM ROCKi until they attain 70% to 80% confluency.

Passaging of iPSC

13. To passage the iPSCs, remove medium from wells that are 70% to 80% confluent, add 1 mL Versene, and incubate at 37°C for 3 min.
14. Carefully remove Versene without disturbing the loosely adherent iPSC colonies.
15. Add 1 mL iPSC medium and gently dislodge the iPSCs, taking care not to overly disrupt the colonies.
16. Seed the cells to another rh-laminin 521-coated 6-well plate for downstream experiments.

EVALUATION OF CHROMOSOMAL LOSS DURING REPROGRAMMING IN iPSCs BY KARYOTYPING

BASIC PROTOCOL 3

Because chromosomal aberrations can occur during the reprogramming of somatic cells into iPSCs (Liu et al., 2020), karyotyping was conducted on the iPSC lines to assess any chromosomal loss during the reprogramming process. The experiment was done based on the protocol developed by Dr. Patricia Labosky, courtesy of Raluca Verona from the laboratory of Dr. Marisa Bartolomei, Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine. The chromosome numbers were counted to ensure no chromosomal loss occurred while reprogramming into iPSCs.

Materials

Required reagents and consumables for Basic Protocol 3 are listed in Table 3.

Preparation of the cells

1. For karyotyping, seed the cells into a rh-laminin 521-coated 6-well plate and feed until they reach 70% to 80% confluency.
2. On the day of karyotyping, feed the cells with fresh iPSC medium containing 0.02 mg/mL demecolcine for 1 hr in the CO_2 incubator.
3. Wash the cells with 1× PBS and dislodge them from the culture dish using 1 mL TryPLE.
4. Collect the cells by centrifugation at 70 × *g* and room temperature for 5 min.
5. Remove the supernatant carefully without disturbing the pellet and add 1 mL of 0.56% KCl dropwise.
6. Gently flip the tubes several times to enhance the single-cell suspension. Add 4 mL of 0.56% KCl to each tube and incubate for 6 min at room temperature, which will allow the cells to swell.

Table 3 Reagents and Consumables for Basic Protocol 3

Reagents		
Item	Company	Cat. no.
Essential 8 (E8) flex medium	Thermo Fisher Scientific	A2858501
Demecolcine	MilliporeSigma	D-7385
Potassium chloride (KCl)	MilliporeSigma	P5405
TryPLE	Gibco	12605-010
Methanol	Fisher Chemical	A452SK
Acetic acid	MilliporeSigma	64-19-7
Giemsa	MilliporeSigma	SLCN5714
Consumables		
Item	Company	Cat. no.
Laminar flow hood	Thermo Fisher Scientific	1300 SERIES A2
Centrifuge	Thermo Fisher Scientific	Sorvall ST 8
6-well cell culture dishes	Fisherbrand	FB012927
CO_2 incubator	Thermo Fisher Scientific	HERACELL VIOS 160i
Superfrost plus microscope slides	Fisherbrand	12-550-15
Inverted phase contrast microscope	Nikon	ECLIPSE Ts2

7. Spin down the cells at 30 × *g* and room temperature for 5 min, then carefully remove the supernatant.
8. Fix the cells using 1 mL methanol:acetic acid at a ratio of 3:1.
9. Flip the tubes several times to resuspend the cells in fixative and add 4 mL fixative.
10. Incubate at room temperature for 5 min and collect the pellets by centrifugation at 30 × *g* and room temperature for 5 min.
11. Repeat the fixation step (steps 8 to 10) three more times.
12. Resuspended the cells in 0.5 mL fixative

Slide preparation and chromosome visualization

13. Prepare the glass slides by soaking them in methanol:acetic acid (3:1) for 10 min, then keep them in ice-cold water until ready for use.
14. Remove the slides from the water and remove the excess liquid.
15. Place the slides on the floor and drop the prepared cell suspension (step 12) onto them from a minimum height of 5 feet to break the cells and improve chromosome spread. For better quality control, use at least 4 slides per sample.
16. Allow the slides to dry at room temperature and stain with Giemsa dye (1:20) for 30 min.
17. Wash the dye with water and photograph the spread-out chromosomes using a microscope at 60× magnification.

GENERATION OF 3D ROs

BASIC PROTOCOL 4

Since the ROs are derived from a pluripotent stem cell source, they need to undergo a stepwise differentiation protocol using small molecules (Fligor et al., 2020). The characterized and karyotyped iPSCs derived from the non-glaucomatous and glaucomatous donors, at 60% to 70% confluency from passage 5 (P5), were used for RO generation. The generated ROs were then used for downstream RGC generation and evaluation of apoptotic marker expression. Further 2D cultures were generated by dissociating day 30 ROs. These post-differentiation ROs were then tested for apoptotic marker expression. Detailed methods for the generation of the ROs have been provided below.

NOTE: Ensure the organoids are fed with appropriate differentiation media with small molecules every other day, as described below.

Materials

Required reagents and consumables for Basic Protocol 4 are listed in Table 4.

Generation of 3D ROs

1. Use 60% to 70% confluent iPSC cells from P5 to generate ROs.
2. Remove the iPSC medium and add 1 mL TryPLE to dislodge the cells as a single-cell suspension from the culture dish.
3. After 3 min incubation at 37°C, arrest TryPLE activity by adding 1 mL serum containing basal 3D culture medium (20% Knockout serum, 10% human serum, 0.1 mM MEM-A, 1 mM Pyruvate, 0.1 mM β-mercaptoethanol, and 1 mL Primocin) (Table 11).
4. Ensure a single-cell suspension by pipetting the cells up and down at least 10–15 times using a 1 mL pipette tip.
5. Collect the cells by brief centrifugation at 70 × *g* and room temperature for 5 min.
6. Carefully remove the supernatant and resuspend the cells in 1 mL basal medium.
7. Count the cells using a hemocytometer and seed 10^4 cells per well to form ROs in a round-bottom, low-adhesion 96-well plate.
8. On day 0, feed the cells with basal medium containing 5 mM iWR1E and 20 mM ROCKi.
9. Add 1% Matrigel from day 1 to day 12, changing the medium every other day.
10. On day 13, transfer all ROs from the 96-well plate to a cell culture dish with a non-adherent surface.
11. Feed the cells only with basal medium containing 1% Matrigel from day 13 to day 15.
12. From day 16 to day 18, add 3 µM CHIR and 100 nM SAG to the basal media containing 1% Matrigel.
13. From day 19 to day 30, replace the 3D medium with NR medium (DMEM/F12, Glutamax, and N2 supplements) (Table 12).
14. Add 10 µM DAPT to the NR medium from day 31 to day 40, then continue with NR medium only after day 40.
15. Use the ROs at day 30 for the isolation of RGCs and downstream experiments.

Table 4 Reagents and Consumables for Basic Protocol 4

Reagents		
Item	Company	Cat. no.
TryPLE	Gibco	12605-010
Matrigel, growth factor reduced	Corning	354230
IWR1e	Sigma Aldrich	10161-5MG
ROCKi	MilliporeSigma	6880001-1MG
CHIR	MilliporeSigma	SML1094-5MG
SAG	MilliporeSigma	SML-13‘4-‘MG
DAPT	MilliporeSigma	SML-13‘4-‘MG
DMEM	Corning	15-017-CV
Human serum	MilliporeSigma	H422-100 mL
Knockout serum	Gibco	10828028
MEM-α	Thermo Fisher Scientific	12571063
Primocin	Invivogen	ant-pm-1
β-mercaptoethanol	MilliporeSigma	M6250-10 mL
Sodium pyruvate	Gibco	11360070
DMEM/F12	Gibco	10565018
N2 Supplement	Thermo Fisher Scientific	17502048
Glutamax	Thermo Fisher Scientific	3550061
Consumables		
Item	Company	Cat. no.
Laminar flow hood	Thermo Fisher Scientific	1300 SERIES A2
Hemocytometer	Hausser Scientific	3200
15-mL centrifuge tube	Corning	430766
Centrifuge	Thermo Fisher Scientific	Sorvall ST 8
6-well cell culture dishes	Fisherbrand	FB012927
96-well round bottom dishes	Costar	7007
Cell culture dish with cell-repellent surface	Greiner Bio-one	664970
CO_2 incubator	Thermo Fisher Scientific	HERACELL VIOS 160i
Inverted phase contrast microscope	Nikon	ECLIPSE Ts2

BASIC PROTOCOL 5

DISSOCIATION AND CULTURING OF RGCs FROM 3D ROs

Cultured adult RGCs provide a useful model system for studying downstream glaucomatous pathology, high throughput testing of therapeutics, and future transplantation studies. Previously, primary RGC cultures have been derived from adult retina and embryonic pluripotent stem cell differentiation (Gill et al., 2016; Gudiseva et al., 2021; Lilley & Robbins, 2005; Rabesandratana et al., 2020; Winzeler & Wang, 2013; Wu et al., 2021) but there have been numerous challenges which include long-term viability, access to human tissue, and ethical dilemma of utilizing embryonic cells. Being an ethically viable source of RGCs, iPSC-derived RGC generation provides a useful cell culture model that can be reprogrammed from a wide variety of somatic cells (Rabesandratana et al., 2020; Tanaka

Table 5 Reagents and Consumables for Basic Protocol 5

Reagents

Item	Company	Cat. no.
Complete neurobasal media prepared using the following components (i-xii).		
i. Basal neurobasal medium	Gibco	21103-049
ii. 10× B27 supplements	Thermo Fisher Scientific	17504044
iii. 100 µg/mL Penicillin-streptomycin	Gibco	5140-122
iv. 2 mM Glutamax	Thermo Fisher Scientific	3550061
v. 1 mM Sodium pyruvate	Gibco	11360070
vi. 100× Insulin-Transferrin-Selenium (ITS -G)	Thermo Fisher Scientific	4140004
vii. 60 ng/mL Progesterone	MilliporeSigma	P6149-10MG
viii. 16 µg/mL Putrescine	MilliporeSigma	P5780-5MG
ix. 100 ng/mL Thyroxine	MilliporeSigma	T1775-100MG
x. 40 ng/mL Triiodothyronine	MilliporeSigma	T2877-100MG
xi. 5 µM/mL Forskolin	MilliporeSigma	F3917-10MG
xii. Human serum	MilliporeSigma	H422-100 mL
Papain	MilliporeSigma	P4762-50MG
poly-D-lysine	Thermo Fisher Scientific	A3890401

Consumables

Item	Company	Cat. no.
Laminar flow hood	Thermo Fisher Scientific	1300 SERIES A2
Hemocytometer	Hausser Scientific	3200
15-mL centrifuge tube	Corning	430766
Centrifuge	Thermo Fisher Scientific	Sorvall ST 8
8-well cell culture dishes (Lab-TEK II) CC2 glass slides sterile	Thermo Fisher Scientific	154941
96-well round-bottom dishes	Costar	7007
CO_2 incubator	Thermo Fisher Scientific	HERACELL VIOS 160i
Inverted phase contrast microscope	Nikon	ECLIPSE Ts2

et al., 2015). The current protocol details the successful generation of RGCs from day 30 ROs that were derived from non-glaucoma and glaucoma patient keratocytes. The protocol uses enzymatic dissociation, followed by culturing with complete neurobasal medium as previously described (Pang et al., 2007; Peng et al., 2022; Sharma et al., 2015).

Materials

Required reagents and consumables for Basic Protocol 5 are listed in Table 5.

1. Place the plate of ROs under a microscope in the hood and collect the organoids using a sterile 1-mL cut tip.
2. Transfer the organoids to a 15-mL centrifuge tube and remove excess medium.
3. Add 1 mL papain to the organoids and incubate in a 37°C water bath for 4 min.

4. Add 2 mL complete neurobasal medium and gently dissociate the ROs using a 1-mL tip.
5. Collect the dissociated cells by brief centrifugation at 70 × g and room temperature for 4 min.
6. Resuspend the cell pellet in 1 mL complete neurobasal media and count the cells using a hemocytometer.
7. Culture each well of a rh-Laminin 521-coated Lab-TEK II CC2 glass slide containing ~2×10^4 cells.
8. Transfer to the incubator and allow to adhere overnight.
9. Carefully remove the non-adherent and floating cells, ensuring that the loosely adherent cells remain undisturbed.
10. Supplement the cells with fresh neurobasal RGC culture medium every day until day 7 and collect the cells for downstream experiments.

SUPPORT PROTOCOL 1

IMMUNOSTAINING FOR PHENOTYPIC CHARACTERIZATION OF CELLS

It is important to characterize the cells to ensure that they are RGCs and suitable for downstream applications. Keratocytes, iPSCs, and RGCs were characterized via immunofluorescence. The dissociated RGCs were also co-stained with RBPMS and cleaved caspase-3 (CASP3) to assess whether the dissociated RGCs from glaucomatous donors possess any glaucomatous phenotypes. The detailed protocol is described below.

Materials

Required reagents and consumables for Support Protocol 1 are listed in Table 6.

Immunofluorescence staining of keratocytes, iPSCs, and dissociated RGCs

1. Seed the cells at a density of 1×10^5 in a sterile glass coverslip.
2. Once the cells are ready to be processed, remove the medium and wash with 1× PBS.
3. Fix the cells in 4% paraformaldehyde for 10 min.
4. Wash three times with 1× PBS and add SuperBlock™ T20 blocking buffer for 1 hr.

Table 6 Reagents and Consumables for Support Protocol 1

Reagents		
Item	Company	Cat. no.
1× Phosphate-buffered saline (PBS)	Corning	21-040-CV
4% paraformaldehyde	Thermo Fisher Scientific	30525-89-4
SuperBlock™ T20	Thermo Fisher Scientific	YK378727
Antibodies (Tables 9 and 10)		
ProLong™ Gold antifade reagent with DAPI	Thermo Fisher Scientific	P36935
Consumables		
Item	Company	Cat. no.
15-mL centrifuge tube	Corning	430766
Fluorescent microscope	Nikon	ECLIPSE Ti2

5. Select the appropriate primary antibodies for characterizing keratocytes (Keratocan), iPSCs (TRA-1-60), and RGCs (RBPMS, BRN3A). Incubate the cells with antibodies (Table 9) in blocking buffer overnight at 4°C.
6. Wash three times with 1× PBS and incubate with respective secondary antibodies for 2 hr at room temperature (Table 10).
7. Mount the slides using ProLong™ Gold antifade reagent with DAPI after washing three times with 1× PBS.
8. Visualize the cells under a fluorescent microscope at 20× magnification.

SECTIONING OF 3D ROs AND IMMUNOSTAINING

SUPPORT PROTOCOL 2

Embedding, cryosectioning, and immunostaining of the day 30 ROs were carried out to evaluate whether the iPSC derived ROs from glaucomatous and non-glaucomatous donors were successfully differentiated into RGCs. In addition to measuring neurodegeneration of glaucomatous RGCs, co-staining of RBPMS with cleaved CASP3 on day 30 ROs was performed. The detailed protocol is described below.

Materials

Required reagents and consumables for Support Protocol 2 are listed in Table 7.

1. Collect day 30 ROs and fix in 4% paraformaldehyde for 15 min.
2. Rinse the ROs with 1× PBS, then sequentially add 1 mL of 10%, 20%, and 30% sucrose every 2 hr until the ROs are fully submerged at 30% sucrose.
3. Transfer the fixed and sucrose-treated ROs to a cryomold containing OCT compound and allow the tissue to freeze at −80°C.
4. Using a cryotome, cryosection the ROs to 30 µm thickness and place the sections on a Superfrost plus slide. Store the slides at −80°C until ready for use.
5. For immunostaining, take the slides from −80°C and place them on a 60°C heating plate for 30 min.

Table 7 Reagents and Consumables for Support Protocol 2

Reagents		
Item	Company	Cat. no.
OCT compound	Fisher healthcare	4585
1× Phosphate-buffered saline (PBS)	Corning	21-040-CV
4% paraformaldehyde	Thermo Fisher Scientific	30525-89-4
SuperBlock™ T20	Thermo Fisher Scientific	YK378727
Antibodies (Tables 9 and 10)		
OCT compound	Fisher healthcare	4585
ProLong™ Gold antifade reagent with DAPI	Thermo Fisher Scientific	P36935
Consumables		
Item	Company	Cat. no.
Cryomold	Tissue-Tek	4565
Cryostat	LEICA	CM3050 S
Fluorescent microscope	Nikon	ECLIPSE Ti2

6. After heating the slides, outline the tissue area using a PAP pen and rinse them in 1× PBS. Then, fix the slides in 4% paraformaldehyde at room temperature for 10 min.
7. Wash the slides three times with 1× PBS and add SuperBlock™ T20 blocking buffer for an hour at room temperature.
8. Dilute the primary antibodies: RBPMS, THY1, ISLET-1, or cleaved CASP3 (Table 9) in blocking buffer and incubate the RO section overnight at 4°C.
9. After incubation, wash slides three times in 1× PBS and incubate for 2 hr with appropriate secondary antibodies (Table 10) at room temperature.
10. Mount the slides using ProLong™ Gold antifade reagent with DAPI after washing three times with 1× PBS.
11. Visualize the cells under a fluorescent microscope at 20× magnification.

SUPPORT PROTOCOL 3

WESTERN BLOTTING FOR CLEAVED CASP3 AND THY1

Western blotting was used to assess the protein expression of cleaved CASP3 and the RGC marker RBPMS in day 30 ROs. Each level was quantified by band intensity using the β-actin housekeeping gene for reference.

Materials

Required reagents and consumables for Support Protocol 3 are listed in Table 8.

1. Collect the day 30 ROs from glaucomatous and non-glaucomatous donors and lyse each RO in 25 µl RIPA lysis buffer.
2. Ensure complete lysis of the RO by pipetting up and down using a 100 µl pipette tip under ice-cold conditions.

Table 8 Reagents and Consumables for Support Protocol 3

Reagents		
Item	Company	Cat. no.
RIPA	Pierce	89900
1× Phosphate-buffered saline (PBS)	Corning	21-040-CV
Novex™ WedgeWell 4%–20% Tris-Glycine Gel	Thermo Fisher Scientific	XP04200BOX
SuperBlock™ T20	Thermo Fisher Scientific	YK378727
2× Laemmli buffer	Novex	2037315
Antibodies (Tables 9 and 10)		
Consumables		
Item	Company	Cat. no.
15-mL centrifuge tube	Corning	430766
Centrifuge	Thermo Fisher Scientific	Sorvall ST 8
PVDF transfer membrane	Thermo Fisher Scientific	88585
Mini Blot Module	Thermo Fisher Scientific	B1000
Chemidoc system	BioRad	ChemiDoc™ MP Imaging Sytem

Table 9 List of Primary Antibodies Used for Immunostaining

Antibody	Cell type	Dilution	Host	Company	Cat. no.
Keratocan	Keratocyte	1:300	Rabbit	Thermo Fisher Scientific	BS-11054R
TRA-1-60	iPSCs	1:300	Mouse	Abcam	AB16288
RBPMS	RGCs	1:300	Mouse	Novus	NBP2-03905
THY1	RGCs	1:500	Mouse	Abcam	AB181469
ISLET 1	RGCs	1:500	Rabbit	Abcam	AB109517
BRN3A	RGCs	1:100	Mouse	EMD Millipore	MAB1585
Cleaved CASP3	Apoptosis	1:300	Rabbit	Abcam	AB2302
β-Actin	Housekeeping	1:1000	Mouse	Santa Cruz Biotechnology	sc-47778

Table 10 List of Secondary Antibodies Used for Immunostaining

Antibody	Dilution	Company	Cat. no.
Goat anti-Mouse IgG, Alexa Flour 568	1:300	Invitrogen	A10037
Goat anti-Mouse IgG, Alexa Flour 488	1:300	Invitrogen	A32723
Goat anti-Rabbit IgG, Alexa Flour 568	1:300	Invitrogen	A11036
Goat anti-Rabbit IgG, Alexa Flour 568	1:300	Invitrogen	A11036
Goat anti-Rabbit IgG, HRP linked	1:1000	Santa Cruz Biotechnology	SC-2004
Goat anti-Mouse IgG, HRP linked	1:1000	Santa Cruz Biotechnology	SC-2005

Table 11 Table Describing the Preparation of 3D Media

Reagent	Required volume	Company	Cat. no.
DMEM	350 mL	Corning	15-017-CV
Knockout serum	100 mL	Gibco	10828028
Human serum	50 mL	MilliporeSigma	H422-100 mL
MEM-α	5 mL	Thermo Fisher Scientific	12571063
Sodium Pyruvate (100 mM)	5 mL	Gibco	11360070
β-mercaptoethanol	4.6 μl	MilliporeSigma	M6250-10 mL
Primocin	1 mL	Invivogen	ant-pm-1

3. Collect the supernatant by centrifugation at 20,000 × g and 4°C for 5 min.
4. Gently mix 15 μg protein with 2× Laemmli buffer and allow for denaturation at 95°C for 10 min on a heating block.
5. Load the protein samples, separate by 4−20% SDS PAGE at constant voltage, and transfer to a PVDF membrane using the Invitrogen wet transfer system.
6. Wash the blot with 1× PBS and block with SuperBlock™ T20 blocking buffer.
7. Incubate the PVDF membrane with primary antibodies diluted in blocking buffer overnight at 4°C with gentle agitation.
8. Wash the membrane three times with 1× PBS and add HRP-conjugated secondary antibodies for 2 hr at room temperature.

Table 12 Table Describing the Preparation of NR Media

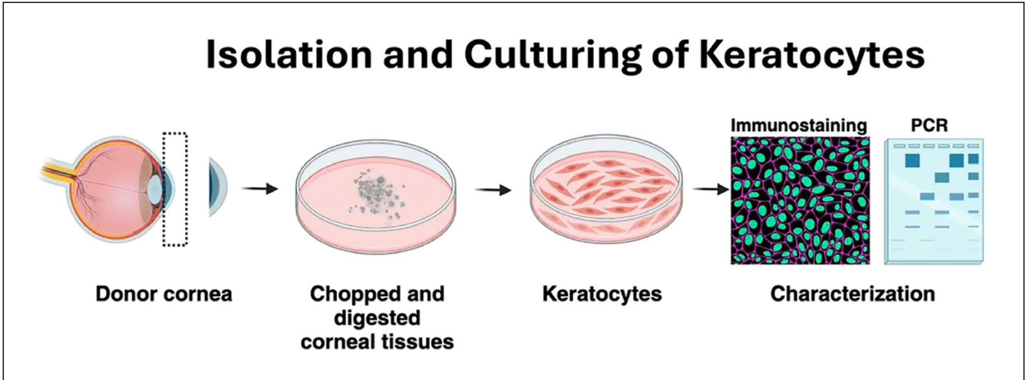
Reagent	Required amount	Company	Cat. no.
DMEM/F12	500 mL	Gibco	10565018
N2 Supplement	5 mL (10×)	Thermo Fisher Scientific	17502048
Glutamax	5 mL	Thermo Fisher Scientific	3550061
Primocin	1 mL	Invivogen	ant-pm-1

9. Wash with 1× PBS thrice and develop using the chemiluminescent method with β-actin as the normalizing control.

COMMENTARY

Differentiating retinal cell types from somatic cells using iPSCs is a powerful tool for *in vitro* studies, enabling drug screening, precision medicine development, and advancing future transplantation research in human diseases. The generation of RGCs, the terminal visual neurons primarily impacted by glaucoma, has gained significant attention in iPSC research. The RGCs are the first retinal cell type to arise from retinal progenitor cells during development (Nguyen-Ba-Charvet & Rebsam, 2020). Similarly, under *in vitro* conditions, they are the first neural cell type to emerge in the retinal organoids (Harkin et al., 2024). Several groups have demonstrated the generation of RGCs from iPSCs based on organoid-based differentiation within 30 to 50 days (Eiraku et al., 2011; Gill et al., 2014; Li & Luo, 2024; Parameswaran et al., 2010; Rabesandratana et al., 2020; Riazifar et al., 2014; Tanaka et al., 2015).

The protocol described in this manuscript details the methodology to generate RGCs from non-glaucomatous and glaucomatous donor keratocyte-derived iPSCs (Video 1). It also includes an assessment of disease characteristics in the derived ROs and RGCs through apoptotic marker expression. Given that the source of somatic cells can influence iPSC generation and may retain epigenetic characteristics closely associated with a disease (Kim et al., 2010; Young et al., 2012), we used ocular fibroblasts, specifically keratocytes as our parental cell source to generate iPSC derived RGCs. Starting from the de-identified human donor corneas, the keratocytes were cultured, characterized and passaged for downstream applications (Fig. 1). Although previous studies successfully generated iPSCs from ocular keratocytes using integrated (Chien et al., 2012; Joseph et al., 2016) and episomal vector-based systems (Bikkuzin et al., 2019), we employed non-integrating Sendai virus-based reprogramming, making it more suitable for clinical application. To our knowledge, no prior studies have used Sendai viral vectors to generate iPSCs from keratocytes. Starting from well-characterized keratocytes, iPSC colonies were generated between day 15 and day 25 of reprogramming. The individual iPSC clones were picked, propagated, and characterized, and no chromosomal loss during reprogramming was confirmed by karyotyping (Fig. 2). Starting from the undifferentiated mass of iPSCs, cells were successfully differentiated in a stepwise



Video 1 Step-by-Step Protocol for Retinal Ganglion Cell Generation. The video illustrates the complete process of isolating keratocytes from donor corneas and differentiating them into retinal ganglion cells

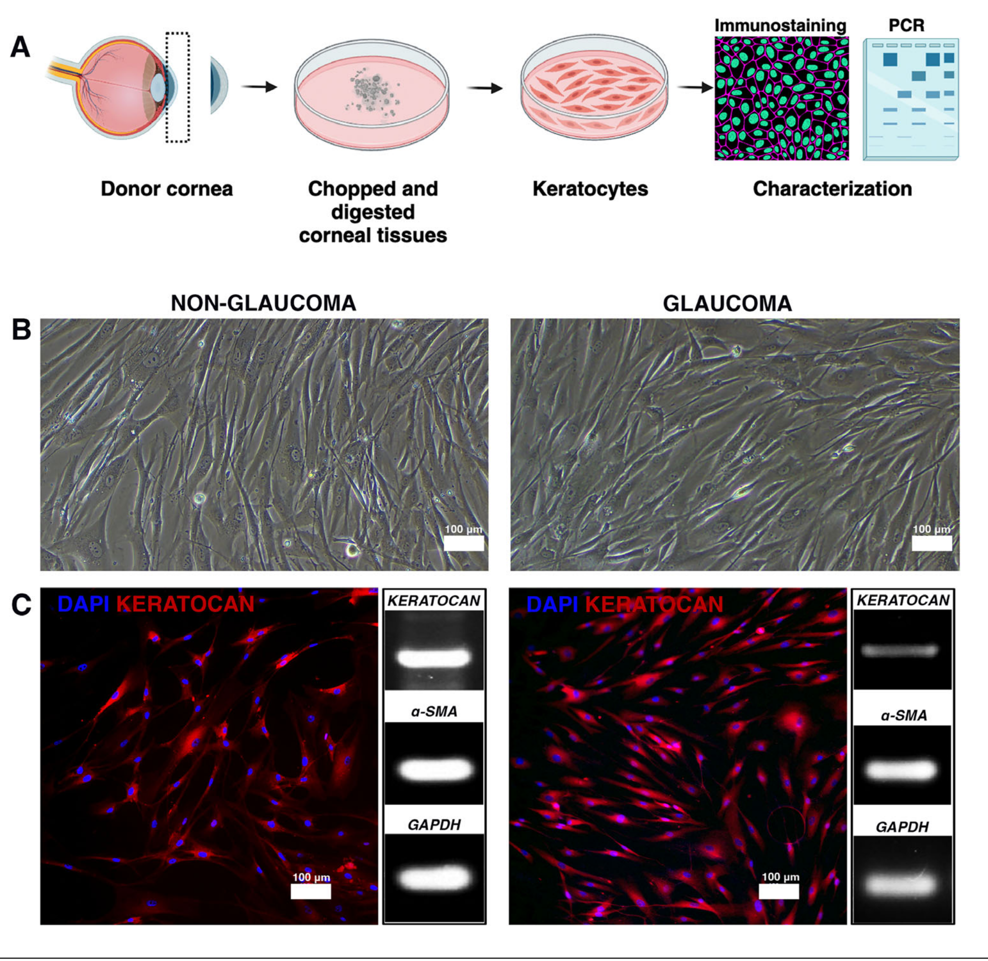


Figure 1 Isolation and culture of keratocytes from non-glaucomatous and glaucomatous donor corneas. (**A**). Schematic of the experimental paradigm. (**B**). Phase contrast images of cultured keratocytes from non-glaucomatous and glaucomatous donors. (**C**). Immunostaining and PCR characterization of the keratocytes using markers keratocan and *α-SMA*. *GAPDH* served as a housekeeping gene for PCR. α-SMA: Alpha smooth muscle actin, PCR: Polymerase chain reaction, *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase, Magnification: 20×.

manner to generate the RGCs. The RGCs were observed as early as 30 days of retinal differentiation within the ROs. Given this manuscript's primary focus on RGC generation, we collected ROs on day 30, cryosectioned them, and stained them with various RGC-specific markers. The ROs showed positive staining for markers such as RBPMS, ISLET1, and THY1, confirming successful RGC generation from non-glaucomatous and glaucomatous donors (Fig. 3).

Our generated iPSC-derived RGCs are a reliable model for various experimental conditions and human therapeutic interventions. Various methods have been previously employed to derive and isolate RGCs from ROs, including transferring day 24 ROs in suspension culture to a 2D matrix (Tanaka et al., 2015), immunopanning (Edo et al., 2020), or enzymatic dissociation of ROs (Langer et al., 2018; Rabesandratana et al., 2020). The current protocol used enzymatic dissociation of day 30 ROs and plating-derived RGCs in poly-D-lysine/rh-laminin coated glass slides. These RGCs were then cultured for 2-3 weeks using RGC culture media (Hameed et al., 2024; Pang et al., 2007; Peng et al., 2022; Sharma et al., 2015). The dissociated RGC cultures at day 7 showed positive expression for RGC-specific markers such as BRN3A, RBPMS, and THY1 by immunostaining and PCR assays (Fig. 4). To assess whether neurodegenerative phenotypes could be detected in glaucomatous ROs and in isolated RGCs, we observed the expression of cleaved CASP3, given that caspase-dependent apoptosis is a well-established mechanism of RGC death in glaucoma (Sanchez-Migallon et al., 2016; Thomas et al., 2017). Previous studies have shown that glaucoma RGCs derived

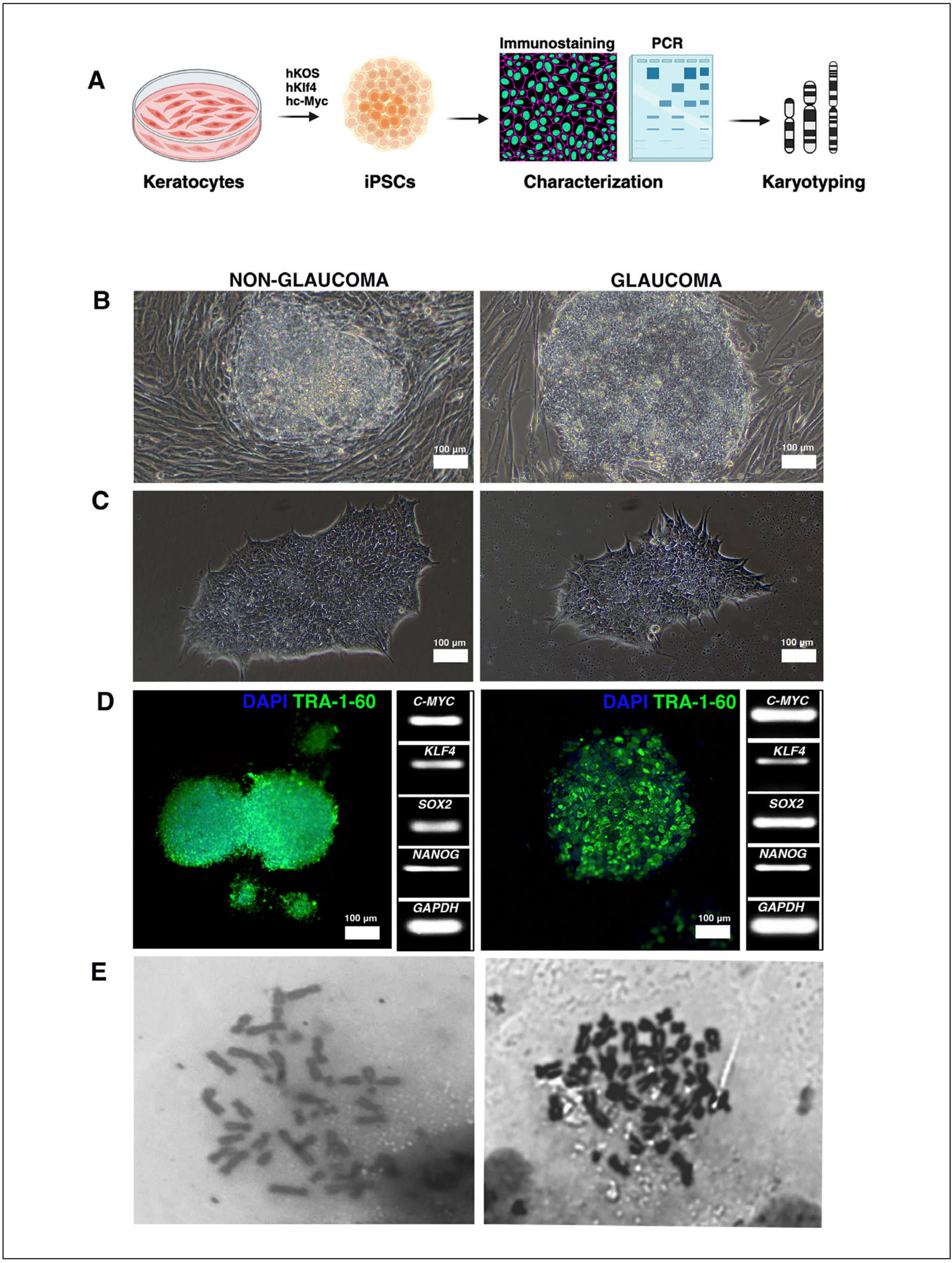


Figure 2 Reprogramming of donor keratocytes into induced pluripotent stem cells. (**A**). Schematic of the experimental paradigm. (**B**). Generation of iPSC clones. (**C**). Propagation of individual clones in a rh-laminin-coated plate. (**D**). Immunostaining of the iPSCs using marker TRA-1-60 and PCR characterization with *C-MYC, KLF4, SOX2, NANOG*, and *GAPDH* serving as a housekeeping control gene. (**E**). Phase contrast images of karyotyping depicting no chromosomal loss while reprogramming. iPSC: induced pluripotent stem cells, TRA-1-60: T cell receptor alpha locus, *C-MYC*: cellular Myc, *KLF4*: Kruppel-like factor 4, *SOX2*: SRY-box 2, *NANOG*: Nanog homeobox, *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase, Magnification: 20×.

from iPSCs bearing OPTN (E50K) mutation and *SIX6* risk allele have elevated CASP3 expression (Teotia et al., 2017; VanderWall et al., 2020), confirming a neurodegenerative phenotype. In our method, iPSCs derived from glaucoma subjects showed significantly elevated cleaved CASP3 expression in both ROs and derived RGCs (Fig. 5). Protein expression analysis of the ROs, using cleaved CASP3 and the RGC marker THY1, revealed a significant increase in cleaved CASP3 ($p < 0.05$) along with a significant

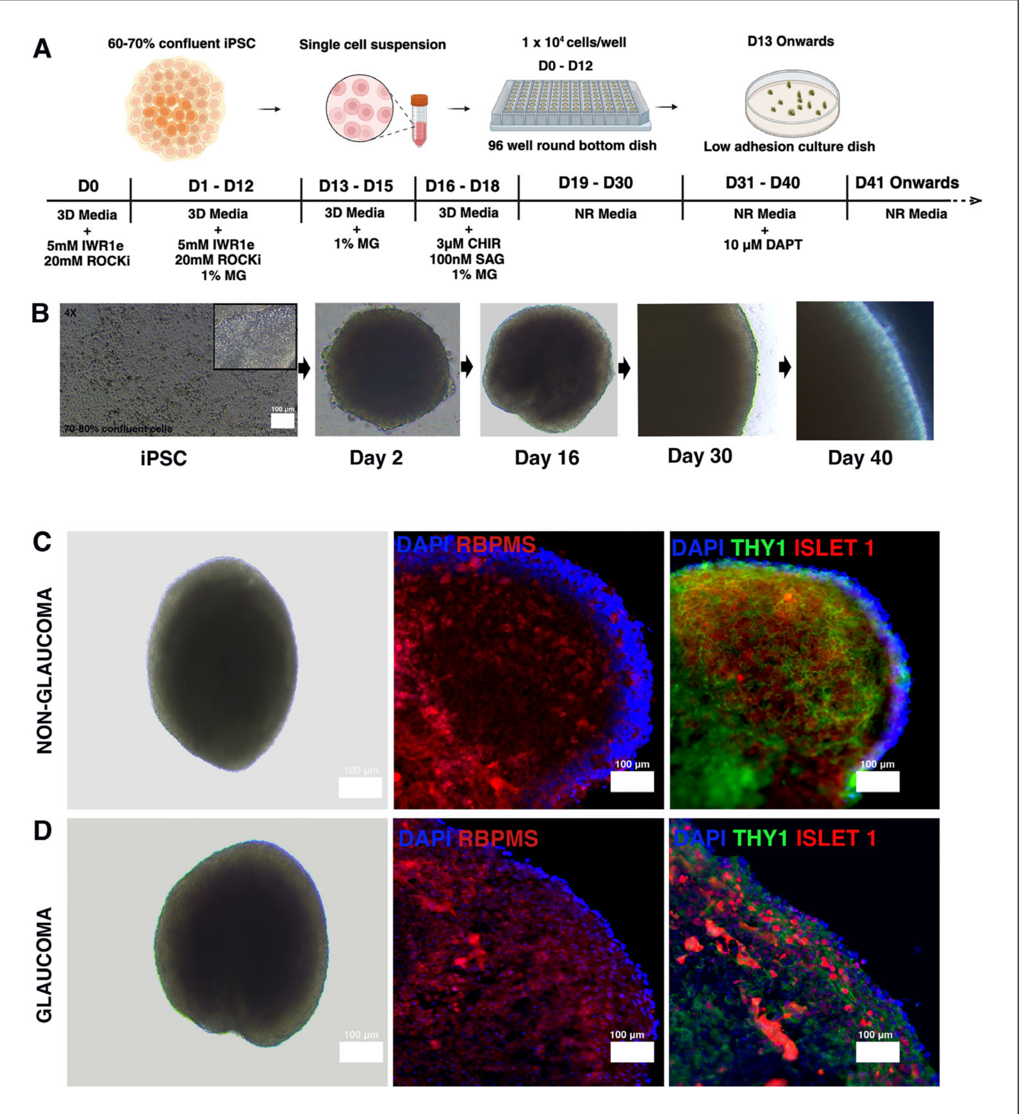


Figure 3 Differentiation of induced pluripotent stem cells into retinal organoids. (**A**). Schematic of the experimental paradigm. (**B**). The stepwise differentiation of iPSCs into ROs demonstrates neuro-retinal formation on day 2, which becomes more pronounced by day 16 and continues to thicken with lamination by day 30 and day 40. Phase contrast and immunostaining images of day 30 (**C**) non-glaucomatous and (**D**) glaucomatous organoids, labeled with RGC markers RBPMS, ISLET-1, and THY1, and counterstained with nuclear marker DAPI. iPSC: induced pluripotent stem cells, RGC: Retinal ganglion cells, ROs: Retinal organoids, RBPMS: RNA binding protein with multiple splicing, ISLET-1: Insulin gene enhancer protein 1, THY1: thymus cell antigen 1, DAPI: 4′,6-diamidino-2-phenylindole, Magnification: 20×.

reduction in THY1 positive RGCs ($p < 0.05$) in glaucomatous ones (Fig. 5). These findings confirm a distinct neurodegenerative signature in both the glaucomatous ROs and their derived RGCs. This protocol confirms that keratocytes can be reprogrammed into iPSCs and subsequently differentiated into RGCs while retaining certain epigenetic features passed on to the iPSCs. This approach shows strong potential as a robust method for deriving and studying glaucomatous RGC phenotypes and for screening candidate therapeutic compounds. Thus, the manuscript highlights that keratocytes harbor epigenetic or disease-characteristic features linked to ocular pathologies, making them an effective cellular source for deriving RGCs to model and investigate the mechanisms of diseases such as glaucoma.

Critical parameters

Culturing keratocytes

To obtain a pure population of keratocyte culture, it is crucial to carefully remove the epithelial and endothelial layers to prevent their contamination.

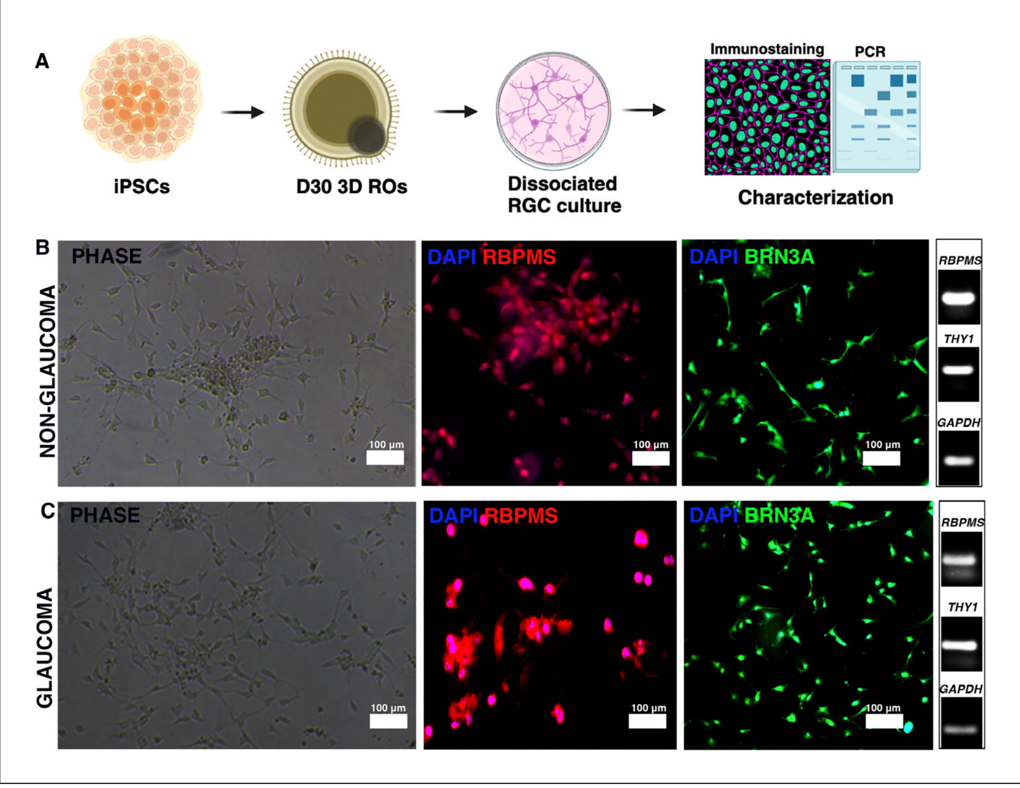


Figure 4 Generating retinal ganglion cells from retinal organoids. (**A**) Schematic of the experimental paradigm. (**B**) Non-glaucomatous and (**C**) glaucomatous depicting phase contrast images of dissociated RGCs and their characterization by immunostaining (RBPMS, BRN3A, DAPI) and PCR (*RBPMS, THY1*). *GAPDH* is used as a housekeeping gene for PCR. RGC: Retinal ganglion cells, ROs: Retinal organoids, RBPMS: RNA binding protein with multiple splicing, BRN3A: brain-specific homeobox/POU domain protein 3A, *THY1*: thymus cell antigen 1, *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase, DAPI: 4',6-diamidino-2-phenylindole, Magnification: 20×.

Reprogramming of keratocytes into iPSCs

It is important to culture keratocytes for reprogramming on dishes coated with rh-laminin and then switch to a second set of laminin-coated dishes on day 5 of reprogramming.

Passaging of iPSCs

Excessive pipetting must be minimized to prevent the breakage of iPSC colonies. Additionally, non-adherent cells must be removed 3 hr after seeding to maintain a healthy culture.

3D ROs

All the supplements need to be aliquoted to avoid repeat freeze-thawing and stored in the recommended conditions.

RGC dissociation and culture

Mild enzymatic dissociation of ROs is essential for obtaining RGCs. Minimizing pipetting is also important, as excessive pipetting may kill the RGCs.

Troubleshooting

Table 13 lists the practical problems that can be encountered from iPSC generation to RGC culture, along with their possible causes and solutions.

Understanding Results

Based on this protocol, it should be possible to isolate, culture, and characterize keratocytes from human donors (Fig. 1). The protocol outlines the process for successfully reprogramming keratocytes into iPSCs with no chromosomal loss and characterization of RGCs using immunostaining and PCR-based markers (Fig. 2). The iPSCs underwent stepwise differentiation into ROs with enriched RGCs by day 30. On day 1 post differentiation protocol, iPSCs cluster together to initiate organoid formation in differentiation medium. By day 2, a brighter neuroretinal ring forms, which becomes more pronounced by day 16 and thickens further with differentiation. RGCs are the primary neuronal cell type generated within the ROs, and their enrichment becomes evident by day 27. Immunostaining with RGC markers clearly demonstrates the abundance of RGCs in day 30 ROs derived from both glaucomatous and non-glaucomatous cell sources (Fig. 3). The

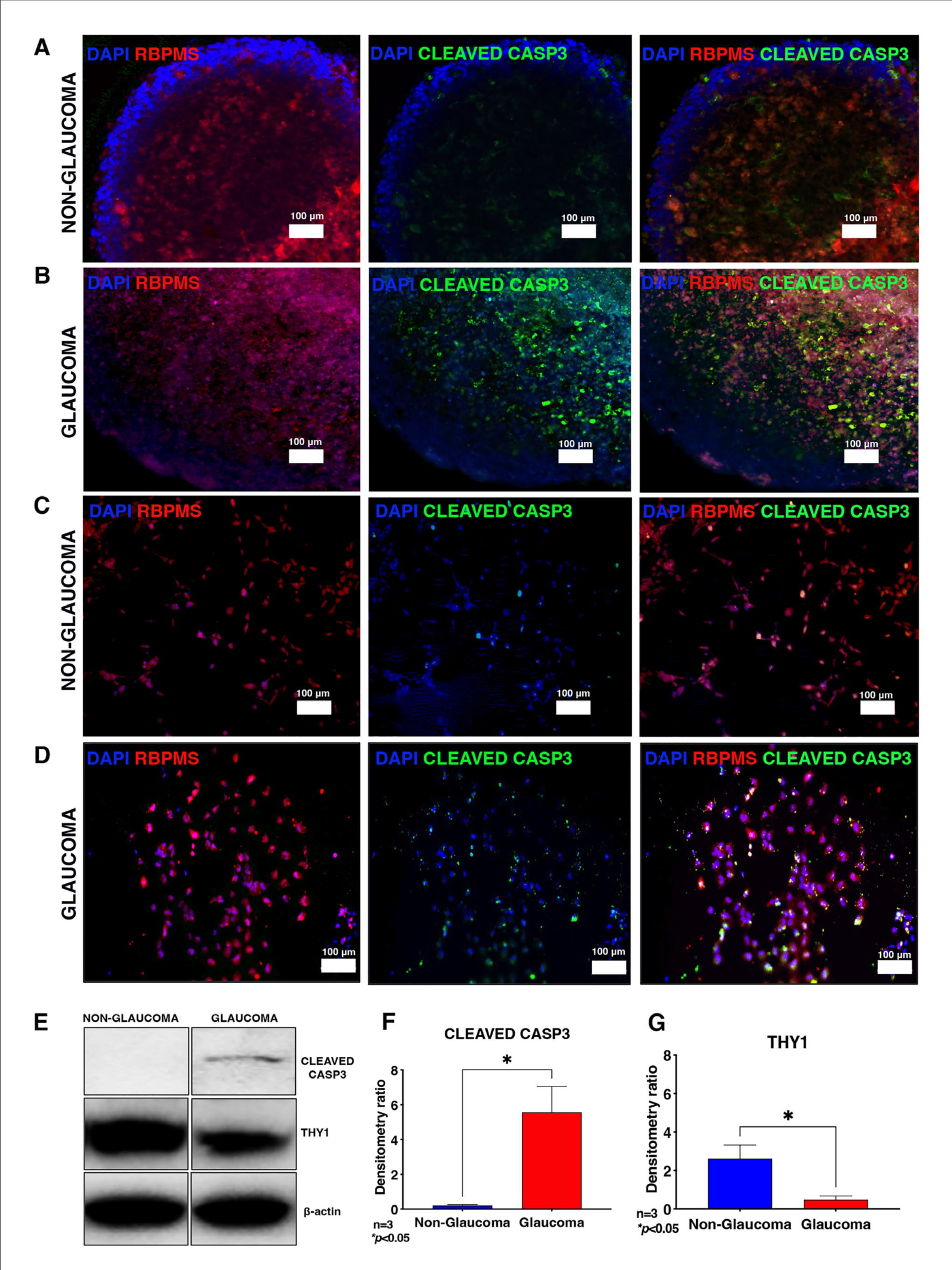


Figure 5 Assessment of neurodegenerative phenotype in the retinal organoids and dissociated retinal ganglion cells. (**A**). Non-glaucomatous and (**B**) glaucomatous day 30 ROs showing expression of RGC marker RBPMS and apoptotic marker cleaved CASP3. The dissociated culture of RGCs from (**C**) non-glaucomatous and (**D**) glaucomatous day 30 ROs expressing RGC marker RBPMS and apoptotic marker cleaved CASP3. (**E**) Western blotting depicting decreased expression of RGC marker THY1 with increased expression of cleaved CASP3 in glaucomatous ROs as compared to non-glaucomatous ROs. Densitometric quantification shows significantly increased expression of (**F**) cleaved CASP3 (*$p < 0.05$) and decreased expression of (**G**) THY1 (*$p < 0.05$) in glaucomatous ROs as compared to non-glaucomatous ROs. β-actin was used as a housekeeping gene. RGC: Retinal ganglion cells, ROs: Retinal organoids, CASP3: Caspase 3, RBPMS: RNA binding protein with multiple splicing, THY1: thymus cell antigen. Magnification: 20×.

Table 13 List of Potential Problems, Causes, and Remedies to Resolve Issues

Problem	Possible causes	Solution
1. No iPSC formation	Transduction in non-laminin-coated dishes	Ensure keratocytes are seeded in rh-laminin 521 from day 1 of transduction
	Not using required viral titers	Use recommended transduction volume specified in the reprogramming kit
	Reduced vector efficiency caused by storage at suboptimal conditions or repeated freeze thawing.	Store the reprogramming kit at −80°C
		Ensure viral vectors are aliquoted for individual reaction and avoid reusing them
2. No organoid formation	Seeding of a smaller number of cells	Seed 1×10^4 cells/ well in a 96-well plate
	Variability of clones	Screen the clones and select the organoid-forming clone
	Degrading of small molecules in the 3D differentiation media due to suboptimal storage	Aliquot the small molecules and store under recommended conditions
		Avoid frequent freeze-thawing of small molecules used in 3D diff media.
3. Fragmented organoids	Poor transferring of organoids to non-adherent surface dishes	Use 1 mL cut tips while transferring organoids.
	Wrong medium composition	Adhere to the medium composition and final concentrations of small molecules
4. Loss of organoids	Aspiration of organoids during medium exchange	Be gentle while aspirating the medium and avoid aspirating organoids
5. RGC death	Extended exposure to papain	Limit papain treatment only for 4 min
	Vigorous pipetting of organoids	Do not dissociate the organoids extensively
	Not using recommended culture dishes	Recommended to use rh-Laminin 521 coated Lab-TEK II CC2 glass slide or RGC compactable dishes
	Incorrect medium composition and suboptimal storage	Adhere to the medium composition and final concentrations of medium components
		Store all medium components under the recommended conditions

protocol describes the 2D culture of RGCs by enzymatically dissociating day 30 ROs, their characterization using specific markers confirming the RGC phenotype (Fig. 4). The protocol not only assesses the feasibility of generating RGCs from donor keratocytes but also evaluating whether the neuroregenerative phenotype is exhibited in keratocyte-derived ROs and RGCs from glaucoma donors. To this end, ROs and dissociated RGCs were co-stained with cleaved CASP3 and RBPMS, revealing increased CASP3 activation with RGC loss in glaucomatous ROs and RGCs compared to non-glaucomatous ones (Fig. 5). Overall, the paper emphasizes that keratocytes harbor the donor's epigenetic characteristics, and their reprogramming provides valuable insights into disease phenotypes. However, one limitation of the study was the use of only Caucasian samples due to the extensive availability of donor tissue from these donor types. Our future endeavors will include plans to address racial demographic comparisons for reprogramming by comparing Caucasian populations with individuals from other racial backgrounds.

Time Considerations

The culture of keratocytes from donor corneas and achieving a sufficient number of cells through reprogramming requires a minimum of 30 days. The duration for reprogramming and iPSC generation varies significantly between donors. In non-glaucomatous keratocytes, clonal generation typically occurs within 15 to 20 days, whereas stable clones from glaucomatous donors may require 20 to 30 days. An additional 15 to 20 days are needed for propagation and characterization of these clones, after which they are ready for downstream organoid generation. Using our protocol, ROs containing RGCs can be generated within 30 days.

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Author Contributions

Shahna Shahul Hameed: Data curation; formal analysis; investigation; methodology; validation, visualization; writing—original draft. **Tasneem Sharma:** Conceptualization; data curation; funding acquisition; project administration; resources; software; supervision; writing—review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

This published article and the associated supplementary information files include all the data generated and analyzed during this study.

Supporting Information

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Supplementary Table 1. Demographics of the human donor eyes: This information provides details about the donor tissues used in the present manuscript.

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