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Reprogramming retinal neurons and standardized quantification of their differentiation in 3-dimensional retinal cultures

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Abstract

Postmitotic differentiated neurons are among the most difficult cells to reprogram into induced pluripotent stem cells (iPSCs) because they have poor viability when cultured as dissociated cells. Other protocols to reprogram postmitotic neurons have required the inactivation of the p53 tumor suppressor. We describe a method that does not require p53 inactivation and induces reprogramming in cells purified from the retinae of reprogrammable mice in aggregates with wild-type retinal cells. After the first 10 days of reprogramming, the aggregates are then dispersed and plated on irradiated feeder cells to propagate and isolate individual iPSC clones. The reprogramming efficiency of different neuronal populations at any stage of development can be quantitated using this protocol. Reprogramming retinal neurons with this protocol will take 56 days, and these retina-derived iPSCs can undergo retinal differentiation to produce retinae in 34 days. In addition, we describe a quantitative assessment of retinal differentiation from these neuron-derived iPSCs called STEM-RET. The procedure quantitates eye field specification, optic cup formation, and retinal differentiation in 3-dimensional cultures using molecular, cellular and morphological criteria. An advanced level of cell culture experience is required to carry out this protocol.

Keywords

Retina; induced pluripotent stem cell; reprogramming; STEM-RET; retinal differentiation; retinal development; retinogenesis; 3D culture

*These authors contributed equally to this work.

SUPPLEMENTAL INFORMATION

No supplemental information

AUTHOR CONTRIBUTIONS STATEMENT

D.H. and M.D. developed the concepts, designed and performed experiments, analyzed data and developed the STEM-RET analysis. D.H. and M.B. wrote the paper, designed figures and performed data analysis. M.D. and L.G. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

INTRODUCTION

Stem cells are a powerful tool for investigating the molecular and cellular mechanisms of development, modeling human disease and in some circumstances, they may serve as a renewable source of cells for transplantation^{1,2}. Recently, retinal pigment epithelial cells derived from embryonic stem cells (ESCs) have been successfully transplanted into patients with macular degeneration and Stargardt's macular dystrophy¹. In addition, photoreceptor precursors have been successfully integrated into the retina in mice^{3,4}. While both human and murine stem cells can produce retinæ in three-dimensional cultures^{5–7}, there are now data suggesting that the source of stem cells may influence the quantity and quality of retinæ produced *in vitro*⁸. Specifically, epigenetic memory may influence the subsequent differentiation of stem cells along different lineages⁹. This is important because it could influence studies on development, disease modeling and the efficacy of cell-based therapies. In this protocol, we describe a new method for reprogramming retinal neurons into induced pluripotent stem cells (iPSCs) and a quantitative scoring system called STEM-RET to compare retinal differentiation across diverse stem cell lines.

Development of the Protocol

Reprogramming retinal neurons—Postmitotic neurons are difficult to reprogram into iPSCs because the dissociated cells are difficult to maintain or fail to undergo reprogramming¹⁰. Kim *et al.* overcame these barriers to reprogramming mature cortical neurons by inactivating the p53 tumor suppressor¹⁰. However, p53 inactivation could result in tumorigenesis of the stem cells or their differentiated lineage. Therefore, iPSCs made in this fashion are unsafe for therapeutic use. As with cortical neurons, purified rod photoreceptors cannot be reprogrammed when plated as single cells⁸. To overcome this limitation, we developed a mosaic culture system to reprogram retinal neurons that does not require p53 inactivation (Fig. 1).

Our unique system for reprogramming retinal neurons was based on the reprogrammable mouse developed by Konrad Hochedlinger¹¹. The advantage of this system is that expression of the reprogramming factors, Oct3/4, Klf4, Sox2, and cMyc, is inducible by adding doxycycline to the culture medium. We crossed the reprogrammable mouse strain to a transgenic line (Nrl-GFP) that expresses GFP in postmitotic rod photoreceptors¹². We purified the rod photoreceptors from the retina of the progeny via FAC sorting for GFP and induced expression of the reprogramming factors by adding doxycycline. The reprogrammable rod photoreceptors were combined with an excess of wild-type retinal neurons in cell pellets and grown in culture on polycarbonate filters for 10 to 14 days before being plated onto irradiated mouse embryonic fibroblast (MEF) feeder cells in the presence of LIF. Using this system, we developed iPSCs from adult and early postnatal rod photoreceptors⁸. This efficient, quantitative method of reprogramming retinal neurons is compatible with conventional tissue culture methods and it may be possible to extend our method to other neuronal populations.

Quantitative retinal differentiation—Following reprogramming, retinal differentiation is achieved via 3-dimensional cultures of stem cell aggregates (Fig 2A). For murine stem

cells, eye field specification occurs within the first 7 days of culture (Fig 2B–C), optic cups are formed from day 7 to day 10 (Fig 2D), and retinal differentiation and maturation occur from day 10 to day 28⁶.

An epigenetic memory of the cellular origins of iPSCs is retained after reprogramming and this may influence retinal differentiation, meaning reprogrammed cells from a specific lineage will differentiate more readily into a similar lineage^{8,9}. We developed a standardized procedure called STEM-RET in order to quantify differences in retinal differentiation proficiency between stem cell lines. It is important to emphasize that the optic cup stage is defined as the presence of neural retina and retinal pigment epithelium in one aggregate in the stem cell cultures. To quantitate these parameters across different stem cell lines, we developed a standardized procedure called STEM-RET. Our scoring system incorporates molecular, cellular, and morphological criteria for quantifying retinogenesis at the eye field specification, optic cup and maturation stages in 3-dimensional cultures. We used the previously described ESC line Eb5 Rx–GFP cell line⁶, which expresses a GFP in the early retina developmental gene Rx, to develop a benchmark for retinal differentiation.

Applications

Our protocol for reprogramming retinal neurons and quantitating their retinal differentiation allows investigators to produce stem cells from diverse lineages and to identify the best source of stem cells for studying retinal development, disease, and for cell-based therapies for human retinopathies. While STEM-RET was developed using murine stem cells, it can readily be adapted for human stem cells using a longer culture period⁷. While we use GFP transgenic reporter lines in our study to purify subsets of murine retinal cells, cell surface markers would have to be used to perform similar studies with human retina¹³. We envision that the STEM-RET protocol could be used to quantitatively compare different stem cell lines to identify those that are the most efficient at making photoreceptors in culture for subsequent transplantation or disease modeling.

Advantages and limitations

Our STEM-RET protocol provides a clear, standardized method to quantify retinogenesis *in vitro* from different stem cell lineages regardless of origin. By cross-breeding mouse strains that express cell lineage specific GFP with a doxycycline inducible reprogrammable mouse, iPSCs can be derived from postmitotic neurons without disrupting p53¹⁰. However, this is technically challenging and requires close attention to detail and some advanced cell culture techniques. As these methods have been developed for mouse retinal cells, some of the methods described here may not be directly applicable for differentiation of human retinal neurons. We would recommend utilizing, cell surface markers instead of GFP transgenic reporter lines in order to purify cellular populations¹³. Furthermore, explant pellet cultures might not be a feasible option for non-inducible, vector-based reprogramming protocols.

Experimental Design

Controls—Our STEM-RET protocol was designed using the Eb5 Rx–GFP cell line as a control in our experiments. The use of this cell line is optional and can be substituted with another ESC line from a pigmented animal. This cell line can be used for training and to

ensure that the protocol is working properly and can be used as a positive control for the stem cell characterization experiments. With Eb5 Rx–GFP cells, begin with step 36 and perform preplating for a FAC sort for SSEA1 before plating for retinal differentiation. The Eb5 Rx–GFP cell line is derived from albino animals and will not produce pigmented tissue. Therefore, optic cups should be identified and excised based on GFP expression.

Reprogrammable mice—Reprogrammable mice are utilized because of the dramatic increase in reprogramming efficiency compared to traditional viral induction¹¹. Breed *Cola1-OKSM* mice with *Rosa26-M2rtTA* mice to create reprogrammable mice¹⁴. CRITICAL We found no difference in reprogramming efficiency of retinal neurons from mice that were homozygous and heterozygous for *Cola1-OKSM*. !CAUTION Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children’s Research Hospital’s Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Explant pellet cultures—Our protocol reprograms retinal neurons using retinal explant pellet cultures that consist of a heterogeneous mix of reprogrammable and wild-type cells. The wild-type cells are used as a substrate to facilitate the reprogramming of isolated retinal neurons from reprogrammable mice. !CAUTION Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children’s Research Hospital’s Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Optimizing retinal pellet and 6-well plate cell densities—In order to ensure reprogramming of retinal neurons, a limiting dilution series is performed to identify the optimal cell concentrations for the retinal pellet cultures. We describe these optimization steps and suggested dilutions in detail when describing the steps to create a retinal pellet and retinal explant cultures.

FAC sorting of retinal cells—Reprogrammable mice can be bred to mice that express GFP or other fluorescent proteins so the offspring produce a population of GFP-labeled cells. This allows isolation and reprogramming of a specific subpopulation of cells. Following dissociation of the retina into single-cell suspension prior to sorting.

Irradiated MEFs—We use irradiated MEFs (ir-MEFs) as a substrate to inhibit the differentiation of stem cells while facilitating their growth. The ir-MEFs used in our experiment were produced in our lab from embryonic day 13.5 (E13.5) wild-type mice using established protocols¹⁵. Irradiated MEFs are commercially available and we recommend using irradiated MEFs from Thermofisher (cat. no. A24903). !CAUTION Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children’s Research Hospital’s Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

FAC sorting for SSEA1 of stem cell lines—Stem cells will undergo spontaneous differentiation during routine passaging even under optimal growth conditions. To minimize the effects of this differentiated cell population, enrichment for the mouse stem cell marker SSEA1 is recommended¹⁶. Sorting for SSEA1+ cells creates a standardized cell stock that reduces variability and enables comparison between stem cell lines via the STEM-RET protocol.

Characterization of stem cell lines—To establish pluripotency in retinal-derived subclones, we characterize the stem cell lines based on karyotype, doxycycline independence, alkaline phosphatase expression, immunofluorescence staining for stem cell markers, teratoma formation, and qPCR analysis of stem cell markers. Karyotyping is used to confirm that cells have not developed major chromosomal abnormalities. Doxycycline independence confirms the endogenous OKSM genes are active. Alkaline phosphatase expression, immunofluorescence, and qPCR are used to confirm the expression of pluripotent stem cell markers. If a cell line fails any of the steps during characterization, the cell line is not considered pluripotent.

CD1-Nude mice are used to test pluripotency of stem cell lines through teratoma formation as part of stem cell characterization. Stem cells (2.0×10^6 cells) are injected subcutaneously in the flank of the animal¹⁷. Teratomas will develop between 6–12 weeks and the presence of all three germ layers confirms the pluripotency of a stem cell line¹⁷. **CRITICAL** Two adult mice are required for each cell line characterization. **!CAUTION** Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children's Research Hospital's Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

96-well plates for retinal differentiation—In order to create healthy retinal spheres during retinal differentiation, it is important to plate stem cells onto low-attachment 96 well plates. Nunclon Sphera plates are specially designed to prevent attachment and to allow aggregation of cells.

Matrigel protein concentration for retinal differentiation—Matrigel provides key laminin/enactin protein to the cells during retinal differentiation. The protein concentration of laminin/entactin varies between lot preparations. It is very important to use matrigel with laminin/entactin protein concentrations between 9.1 and 10.1 mg/ml, as determined by the certificate of analysis provided by the manufacturer.

Delineation of optic cups—A critical step in the process of retinal differentiation is identification and isolation of optic cups. On day 10 of retinal differentiation, optic cups are identified and pinched off of the neuronal aggregates (Fig 2D). We identify optic cups by adjacent pigmented regions of the retinal spheres because these regions contain retinal tissue at equivalent maturation to the optic cup developmental stage *in vivo* (see Fig 2D for a pigmented example). Pigmentation of the retinal pigment epithelium coincides with optic cup formation, and although we rarely observe invagination of the tissue, pigmented regions contain retinal tissue at equivalent maturation to the optic cup developmental stage *in vivo*.

Therefore, we identify the aggregates of pigmentation and adjacent retinal tissue as “optic cups”. For the albino Eb5 Rx–GFP cell line, optic cups can be identified by the expression of GFP, and optic cups can be excised in albino, non-GFP lines based on tissue morphology (see Fig 2D for a non-pigmented example).

Calculation of STEM-RET—STEM-RET combines the quantitative scores for eye field specification, optic cup formation, and the combined scores retinal differentiation⁸. Retinal differentiation is comprised of qPCR (RDQ), immunofluorescence (RDIF), and electron microscopy (RDEM). The STEM-RET scoring system enables an unbiased, quantitative approach to determine retinogenesis of stem cells lines independent of origin.

REAGENTS

- *Cola1–OKSM* mice (Jackson Laboratory, stock no. 011001) !CAUTION Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children’s Research Hospital’s Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.
- *Rosa26–M2rtTA* mice (Jackson Laboratory, stock no. 006965) !CAUTION Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children’s Research Hospital’s Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.
- *C57BL/6J* wild-type mice (Jackson Laboratory, stock no. 000664) !CAUTION Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children’s Research Hospital’s Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.
- *CD1–Nude* mice (Jackson Laboratory, stock no. 002019) !CAUTION Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children’s Research Hospital’s Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.
- ABC Kit, Vectastain (Vector Laboratories, cat. no. PK-4000)
- Acetic acid (Fisher Scientific, cat. no. A38S) !CAUTION Acetic acid is hazardous. Wear protective gloves.
- Antibodies (refer to Table 1 for list of antibodies used for stem cell and retinal characterization)

- AP stain kit (Millipore, cat. no. SCR004)
- 2-mercaptoethanol (Sigma-Aldrich, cat. no. M7522) !CAUTION 2-mercaptoethanol is hazardous. Wear protective gloves.
- Bovine serum albumin (BSA) (Sigma-Aldrich, cat. no. A7906)
- Calcium chloride (CaCl₂) (Sigma-Aldrich, cat. No. C4901)
- Colcemid solution, 10 µg/ml (Thermo Fisher Scientific, cat. no. 15212-012) ! CAUTION Colcemid is hazardous. Wear protective gloves.
- Collagenase (Thermo Fisher Scientific, cat. no. 17104-019)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat. no. D2650) !CAUTION DMSO is hazardous. Wear protective gloves.
- Direct-zol RNA preparation kit (Zymo Research, cat. no. R2052)
- DNase, lyophilized (Sigma-Aldrich, cat. no. D4513)
- DMEM/F12 with GlutaMAX (Thermo Fisher Scientific, cat. no. 10565-018)
- DMEM with glutamine (Lonza, cat. no. 12-614f)
- Doxycycline hydrochloride (Research Products International, cat. no. D43020-25.0)
- Eb5 Rx–GFP murine ES cell line (Riken, AES0145) !CAUTION The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
- Fetal bovine serum (FBS) (Hyclone, cat. no. SV30014.03)
- Gelatin from porcine skin (Sigma-Aldrich, cat. no. G2500-100G)
- Glasgow Minimum Essential Medium (GMEM) (Sigma-Aldrich, cat. no. G5154)
- GlutaMAX supplement, 100× (Thermo Fisher Scientific, cat. no. 35050-061)
- Glutaraldehyde, 25% (Electron Microscopy Sciences, cat. no. 16530)
- HEPES, 1 M (Thermo Fisher Scientific, cat. no. 15630)
- High-Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, cat. no. 4387406)
- Human gamma globulin (Sigma-Aldrich, cat. no. G4386)
- Insulin, 10 mg/ml (Sigma-Aldrich, cat. no. I9278)
- Knockout serum replacement (KSR) (Thermo Fisher Scientific, cat. no. 10828-028)
- Leukemia inhibitory factor (LIF) (Millipore, cat. no. ESG1107)
- Mouse embryonic fibroblasts (MEFs) !CAUTION The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

- Matrigel, growth factor reduced, LDEV-free (Corning, cat. no. 354230) Thaw on ice at 4°C overnight before use. ▲ CRITICAL Variations in protein concentration between batches of matrigel will affect retinal differentiation with STEM-RET. The protein concentrations between 9.1 and 10.1 mg/ml are optimal.
- Methanol (Fisher Scientific, cat. no. A412) !CAUTION Methanol is hazardous. Wear protective gloves.
- N2 supplement (Thermo Fisher Scientific, cat. no. 17502-048)
- Nonessential amino acids, 100× (NEAA) (Thermo Fisher Scientific, cat. no. 11140)
- OCT cryoblock reagent (Tissue-Tek, cat. no. 4583)
- Paraformaldehyde, 16% (PFA) (Electron Microscopy Sciences, cat. no. 15710) ! CAUTION PFA is hazardous. Wear protective gloves and work in a fume hood.
- Penicillin-streptomycin, 100× (Thermo Fisher Scientific, cat. no. 15140-122)
- Phosphate-buffered saline solution without calcium or magnesium (PBS−/−) (BioWhittaker, cat. no. 17-516F)
- Phosphate-buffered saline solution with calcium and magnesium (PBS+/+) (BioWhittaker, cat. no. 17-513F)
- Potassium chloride (0.075M in water) (Fisher Scientific, cat. no. P217-500)
- Retinoic acid (Sigma-Aldrich, cat. no. R2625)
- Sodium cacodylate trihydrate, sodium dimethyl arsenate (Electron Microscopy Sciences, cat. no. 12310)
- Sodium chloride (NaCl) (Sigma-Aldrich, cat. no. S5886)
- Sodium pyruvate, 100mM (Thermo Fisher Scientific, cat. no. 11360)
- Soybean trypsin inhibitor, powder (Sigma-Aldrich, cat. no. T6522)
- Sucrose (Sigma-Aldrich, cat. no. 84097)
- Taurine (Sigma-Aldrich, cat. no. T8691)
- TaqMan Custom Array Cards, format 24 (Applied Biosystems, cat. no. 4342249) (refer to Table 2 for the list of TaqMan array genes used for stem cell and retinal characterization)
- TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, cat. no. 4304437)
- Trizma Base (Sigma-Aldrich, cat. no. T1503)
- Triton X-100 (Sigma-Aldrich, cat. no. T9284)
- Trizol (Thermo Fisher Scientific, cat. no. 15596018) !CAUTION Trizol is extremely hazardous. Wear protective gloves and a lab coat. Work in a fume hood.

- Trypsin-EDTA, 0.25% (Thermo Fisher Scientific, cat. no. 25200-056)
- Trypsin, lyophilized (Sigma-Aldrich, cat. no. T2271)
- Trypsin (Cellgro, cat. no. 25052-CI)
- Trypsin-versene (BioWhittaker, cat. no. 17-161F)
- Trypsin, 2.5% (Thermo Fisher Scientific, cat. no. 15090-046)
- TSA Plus Cyanine 3 System (PerkinElmer, cat. no. NEL744001KT)
- Tween 20 (Sigma-Aldrich, cat. no. P7949-100ml)

EQUIPMENT

- 6-well, 12-well, and 24-well plates (Corning, cat. no. 3506, 3512, 3527)
- 10-cm dish (Corning cat. no. 353003)
- 10-cm Petri dish (Fisher Scientific, cat. no. FB0875712)
- 96-well plate, round bottom (Corning, cat. no. 3799)
- 96-well plate, Nunclon Sphera, round bottom (Thermo Scientific Nunc, cat. no. 174925) ▲ CRITICAL Low-attachment cell culture plates required for 3D retinal differentiation.
- Cell strainer, 40- μ m mesh, nylon (Falcon, cat. no. 352340)
- Centrifuges (Hermle Z400K and Z233M-2, Labnet)
- Conical tubes (15-ml and 50-ml) (Falcon, cat. no. 352097 and 352098)
- Cryostat (Leica CM3050-S)
- Cryotubes (Thermo Scientific, cat. no. 377267)
- Eppendorf tubes, siliconized (1.5-ml, Fisher Scientific cat. no. 02-681-320)
- Filter flasks, 0.22- μ m, cellulose acetate (250-ml and 500-ml) (Corning, cat. no. 430769 and 430767)
- Forceps, #55 (Fine Science Tools, cat. no. 11255-20)
- Freezer (-20°C and -80°C) and refrigerator (4°C) (Kenmore Heavy Duty Commercial, Thermo Scientific)
- Flow cytometer for FAC sorting (BD Biosciences FACS Aria III sorter)
- Glass jars (250 ml and 500 ml) (Corning, cat. no. 1395-250, 1395-500)
- Glass Pasteur pipettes (Corning, cat. no. 7095B-9)
- Hemocytometer (Fisher Scientific, cat. no. 0267110)
- Incubator (humidified, 37°C with 5% CO_2) and high-oxygen incubator (humidified, 37°C with 5% CO_2 and 40% O_2) (HERAcell 150i, Thermo Fisher Scientific)

- Lab oven (80°C) (30 GCE-LT, Quincy Lab, Inc.)
- Laminar flow hood (SterilGARD III Advance, The Baker Company)
- Microscope camera (Digital Sight DS-L3, Nikon)
- Microscope slides, 8-chamber, plastic (Thermo Fisher Scientific, cat. no. 177445)
- Microscope slides, Superfrost Plus, glass (Fisher Scientific, cat. no. 12-550-15)
- Microscope, transmission electron (FEI Tecnai F 20 TEM FEG electron microscope with A ATM KR41 camera)
- MilliQ water filter system (Millipore)
- Needle, 16-gauge (BD Biosciences, cat. no. 305197)
- Pipet-Aid XP (Drummond)
- Pipettes and pipette tips (Alphapette pipettes, Molecular Technologies; Avant Guard barrier tips, cat. nos. AV10, AV100, AV200, AV1000)
- Pipette tips, MultiFlex (Fisher Scientific, cat. no. 05-408-151)
- Real-time PCR system (7900HT Fast, Thermo Fisher Scientific)
- Serological pipettes (5 ml, 10 ml, and 25 ml) (Falcon, cat. no. 357543, 357551, 357525)
- Tissue homogenizer (Polytron PT10-35GT).
- Water bath, 37°C (Precision 280 series microprocessor controlled)
- Whatman membranes (Nuclepore, cat. no. 110410)

REAGENT SETUP

Retina-trypsin (100×)

Trypsin for isolating retinal neurons is prepared by adding 100 mg of trypsin (Sigma-Aldrich) to 10 ml PBS^{−/−} (10 mg/ml). Aliquots can be stored at −20°C for up to 1 month.

Soybean trypsin inhibitor (STI)

Soybean trypsin inhibitor is prepared by adding 100 mg to 10 ml PBS^{−/−} (10 mg/ml). Aliquots can be stored at −20°C for up to 1 month.

DNase

Dnase is prepared by adding 6 ml PBS^{+/+} to one vial of lyophilized DNase. Aliquots can be stored at −20°C for up to 1 month.

Doxycycline (DOX)

Doxycycline for inducing reprogramming is prepared by adding 10 mg of doxycycline hydrochloride to 1 mL of sterile PBS^{−/−}. Aliquots can be stored at −20°C for up to 2 years

in a light-sealed container. Avoid multiple freeze-thaws. Add 2 μ l for every 10 ml of medium for a final concentration of 2 μ g/ml.

Complete DMEM (cDMEM)

To prepare cDMEM, add 50 ml FBS and 5 ml penicillin-streptomycin to 445 ml DMEM with glutamine. Sterilize the medium by filtering it through a 0.22- μ m filter flask. The medium can be stored at 4°C for up to 1 month. Warm the medium in a 37°C in a water bath before use.

Explant culture medium

To prepare retinal explant culture medium, add 25 ml FBS, 2.5 ml HEPES, 2.5 ml penicillin-streptomycin, and 125 μ l insulin to 219.9 ml DMEM/F12 with GlutaMAX. Sterilize the medium by filtering through a 0.22- μ m filter flask. The medium can be stored at 4°C for up to 1 month. Before use, warm the medium in a 37°C in a water bath and add LIF (2 μ l/10 ml) and DOX (2 μ l/10 ml).

BSA cushion medium (4% BSA in DMEM/F12, wt/vol)

To prepare BSA cushion medium, add 2 g bovine serum albumin to 50 ml of DMEM/F12 with GlutaMAX. The solution can be stored at 4°C for up to 1 month.

Gelatin solution (0.1% in water, wt/vol)

To prepare a gelatin solution, dissolve 0.5 g gelatin in 100 ml MilliQ water to obtain a 5 $\times$ gelatin solution. Sterilize the 5 $\times$ solution by autoclaving and allow it to cool to room temperature (25°C). Add the 100 ml 5 $\times$ gelatin solution to 400 ml sterile-filtered MilliQ water and mix well. The gelatin solution can be stored at 4°C for up to 1 month.

2-mercaptoethanol solution (0.1 M)

To prepare a 0.1 M 2-mercaptoethanol solution, add 100 μ l of 2-mercaptoethanol to 14.2 ml of sterile PBS–/–. This solution should be made fresh before use in ESC maintenance medium and retinal differentiation medium.

ESC maintenance medium

To prepare ESC maintenance medium, add 5 ml FBS, 50 ml KSR, 5 ml GlutaMAX, 5 ml NEAA, 5 ml sodium pyruvate, and 0.5 ml 2-mercaptoethanol solution (0.1 M) to 429.5 ml of GMEM. Sterilize the medium by filtering it through a 0.22 μ m filter flask. The medium can be stored at 4°C for up to 1 month. Before use, warm the medium in a 37°C water bath and add LIF (2 μ l/10 ml) and DOX (2 μ l/ 10 ml).

Collagenase solution

To prepare collagenase solution, add 100 mg collagenase to 10 ml sterile PBS–/– (10 mg/ml). This solution should be made fresh before use in ESC dissociation solution.

ESC dissociation solution

To prepare ESC dissociation solution, add 20 ml KSR, 10 ml 2.5% trypsin, 10 ml collagenase solution, and 100 μ l CaCl_2 (1 M in MilliQ water) to 60 ml of PBS $^-/-$. Sterilize the solution by filtering it through a 0.22- μ m filter flask. Aliquots can be stored at -20°C for up to 2 month. Before use, warm the solution to 37°C in a water bath.

Retina differentiation medium

To prepare retinal differentiation medium, add 1.5 ml KSR, 1 ml GlutaMAX, 1 ml NEAA, 1 ml sodium pyruvate, and 100 μ l 2-mercaptoethanol solution (0.1 M) to 95.4 ml GMEM. Sterilize the medium by filtering through a 0.22- μ m filter flask. The medium can be stored at 4°C for up to 1 month.

Matrigel solution

To prepare matrigel solution, thaw matrigel overnight on ice at 4°C . Mix 240 μ l matrigel and 1.8 ml cold retinal differentiation medium in a 15-ml conical tube on ice. Use immediately.

Taurine solution (100 mM)

To prepare a 100 mM taurine stock solution, add 125.15 mg taurine per 10 ml in PBS $^-/-$. Aliquots can be stored at -20°C for up to 1 year. Add 100 μ l of 100 mM stock solution of taurine for every 10 ml medium for a final concentration of 1 mM.

Retinoic acid solution (0.5 mM)

To prepare a 0.5mM retinoic acid stock solution, add 7.511 mg to 50 ml DMSO in a dark room. Aliquots can be stored at -20°C in a light-sealed container for up to 1 year. Add 10 μ l of 0.5 mM stock solution of retinoic acid for every 10 ml of medium for a final concentration of 0.5 μ M.

Maturation medium 1 (MM1)

To prepare MM1, add 1 ml penicillin-streptomycin and 1 ml N2 supplement to 98 ml DMEM/F12 with GlutaMAX. Sterilize the medium by filtering it through a 0.22- μ m filter flask. The medium can be stored at 4°C for up to 1 month. Before use, warm the medium to 37°C in a water bath.

Maturation medium 2 (MM2)

To prepare MM2, add 1 ml penicillin-streptomycin, 1 ml N2 supplement, and 10 ml FBS to 88 ml DMEM/F12 with GlutaMAX. Sterilize the medium by filtering it through a 0.22- μ m filter flask. The medium can be stored at 4°C for up to 1 month. Before use, warm the medium to 37°C in a water bath.

Alkaline phosphatase (AP) rinse buffer

To prepare the alkaline phosphatase rinse buffer, add 100 ml 1 M Tris (pH 8), 75 ml NaCl (5 M in water), and 25 ml 20% Tween to 300 ml MilliQ water. Adjust the pH of the solution to 7.4. The buffer can be stored at room temperature for up to 1 month.

Sodium cacodylate stock (0.2 M, pH 7.2–7.4)

To prepare the sodium cacodylate stock, add 4.28g sodium cacodylate to 100ml water, stir for 30 min and adjust the pH to 7.4. The stock can be stored at 4°C for up to 1 month.

Electron microscopy fixative (2.5% glutaraldehyde, 2% PFA in 0.1 M sodium cacodylate)

To prepare the electron microscopy fixative, mix 50 ml sodium cacodylate stock (0.2 M), 10 ml glutaraldehyde (25%), 12.5 ml PFA (16%), and 27.5 ml MilliQ water. !CAUTION Electron microscopy fixative is hazardous. Wear protective gloves and work in a fume hood.

30% sucrose-PBS solution (wt/vol)

To prepare the 30% sucrose-PBS solution, add 3 g sucrose to 10 ml PBS–/– and vortex to dissolve the solution. The stock solution can be stored at 4°C for up to 3 months.

PROCEDURE**Reprogramming retinal cells Retinal dissection ● TIMING 30 min**

- 1 Euthanize reprogrammable mice ($n=3-6$) by CO₂ asphyxiation followed by cervical dislocation or decapitation. A piece of tissue (snout, ear, or tail) can be collected at this time for genotyping, if necessary. !CAUTION Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children's Research Hospital's Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.
- 2 Enucleate the eyes and place them in 10-cm dishes with PBS–/–.
- 3 Under a dissecting microscope, use a 16-gauge needle to puncture the eye along the sclera.
- 4 Use #55 forceps to carefully open the eye, exposing the retina. Remove the lens, optic nerve, and ciliary muscle, if still attached.

Retinal dissociation ● TIMING 1 h

- 5 Transfer up to 4 retinæ per tube to 1.5-ml siliconized Eppendorf tubes with 100 µl of sterile PBS–/– per retina.
- 6 Add 10 µl retina-trypsin (100×) per retina and incubate for 5 min in a 37°C water bath.
- 7 Triturate 5 to 7 times with a P1000 barrier pipette to dissociate the retinæ and incubate for an additional 3 min at 37°C.
- 8 Add 10 µl DNase and 10 µl STI per retina. Gently pipette the solution 3 or 4 times with a P1000 to mix it and incubate for 5 min in a 37°C water bath. (Fig. 3A)

- 9 Pipette the cell suspension into a 50-ml conical tube through a 40- μ m mesh cell strainer. Wash the cell strainer with PBS—/— to bring the total volume to 3 ml.
- 10 Add 5 ml BSA cushion medium to a 15-ml conical tube. Tilt the tube at a 45° angle and slowly pipette the filtered cell suspension on top of the BSA cushion with a P1000 pipette to create a layered solution.
- 11 Centrifuge the solution at 500 *g* for 10 min at 4°C to separate the cells from the debris.
- 12 Aspirate the supernatant and resuspend the cell pellet in 750 μ l explant culture medium. Fluorescence-activated cell (FAC) sort the dissociated retina to enrich for a specific retinal subpopulation¹⁸. We recommend using the following settings: 100 μ m nozzle size, 20 psi, 35,000 kHz, and 4°C. After the sort, centrifuge the FAC-sorted cells at 500 *g* for 5 min at 4°C, aspirate the supernatant, and resuspend the cells in 750 μ l explant culture medium.
- 13 While sorting the samples, dissociate the wild-type retinæ of 4 to 6 C57BL/6J postnatal (days 0–5) pups for 16–24 explant pellet cultures according to steps 1 through 8 (without a BSA cushion). Bring the total volume of the cells to 10 ml in explant culture medium.
- 14 Count the cells from both the reprogrammable and wild-type retinæ and proceed directly to step 15.

Making retinal pellets ● TIMING 1 h

- 15 Add 2 ml explant culture medium to each well of a sterile 12-well plate.
- 16 Float a Whatman polycarbonate membrane with the glossy side up in each well.
- 17 Add the FAC-sorted cell population from Step 14 to a 1.5 ml siliconized Eppendorf tubes at a suitable concentration. A limiting dilution series of reprogrammable cells should be performed to determine the optimal cell concentration for reprogramming cells in the retinal explant culture. We recommend plating 60, 600, 6000, and 60,000 reprogrammable cells per pellet culture to determine the optimal concentration⁸. ? TROUBLESHOOTING
- 18 Add 1.5e⁶ wild-type retina cells from step 14 to each Eppendorf tube containing reprogrammable cells from step 17. The total volume of this cell suspension should not exceed 1.5 ml.
- 19 Centrifuge the cell suspension at 14,000 *g* for 15 s at room temperature (25°C) to create a retinal cell pellet (Fig. 3B).
- 20 Partially remove the supernatant from each tube, leaving 500 μ l in the tube. With a MultiFlex tip on a P200 pipette, fill the tip with 135 μ l of medium. Gently nudge the pellet off the bottom of the tube by delicately kneading the pellet, peeling away the edges and pushing them toward the center of the pellet (Fig. 3B–C). Gently expel the medium in the pipette tip to dislodge the pellet from the side of the tube. With a P1000 pipette, gently remove the pellet with 50 to 100 μ l

of media and deposit it on a floating Whatman membrane in the 12-well plate (Fig. 3D–F). ? TROUBLESHOOTING

- 21 Incubate pellet cultures at 37°C with 5% CO₂.

Retinal explant reprogramming ● TIMING 9 days

- 22 Feed the pellets daily by placing 10 µl of conditioned explant culture medium from each individual well on top of each pellet. ▲ CRITICAL STEP Daily feeding of retinal explants is required to maintain optimal oxygen and nutrient concentrations. If the pellets are not fed daily, the cells will die and not reprogram.
- 23 On days 4 and 8 of reprogramming, remove 1 ml explant medium from the wells and replace with 1 ml fresh, warmed explant culture medium.

Dispersal of retinal explant cultures ● TIMING 30 min + 5 days

CRITICAL Prepare a 6-well plate with ir-MEFs the day before splitting/passaging stem cells (see Box 1 for procedure).

Box 1

Preparation of ir-MEF feeder layer ● TIMING 30 min

CRITICAL

The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

1. Coat tissue culture plates with gelatin solution and incubate at 37°C for at least 15 min. Before seeding ir-MEFs, aspirate excess gelatin.
2. Thaw cryotubes of ir-MEFs in a 37°C water bath and transfer the cells to a 50-ml conical tube.
3. Add 10 ml cDMEM for every tube of thawed cells.
4. Centrifuge the cells at 250 *g* for 5 min at room temperature.
5. Aspirate the supernatant and resuspend in cDMEM. Seed ir-MEFs on gelatin-coated plates according to the cell concentrations shown **below**:

Plate type	Ir-MEFs/well	Volume/well
24-well	72,000	0.5 ml
12-well	200,000	1 ml
6-well	400,000	2 ml
10 cm dish	2.7-3e ⁶	10 ml

6. Incubate the cells overnight at 37°C with 5% CO₂ for use the next day. ▲ CRITICAL STEP The health of ir-MEFs is very important for maintaining

stem cells. Ir-MEF plates should be checked before use for healthy, confluent ir-MEFs and should be used within 5 days of plating.

- 24 After 9 days of culture move membranes with explant cultures from the tissue culture dish to a sterile lid or dish. Use forceps to carefully nudge the explants off the membranes onto the sterile lid or dish. Wash the membranes with 200 μ l PBS–/– and transfer explants and PBS–/– into a 1.5-ml siliconized Eppendorf tubes. **CRITICAL STEP** Each individual membrane should be placed into an individual tube and dispersed separately for the remainder of the procedure. It is recommended to determine the optimal cell concentration in the retinal pellets for each individual line (Step 27).
- 25 Trypsinize and DNase treat the cells. Follow steps 6 to 8 as if there were 2 retinae in each tube. Do not centrifuge the dissociated cells from the explants or dilute the solution.
- 26 Take 10 μ l from the tube for a cell count and add 750 μ l ESC maintenance medium.
- 27 Seed the cells from each retinal pellet into 1 well of a 6-well plate with an ir-MEF feeder cell layer and 2 ml ESC maintenance medium per well. For initial experiments, we recommend plating cells in a limiting dilution series of 20, 200, 2000, and 20,000 cells per well to determine the optimal cell concentration that will result in distinct colonies. ? TROUBLESHOOTING
- 28 Incubate the plates at 37°C with 5% CO₂.
- 29 Daily, aspirate the medium and exchange with fresh ESC maintenance medium for 5 days.

Picking colonies ● TIMING 2 h

CRITICAL Prepare 24-well plates with ir-MEFs the day before splitting/passaging stem cells (see Box 1 for procedure).

- 30 Fill a 96-well round bottom plate with 40 μ l of warmed trypsin-versene per well.
- 31 Exchange the cDMEM from one or two 24-well culture plates containing ir-MEF feeder layers with ESC maintenance medium.
- 32 Using a sterile P10 pipette tip and an inverted microscope set at 4x magnification; gently nudge an individual colony to dislodge it from the ir-MEF feeder layer. Then aspirate the dislodged stem cell colony with 7 μ l of media and transfer from the 6well plate to individual wells in the 96-well plate from step 30. ▲ **CRITICAL STEP** This step can be technically challenging and will require practice. Pick sets of 6 to 8 colonies at a time in order to minimize the amount of time colonies spend in trypsinversene. Each colony should be placed in a single well of the 96-well plate. ? TROUBLESHOOTING
- 33 Using an inverted microscope, check that the colonies have been successfully transferred to the 96-well plate and then pipet 2–3 times to dissociate the colony

and transfer the entire contents of the 96-well to a separate well of a 24-well plate containing ir-MEFs and ESC maintenance media prepared in step 31. Repeat this step for a total of 24 to 48 colonies per cell line.

- 34 Incubate the plates at 37°C with 5% CO₂. ▲ CRITICAL STEP Each of the wells is now considered a unique stem cell subclone line. This is considered the first passage (p1) of these lines.
- 35 Exchange the medium daily with fresh ESC maintenance medium. Subclones can be grown until there are 200,000 to 300,000 iPSCs per well or up to 6 days. The lines should then be passaged onto 12-well tissue culture plates with an ir-MEF feeder cell layer (see Box 1 for ir-MEF procedure).

Passaging stem cell lines to passage 20 (p20) to expand stocks ● TIMING 40 days

CRITICAL All exchanges and splits should occur around the same time of day (with 24 h between exchanges). For routine passaging, we grow stem cell lines on 12-well tissue culture plates with an ir-MEF feeder cell layer and typical stem cells are split every other day. Prepare a 12-well plate with ir-MEFs the day before splitting/passaging stem cells (Box 1).

Medium exchange : The day following initial seeding

- 36 Observe cell density and differentiation. Aspirate the used ESC maintenance medium from each well. ?TROUBLESHOOTING
- 37 Gently pipette 1 ml fresh ESC maintenance medium into each well. CRITICAL STEP We recommend maintaining the cells with doxycycline until reprogramming is confirmed.
- 38 Incubate the plates at 37°C with 5% CO₂.
- 39 Repeat steps 36–38 daily.

Splitting/passaging: Two days following initial seeding or when colonies begin to merge together

- 40 Aspirate the medium from the well and wash with 1 ml PBS–/–.
- 41 Aspirate the PBS–/–.
- 42 Add 90 µl 0.25% trypsin-EDTA to each well for 2 min at room temperature or until the cells begin to lift off the plate. ? TROUBLESHOOTING
- 43 Add 1 ml ESC maintenance media per well and dissociate the cells by triturating 3 or 4 times with a P1000 barrier tip. ▲ CRITICAL STEP A single cell suspension is desirable to ensure colony growth is evenly distributed. ? TROUBLESHOOTING
- 44 Transfer the cell suspension to a 15-ml conical tube and add 3 ml ESC maintenance medium.
- 45 Centrifuge the cells at 250 *g* for 3 min at room temperature.

- 46 Aspirate the supernatant, resuspend the cells in 2 ml ESC maintenance medium, and count the cells.
- 47 Plate 100,000 cells per well on a 12-well plate with an ir-MEF feeder cell layer. ? TROUBLESHOOTING

Freezing stocks—Freeze down the stem cells at passages p5, p10, p15 and p20.

- 48 To freeze cells, repeat steps 40–45 as if the cells are being passaged.
- 49 Aspirate the supernatant and resuspend the cells in ESC maintenance medium with 10% DMSO at 100,000 cells/ml.
- 50 Aliquot 1 ml of the cell suspension into each cryotube and store overnight at -80°C .
- 51 For long-term storage, transfer cryotubes to liquid nitrogen. ■ PAUSE POINT Cells can be stored in liquid nitrogen indefinitely.

Expanding stem cell lines to 10-cm dishes ● TIMING 7 days

CRITICAL Prepare a 12-well plate and 10 cm plate with ir-MEFs the day before splitting/passaging stem cells (see Box 1 for procedure).

- 52 Thaw one vial of stem cells in a 37°C water bath and transfer the cells to a 15-ml conical tube. Add 9 ml ESC maintenance medium.
- 53 Centrifuge the cells at 250 *g* for 5 min, aspirate the medium, and resuspend the cells in 2 ml ESC maintenance medium.
- 54 Add the cell suspension to 2 wells of a 12-well plate with ir-MEF feeder cells and incubate at 37°C with 5% CO_2 .
- 55 Passage and expand the cells several more times onto 10-cm culture dishes (see steps 36–47). CRITICAL STEP An example of a typical cell expansion: Passage 2 wells of a 12-well to 4 wells of a 12-well dish (100,000 cells/well), 4 wells of a 12well dish to one 10-cm culture dish (2×10^6 cells/dish), and one 10-cm culture dish to two 10-cm culture dishes (2×10^6 cells/dish). This propagation series will take three passages and should provide a sufficient quantity of cells for future experiments.

Preplating to separate stem cells from ir-MEFs ● TIMING 2 h

- 56 Coat a sterile 10-cm dish with 5 ml gelatin solution and incubate at 37°C for at least 15 min. One gelatin-coated 10-cm dish is required for each 10-cm dish with stem cells from step 55. Steps 57–71 are written for a single 10-cm plate. Repeat each step for each additional plate.
- 57 Rinse the stem cells from one to two 10-cm dishes with PBS–/–, aspirate, and add 1 ml ESC dissociation solution. Incubate the cells at room temperature for 3 to 4 min or until the cells begin to loosen from the plate. ▲ CRITICAL STEP

ESC dissociation solution will breakup the ir-MEF feeder layer while maintaining the stem cell colonies. ? TROUBLESHOOTING

- 58 Add 5 ml ESC maintenance medium to stop the digestion reaction.
- 59 Transfer the cell suspension to a 15-ml conical tube and centrifuge at 250 *g* for 5 min at room temperature.
- 60 While centrifuging the cells, aspirate the gelatin from the 10-cm plate from step 56 and add 3 ml ESC maintenance medium to wash the excess gelatin from the plate.
- 61 Aspirate the supernatant from the cells from step 59 and resuspend the cells in 5 ml ESC maintenance medium (half the volume that is normally used to plate cells on a 10-cm dish). ▲ CRITICAL STEP The key to successfully separating the ir-MEFs is to provide a broad surface area to which the cells can attach. The ir-MEFs will quickly attach to the fresh 10-cm plate while the stem cell colonies will remain in suspension.
- 62 Aspirate the 3 ml ESC maintenance medium from the plate and add the 5 ml cell suspension. Incubate the cells at 37°C with 5% CO₂ for 30 min to allow the ir-MEFs to adhere to the plate.
- 63 After 30 min, tilt the plate to pool the medium and rinse the plate 3 or 4 times with the medium to collect stem cell colonies. ▲ CRITICAL STEP Do NOT scrape the plate.
- 64 Transfer the supernatant into a 15-ml conical tube and discard the 10-cm plate with ir-MEFs.
- 65 Centrifuge the stem cell colonies at 250 *g* for 5 min at room temperature.
- 66 Aspirate the supernatant and resuspend the stem cells in 5 ml PBS–/–.
- 67 Centrifuge the stem cell colonies at 250 *g* for 5 min at room temperature.
- 68 Aspirate the supernatant and resuspend the cells in 1 ml 0.25% trypsin-EDTA for exactly 1 min room temperature. Gently agitate to resuspend the cells every 20 to 30 s. ▲ CRITICAL STEP This short trypsin digestion creates a single-cell suspension. The cell viability will greatly decrease if the cells are left in trypsin for longer than 1 min.
- 69 Add 5 ml ESC maintenance medium to stop the reaction and gently triturate 5 or 6 times with a P1000 barrier tip to create a single-cell suspension.
- 70 Centrifuge the cells at 250*g* for 5 min.
- 71 Aspirate the supernatant. Continue directly to step 72 (for FAC sorting for SSEA1), step 79 (for characterization), or step 84 (for retinal differentiation).

FAC sort to enrich for SSEA1+ undifferentiated stem cells

▲ CRITICAL STEP Routine passaging can cause spontaneous differentiation even under optimal growth conditions. Sorting viable cells for SSEA1, or with another appropriate

marker, creates a standardized cell stock that reduces variability and enables comparison between stem cell lines via the STEM-RET protocol.

- 72** After step 71, resuspend the cells from each 10-cm plate in 500 μ l human gamma globulin blocking reagent and transfer them to a 40- μ m mesh cell strainer on a 50-ml conical tube. Add 3 ml ESC maintenance medium to wash the cells through the filter.
- 73** Add 20 μ l PE-conjugated SSEA1 antibody or 2 μ l rat IgG PE isotype control antibody (Table 1) and incubate on ice for 15 min.
- 74** Count the cells. ? TROUBLESHOOTING
- 75** Centrifuge the cells at 250 *g* for 5 min at room temperature and resuspend them in 500 μ l ESC maintenance medium for FAC sorting¹⁸. Perform a DAPI counterstain for nonviable cells. We recommend the following settings: 85 μ m nozzle size, 35 psi, 55,000 kHz, and 4°C.
- 76** After sorting, freeze the viable SSEA1+ cells at 400,000 to 500,000 cells per ml (1 ml per cryovial) by following steps 48–51. ? TROUBLESHOOTING For long-term storage, transfer cells to liquid nitrogen. ■ PAUSE POINT Cells can be stored in liquid nitrogen indefinitely.

Characterizing stem cell lines

- 77** Thaw a vial of SSEA1+ cells and seed a 12-well plate with 100,000 cell/well. Expand the cells as outlined in step 55 until cells are plated onto one 10-cm dish.
- 78** Perform steps 56–71 to separate stem cells from the ir-MEFs and produce a single-cell suspension.
- 79** After step 71, resuspend the cells in 5 ml ESC maintenance medium and filter cells through a 40- μ m mesh cell strainer into a 50-ml conical tube.
- 80** Count the cells. A total of 2.1×10^6 cells is necessary to complete all sections A through F below. Aliquot the cells required for each section into 15-ml conical tubes.
- 81** Centrifuge the cells at 250 *g* for 5 min and resuspend them according to the steps in sections A through F.

(A) Karyotyping ● TIMING 4 days—CRITICAL Prepare a 12-well plate with ir-MEFs the day before karyotyping

- (i)** Day 1: Resuspend 200,000 cells from Step 81 in 2 ml ESC maintenance medium with LIF and DOX.
- (ii)** Plate the stem cells at 100,000 cells per well into 2 wells of a 12-well plate with an ir-MEF feeder cell layer.
- (iii)** Incubate at 37°C with 5% CO₂ overnight.

- (iv) Day 2: Exchange the medium in each well with 1 ml fresh ESC maintenance medium (steps 36–39).
 - (ii) Day 3: Exchange the medium with 1 ml fresh ESC maintenance medium and add 20 μ l colcemid solution per well. Incubate the cells for 4 to 5 h at 37°C.
 - (iii) Rinse the wells with 500 μ l PBS–/–, aspirate the PBS–/–, and add 500 μ l trypsin (Cellgro) for 5 min at room temperature or until the cells begin to lift off of the plate.
 - (iv) Gently transfer the cells to a 15-ml conical tube. Wash the plate with 500 μ l PBS –/– and transfer the cell suspension to the 15-ml conical tube. Centrifuge them at 1600 *g* for 5 min at room temperature.
 - (v) Remove supernatant and resuspend the cells in 4 ml potassium chloride solution (0.075 M).
 - (vi) Incubate the cell suspension for 8 min at room temperature and then centrifuge it at 765 *g* for 5 min at room temperature.
 - (vii) Aspirate the supernatant and add 2.5 ml 3:1 (vol:vol) methanol:acetic acid fixative.
 - (viii) Incubate for 15 min at room temperature.
 - (ix) Centrifuge the cells at 765 *g* for 6 min at room temperature, aspirate the supernatant and add 2.5 ml 3:1 (vol:vol) methanol:acetic acid fixative.
 - (x) Prewash slides by wiping with 70% methanol to remove any debris and store in MilliQ water. Plate the cells onto prewashed slides and allow them to air dry for 24 to 48 h before performing Giemsa-trypsin staining.
 - (xi) Image and analyze the chromosomes to identify any abnormalities⁸. ?
- TROUBLESHOOTING

(B) DOX independence assessment ● TIMING 10 days

- i. Day 1: Resuspend one set of 100,000 cells from Step 81 in 1 ml ESC maintenance medium with LIF and DOX. Resuspend another set of 100,000 cells in 1 ml ESC maintenance medium without DOX (i.e., containing LIF only).
- ii. Plate each set of stem cells into a well of a 12-well plate with an ir-MEF feeder cell layer (see Box 1 for ir-MEF preparation).
- iii. Maintain cells with passage or exchange the medium daily (Steps 36–47) up to 5 passages. Maintain and passage one set of cells with ESC maintenance medium containing LIF and DOX. Maintain the other set of cells with ESC maintenance medium containing LIF but no DOX.
- iv. After 5 passages, image the colonies under a microscope and note any morphological differences and signs of differentiation between the cells maintained with doxycycline and those maintained without doxycycline. If differences are noted, follow up with additional AP staining (section 81C) or

immunofluorescence staining (section 81D) to confirm differentiation. Stem lines that differentiate when doxycycline is removed are not considered fully reprogrammed⁸. ? TROUBLESHOOTING

(C) Alkaline phosphatase (AP) staining ● TIMING 5 days

- i. Day 1: Resuspend 100,000 cells from Step 81 in 1 ml ESC maintenance medium **without LIF or DOX (LIF/DOX-)**.
- ii. Add ESC maintenance medium with LIF and DOX (LIF/DOX+) to 2 wells of a 12-well plate with an ir-MEF feeder layer, and add ESC maintenance medium **without LIF or DOX (LIF/DOX-)** to 2 other wells.
- iii. Plate 50 µl and 100 µl stem cells in each set of wells (5000 and 10,000 cells per well, respectively). One well for each cell concentration should be maintained with ESC maintenance medium with LIF and DOX (LIF/DOX+) and the other with ESC maintenance medium **without LIF or DOX (LIF/DOX-)**. **CRITICAL STEP** Removing LIF and DOX from the medium initiates differentiation of stem cells.
- iv. Incubate at 37°C with 5% CO₂.
- v. Days 2–4: Perform full medium exchanges daily for 5 days with the corresponding medium (LIF/DOX+ or LIF/DOX-) according to steps 36–39.
- vi. Day 5: Wash the cells with PBS–/– and AP rinse buffer. Fix the cells with 4% PFA for 1 min. **▲ CRITICAL STEP** AP enzymatic activity is sensitive to fixation. Do not fix the cells for longer than 1 min.
- vii. Use the Millipore AP stain kit according to the manufacturer's instructions. Wash the cells with AP rinse buffer and image the colonies to assess the loss of AP stain resulting from the removal of LIF and DOX from the medium⁸. ? TROUBLESHOOTING

(D) Immunostaining for stem cell markers ● TIMING 3 days

- i. Day 1: Resuspend 200,000 cells from Step 81 in 2 ml ESC maintenance medium with LIF and DOX.
- ii. Add 500 µl gelatin solution to 8-chamber plastic microscope slides and incubate at 37°C for 15 min. Each cell line will need 4 wells. Before plating, aspirate the gelatin solution and wash once with ESC maintenance medium to remove excess gelatin.
- iii. Aspirate the ESC maintenance medium from 4 wells of the microscope slide.
- iv. Plate 360 µl cell suspension per well (36,000 cells) and incubate at 37°C with 5% CO₂.
- v. Day 2: Aspirate the media and wash the cells with PBS–/–. Fix the cells with 4% PFA for 30 min. **■ PAUSE POINT** Cells can be stored in 4% PFA at 4°C for up to a week.

- vi. Rinse the cells 3 times with PBS–/– and block for 3 to 4 h at room temperature in the appropriate blocking serum with 0.5% Triton X-100 (see Table 1 for information about cMyc, Nanog, Oct3/4 and SSEA1 antibodies).
- vii. Incubate the cells with primary antibodies overnight at 4°C.
- viii. Day 3: Rinse the cells twice with PBS–/– and incubate them for 2 h with secondary antibody at room temperature.
- ix. Rinse the cells twice with PBS–/– and incubate for 30 min with ABC kit at room temperature.
- x. Visualize the immunostaining by incubating the cells for 10 min with cyanine–3–conjugated–tyramide reagent before performing DAPI counterstaining of nuclei for 10 min at room temperature⁸.

(E) Teratoma formation ● TIMING up to 3 months

- i. Resuspend 1,200,000 cells from Step 81 in 600 µl matrigel.
- ii. Inject 100 µl cell suspension into each flank of 3 adult CD1-Nude mice (200,000 cells/injection)¹⁷. !CAUTION Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children's Research Hospital's Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.
- iii. Monitor the growth of the teratoma for up to 12 weeks post injection, until tumor size reaches 20% of body weight, or an endpoint as determined by the institution. ▲ CRITICAL STEP The endpoint for each mouse should be in accordance with the guidelines and regulations of the relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children's Research Hospital's Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.
- iv. Euthanize the mouse by CO₂ asphyxiation followed by cervical dislocation or decapitation. Resect the teratomas and fix in 4% PFA. !CAUTION Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children's Research Hospital's Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. ■ PAUSE POINT Fixed tissue can be stored at 4°C for several weeks.
- v. Embed the teratomas in paraffin and section (200 µm sections).
- vi. Perform hematoxylin and eosin staining. For immunohistochemical staining (Step 81D(vi–x)), use primary antibodies for cytokeratin–oscar, desmin, and synaptophysin (Table 1)⁸.

(F) qPCR analysis of stem cell markers ● TIMING 2 h

- i. Resuspend 1×10^6 cells from Step 81 in 800 μ l Trizol reagent. ■ PAUSE POINT Samples can be flash frozen and stored at -80°C for up to 1 year. ! CRITICAL STEP Trizol reagent is a hazardous reagent. Wear protective eyewear, gloves, and a labcoat. All work done with Trizol reagent should be done in a fume hood.
- ii. If frozen, thaw samples on ice, then homogenize at 17,000 rpm for 30 s with a tissue homogenizer.
- iii. Purify the RNA with a Direct-zol kit following the manufacturer's instructions.
- iv. Synthesize cDNA from the RNA with the High-Capacity RNA-to-cDNA kit following the manufacturer's instructions. Dilute the cDNA to 10 ng/ μ l for each sample.
- v. Prepare the cDNA sample for analysis with a TaqMan Custom Array Card by combining 200 ng cDNA (20 μ l), 30 μ l RNase/DNase free water, and 50 μ l TaqMan Universal PCR MasterMix.
- vi. Load 100 μ l of the cDNA sample into one lane of TaqMan Array card for stem cell markers (Table 2). Perform real time qPCR analysis with a 7900 HT Fast real-time PCR system. Normalize the samples to GAPDH.⁸

Retinal differentiation of stem cells Plating for retinal differentiation ● TIMING 30 min + 6 days

CRITICAL Prepare a 12-well plate and 10 cm plate with ir-MEFS the day before splitting/passaging stem cells (see Box 1).

- 82 Thaw 1 cryovial of SSEA1+ cells, expand the cells to a 10-cm dish (steps 52–55), and perform replating to remove the ir-MEF feeder cells (steps 56–71). ▲ CRITICAL STEP Replating is important for removing ir-MEFs as contamination by ir-MEFs will negatively affect the retinal differentiation of stem cells.
- 83 After step 71, resuspend the cells in 5 ml ESC maintenance medium and filter them through a 40- μ m mesh cell strainer into a 50-ml conical tube.
- 84 Adjust the volume of the cell suspension to 10 ml and count the cells.
- 85 Take 300,000 stem cells and centrifuge them at 250g for 3 min at room temperature. Aspirate the supernatant and resuspend the cells in 3 ml of retinal differentiation medium. Perform another cell count to confirm the concentration of the cells.
- 86 Adjust the volume of the cell suspension to give a concentration of 30,000 cells/ml in retinal differentiation medium.
- 87 Dispense the diluted cell suspension into Nunclon Sphera 96-well plates at 100 μ l/well.

- 88** Incubate the plates at 37°C with 5% CO₂ overnight. Thaw matrigel overnight on ice at 4°C.
- 89** Day 1: On ice, prepare matrigel solution with **cold** retinal differentiation medium. Pipette 3 to 4 times with a P1000 barrier tip to mix. **▲ CRITICAL STEP** For the best results, add matrigel solution within 18 h of plating the stem cells.
- 90** Dispense 20 µl matrigel solution into each well of Nunclon Sphera 96-well plates containing stem cell aggregates.
- 91** Day 2 to 7: Incubate the stem cell aggregates at 37°C with 5% CO₂. Image the cells daily to track changes in morphology⁸. Spheres will appear lumpy at first and then develop thick, laminated edges and outcroppings over the next 7 days (Fig. 2B). **? TROUBLESHOOTING**

Day 7: Eye field specification ● TIMING 1–2 h + 3 days

- 92** Score the individual spheres with a phase contrast light microscope⁸. For examples of different type of spheres, see Fig. 2C. Score the spheres as follows:

Score	Description of Spheres
A	Spheres have thick, laminated edges, one or more optic vesicle outcroppings, and no or limited nonretinal cells are present
B	Spheres have thick, laminated edges, one or more optic vesicle outcroppings, and moderate to high numbers of nonretinal cells are present
C	Spheres have differentiated into nonretinal anterior neural tissue, or retinal features are obscured or potentially contaminated by nonretinal tissue
X	Spheres have thin, laminated edges in a defined sphere form. Spheres appear to have stalled during retinal morphogenesis

- 93** Record the number of spheres in each category for further analysis of eye field specification (see **step 105A**). Transfer the “A” and “B” spheres to a 15-ml conical tube for continuation of the STEM-RET protocol. **▲ CRITICAL STEP** The presence of non-retinal cells did not affect our ability to isolate individual optic vesicles. **? TROUBLESHOOTING**
- 94** Wait 5 min to allow the spheres to fall out of suspension and settle on the bottom of the tube.
- 95** Carefully discard most of the supernatant and transfer the spheres to a 10-cm Petri dish with 10 ml of prewarmed MM1.
- 96** Incubate the spheres at 37°C with 5% CO₂ and 40% O₂ for 3 days. **▲ CRITICAL STEP** Retinal spheres require incubation in a high-oxygen incubator for the remainder of the procedure.

Day 10: Excision of optic cups ● TIMING 45 – 60 min + 4 days

- 97 Image and count the total number of retinal spheres and the number of retinal spheres with pigmentation or visible retinal outcroppings (Fig. 2D). **CRITICAL STEP** pigmented regions contain retinal tissue at equivalent maturation to the optic cup developmental stage *in vivo* therefore; we identify the aggregates of pigmentation and adjacent retinal tissue as “optic cups”. See the **Experimental Design** section for details.
- 98 With two #55 forceps, pinch off the optic cup outcroppings. Reserve the optic cup outcroppings and discard the nonretinal pieces. Some spheres may have multiple outcroppings that can be pinched, whereas others may have pigmentation but no outcroppings that can be excised. **▲ CRITICAL STEP** The excision of optic outcroppings is technically challenging and will require practice. It is important to excise only retinal tissue, as nonretinal tissue will proliferate and overtake the retinal sphere.
- 99 Record the number of optic cups excised and the number of pigmented spheres for analysis (see **step 105B**). ? TROUBLESHOOTING
- 100 Transfer the optic cups to a fresh 10-cm Petri dish with 10 ml MM2 with retinoic acid solution and taurine solution added.
- 101 Incubate the optic cups at 37°C with 5% CO₂ and 40% O₂ (high oxygen) for 4 days.

Day 14 to 25: Medium Exchanges ● TIMING 14 days

- 102 Day 14: Carefully remove the medium and replace it with prewarmed 10 ml MM2 with taurine solution added (**no retinoic acid**). Continue to incubate the spheres at 37°C with 5% CO₂ and 40% O₂ (high oxygen)
- 103 Days 17, 20, 23, and 25: Carefully remove half of the medium (5 ml) and replace it with 5ml fresh MM2 with taurine solution added (**no retinoic acid**). Continue to incubate the spheres at 37°C with 5% CO₂ and 40% O₂ (high oxygen)

Day 28: Harvesting retinal spheres ● TIMING 30–60 min + 2–3 days

- 104 The optic cup outcroppings are processed to quantify retinal differentiation. Pinched outcroppings are imaged for GFP expression, if applicable, and transferred to Trizol for gene expression analysis (A), fixed for electron microscopy morphological analysis (B), or fixed for immunostaining (C).

(A) Trizol preservation of spheres for gene expression analysis

- i. Transfer 2 retinal spheres to a siliconized Eppendorf tube with 800 µl Trizol reagent. Flash freeze the samples and store at –80°C. **■ PAUSE POINT** Trizol samples can be stored at –80°C for up to 1 year. **!CAUTION** Trizol reagent is a hazardous reagent. Wear protective eyewear, gloves, and a labcoat. All work done with Trizol reagent should be performed in a fume hood.

- ii. Follow steps 81F(ii–v).
- iii. Load 100 µl of the cDNA sample into one lane of TaqMan Array Card for **retinal markers** (Table 2). Perform real time qPCR analysis with a 7900 HT Fast real-time PCR system. Normalize the samples to GAPDH. See **step 105C(i)** for RDQ analysis.

(B) Electron microscopic analysis of retinal features

- i. Transfer the retinal spheres to a siliconized Eppendorf tube with 1 ml of electron microscopy fixative and fix the spheres for 1.5 h at room temperature. ! CAUTION The electron microscopy fixative is a hazardous material. Wear protective eyewear, gloves, and a labcoat.
- ii. Dehydrate the samples through a graded ethanol-to-propylene oxide wash, infiltrate them, and embed them in epoxy resin.
- iii. Allow the epoxy resin to polymerize overnight at 80°C in a lab oven. ■ PAUSE POINT After the samples have been embedded in resin, they can be stored at room temperature indefinitely.
- iv. Cut semi-thin sections (0.5 µm) with a microtome and stain them with toluidine blue. Cut ultrathin sections (80 nm) with a microtome and image them with a transmission electron microscope (TEM). See **step 105C(iii)** for RDEM analysis and Table 3 for the list of retinal features. Fig. 4 shows an example of each of the retinal features in a normal adult mouse retina.

(C) Immunofluorescence labeling of retinal markers

- i. Transfer the retinal spheres to a siliconized Eppendorf tube with 1 ml 4% PFA and fix the tissue overnight at 4°C. !CAUTION 4% PFA solution is a hazardous material. Wear protective eyewear, gloves, and a lab coat. ■ PAUSE POINT Fixed samples can be stored at 4°C for up to 1 week.
- ii. Cryo-protect the samples by rinsing them once with PBS–/– and submerging the tissue in a 30% sucrose-PBS solution at 4°C overnight
- iii. Position the spheres in OCT solution and flash freeze them. ■ PAUSE POINT Frozen blocks can be stored at –80°C indefinitely.
- iv. Cut sections (10 µm) with a cryostat and allow them to air-dry on Superfrost Plus microscope slides.
- v. Immunostain the section with antibodies for calbindin, syntaxin, PKC–α, glutamine synthetase, pax6, chx10, and recoverin (see Table 1, **in bold**) and analyze them. See **step 105C(iv)** for RDIF analysis and Fig. 5 for examples of positive immunostaining.

Calculating STEM-RET scores TIMING 1–2 hours

CRITICAL For statistical power, run each cell line through the STEM-RET protocol in triplicate. Examples of STEM-RET scoring results can be found in our previous publication⁸

- 105** To calculate STEM-RET scores three aspects of growth are calculated. Follow section A to score *Eye field specification*, section B to score *Optic cup formation*, section C to score *Retinal differentiation*

(A) Eye field specification—CRITICAL This integrated score of eye field induction efficiency (EFE), eye field induction specificity (EFS), and eye field proliferation (EFP) is calculated at day 7 (Step 92).

- i. Calculate eye field induction efficiency (EFE) by counting the number of A and B spheres and dividing by the total number of spheres
- ii. Calculate eye field induction specificity (EFS) by counting the number of A spheres and dividing by the total number of A, B and C spheres
- iii. Calculate eye field proliferation (EFP) by counting the number of A, B and C spheres divided by the total number of spheres
- iv. Calculate the eye field specification score by taking the average of EFE, EFS and EFP. See table 4 for the recommended value range observed in r-iPSCs and control Eb5 Rx—GFP

(B) Optic cup formation—CRITICAL This is an integrated score of optic cup efficiency (OCE) and optic cup frequency (OCF) on day 10 (Step 97).

- i. Calculate OCE as follows (number of pigmented spheres) / (total number of spheres)
- ii. Calculate OCF as follows: (total number of pinches) / (total number of spheres)
- iii. Calculate the optic cup formation score by taking the average of OCE and OCF. See table 4 for the recommended value range observed in r-iPSCs and control Eb5 Rx—GFP

(C) Retinal differentiation

- i. At day 28 (Step 104) assess retinal differentiation by performing qPCR analysis of 15 retinal genes (RDQ) (Table 2 –retinal differentiation, **in bold**), transmission electron microscopic analysis for 18 morphological marks (RDEM) (Table 3; Fig. 4), and immunofluorescence labeling of 9 retina-specific antibodies (RDIF) (Table 1, **in bold**; Fig. 5).
- ii. To calculate RDQ: All qPCR data for stem cell derived spheres, E14.5 normal mouse retina and P12 normal mouse retinae are normalized to *Gapdh* expression as an internal control. The stem cell derived sphere expression and P12 expression are then normalized to E14.5 expression. Finally, the expression in the sphere is calculated relative to P12 retina. The data across genes are then averaged to provide an integrated score. If the stem cell derived retina expresses a gene at the same level at a P12 retina, the score for that gene will be 1.0.
- iii. To calculate RDEM: Each sphere is evaluated for the presence of retinal features in a blinded manner (Table 3, Fig. 4). Each mark is scored either 1 (present) or 0

(absent). The score for all of the marks are then averaged so that a final score of 1.0 is equivalent to mature murine retina.

- iv. To calculate RDIF: Retinal spheres are assessed using a blinded scoring system (Table 2). Each antibody is scored 1.0 (>5 cells/section), 0.5 (<5 cells/section), or 0 (absent). The scores for all of the antibodies are then averaged so that a final score of 1.0 is equivalent to mature retina. Fig. 5 shows examples of positive immunostaining for each antibody.

TROUBLESHOOTING

See Table 1 for troubleshooting guidance.

● TIMING

Step(s)	Section	Timing
1–4	Retinal dissection	30 min
5–14	Retinal dissociation	1 h
15–21	Making retinal explants	1 h
22–23	Retinal explant reprogramming	9 days
Box 1	Preparation of ir-MEF feeder layer	30 min
24–29	Dispersal of retinal explant cultures	30 min
30–35	Picking colonies	2 h
36–51	Passaging stem cell lines to passage 20 (p20)	40 days
52–55	Expanding stem cell lines to 10-cm dish	7 days
56–71	Preplating to separate stem cells and ir-MEFs	2 h
81A	Karyotyping	4 days
81B	DOX independence assessment	10 days
81C	Alkaline phosphatase (AP) staining	5 days
81D	Immunostaining for stem cell markers	3 days
81E	Teratoma formation	Up to 3 months
81F	qPCR analysis of stem cell markers	2 h
82–91	Plating for retinal differentiation	30 min + 6 days
92–96	Eye field specification	1–2 h + 3 days
97–101	Excision of optic cups	45–60 min + 4 days
102–103	Medium exchanges	14 days
104	Harvesting retinal spheres	30–60 min + 3 days
105	Calculating STEM-RET scores	1–2 h

ANTICIPATED RESULTS

With our protocol, murine retinal neurons can be successfully reprogrammed with reprogramming efficiency dependent on the cell type, developmental stage, and size of the neuronal population. While our experiments used transgenic mice to reprogram retinal

neurons, our technique to culture retinal neurons as explant cultures can be used with other reprogramming protocols. In reprogramming retinal neurons, we would pick 24 to 36 colonies per experiment to establish 10 to 12 retinal derived iPSC subclone lines. iPSCs lines that failed to establish typically stalled or failed to proliferate during the early passages after picking (p0–p5). After 20 passages, retina-derived iPSC lines should be pluripotent, independent of doxycycline, alkaline phosphatase positive, and expressing endogenous pluripotent markers similar to embryonic stem cells. An example of stem cell characterization results can be found in our previous publication⁸. The variability we observed during these experiments was primarily due to differences in the reprogramming efficiency of retinal neurons from different origins and the health of retinal explants cultures. Cell lines that fail to meet these criteria for fully reprogrammed pluripotent stem cells were discarded and not used in our experiments.

Monitoring the colony morphology, karyotype, and general culture health is important for quality control. There will be some spontaneous differentiation in culture, and differentiated cells produce variability in the reproducibility of the STEM-RET protocol. While we observed differences in the outcome of the STEM-RET line between cell lines; when each line was run in multiple, independent experiments, a line would produce a consistent result. It is optimal to begin experiments with standardized, undifferentiated cell stocks to reduce the variability added by differentiated cells. We recommend FAC sorting for SSEA1, which enriches for undifferentiated mouse stem cells and provides a standardized stock for experiments. In our experiments, the percentage of SSEA1+ cells for a given stem cell line ranged from 30 to 75% of the total number of cells. This variability was dependent on the retinal origin of the cell line and culture conditions during passage.

With our STEM-RET experiments, we observed a range of spheres (19–53%) that scored “A” or “B” on day 7. On day 10, 14–91% of the spheres were pigmented, this was dependent on the iPSC line but was consistent in multiple experiments. When comparing different retinal-derived iPSC lines and the Eb5 Rx–GFP line, we observe a range of scores (Table 3). A few cell lines we tested were determined pluripotent, however, they were not ideal for STEM-RET and did not develop retinal tissue. For stem cells that produced retina, the RDQ, RDEM, and RDIF scores were similar to that of normal mature mouse retina.

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Fig. 1. Diagram of reprogramming protocol using retinal pellet explants

Timeline to reprogram retinal neurons and establish induced pluripotent stem cell (iPSCs) lines (Steps 1–48). Arrowheads annotate the timeline where additional procedural steps occur. Fluorescence-activated cell sorting (FACS), irradiated mouse embryonic fibroblast (ir-MEFs).

Fig. 2. STEM-RET differentiation protocol with representative retinal spheres

A) Diagram of the STEM-RET differentiation protocol with corresponding developmental time points, eye field specification (days 0–7), optic cup formation (days 7–10), and retinal differentiation (days 14–28). Arrowheads indicate additional manipulation or media exchanges. Retinal differentiation media (RD), maturation media 1 (MM1), maturation media 2 (MM2), retinoic acid (RA), taurine (T). **B)** Representative micrographs of spheres during eye field specification from day 1 to day 7. Black arrows indicate regions of early eye field formation. Scale bar = 200 μ m. **C)** Representative micrographs of spheres scored on day 7. Black arrows indicate regions of eye field formation. See step 92 for scoring criteria for spheres A, B, C, and X. These data are used for the eye field formation calculations in step 105A. Scale bar = 200 μ m. **D)** Representative micrograph of pigmented and non-pigmented spheres at day 10. Dash lines indicate cuts to excise retinal tissue. Arrow indicates an optic cup with an invagination. Scale bars = 1 mm

Fig. 3. Preparation of a retinal pellet explant

A) Photograph of an Eppendorf tube with dissociated retinal cells (step 8) **B–C)**

Photographs showing placement of a pipet tip to dislodge a retinal pellet after centrifugation

(step 19). **D)** Photograph of a retinal pellet in a P1000 pipette (step 20). **E)** Photograph of a

retinal pellet transferred to a Whatman membrane. **F)** Photograph of a retinal pellet explant

on a Whatman membrane.

Fig. 4. Representative micrographs of retinal features used in RDEM scoring

See Table 3 for detailed descriptions of each feature. **A)** Montage of electron micrographs from an adult wild-type retina with outer segments (OS), inner segments (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), horizontal cells (blue), Müller glia cells (pink), ganglion cells (green), and inner limiting membrane (purple) located at the basal surface. Scale bar = 25 μ m. **B)** The junction between rods and Müller cells (pink) forming the outer limiting membrane (white arrows) with mitochondria (mito) and inner segments (IS). Scale bar = 1 μ m. **C)** The distal terminals of rods with mitochondrion (mito) featuring an invagination between rod, bipolar (yellow), and horizontal cells (blue) cells. White arrows indicate ribbon synapses with numerous synaptic vesicles (encircled in white circle). Scale bar = 500 nm. **D)** Horizontal cell (blue) with process (arrow). Scale bar = 10 μ m. **E)** Example of dense core vesicles (arrows). Scale bar = 500 nm. **F)** Ganglion cells (green) and Müller cell foot plates (purple)

that form the inner limiting membrane. Scale bar = 2 μm . !CAUTION Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children's Research Hospital's Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

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Fig. 5. Representative micrographs of immunofluorescence used in RDIF scoring

Cross section of adult mouse retina (left) and day-28 retinal tissue derived from EB5 Rx – GFP stem cells (right). Nuclei in the outer nuclear layer (ONL) and inner nuclear layer (INL) are counter stained with DAPI (blue). Antibodies are scored 1.0 (>5 positive cells/section), 0.5 (<5 positive cells/section), or 0 (absent). Scale bars = 10 μ m. **A)** Rhodopsin (red); rod photoreceptors (arrows). **B)** Recoverin (red); photoreceptors (arrows). **C)** Pax6 (red); amacrine neurons (arrows) in mature retinæ. **D)** PKC- α (red); bipolar neurons (arrows). **E)** Cone arrestin (red); cone photoreceptors (arrows). **F)** Glutamine synthetase (red); Müller glial cells (arrows). **G)** Calbindin (red); horizontal neurons (arrows). **H)** Chx10 (red); bipolar neurons (arrows) in mature retinæ. **I)** Syntaxin (red); amacrine neurons (arrows). !CAUTION Any experiments involving live mice must conform to relevant Institutional and National regulations. Our animal protocols were approved by the St. Jude Children’s Research Hospital’s Institutional Animal Care and Use Committee, which is

accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

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Table 1**Antibodies**

Retina-specific antibodies in **bold** are used on retinal sphere sections (section 104C). The number of positively stained cells is used to calculate RDIF (see step 105Civ for scoring details). cMyc, Nanog, Oct3/4 and SSEA1 antibodies are used for stem cell characterization (section 81D). Synaptophysin, cytokeratin-OSCAR, and desmin antibodies are used for assessing teratoma formation (section 81E). Secondary antibodies are in *italics*. The PE-conjugated SSEA1 and isotype antibodies are used for FAC sorting for SSEA1 (step 73).

Antibody	Host	Supplier	Cat. no.	Dilution
Calbindin-D- 28K	Mouse	Sigma-Aldrich	C9848	1:100
Syntaxin	Mouse	Sigma-Aldrich	S0664	1:500
PKC-α	Mouse	Millipore	05-154	1:5000
Cone arrestin	Rabbit	Millipore	AB15282	1:5000
Glutamine synthetase	Mouse	BD Biosciences	610518	1:100
Pax6	Mouse	Developmental Studies Hybridoma Bank	Pax6	1:100
Chx10	Sheep	Exalpha Biologicals	X1180P	1:500
Recoverin	Rabbit	Millipore	AB5585	1:5000
Rhodopsin	Mouse	Gift from Dr. Robert Molday	-	1:100
cMyc	Mouse	Santa Cruz	SC-40	1:200
Nanog	Rabbit	Repro Cell	RCAB0002P-F	1:500
Oct3/4	Mouse	BD Biosciences	611202	1:500
SSEA1	Mouse	Millipore	MAB4301	1:500
Synaptophysin	Rabbit	Spring Bioscience	E2172	1:400
Cytokeratin- OSCAR	Mouse	BioLegend	908203	1:100
Desmin	Rabbit	Thermo Scientific	RB-9014	1:500
<i>Anti-GFP</i>	Chicken	Abcam	AB13970	1:500
<i>Biotinylated anti-chicken</i>	Goat	Vector	BA-9010	1:1000
<i>Biotinylated anti-mouse</i>	Donkey	Vector	BA-2000	1:500
<i>Biotinylated anti-rabbit</i>	Goat	Vector	BA-1000	1:500
<i>Biotinylated anti-sheep</i>	Rabbit	Vector	BA-6000	1:500
SSEA1, PE- conjugated*	Mouse	BD Biosciences	560142	1:175
IgG PE isotype*	Rat	BD Biosciences	562309	1:1500

Table 2**TaqMan Custom Array Genes**

The stem cell characterization genes (left) are used to assess the pluripotency of the stem cell lines (section 81F). Data should be normalized to *Gpi1*, *Gapdh*, or *18S* and scored relative to Eb5 Rx–GFP or another embryonic stem cell line. The retinal differentiation genes (right) are used to assess the degree of retinal maturation in STEM-RET (step 104Aiii). Retina-specific TaqMan probes in **bold** are used to perform qPCR analysis on the retinal spheres and normalized to *Gpi1*, *Gapdh*, or *18S* gene expression. The relative fold expression of the **bold** genes is used to calculate the RDQ (step 105Cii).

Stem cell characterization	
Gene	Accession no.
Meg3	Mm00522599_m1
Nanog	Mm02384862_g1
Pou5f1	Mm03053917_g1
Sox2	Mm03053810_s1
Utf1	Mm00447703_g1
Myc	Mm00487804_m1
Cd9	Mm01182922_g1
Gapdh	Mm03302249_g1
18S	Hs99999901_s1
Gpi1	Mm04213227_s1

Retinal differentiation	
Gene	Accession no.
Rho	Mm01184405_m1
Rcvrn	Mm00501325_m1
Opn1sw	Mm00432058_m1
Lhx9	Mm00495308_m1
Pou4f2	Mm00454754_s1
Gpi1	Mm04213227_s1
Stx1a	Mm00444008_m1
Calb1	Mm00486647_m1
18S	Hs99999901_s1
Prkca	Mm00440858_m1
Nrl	Mm00476550_m1
Crx	Mm00483994_m1
Arr3	Mm00504636_g1
Glul	Mm00725701_s1
Rlbp1	Mm00445129_m1
Nefl	Mm01315667_gH
Tfap2b	Mm00493468_m1
Grm6	Mm00841148_m1

Retinal differentiation	
Gene	Accession no.
Gapdh	Mm03302249_g1

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Table 3

Electron microscopy criteria

Retinal layer/cell type	Mark	Location in Fig. 4	Description
Rods	Presence of outer segments	Fig. 4A- OS	Stacked membrane discs contained within rod outer segment plasma membrane
Rods	Presence of inner segments	Fig. 4A,B- IS, mitochondria (mito)	Contain mitochondria and connecting cilium
Outer limiting membrane	Presence of linear array of cell-cell junctions	Fig. 4B- White arrows pointing to dark junctions	Adherens junctions between photoreceptors and Müller cell plasma membranes
Outer limiting membrane	Presence of Müller cell processes	Fig. 4B- Müller cell projections (pink) around a rod (grey)	Distal projections of Müller cells adjacent to inner segments of photoreceptors
Rods	Presence of condensed chromatin	Fig. 4A- Dark nuclei in the outer nuclear layer (ONL).	Dense chromatin within rod nuclei
Rods	Presence of ribbon synapses	Fig. 4C- White arrows pointing to grey ribbon synapses. Horizontal (blue) and bipolar (yellow) cells	Presynaptic ribbons within rod terminals contacting horizontal and bipolar cells
Rods	Presence of synaptic vesicles	Fig. 4C- Numerous synaptic vesicles, two shown in the white circle.	High concentrations of small, round uniform vesicles within photoreceptor terminals
Rods	Presence of synaptic mitochondrion	Fig. 4C- Single, large mitochondrion	Single prominent mitochondrion with circular profile
Rods	Presence of synaptic invaginations	Fig. 4C- Invagination of rod terminal (grey) by bipolar (yellow) and horizontal (blue) cells	Postsynaptic triad formation of horizontal cell neurites and bipolar cell dendrites within photoreceptor terminals
Outer plexiform layer	Presence of outer plexiform layer structure	Fig. 4A- OPL	Consists of photoreceptor terminals and their connections with bipolar and horizontal cells
Horizontal cells	Large cytoplasm-to-nucleus ratio.	Fig. 4A,D- Horizontal cells (blue)	Large, pale-staining nucleus surrounded by substantial cytoplasm
Horizontal cells	Presence of horizontal processes	Fig. 4D- Horizontal cell (blue) with horizontal process (white arrow)	Broad, tapering processes originating from horizontal cell body and projecting horizontally within outer plexiform layer
Horizontal/amacrine/bipolar/and Müller cells	Cell bodies form an inner nuclear layer	Fig. 4A- INL; Horizontal cell (blue), amacrine/bipolar cells (grey), and Müller cells (pink)	Presence of horizontal, amacrine/ bipolar, and Müller cell bodies within discrete layer
Amacrine/bipolar cells	Presence of dense core vesicles	Fig. 4E- Two dense core vesicles (white arrows)	Found in dopamine/ catecholamine synapses between amacrine and bipolar cells
Ganglion cells	Cell body size similar to mature murine ganglion cells	Fig. 4A,F- Ganglion cells (green)	Characteristically large soma with limited cytoplasm
Ganglion cells	Cell bodies localized to basal region	Fig. 4A,F- Ganglion cells (green)	Located between the inner plexiform layer and inner limiting membrane
Inner limiting membrane	Presence of uniform inner surface created by tightly packed cell processes.	Fig. 4A,F- Müller cell foot plates (purple) forming the inner limiting membrane	Consists of contiguous Müller cell foot plates projecting distally from Müller cell bodies

Table 4
Range in STEM-RET scores for r-iPSCs and Eb5 Rx-GFP

Variability in retinal differentiation efficiency observed in rod-derived iPSC lines and the ESC line EB5 Rx-GFP⁸. Eye field scores were calculated using step 105A, and optic cup scores were calculated using step 105B. A score of 1.00 indicates the highest degree of efficiently to produce retinal tissue at each stage. The lower the score, the less efficient the cell line is at producing retinal tissue.

	Retinal-derived iPSCs	Eb5 Rx-GFP
Eye field induction efficiency (EFE)	0.04–0.77	0.60–0.96
Eye field induction specificity (EFS)	0.0–0.19	0.0–0.49
Eye field proliferation (EFP)	0.46–1.0	1.0
Optic cup efficiency (OCE)	0.15–0.95	0.0 (albino)
Optic cup frequency (OCF)	0.09–0.93	0.23–0.75

Table 1**TROUBLESHOOTING**

Step(s)	Problem	Possible reason(s)	Solution(s)
17	Not enough cells for a complete dilution series.	Low cell population at developmental stage.	Combine the retinæ or exclude the higher concentrations of dilution series.
20	Pellet breaks apart.	This technique is challenging and requires practice.	With a P1000, triturate 2 or 3 times to disperse pellet and repeat the centrifugation (step 19). Note: This should only be done twice to reduce cell death
27	Low number of cells recovered.	Inefficient reprogramming. Cell died during reprogramming. Incomplete recovery of explant pellet.	Increase the concentration of reprogrammable neurons in the explants. Minimize the number of centrifugations in step 19. Feed explants daily and change medium on days 4 and 8. Immerse the membrane in medium and remove the explant. Centrifuge the cells at 250g, aspirate the supernatant, and resuspend the cells in PBS-/-.
32	No colonies formed.	Inefficient reprogramming. Cell numbers too low.	Repeat the experiment with higher concentrations of cells in step 17.
42	Low number of cells.	Slow-growing Too few cells plated	Allow for an additional day of growth or seed cells at a higher concentration.
47	Colonies look unhealthy.	Over digestion with trypsin. ESC maintenance medium is expired. Cells not attended to every 24 h.	Observe cells under the microscope. Stop the enzyme reaction when colonies begin to dislodge. Check the medium components for expiration and make fresh medium. Feed and split cells every 24 h.
47	Large colonies after passage.	Under digestion with trypsin.	When passaging cells, record the amount of time in trypsin and triturate with a P1000 2 or 3 times to create a single-cell suspension.
57	Colonies break apart.	Over digestion with ESC dissociation solution.	Shorten the exposure to digest solution. Observe the reaction under microscope. Colonies should dislodge from plate but not break apart. Stop the reaction by adding ESC maintenance media (step 42).
73	Low number of cells/too many MEFs.	Preplating time too long/short.	Extend or shorten preplating time as needed. Time may vary between cell lines.
76	Poor yield of SSEA1+ cells.	Cell differentiating while culturing.	Check medium components for expiration and make fresh medium. Monitor cultures for differentiation. Use gelatinized plates with ir-MEF feeder layer.
81 A(xi)	Abnormal chromosomes.	Stem cells are known to develop chromosomal abnormalities.	Run the STEM- RET protocol but monitor the cells closely. Most common chromosomal abnormalities (+8, +11, +14) do not affect STEM-RET.
81 B(v)	Cell line is not DOX independent.	Incomplete reprogramming.	Discard the cell line.
81 C(vii)	Colonies do not stain positive	Cells were over-fixed Kits reagents have degraded	It is critical to limit fixation to 1 minute Kit reagents are light sensitive – Order new kit reagents
91	Poor lamination.	Matrigel concentration or cell line not ideal for STEM-RET.	Try repeating the experiment with a more appropriate concentration of matrigel.
91	Aggregates fail to form.	Severe problems with morphology, differentiation, or chromosomal abnormalities.	The cell line is not ideal for STEM- RET.
93	Few or no “A” or “B” spheres.	Matrigel concentration or cell line not ideal for STEM-RET.	Adjust the matrigel concentration. The cell line is not ideal for STEM- RET.

Step(s)	Problem	Possible reason(s)	Solution(s)
99	Poor to no pinches.	Incorrect scoring at day 7.	Reevaluate the scoring criteria at day 7 for “A” and “B” spheres.