Supplemental Methods

Pluripotent stem cell culture and differentiation

All work performed had approval from the Children's Hospital Los Angeles (CHLA) Stem Cell Research Oversight Committee and/or the CHLA Institutional Review Board. WA09 (WiCell Research Institute, Madison, WI) hESCs were maintained on mouse embryonic fibroblasts in DMEM/F12, 20% KSR, 2 mM glutamine, 100 μM NEAA, 100 μM BME, and 4 ng/ml bFGF and passaged manually following collagenase treatment every four or five days. Induced pluripotent stem cell lines (iPSCs) were produced from peripheral blood mononuclear cells at Cellular Dynamics International (CDI, Madison, WI) using a proprietary seven factor episomal methodology based on Yu et al.[1] All exhibited normal karyotypes and expressed standard pluripotency markers. iPSC lines were maintained in either Essential 8 media (Invitrogen-ThermoFisher Scientific) with a vitronectin (VTN-N, Invitrogen-ThermoFisher Scientific) substrate or TeSRE8 media (Stem Cell Technologies, Cambridge, MA) with a Vitronectin XF (Stem Cell Technologies) substrate and passaged every 5-7 days with EDTA or ReLeSR (Stem Cell Technologies). Multiple iPSC lines were used. For FLIM and IF: C229.1 (DIC205), C232.2 (DIC121), C229.3 (DIC 100) and 348.3 (DIC31) and for OCT: C229.3 (DIC 169) and C275.4 (DIC 92). All lines were derived from normal children except 348.3 which was derived from a child diagnosed with optic nerve hypoplasia.

For production of retinal organoids, WA09 cells were differentiated to neural retina (not optic cup) in three dimensional culture as described[2] except hESC colonies were dissociated with Accutase (Thermo Fisher Scientific) and reaggregated in Lipidure-coated U well 96-well plates (Nof America) using 5000 cells per well in 30 μl Aggrewell media (Stem Cell Technologies) + 10 μM Y27632 (Tocris). Media was changed to retinal differentiation media[2] on day 1 and Growth Factor Reduced Matrigel (Corning) plus 3 μM IWR1 (Cayman Chemical, Ann Arbor, MI) were added on day 2. Matrigel was added only on day 2 to 0.5%; the concentration varied by lot, but this was generally about 50 μg/ml. Tissue was incubated at 37°C, 5% CO_2, 20% oxygen until use. Usually the entire aggregate formed retinal tissue with the apical surface on the exterior, so the excision step described[2] was generally unnecessary. Independent HESC differentiation runs were used for each of the days in culture represented. iPSC lines were differentiated according to a published method[3] with the following modifications: aggregates of

12000 cells were formed on day 0 in Aggrewell media (Stem Cell Technologies) containing ISL (3 uM IWR1, SB431542 (Cayman Chemical), LDN193189 (SIGMA)) instead of gfCDM plus knockout serum replacement (KSR); the media was changed to gfCDM/KSR containing ISL on day 1. On day 6, in conjunction with the 1.5 nM BMP (R and D Systems, Minneapolis, MN) described[3], 3 uM IWR1 was added. No induction reversal step was performed on days 18-24 and instead organoids were maintained in "NR –differentiation medium" lacking retinoic acid and taurine during this period. Supplementation with 0.5 uM retinoic acid (Cayman Chemical) and 0.1 mM taurine (SIGMA) began on day 30.

Immunofluorescent staining

Retinal organoids were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at room temperature (RT) for 12-15 min, cryoprotected in 30% sucrose in PBS overnight at 4°C, and embedded in OCT (Tissue-Tek). Cryosections (10 μm) were placed on positively charged slides. Blocks and slides were stored at -80°C. Slides were heated at 37°C for 15 min prior to staining, post fixed 5 min in 4% PFA, and rinsed 3 x 5 min in TBS + 0.05% Triton X-100 (TBST). Tissue sections were blocked for 1 hr with blocking buffer (5% normal donkey serum and 3% bovine serum albumin (BSA) in TBS with 0.2% Triton X-100) at RT. Sections were incubated with primary antibodies diluted in blocking buffer overnight at 4°C, washed 3 X 5 min in TBST, incubated with secondary antibodies diluted in 3% BSA in TBS with 0.2% Triton X-100 for 1 hr at RT in the dark, washed 3 X 5 min in TBST with DAPI (1:1000) included in the last wash, and cover-slipped using ProLong Diamond (Invitrogen-Thermo Fisher). Primary antibodies (goat anti-VSX2 (CHX10), rabbit anti-CRX, and goat anti-BRN3 (Santa Cruz Biotechnology, Santa Cruz, CA sc-21690, sc-30150, and sc-6026), mouse anti-PAX6 (Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-Ki67(BD Biosciences, San Jose, CA), and rabbit anti-Cleaved Caspase 3 (Cell Signaling Technology, Danvers, MA) were visualized using Alexa Fluor 488- or 568-conjugated secondary antibodies. Fluorescence images were acquired with the Nikon Eclipse Ti microscope at 20X magnification.

Hyperspectral Imaging

Spectral Images were acquired with a Zeiss LSM-780 inverted microscope, using a 32 channel Zeiss QUASAR detector, coupled to a Ti:Sapphire laser system (Coherent Chameleon Ultra II),

using a 32× 0.85 numerical aperture water immersion objective (Zeiss Korr C-Apochromat) optimized for 2-photon imaging. Organoids were excited at 740 nm with an average power of 10 mW. The emitted fluorescence signal was dispersed by a diffraction grating, and multiple emission bands, each with approximately 9 nm bandwidth, were collected by the array of 32 detectors through de-scanned (confocal) detection. The emission bandwidth was from 420 nm to 690 nm.

Fluorescence Lifetime Imaging

Fluorescence lifetime images were acquired with a Zeiss LSM-780 inverted microscope coupled to a Ti:Sapphire laser system (Coherent Chameleon Ultra II) and an A320 FastFLIM FLIMbox (ISS, Champaign, IL)[4], using a 32× 0.85 NA water immersion objective optimized for 2-photon imaging. For image acquisition, the following settings were used: image size of 256×256 pixels and scan speed of 12.6 μs/pixel. A dichroic filter (690 nm) was used to separate the fluorescence signal from the laser light. Fluorescence was detected by a hybrid photomultiplier tube (Hamamatsu R10467U-40) with a 460/80 nm band-pass filter in front of the detector. FLIM data were acquired by the VistaVision software from ISS (Champaign, IL), and processed by SimFCS software (Laboratory for Fluorescence Dynamics, Irvine, CA). The excitation wavelength was 740 nm, and the average excitation power was ~ 5 mW. FLIM calibration was performed by measuring the known lifetime of coumarin 6 with a single exponential of 2.55 ns.[5]

Additional References

1. Yu J, Chau KF, Vodyanik MA, Jiang J, Jiang Y. Efficient feeder-free episomal reprogramming with small molecules. *PLoS One* 2011;6:e17557.
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3. Kuwahara A, Ozone C, Nakano T, Saito K, Eiraku M, Sasai Y. Generation of a ciliary margin-like stem cell niche from self-organizing human retinal tissue. *Nature communications* 2015;6:6286.
4. Arnesano C, Santoro Y, Gratton E. Digital parallel frequency-domain spectroscopy for tissue imaging. *J Biomed Opt* 2012;17:96014-96011.
5. Sun Y, Day RN, Periasamy A. Investigating protein-protein interactions in living cells using fluorescence lifetime imaging microscopy. *Nat Protoc* 2011;6:1324-1340.