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Supplemental Information

Accelerated and Improved Differentiation of Retinal Organoids from Pluripotent Stem Cells in Rotating-Wall Vessel Bioreactors

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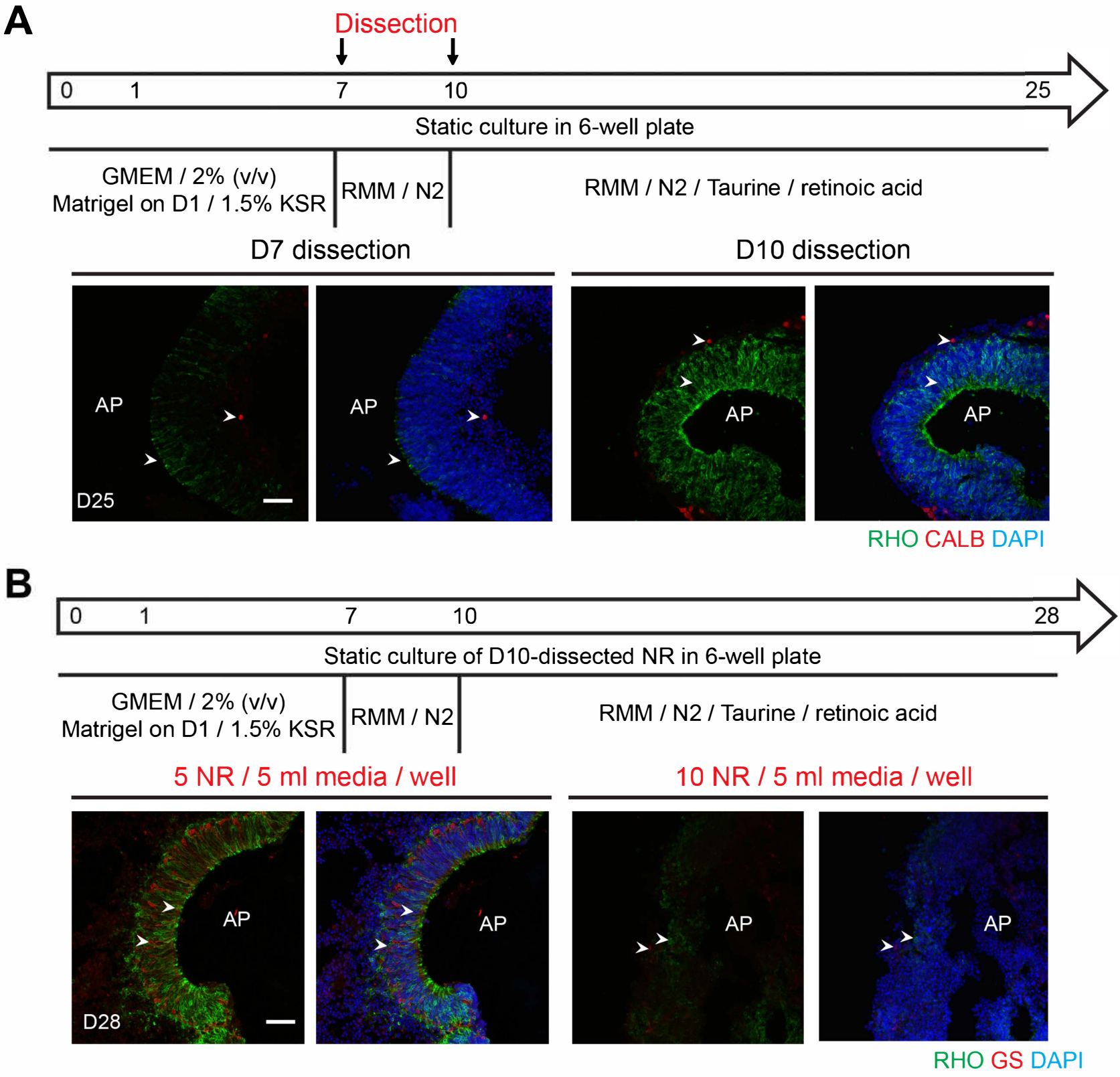


Figure S1 (related to Figure 1E)

Optimization of organoid culture conditions by: (A) optic cup dissection day and (B) neural retina (NR) seeding density. N umbers in arrows show the differentiation day. GMEM: Glasgow minimum essential medium; KSR: knockout serum replacement; RMM: retinal maturation medium constituted of DMEM/Fl2 with GlutaMAX, lx penicillin/streptomycin, lx 2-mercaptoethanol and lx N2 supplement; FBS: fetal bovine serum; N2: N2 supplement. Rhodopsin (RHO, green) is a marker for rod photoreceptors in (A) and (B). Calbindin (CALB, red) is a marker for horizontal and amacrine cells in (A). Glutamine synthetase (GS, red) is a marker for Muller glia in (B). All nuclei were stained with 4',6- diamidino-2-phenylindole (DAPI, blue). Representative figures are shown for each condition. Arrowheads indicate relevant imnunostaining with (A) and (B) RHO, (A) CALB and (B) GS. AP shows the apical side of the organoids. Scale bar: 50 µm.

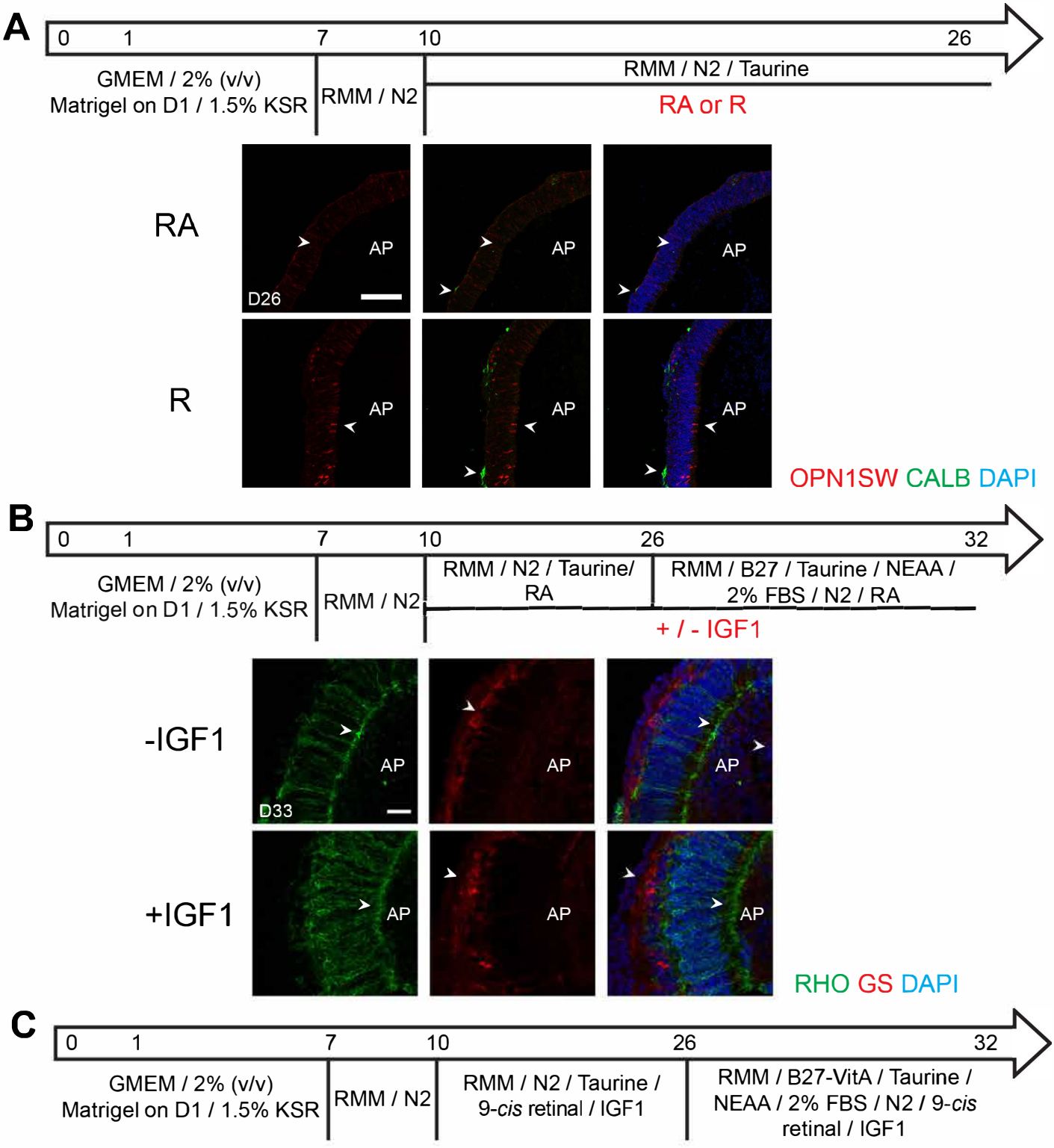
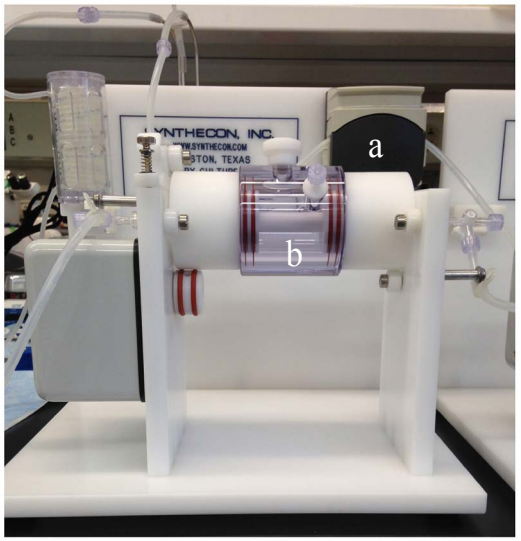


Figure S2 (related to Figure 1E)

Modification of the HIPRO protocol. (A) 9-*cis* Retinal (R) versus *all-trans* Retinoic Acid (RA). S-opsin (OPN1 SW, Red) and paired box protein 6 (PAX6, Green) are markers of S-cone photoreceptors and amacrine/ganglion cells, respectively. (B) Addition of insulin-like growth factor 1 (IGF1). Numbers in arrows show the differentiation day. GMEM: Glasgow minimum essential medium; KSR: knockout serum replacement; RMM: retinal maturation medium constituted of DMEM/F12 with GlutaMAX, 1x penicillin/streptomycin, 1x 2-mercaptoethanol and 1x N2 supplement; RA: *all-trans* retinoic acid; R: 9-*cis* retinal; NEAA: non-essential amino acids; FBS: fetal bovine serum; B27: B27 supplement; N2: N2 supplement; B27-VitA, B27 supplement without Vitamin A; IGF1 : insulin-like growth factor 1. Rhodopsin (RHO, green) and glutamine synthetase (GS, red) are markers for rod photoreceptors and Müller glia, respectively. All nuclei were stained with 4',6-diamid-ino-2-phenylindole (DAPI, blue). Representative figures are shown for each condition. Arrowheads indicate relevant immunostaining with (A) OPN1 SW, CALB, (B) RHO and GS. AP shows the apical side ofthe organoids. Scale bar: (A) 50 µm, and (B) 20 µm.

A



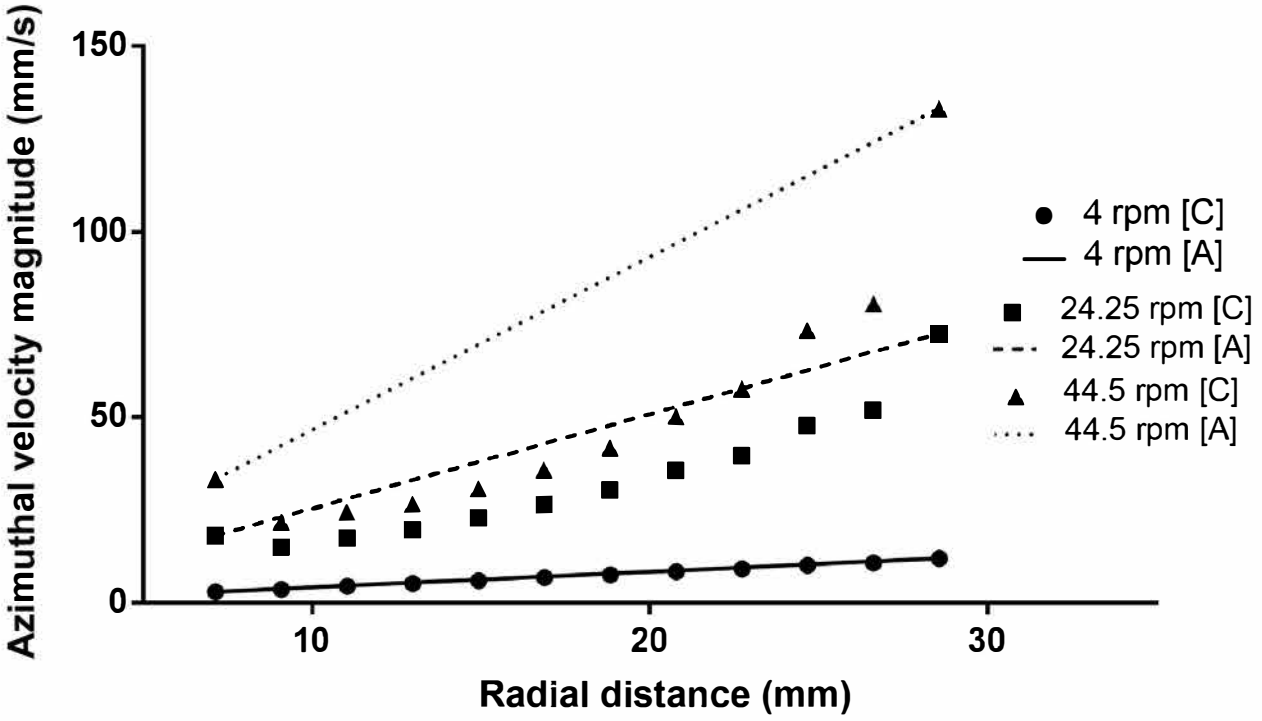
B

(1) $$V_t = \frac{2gr^2(\rho_{NR} - \rho_f)}{9\mu\rho_f}$$

(2) $$\tau_{max} = \frac{3\mu V_t}{2r}$$

Parameter	Description
V_t	Terminal velocity
τ_{max}	Maximum shear stress
g	Acceleration due to gravity
r	Radius of neural retina
ρ_{NR}	Density of neural retina
ρ_f	Density of cell culture medium
μ	Viscosity of cell culture medium

C



RWV Operating Speed (rpm)	Tangential Velocity (mm/s)	Reynolds Number
4.0	11.97	333.73
24.3	72.56	2023.11
44.5	133.16	3712.77

Figure S3 (related to Figure 1F-G)
Characterization of rotating-wall vessel bioreactor (RWV). (A) Commercially purchased RWV from Synthecon with (a) perfusion pump and (b) perfusion vessle. (B) Governing RWV equations regarding: (1) Analytical solution of the NR terminal velocity as a function of NR density and radius. (2) Analytical solution of maximum shear stress applied to NR by cell culture medium as a function of NR terminal velocity and radius. (C) RWV analytical flow profile [A] and CFD simulations [C] across minimum to maximum rotation speeds. Accompanying tangential (azimuthal) velocities and Reynolds Numbers are reported at the largest radial distance in the RWV and correspond to maximum values for all rotation speeds. For experimentation, rotational speeds were chosen under 24.3 rpm to remain below the transition to turbulent flow. Typically the organoids were maintained at radial distances between 10 to 25 mm from the center of the perfusion core during NR development.

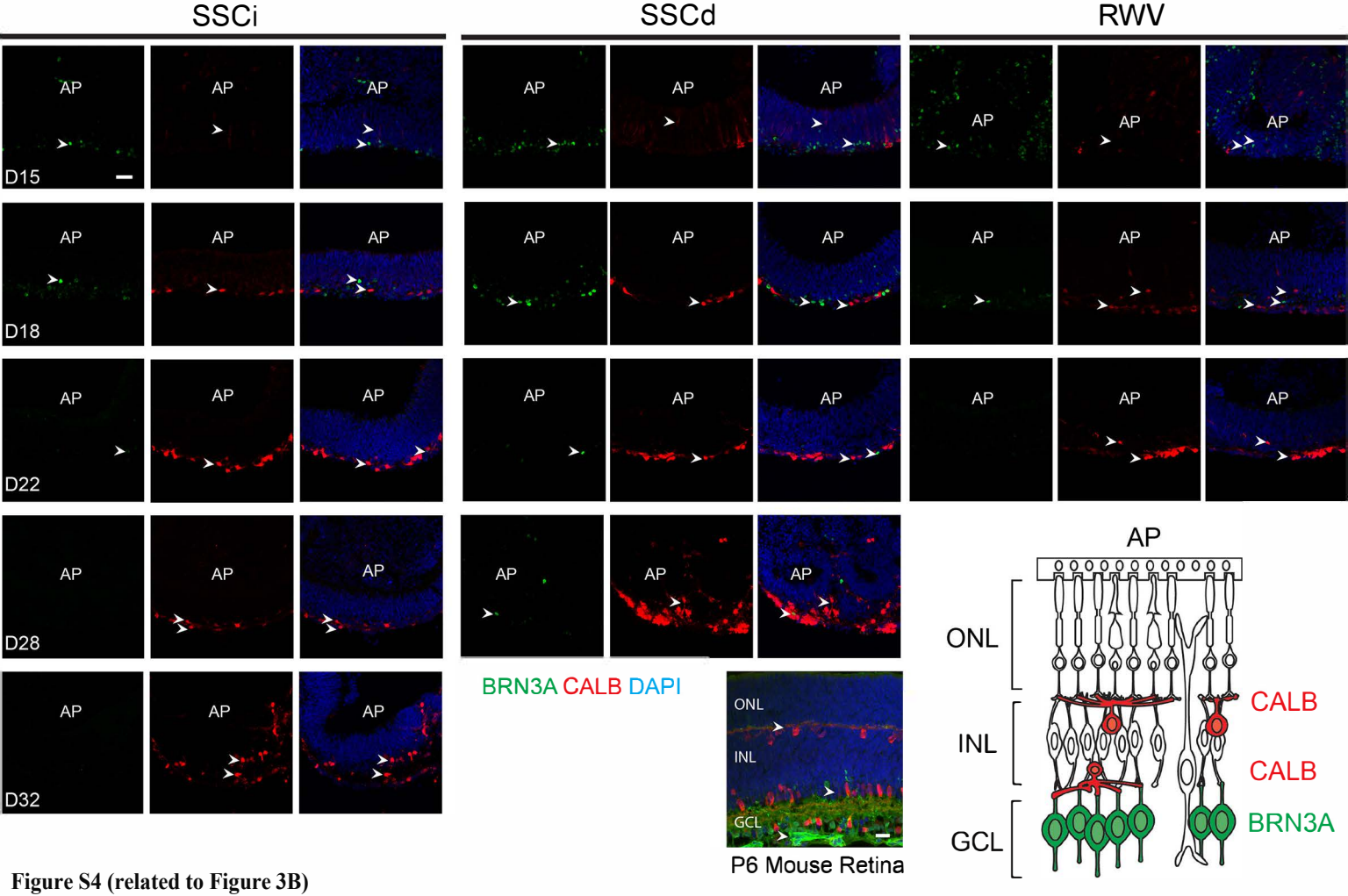


Figure S4 (related to Figure 3B)

Genesis of early-born cell types. Brain-specific homeobox/POU domain protein 3a (BRN3A, Green) and Calbindin (CALB, red) are markers of ganglion cells and horizontal/amacrine cells, respectively. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Representative figures are shown. Arrowheads indicate relevant immunostaining with BRN3A and CALB. AP shows the apical side of the organoids. Scale bar: 50 μm (organoids), and 20 μm (mouse retina).

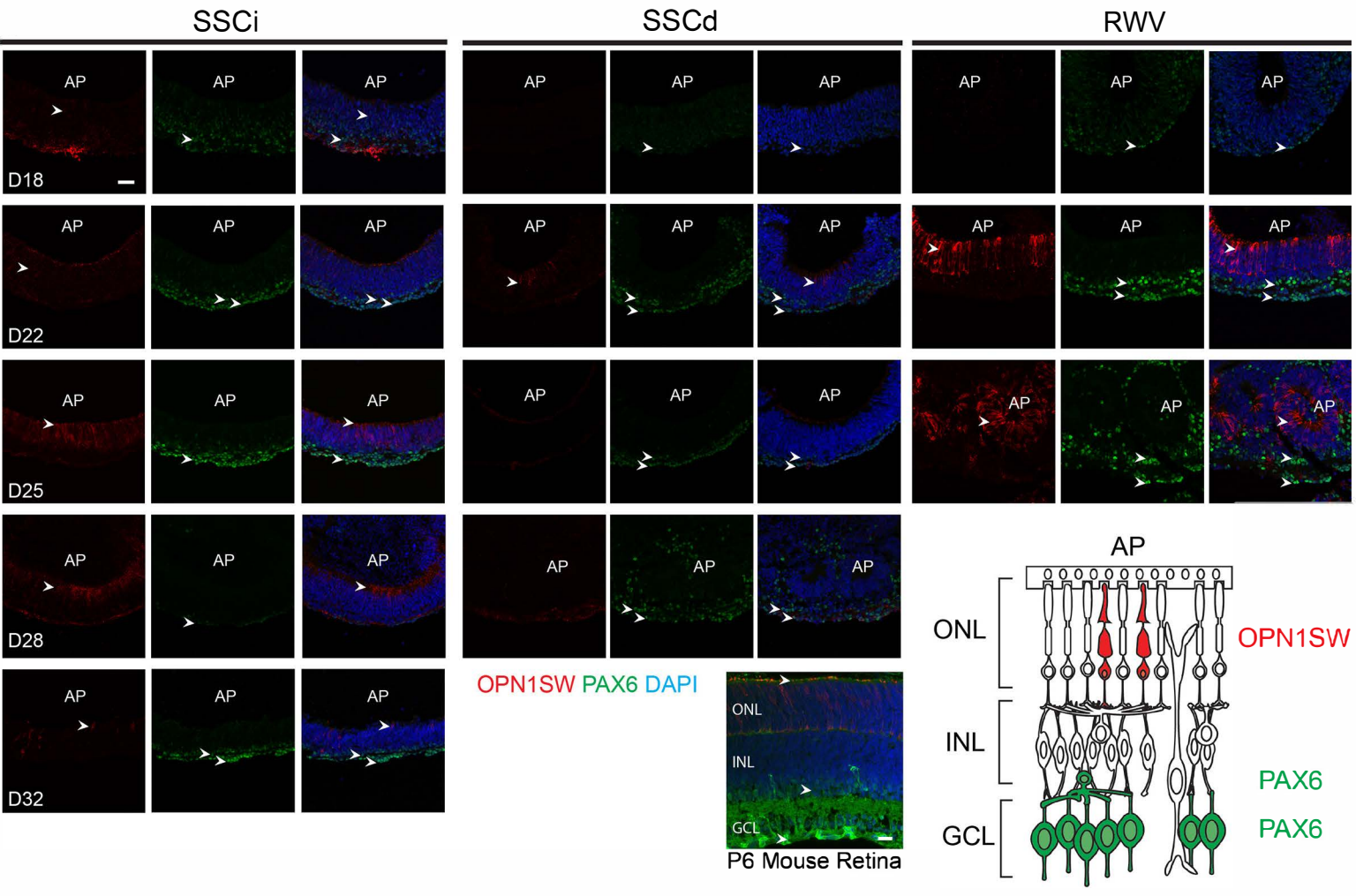


Figure S5 (related to Figure 3C)

Development of cone photoreceptors and amacrine/ganglion cells, followed by the formation of plexiform layers. S-opsin (OPN1SW, Red) and paired box protein 6 (PAX6, Green) are markers of S-cone photoreceptors and amacrine/ganglion cells, respectively. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Representative figures are shown. Arrowheads indicate relevant immunostaining with OPNISW and PAX6. AP shows the apical side of the organoids. Scale bar = 50 μm (organoids), and 20 μm (mouse retina).

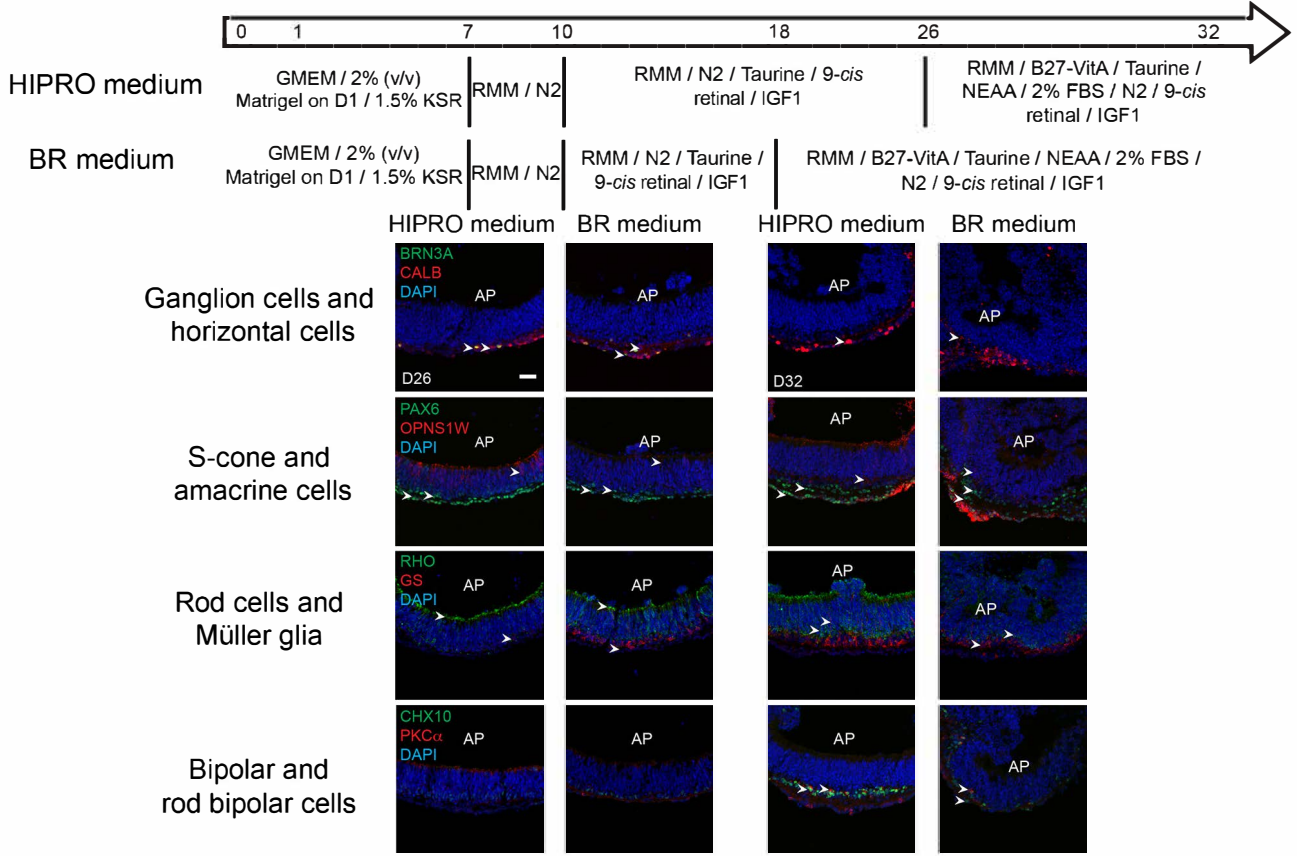


Figure S6 (related to Figure 3-5)

HIPRO medium versus bioreactor (BR) medium for intact static suspension culture (SSCi) organoids. Numbers in arrows show the differentiation day. GMEM: Glasgow minimum essential medium; KSR: knockout serum replacement; RMM: retinal maturation medium constituted of DMEM/F12 with GlutaMAX, 1x penicillin/streptomycin, 1x 2-mercaptoethanol and 1x N2 supplement; NEAA: non-essential amino acids; FBS: fetal bovine serum; N2: N2 supplement; B27-VitA: B27 supplement without Vitamin A; IGF 1: insulin-like growth factor 1. Brain-specific homeobox/POU domain protein 3a (BRN3A, Green), Calbindin (CALB, red), paired box protein 6 (PAX6, Green), S-opsin (OPNISW, Red), Rhodopsin (RHO, green), glutamine synthetase (GS, red), ceh-10 homeodomain-containing homolog 10 (CHXI0, green), and protein kinase C alpha (PKCα, red) are markers for ganglion, horizontal, S-cone photoreceptor, amacrine, rod photoreceptor, Müller glia, bipolar and rod bipolar cells, respectively. Nuclei were stained with 4' ,6-diamidino-2-phenylindole (DAPI, blue). Representative figures are shown. Arrowheads indicate relevant immunostaining with BRN3A, CALB, PAX6, RHO, GS, CHXI0 and PKCα. AP shows apical side of the organoids. Scale bar = 50 μm.

Table S2. Antibody information and dilutions (related to Supplemental Experimental Procedures)

Antibody	Host	Source	Cat. no.	Dilution
ADP-ribosylation factor-like protein 13B (ARL13B)	Rabbit	Abcam	ab83879	1:500
Bassoon	Rabbit	Cell Signaling	6897S	1:200
Brain-specific homeobox/POU domain protein 3A (BRN3A)	Mouse	Millipore	MAB1585	1:200
Calbindin (CALB)	Rabbit	Calbiochem	PC253L	1:1000
Ceh-10 Homeodomain-Containing Homolog (CHX10)	Sheep	Abcam	ab16141	1:200
Glutamine synthetase (GS)	Rabbit	Abcam	ab49873	1:200
Laminin	Rabbit	Sigma-Aldrich	L9393	1:50
Opsin 1 short-wave-sensitive (OPN1SW)	Goat	Santa Cruz	sc-14363	1:200
Paired box protein 6 (PAX6)	Mouse	DSHB	PAX6-c	1:200
Phospho-histone H3 (PH3)	Rabbit	Cell signaling	9701L	1:200
Protein kinase C alpha (PKCα)	Rabbit	Sigma-Aldrich	P4334	1:1000
Rhodopsin (RHO)	Mouse	A gift from Dr. Robert Molday, University of British Columbia, Canada	---	1:500
γ-Tubulin	Mouse	Sigma-Aldrich	T6557	1:500
Orthodenticle homeobox 2 (OTX2)	Rabbit	Sigma-Aldrich	HPA000633	1:200
Synaptophysin	Mouse	Abcam	ab8049	1:200

Supplemental Experimental Procedures

Generation and maintenance of mouse embryonic stem cells

Mouse wild-type (WT) embryonic stem cells (ESCs) were isolated from the inner cell mass (ICM) of 3.5-day blastocyst of *Nrl*-GFP transgenic mice (Akimoto et al., 2006). A second WT ESC clone (R1) (Nagy et al., 1993) was obtained from A. Nagy, Lunenfeld-Tanenbaum Research Institute of Mount Sinai Hospital, Toronto, Canada. ESCs were maintained on feeders of mouse embryonic fibroblasts in the presence of leukemia inhibitory factor (LIF) (Millipore) at 37℃, 5% CO_2 (Ying et al., 2008). ESC clones were characterized by morphology, proliferation rate and expression of pluripotency markers (Chen, 2016). Maintenance medium of ESC culture consists of Knockout DMEM (Life Technologies) supplemented with 1x MEM non-essential amino acids (NEAA) (Sigma), 1x GlutaMAX (Life Technologies), 1x Penicillin-Streptomycin (PS) (Life Technologies), 1x 2-Mercaptoethanol (2-ME) (Life Technologies), 2000 U/ml LIF (Millipore), and 15% ES cell-qualified fetal bovine serum (FBS) (Life Technologies). Cells were passaged using TrypLE Express (Life Technologies) every three days.

Differentiation of ESCs into retinal organoids

ESCs were differentiated into retinal organoids using modified HIPRO protocol, as described (Chen, 2016). After dissociation and removal of feeder cells, 3000 ESCs were plated in each well of PrimeSurface low adhesion U-shaped 96-well plate (Wako) with retinal differentiation medium constituted by Glasgow minimum essential medium (Life Technologies), 1x NEAA (Sigma), 1x sodium pyruvate (Sigma), 1x 2-ME (Life Technologies) and 1.5%(v/v) knockout serum replacement (Life Technologies). At differentiation day (D)1, 120 ul >9.5 mg/ml Matrigel (Corning) was diluted in 900 ul retinal differentiation medium. 20 ul of the diluted Matrigel was added to each well. Retinal organoids were transferred at D7 to a 100 mm Poly(2-hydroxyethyl methacrylate) (Sigma)-coated petri dish with 10 ml retinal maturation medium (DMEM/F12 with GlutaMAX (Life Technologies) with 1x 2-ME (Life Technologies) and 1x PS (Life Technologies). At D10, optic cups of retinal organoids were dissected from intact retinal organoids using a moria nickel-plated pin holder (Fine Science Tools) with 0.25 mm-diameter tungsten needles (Fine Science Tools). Dissected NR in static suspension culture (SSCd) or rotating-wall vessel bioreactors (RWB) were supplied with DMEM/F12 with GlutaMAX (Life Technologies), 1x 2-ME (Life Technologies), 1x PS (Life Technologies), 1x N2 supplement (Life Technologies), 1 mM taurine (Sigma), 500 nM 9-*cis* retinal (Sigma) and 100 ng/ml insulin-like growth factor 1 (IGF1) (Life Technologies). From D18 and onwards, 1x NEAA (Sigma), 1x B27 without Vitamin A (Life Technologies) and 2%(v/v) FBS (Atlanta Biologicals) were added to the culture. Intact organoids in static suspension culture (SSCi) were maintained in the same media, except 1x MEM non-essential amino acid (Sigma), 1x B27 without Vitamin A (Life Technologies) and 2% (v/v) FBS (Atlanta Biologicals) were added from D26 and onwards. Half-media exchanges were carried out every two days for all three conditions. The cultures were incubated in 5% O_2 from D0 to D10 and with 20% O_2 from D10 onwards.

Immunohistochemistry (IHC)

Organoids were fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Science) for 1 hour and cryo-protected in 15% (v/v) sucrose (Sigma) for at least 2 hours, followed by 30% (v/v) sucrose (Sigma) overnight, before embedding in OCT compound (Sakura Finetek). OCT blocks were sectioned at 10 μm thickness and incubated under vacuum at room temperature for at least 30 minutes, before immunostaining or storage at -80℃. Sections were hydrated in Tris-buffered Saline (TBS) (Quality Biological) for 10 minutes and incubated in blocking solution (10% (v/v) normal donkey serum and 1% (w/v) bovine serum albumin (BSA) (Sigma)) for at least 1 hour at room temperature. After incubation with primary antibody, which was diluted in blocking solution (1xTBS, 10% donkey serum and 1% BSA) overnight at 4°C, slides were washed three times for 10 minutes each with 0.05% (v/v) Triton X-100 (Sigma) in TBS, followed by 5-minute wash with TBS. Slides were then stained with appropriate secondary antibodies at room temperature for 1.5 hours. After three 10-minute washes, DAPI (4',6-diamidino-2-phenylindole)(Life Technologies) was added for 5 minutes, followed by three washes, 5 minutes each, in TBS. For comparison of intensity, images in the same section were captured using identical gain and voltage. Fluorescence images were captured using LSM 880 confocal microscope (Zeiss). Information and dilutions of antibodies are summarized in Table S2.

Transcriptome analysis

Gene level quantification of RNA-seq data from *in vivo* mouse retina (Brooks, et al., manuscript in preparation) and *in vitro* organoids was performed as described (Chen, 2016) with Ensembl data release 88 (Aken et al., 2017). The gene level count-matrix was then TMM normalized using the edgeR (3.32.2) (McCarthy et al., 2012; Robinson et al., 2010) package in the R (v3.4.0) (R Development Core Team, 2017) programming environment.

Differential expression (DE) analysis of the organoid data was performed for each time-wise culturing condition using limma (3.32.2) (Ritchie et al., 2015) with voom (Law et al., 2014) and eBayes functions. Genes having a mean of 10 counts-per-million (CPM) expression in at least one group were used in the analysis and those showing Benjamini-Hochberg false discovery rate (FDR) of < 1% and minimum fold change of 5 were considered in significant DE category.

Gene expression clustering was performed on the significantly DE genes using Affinity propagation (AP) algorithm (Bodenhofer et al., 2011; Frey and Dueck, 2007) in a two-step process. The first step consisted of using negative distance as the similarity measure, followed by determination of the exemplars for the 27 clusters identified. In the second step, the exemplars from the first step are clustered using Pearson's correlation as the similarity measure and the 27 clusters were merged to obtain the final 5 super-clusters.

Gene ontology (GO) analysis was performed for each cluster using clusterProfiler (Yu et al., 2012) package. To reduce inherent redundancy associated with GO analysis, sematic similarity analysis was performed using Wang similarity (Wang et al., 2007; Yu et al., 2010) in the GOSemSim (3.32.2) package. Similarity matrices were then clustered using AP with q parameter of 0.85. The leaf term in the GO tree based on GO.db (Carlson, 2017) was selected as the representative.

Statistical Analysis

At least four independent experiments were performed. All data were expressed as mean ± standard error of the mean (S.E.M.), unless specified otherwise. One-way ANOVA was used to compare NR size for the three culture conditions at specific time points and Tukey's test was used as Posthoc test. $p < 0.05$ was considered significant. All statistical analyses were carried out using R programming (R Development Core Team, 2017).

Experimental References

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