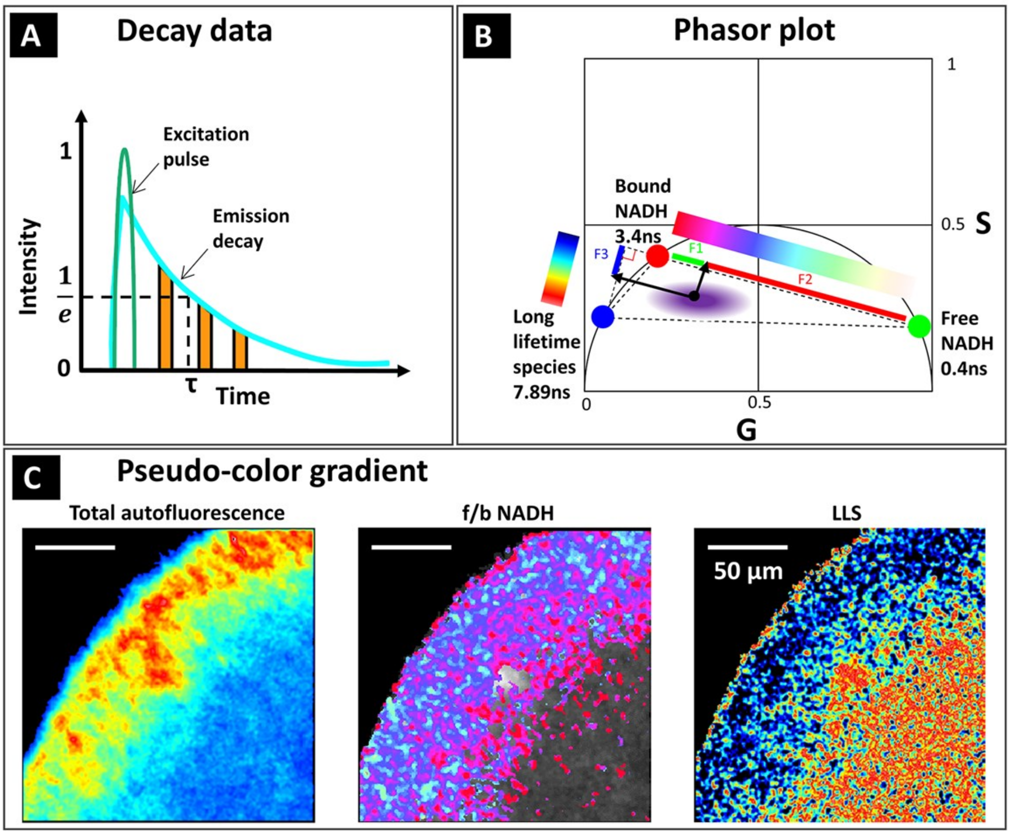

Figure S1: Fluorescence lifetime imaging and analysis using the phasor approach. (A) Fluorescence lifetime was acquired by quantifying emitted fluorescent photon over time after an excitation pulse was supplied to obtain an emission decay curve; (B) Phasor plot produced a 2-dimensional space for intrinsic fluorophors with different lifetimes corresponding with different types of metabolism (oxidative phosphorylation favors bound NADH and glycolysis favors free NADH) and different amounts of oxidative sress (long lifetime species). The free/bound NADH ratio and long LLS ratio were obtained by calculating projecting the 3 dimensional photon count histogram onto the Bound-Free axis and LLS axis respectively; (C) A representative images of RtOg analyzed by the phasor approach. The autofluorescence images encapsulated all total fluorescenee, while the f/b NADH and LLS are pseudocolor images based on the phasor analysis of quantized fluorescent emission. f/b NADH was free to bound NADH ratio. LLS was long lifetime species

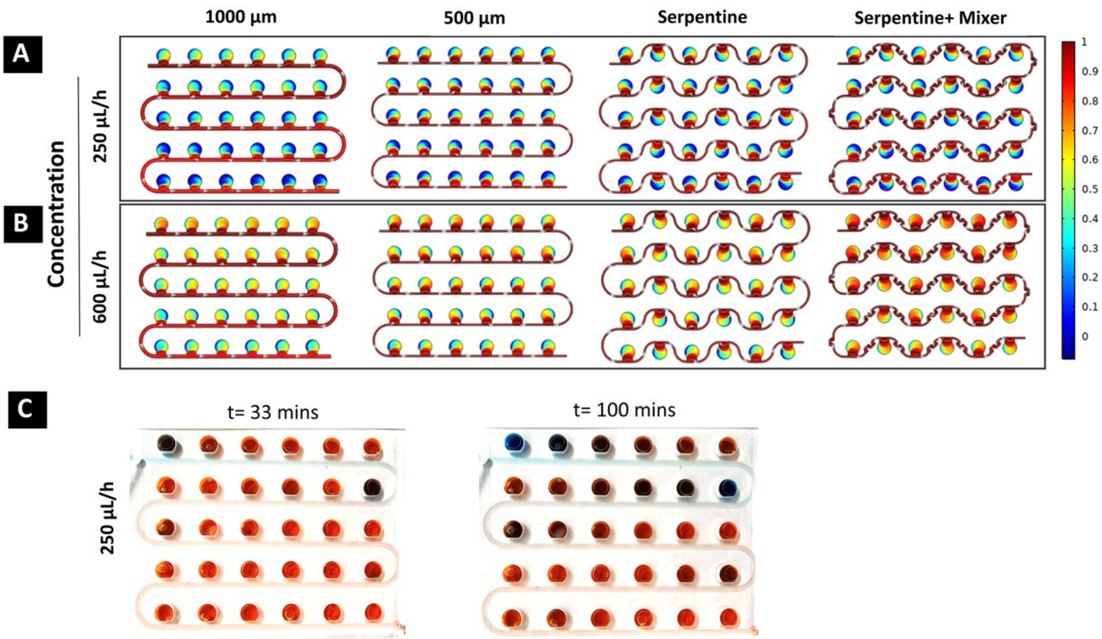

Figure S2: COMSOL simulation and dye test of 5*6 arrays bioreactor. (A) Concentration distribution after 30 minutes of slow flow (250 µL/h); (B) Concentration distribution after 30 minutes of fast flow (600 µL/h); (C) Concentration pattern of the wide channel (1000 µm) design (5*6 array) after 33 minutes and 100 minutes of 250 µL/h flow.

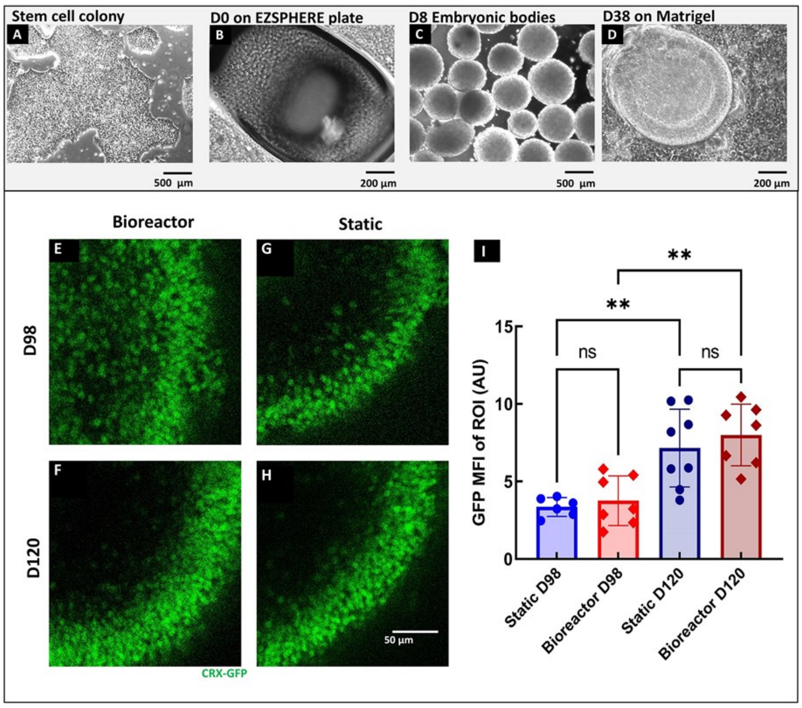

Figure S3: Phase contrast and CRX-GFP fluorescence imaging results. (A) Human embryonic stem cell colony; (B) Day 0 of differentiation, dissociated CRX-GFP stem cells in EZSPHERE microwell plate (well size: 800 µm); (C) Day 8 of differentiation, embryonic bodies ready for seeding on Matrigel; (D) Day 38 differentiation on Matrigel; (E-H) Fluorescence images showed distinct cell nuclear layer corresponding to the CRX-GFP fusion protein localized in nuclei; (I) The mean fluorescence intensity of GFP signals at region of interest (One-way ANOVA test was performed: Static D98, n = 6; Bioreactor D98, n = 7; Static D120 n = 8; Bioreactor D120, n = 7).

RtOgs in both static and bioreactor groups displayed a thick nuclear outer layer which expressed CRX gene on day 120 of differentiation (SFig. 3F, H). The mean fluorescent intensity (MFI) of the selected outer surface region showed no significant difference between static and bioreactor cultured RtOgs on day 98 and 120. However, both groups had a significant increase of MFI over time, which suggests an increase of CRX expression during RtOgs differentiation (SFig. 3I).

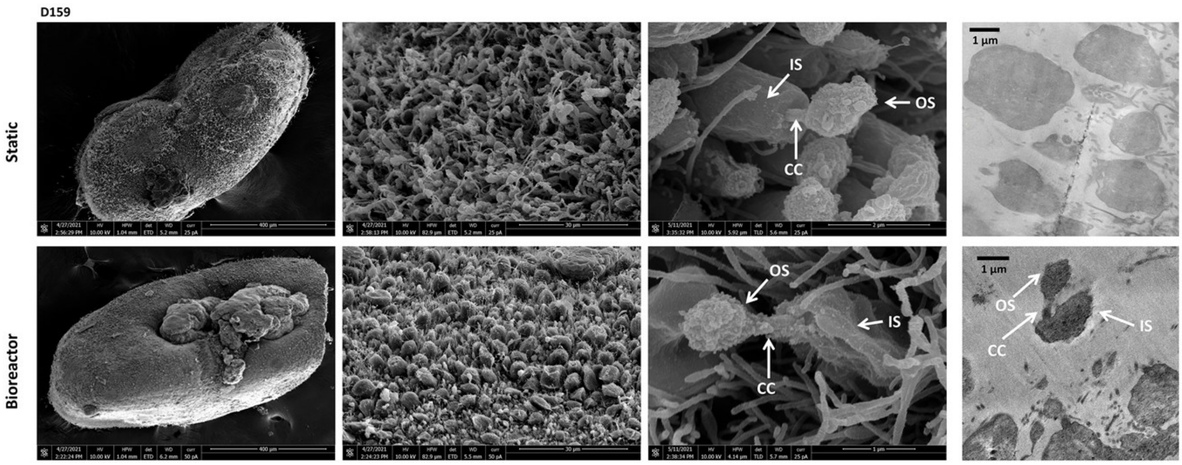

Figure S4: SEM and TEM images of RtOgs on day 159 of differentiation showed outer segment-like structures. Arrow markers: IS – inner segment, OS – outer segment, CC – connecting cilium.

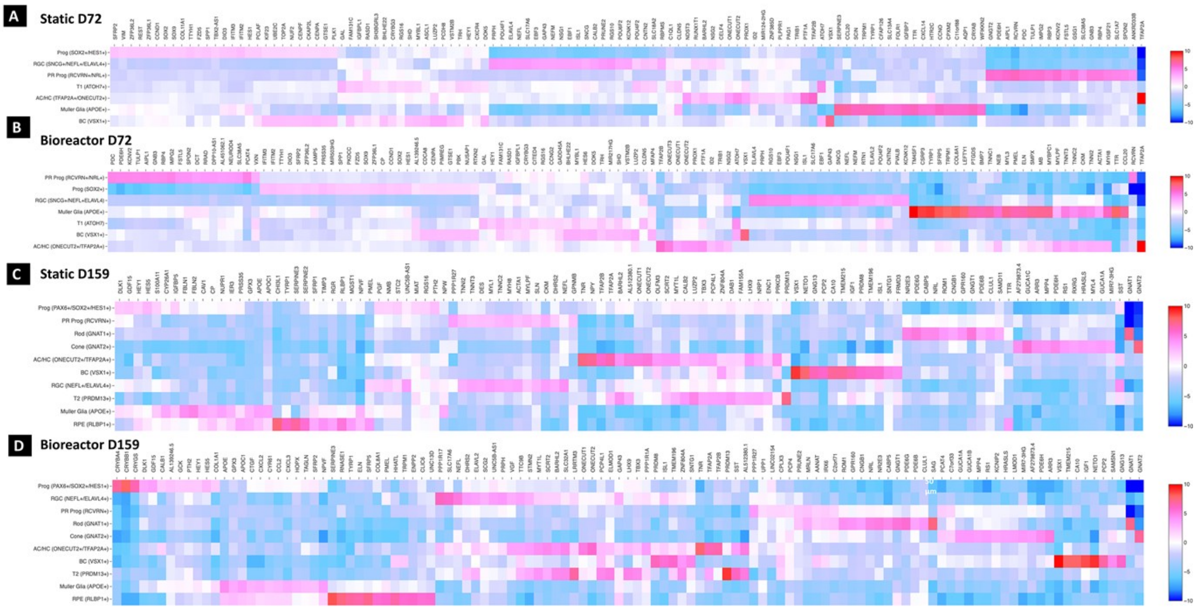

Figure S5: Single-cell RNA gene expression heatmap of different samples. Clustered by cell types using Loupe Browser.

Table S1: Summary of Experimental Groups

Cell line	Total time	Static & Bioreactor	PC	FLIM	IH	GFP	scRNA	qPCR	SEM TEM
CRX-GFP	D38-D72	D41-D72 (31 days)	**D41** **D71**	**D38** **D71**	**D72**		**D72**		
	D87-D124	D87-D124 (37 days)	**D88** **D124**	**D98** **D120**		**D98** **D120**		**D124**	
	D125-D159	D128-D159 (31 days)	**D158**	**D125** **D158**	**D159**		**D159**		**D159**
CSC-14	D70-D105	D70-D105 (35 days)						**D105**	

*PC – phase contrast imaging; FLIM – fluorescence lifetime imaging; IH – immunohistology; scRNA – single-cell RNA sequencing.

Table S2: Information of qPCR primers

Gene name	Official full name	GeneGlobe ID
CHX10 (VSX2)	Visual system homeobox 2	QT00221081
NRL	Neural retina leucine zipper	QT01005165
RAX	Retina and anterior neural fold homeobox	QT00212667
RCVRN	Recoverin	QT00014098
ARR3	Arrestin 3	QT00000182
SAG	S-antigen visual arrestin	QT01007958
PRPH2	Peripherin 2	QT00094094
GNAT	G-protein subunit alpha transducin	QT00235606
GNAT2	G-protein subunit alpha transducin 2	QT00008764
RHO	Rhodopsin	QT01017058
OPN1SW	Opsin 1, short wave sensitive	QT00017304
OPN1MW	Opsin 1, medium wave sensitive	QT00040887
OPN1LW	Opsin 1, long wave sensitive	QT01007356
RPL7	Ribosomal protein L7	QT01670137

Table S3: Information of Antibodies

Antibody	Species	Concentration	Manufacturer	Catalogue #	RRID
Rhodopsin (Rho4D2)	Mouse	1:100	Gift of Dr. Molday [1], University of British Columbia	N/A	AB_2315273 AB_2315274
Human NRL	Goat	1:100	R&D Systems	AF2945	AB_2155098
Recoverin	Rabbit	1:2000	Millipore	AB5585	AB_2253622
Calretinin	Goat	1:100	Novus	AF5065	AB_2068516
OTX2	Rabbit	1:1000	ThermoFisher	701948	AB_2608961
CHX10	Mouse	1:100	Santa Cruz	sc-365519	AB_10842442
RG-opsin	Rabbit	1:1000	Millipore	AB5405	AB_177456
Synaptophysin	Goat	1:100	Novus	AF5555	AB_2198864
PKC alpha	Rabbit	1:200	Oxford Biomedical	PK13	N/A
CRALBP	Rabbit	1:2000	Fitzgerald	70R-19906	N/A

Table S4: Key Reagents and Resources

Reagents or Resource	Source	Identifier
mTeSR 1 media	STEMCELL Technologies	Cat# 85850
ReLeSR	STEMCELL Technologies	Cat# 100-0484
Vitronectin XF™	STEMCELL Technologies	Cat# 07180
Accutase	Nacalai USA, Inc	Cat# NU1267954
Growth factor reduced Matrigel	Corning	Cat# 354230
Dulbecco's modified eagle medium (DMEM)	Gibco	Cat# 12100-038
F12 Nutrient Mixture	Gibco	Cat# 21700-026
N2 supplement	Gibco	Cat# 17-502-048
Minimum essential media non-essential amino acids (NEAA)	Gibco	Cat# 11140-050
L-glutamine 200mM (100X)	Gibco	Cat# 25030-081
Heparin	Sigma-Aldrich	CAS 9041-08-1
B27 supplement (50X) (minus vitamin A)	Gibco	Cat# 1587-010

B27 Plus supplement (50X)	Gibco	Cat# A3582801
Taurine	Sigma-Aldrich	CAS# 107-35-7
Heat inactivated 10% fetal bovine serum (FBS)	Gibco	Cat# 10438-026
bFGF	Peprotech	Cat# 100-18B
Activin-A	Peprotech	Cat# 120-14E
Collagenase IV	Gibco	Cat# 17104019
Anti-cell adherence solution	STEMCELL Technologies	Cat# 07010
Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (10X)	STEMCELL Technologies	Cat# 37354
TRIzol reagent	Fisher	Cat# 15596026
DNase I	Invitrogen TURBO	Cat# AM2238
Phenol/Chloroform/Isoamyl Alcohol	Fisher	Cat# BP1752I-400
RT^2 cDNA synthesis kit	Qiagen	Cat# 330401
ROX qPCR master mix	Qiagen	Cat# 330530
Worthington papain dissociation system	Worthington	http://www.worthington-biochem.com/PDS/cat.html
10X Genomics Chromium Single Cell 3' Reagent Kit v3.1	10X Genomics	N/A
Kapa qPCR Library	Roche	Cat# 07960140001
Histo-VT One	Nacalai	Product# 06380-05
Vectashield Vibrance Antifade Mounting Medium	Vector Labs	Cat# H-1700
Standard clear resin	Formlabs	Cat# RS-F2-GPCL-04
Optimum cutting temperature (OCT) compound (PolarStat Plus, StatLab, McKinney, TX, USA)	Ted Pella Inc.	Product# 27301-1
Critical Commercial Assays		
0.6X SPRIselect	Beckman Coulter	Cat# B23318
Qubit DNA HS assay	Life Technologies	Cat Q32851
Agilent 2100 Bioanalyzer DNA HS	Agilent	Cat# 5067-1504
Experimental Models: Cell Lines		
hESC, CRX-GFP H9	Dr. Majlinda Lako, Newcastle University [2-4], UK	Derived from NIH registration #004

hESCs, CSC-14	AIVITA Biomedical Inc.	NIH registration #0284
Equipment and Culture Plates		
Formlabs Form 3B	Formlabs	N/A
Harrick	Harrick Plasma	N/A
#1.5, 64*50 mm, ClariTex	Ted Pella Inc.	Cat# 260378
Humidified 5% CO_2 incubator	Nuaire	N/A
EZSPHERE 12-well plate (D: 800μm, d: 400μm]	Nacalai USA, Inc	Cat# TCI-4815-903SP-10P
Ultra-low attachment Corning Costar 24-well plate	Corning	Cat# 07-200-602
CoolCLAVE Plus	Genlantis	N/A
50 mL Steriflip-GP sterile centrifuge tube with filter cap pore size 0.22 μm	Millipore Sigma	Cat# SCGP00525
MicroAmp™ optical adhesive film	Thermo Fisher Scientific	Cat# 4311971
ESCO Class II Type A2 biosafety cabinet	ESCO Micro Pte. Ltd.	N/A
Zeiss LSM 780	Carl Zeiss	N/A
Mai Tai multi-photon laser source	Spectra-Physics Mai Tai	N/A
photomultiplier tube	Hamamatsu Photonics	H7422p-40
FastFLIM FLIMbox	ISS	N/A
Nunc® Lab-Tek® II Chambered Coverglass	Thermo Fisher	Cat# 155411
Olympus IX71	Olympus	N/A
QICAM FAST1394 CCD camera	Teledyne QImaging	N/A
Bio-Rad C1000 Thermocycler	Bio-Rad Laboratories	N/A
Dynabeads MyOne SILANE	Life Technologies	N/A
Illumina NovaSeq 6000	Illumina	N/A
Zeiss LSM700	Carl Zeiss	N/A
JEOL 2100	JEOL USA, Inc.	N/A
FEI Magellan 400 XHR	FEI Company	N/A
Software and Algorithms		
COMSOL Multiphysics 5.6	COMSOL, Inc.	N/A
SolidWorks 2020	SolidWorks Corp.	N/A
Graphpad Prism	Graphpad Software LLC	N/A

FASTQC	Babraham Bioinformatics	https://github.com/s-andrews/FastQC1
cellRanger v.3.1.0.	10X Genomics	N/A
Zen 3.3 Software	Zeiss	N/A
Adobe Photoshop	Adobe	N/A
Etomo	University of Colorado, Boulder	N/A

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1. Molday, R.S. and D. MacKenzie, *Monoclonal antibodies to rhodopsin: characterization, cross-reactivity, and application as structural probes.* Biochemistry, 1983. **22**(3): p. 653-60.
2. Collin, J., et al., *Using zinc finger nuclease technology to generate CRX-reporter human embryonic stem cells as a tool to identify and study the emergence of photoreceptors precursors during pluripotent stem cell differentiation.* Stem Cells, 2016. **34**(2): p. 311-321.
3. Collin, J., et al., *Deconstructing retinal organoids: single cell RNA-Seq reveals the cellular components of human pluripotent stem cell-derived retina.* Stem Cells, 2019. **37**(5): p. 593-598.
4. Collin, J., et al., *CRX expression in pluripotent stem cell-derived photoreceptors marks a transplantable subpopulation of early cones.* Stem Cells, 2019. **37**(5): p. 609-622.