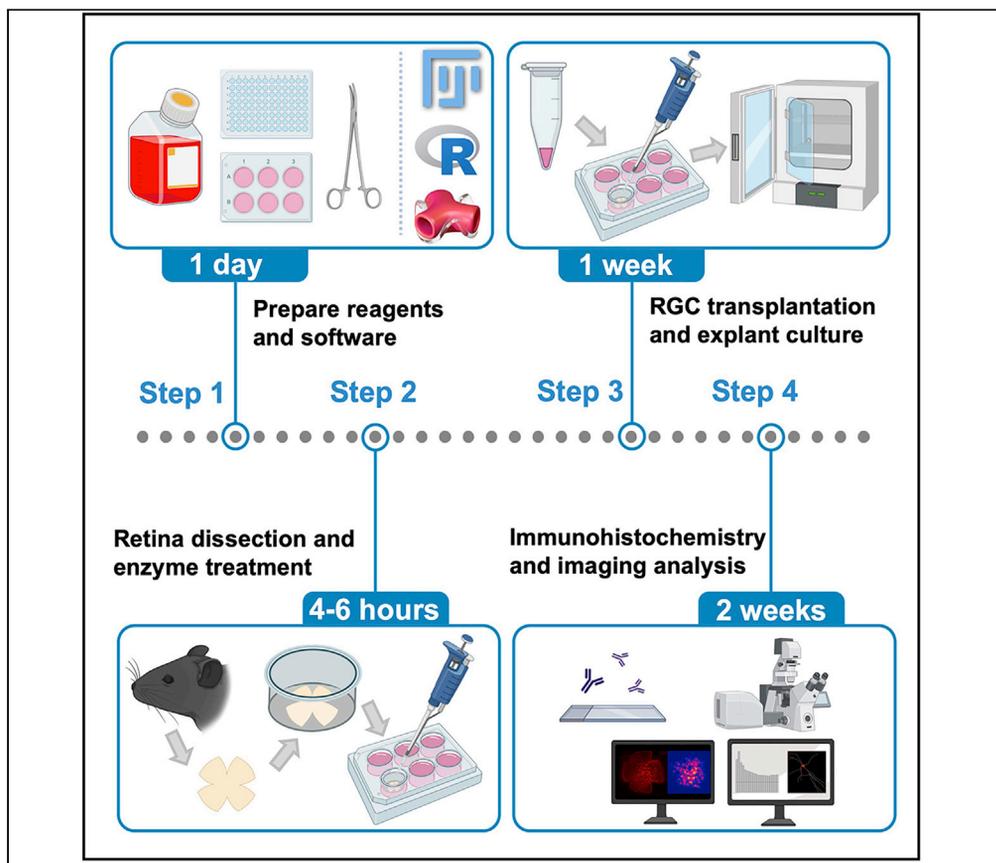


Protocol

Analyses of transplanted human retinal ganglion cell morphology and localization in murine organotypic retinal explant culture



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Highlights

Protocol for murine organotypic retinal explant culture with RGC transplantation

Proteolytic digestion of the internal limiting membrane enhances engraftment

Microscopy-based analyses quantify donor RGC survival and topology

Three-dimensional microscopy reconstructions localize donor neurite engraftment

Retinal ganglion cell (RGC) transplantation has the potential to restore vision in optic neuropathy, but donor neuron survival and retinal integration remain challenging. Here, we present a protocol for *ex vivo* human RGC transplantation on flatmounted murine organotypic retinal explants, providing a robust platform for studying donor RGC survival, dendritic stratification, topographic distribution, donor-host interactions, and pro-engraftment strategies. The protocol includes microscopy-based analyses to evaluate donor cell engraftment and can be adapted to various donor cell types or culture systems.

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Protocol

Analyses of transplanted human retinal ganglion cell morphology and localization in murine organotypic retinal explant culture

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SUMMARY

Retinal ganglion cell (RGC) transplantation has the potential to restore vision in optic neuropathy, but donor neuron survival and retinal integration remain challenging. Here, we present a protocol for *ex vivo* human RGC transplantation on flatmounted murine organotypic retinal explants, providing a robust platform for studying donor RGC survival, dendritic stratification, topographic distribution, donor-host interactions, and pro-engraftment strategies. The protocol includes microscopy-based analyses to evaluate donor cell engraftment and can be adapted to various donor cell types or culture systems.

For complete details on the use and execution of this protocol, please refer to Zhang et al. (2021a, 2021b).

BEFORE YOU BEGIN

The protocol below describes specific steps for culturing human pluripotent stem cell-derived retinal ganglion cells (RGCs) on mouse organotypic retinal explants to model xenographic intravitreal neuronal transplantation, followed by the quantitative characterization of donor neuronal behavior *in situ*. There are numerous methods for stimulating differentiation of stem cells into RGCs (Gill et al., 2016; Sluch et al., 2017; Teotia et al., 2017; Langer et al., 2018) and this protocol is amenable to cellular transplantation from various sources including freshly isolated primary RGCs (Venugopalan et al., 2016; Wu et al., 2018). Therefore, specific steps for donor neuron derivation are not specified but rather left to the end user. In addition, neurons can be co-cultured on retinal explants derived from other species including rat, pig, cow, and post-mortem human tissue. The reader is referred to complimentary sources for derivation of retinal explant cultures from those species (Bull et al., 2011; Wang et al., 2011; Januschowski et al., 2012; Peynshaert et al., 2017). We have documented that proteolytic digestion of the internal limiting membrane (ILM) with the enzyme Pronase facilitates the structural engraftment of donor RGCs into the host retina (Zhang et al., 2021a, 2021b), and so steps to achieve ILM disruption are included here. For complete details on the generation and use of this protocol, please refer to (Zhang et al., 2021a, 2021b).

Institutional permission and oversight information

Use of mice and all animal procedures performed were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University School of Medicine. The experimental use of stem cells was approved by the Institutional Stem Cell Research Oversight Committee of Johns Hopkins University School of Medicine.



Days before dissection

⌚ Timing: 0.5 days

1. Precoat a 96-well plate with poly-L-ornithine (PLO) and laminin for eventual plating of RGCs. A sample of RGCs used for explant transplantation will be cultured in parallel on PLO-laminin coated plates to evaluate batch-specific donor neuron viability independent of recipient retinal explant cultures.
 - a. Dilute PLO with water to yield a working concentration of 20 $\mu\text{g}/\text{mL}$ for polystyrene plates or 50 $\mu\text{g}/\text{mL}$ for glass plates.
 - b. Dilute laminin to a working concentration of 5 $\mu\text{g}/\text{mL}$.
 - c. Add 100 μL of PLO solution to cover the surface of each well. Place the plate in a 37°C incubator for 1 h.
 - d. Aspirate the PLO solution and rinse the wells once briefly with 200 μL of sterile water.
 - e. Aspirate the water from each well and add 100 μL of laminin solution to each well. Keep the plate at 4°C overnight (12 h).
 - f. Coated plates can be stored in the laminin solution at 4°C for 3 weeks. Prior to use, bring the plate to room temperature (20°C) and remove the laminin solution. Aspirate the laminin solution and rinse the wells with sterile PBS immediately before plating the RGCs.
2. Prepare Retinal Explant Culture Media. See the list of supplemental ingredients in the [materials and equipment](#) section.

Preparing dissection hood and euthanasia area

⌚ Timing: 1 h

3. Thoroughly sanitize the tissue culture hood and dissection microscope with 70% ethanol. Place sterile dissection instruments inside the hood. The list of tools required includes:
 - a. Dumont #5 forceps (x2).
 - b. Vannas scissors.
 - c. Bent metal vascular probe.
 - d. 30-gauge needle.
 - e. 1,000 μL and 200 μL pipettes with sterile barrier tips.
 - f. Straight razor blade.
 - g. Whatman filter paper.
 - h. Organotypic culture inserts.
4. In the euthanasia area, prepare 15 mL conical tubes filled with sterile ice-cold DPBS. Label each 15 mL conical tube for one dissected eye.

Preparing culture media and enzymes

⌚ Timing: 1 h

5. On the day of dissection, add 1.5 mL of culture media to each well of a 6-well plate. Each well will hold one culture insert and one organotypic retinal explant. Place the plates in the 37°C incubator with 5% CO_2 to equilibrate the media to physiological pH.
 - a. Stock media is stable at 4°C in the dark for at least 1 month.

⚠ CRITICAL: Add sterile water to the plate in the space outside of the wells to provide localized humidity and prevent excessive evaporation of culture media.

6. Prepare Pronase enzyme prior to dissection. Due to the low final concentration and the small volume of the enzyme solution, perform serial dilution by first making a 60 U/mL solution before diluting to the final 0.6 U/mL solution.

△ **CRITICAL:** Check the original manufacturer’s website for lot-specific units of enzymatic activity per milligram (U/mg). This is used to calculate the mass of enzyme needed for the desired final concentration of 0.6 U/mL.

- a. Using an analytical balance, weigh out 9.23 mg of the Pronase powder (assuming an enzymatic activity of 6.5 U/mg based on Lot# SLCB5965). Use a small piece of sterile aluminum foil as the weighing boat to reduce static. Add the Pronase powder to a 1.5 mL tube, then resuspend the enzyme in 1 mL of sterile BSS. This results in a 60 U/mL solution of Pronase, which can then be diluted to 0.6 U/mL with BSS.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-RFP antibody (1:300)	Rockland Immunochemicals	Cat#600-401-379
Mouse monoclonal anti-human nuclei antibody (1:300)	Millipore	Cat#MAB1281
Goat anti-mouse IgG (H+L), Superclonal™ recombinant secondary antibody, Alex Fluor 488 (1:1000)	Thermo Fisher Scientific	Cat#A28175
Goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 568 (1:1000)	Thermo Fisher Scientific	Cat#A11036
Chemicals, peptides, and recombinant proteins		
B-27 Plus Supplement	Thermo Fisher Scientific	Cat#A3582801
Bovine Serum Albumin	Sigma-Aldrich	Cat#A9418
Balanced Salt Solution (BSS)	Gibco	Cat#14287080
Calcein, AM, cell-permeant dye	Thermo Fisher Scientific	Cat#C1430
DAPI	Thermo Fisher Scientific	Cat#D1306
Dulbecco’s phosphate buffered saline (DPBS)	Thermo Fisher Scientific	Cat#14040133
Fluorescent Mounting Medium	Dako	Cat#S3023
GlutaMAX Supplement	Thermo Fisher Scientific	Cat#35050061
Laminin	Sigma-Aldrich	Cat#L2020
N-2 Supplement	Thermo Fisher Scientific	Cat#17502001
Neurobasal-A Medium	Thermo Fisher Scientific	Cat#10888022
Normal Goat Serum	Thermo Fisher Scientific	Cat#50197Z
Ovomucoid	Sigma-Aldrich	Cat#T9253
Penicillin-Streptomycin	Thermo Fisher Scientific	Cat#15070063
Poly-L-ornithine solution	Sigma-Aldrich	Cat#P4957
Protease from <i>Streptomyces griseus</i> (Pronase)	Sigma-Aldrich	Cat#P8811
Triton X-100	Sigma-Aldrich	Cat#9036-19-5
Experimental models: Cell lines		
Human: H7 ES cell derived RGCs	Laboratory of Don Zack (Johns Hopkins University)	(Sluch et al., 2017)
Experimental models: Organisms/strains		
6- to 12-week-old male and female mice (C57BL/6J)	The Jackson Laboratory	RRID: IMSR_JAX:000664
Software and algorithms		
Fiji/ImageJ	National Institutes of Health (NIH)	https://imagej.nih.gov/ij/
IMARIS	Bitplane	https://www.bitplane.com/imaris
Photoshop	Adobe	N/A
R	R Foundation for Statistical Computing	https://www.R-project.org/
R package: sje30	Stephen Eglén, Ph.D.	https://github.com/sje30/sje30
R package: spatstat	(Baddeley et al., 2015)	https://spatstat.org/

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Other</i>		
30G needle	BD	Cat#304000
1,000 μ L, 200 μ L, 10 μ L pipettes with sterile barrier tips	N/A	N/A
Angled Vessel Probe	Fine Science Tools	Cat#10140-03
Cover glasses	Thermo Fisher Scientific	Cat#12-541B
Crescent Knife	MicroSurgical Technology	Cat#AB-ACU26KL
Dissecting Microscope	Leica Microsystems	Cat#SP-S9D-TS
Dumont Forceps #5	Fine Science Tools	Cat#11251-10
Dumont #7 Curved Forceps	Fine Science Tools	Cat#11271-30
EVOS FL Auto Imaging System	Thermo Fisher Scientific	Cat#AMC1000
LSM 880 Confocal Microscope	Zeiss	N/A
Microscope Slides	Fisher Scientific	Cat#12-550-003
Millex-GV Syringe Filter, 0.22 μ m	Millipore	Cat#SLGV033RS
Organotypic culture inserts	Millipore	Cat#PICMORG50
Straight razor blade	N/A	N/A
Tissue Culture Hood	N/A	N/A
Tissue Culture Incubator	N/A	N/A
Vannas scissors	Fine Science Tools	Cat#15000-08
Whatman filter paper	Whatman	Cat#1001090

MATERIALS AND EQUIPMENT

Prepare Retinal Explant Culture Media, Pronase Solution, and Proteolytic Inhibitor Solution. Sterilize the Retinal Explant Culture Media using a 0.22 μ m filter and store at 4°C in the dark. Pronase Solution should be freshly prepared under aseptic conditions as to avoid filtering low volumes. Proteolytic Inhibitor Solution should be freshly prepared and sterilized using a 0.22 μ m filter; it can be stored at room temperature (20°C) for a few hours.

Retinal Explant Culture Media

Reagent	Stock concentration	Final concentration	Amount
Neurobasal-A Medium	N/A	N/A	47.5 mL
B27 Plus Supplement	50 \times	1 \times	1 mL
N-2 Supplement	100 \times	1 \times	500 μ L
Penicillin-Streptomycin	5,000 U/mL penicillin 5,000 μ g/mL streptomycin	50 U/mL penicillin 50 μ g/mL streptomycin	500 μ L
GlutaMAX Supplement	100 \times	1 \times	500 μ L
Total	N/A	N/A	50 mL

Sterilize using a 0.22 μ m filter, store at 4°C, do not store for more than 1 month.

Pronase Solution

Reagent	Stock concentration	Final concentration	Amount
Protease from <i>Streptomyces griseus</i>	~3.5 U/mg	0.6 U/mL	lot dependent
Balanced Salt Solution	N/A	N/A	lot dependent
Total	N/A	N/A	lot dependent

Create a 0.6 U/mL Pronase Solution by dissolving the lyophilized powder in sterile BSS. Check the exact stock enzyme concentration by referencing the Lot Number on the manufacturer [website](#). We recommend preparing an initial 100 \times concentrate in 1 mL of BSS (i.e., 60 U/mL in 1 mL BSS), and dilute the stock solution to the final concentration of 0.6 U/mL. Final solution can be kept at room temperature (20°C). Prepare enzyme solution fresh for each experiment and discard the unused enzyme solution.

△ CRITICAL: Practice sterile technique while preparing the enzyme solution.

Proteolytic Inhibitor Solution			
Reagent	Stock concentration	Final concentration	Amount
Bovine Serum Albumin	N/A	0.15 mM	150 mg
Ovomucoid	N/A	0.357 mM	150 mg
Balanced Salt Solution	N/A	N/A	15 mL
Total	N/A	N/A	15 mL

Sterilize using a 0.22 μm filter and store at room temperature (20°C) prior to use. Prepare the inhibitor solution fresh for each experiment and discard the unused solution. Prepare enzyme inhibitor solution fresh for each experiment and discard the unused enzyme solution.

STEP-BY-STEP METHOD DETAILS

Animal dissection and retinal explant preparation

⌚ **Timing:** 10–15 min for each pair of eyes; 2–3 h for dissecting a cohort of approximately 15–30 eyes

This section describes the detailed procedure of dissecting the retina and flatmounting the explant in culture. The techniques outlined can be adapted to retinal tissue derived from other species.

1. Euthanize 6- to 12-week-old mice using either deep anesthesia with intraperitoneal ketamine/xylazine or CO₂ asphyxiation followed by cervical dislocation, in accordance with AVMA Guidelines for Euthanasia of Animals.

Note: Consistency in animal age ensures homogeneity in retinal tissue architecture and transplantation results.

- a. Enucleate eyes by clasping behind the globe using Dumont #7 curved forceps and applying brisk outward force.
- b. Immediately place eyes in ice cold DPBS. Transport enucleated eyes to the dissection hood for explantation.

⚠ **CRITICAL:** Euthanize one animal at a time. A skilled dissectionist can flatmount retinas from one pair of eyes in approximately 6–10 min. During retinal dissection, an assistant can expedite the process by euthanizing and enucleating the next animal.

2. Explant the retina from the globe. Begin by using a 30-gauge needle to puncture the sclera at the limbus and then use Vannas scissors to incise the sclera just posterior to the limbus circumferentially (Figures 1A–1D; Methods video S1).
3. Separate the anterior and posterior segments of the globe (Figures 1E and 1F).
 - a. Remove and discard the anterior segment including the crystalline lens (Figure 1G).
 - b. Grasp the sclera of the posterior segment with #5 forceps and evert the posterior eye cup by applying pressure to the optic nerve posteriorly (Figures 1H–1M).

Note: This will separate the neural retina from the retinal pigmented epithelium (Methods video S2).

Note: Small amount of RPE remain attached to the retina will not affect the explant culture or subsequent imaging.

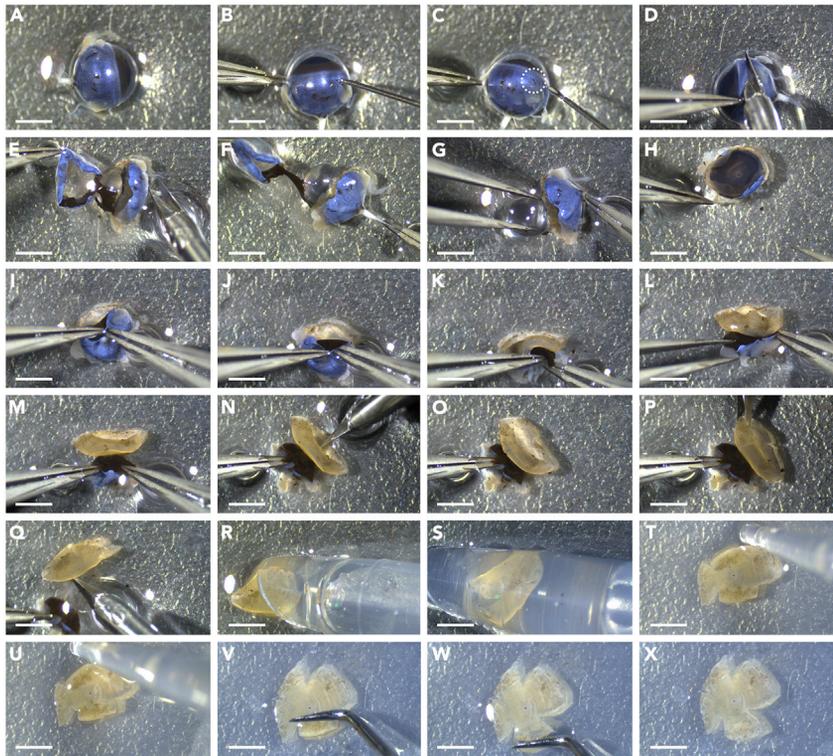


Figure 1. Retinal dissection from an enucleated mouse eye

- (A) An enucleated mouse eye submerged in PBS.
- (B) Grasp the sclera or a stump of residual extraocular muscle with Dumont Forceps #5 and secure the eye in position. Create a puncture in the sclera across the globe using a 30-gauge needle.
- (C) The dotted circle indicates the scleral puncture.
- (D) Grasp the anterior sclera at the edge of the puncture with the Dumont Forceps #5 and create a circumferential incision parallel to and just posterior to the limbus with Vannas scissors.
- (E) Remove the cornea from the posterior eye cup by holding the eye at the limbus with forceps.
- (F) Use a second pair of forceps to hold the posterior cup at the optic nerve to create traction.
- (G) While securing the optic nerve with forceps, pull away the crystalline lens with a second pair of forceps.
- (H) An inside view of an isolated posterior cup shows the neuroretina surrounded by retinal pigmented epithelium and sclera.
- (I) Use a pair of forceps to grab the scleral edge and begin folding back the sclera from the retina.
- (J) Continue to use both pairs of forceps to peel back the sclera from the retina and work circumferentially around the limbal edge.
- (K) Evert the sclera to prolapse the retina.
- (L) Continue to gently separate the retina from the sclera.
- (M) The scleral cup is now completely everted and only attached to the retina at the optic nerve head.
- (N) Secure the sclera with forceps and create four radial incisions within the retina.
- (O) Even with relaxing cuts, the retina still retains a folded shape at this stage.
- (P) Make the final radial incision in the retina. Relaxing incisions are 90 degrees apart.
- (Q) Detach the retina from the sclera by axotomizing RGCs at the optic nerve head with the Vannas scissors.
- (R) Aspirate the free-floating retina using a P1000 pipette with a widened tip.
- (S) A view showing the retina inside the P1000 pipette tip.
- (T) Transfer the retina onto the center of a culture insert and position the retina with the inner retina facing up.
- (U) Aspirate the excess PBS around the retina. The retina edges will start unfolding and flattening.
- (V) An alternative method of unfolding the edge is by using a bent vascular probe to engage the overlying fluid and use surface tension to draw the folded retina peripherally.
- (W) Use the surface tension between the vascular probe and the folded surface to flatten out the retina.
- (X) The flatmounted retina on the culture membrane. Scale bar, 2 mm.

△ **CRITICAL:** To avoid explant culture contamination, perform dissection in a sterilized hood. Additionally, sterilize all dissection tools prior to use and between animals.

△ **CRITICAL:** Avoid iatrogenic damage to the retinal tissue during dissection, as it induces surface disruption and glial reactivity. Do not touch the retinal tissue with dissecting instruments. Instead, peel the non-retinal tissue away from the neural retina to achieve retinal isolation.

4. Prior to separating the retina from the eye cup, create 4 radial relaxing incisions in the retina from the periphery halfway to the optic nerve with Vannas scissors (Figures 1N–1P), which are necessary to mount the semispherical retina onto a flat membrane. Then, detach the retina by cutting the optic nerve posterior to the retina using Vannas scissors (Figure 1Q) (Methods video S3).
5. Carefully transfer the free-floating retinas in PBS onto organotypic culture inserts using a sterile P1000 pipette with tip widened by cutting with a straight razor (Figures 1R and 1S).
 - a. Orient the retina inside a culture insert with the RGC layer (RGCL) facing up (photoreceptors against the membrane). The retina naturally curls with the photoreceptor side outward, and the retinal vessels are visible on the RGCL side.
 - b. Using a P200 pipette, slowly aspirate excess PBS until the retina is flatmounted on the dry filter (Figures 1T and 1U).
 - c. Use surface tension between the pipette tip or vascular probe and the retinal tissue to draw the edges of retinal explant out such that they lie flat, rather than folded (Figures 1V and 1W). Avoid touching the retinal tissue directly with the pipette tip or vascular probe (Methods videos S4 and S5).

△ **CRITICAL:** The natural curvature of the retinal tissue creates a tendency for the tissue to fold inward. The inner retinal surface (RGCL/vitreous side) must face up when flattened (Figure 1X).

△ **CRITICAL:** Cell and tissue culture inserts are available with several options for membrane composition. We have found that hydrophilic PTFE membrane with 0.4 μm pore size (PICM0RG50) works well for culturing organotypic retinal explants.

6. Apply Whatman paper to the undersurface of the organotypic membrane to remove residual fluid and create suction to adhere the retinal tissue to the membrane.
7. Place the culture insert with the flatmounted retina in the 6-well plate.

Note: Avoid introduction of air bubbles underneath the filter during this step.

- a. The culture media underlying the filter feeds the organotypic retinal explants, which remain under a thin air-fluid interface.
- b. Place the culture plate back in the incubator.

Pronase treatment and enzyme inhibition

⌚ **Timing:** 2 h

This section describes the preparation of the Pronase enzyme to be used on flatmounted retinal explant. The enzyme should be prepared prior to dissection; it is stable for several hours at room temperature (20°C).

8. Work with one 6-well plate at a time in the dissection hood while the rest of the plates remain in the incubator.
 - a. Using a P10 pipette, dispense 5 μL of 0.6 U/mL Pronase on to the surface of each retina.

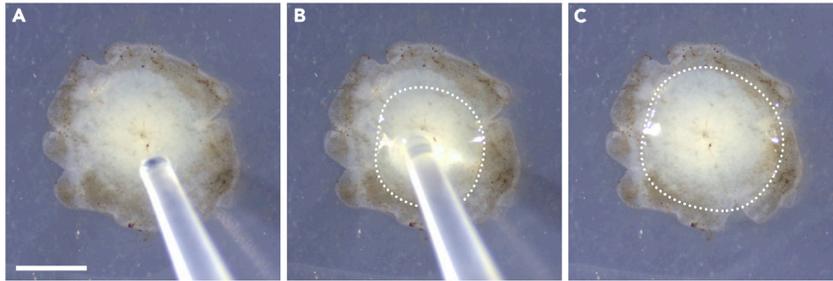


Figure 2. Pipetting technique

(A) Position the pipette tip over the optic nerve head.
 (B) Pipette a droplet directly over the retina; the dotted line demarcates the edge of the droplet.
 (C) The droplet distributes evenly around the central retina. Scale bar, 2 mm.

- b. Directly visualize the enzyme application onto the retinal surface using a dissecting microscope as a centrally-placed droplet will remain confined to the retina due to the surface tension of the air-fluid interface (Methods video S6).
- c. Start a timer for 60 min once the first retina receives the enzyme.
- d. Return the completed 6-well plate back into the incubator before taking out the next plate for enzyme treatment.

△ **CRITICAL:** To dry the external surface of the pipette tip prior to each application, gently wipe the tip with Kimwipe in a single forward motion. Failure to do so will result in the droplet adhering to the side of the pipette tip instead of dispensing onto the retinal surface. While contamination from this step is rare, use a new box of Kimwipe for each experiment to limit contamination. Avoid touching the surface of the Kimwipe that will contact the pipette tip.

△ **CRITICAL:** The pipette should be angled as vertically as possible (Figures 2A and 2B), with pipette tip aimed at the optic nerve head to ensure equal distribution of the droplet on the retinal surface (Figure 2C) (Methods video S6).

9. Prepare the Inhibitor Solution during enzymatic ILM digestion. The complete list of ingredients is in the materials and equipment section.

Note: Since the Inhibitor Solution contains bovine serum albumin (BSA), it should be made fresh, immediately prior to use.

- a. Filter the final Inhibitor Solution with a 0.22 μm syringe filter.
- b. Keep the solution at room temperature (20°C) until use.

10. After 1 h of enzyme treatment, add 300 μL of the Inhibitor Solution to the surface of each retina.

Note: This volume of fluid will not remain confined to the retinal surface and will spill over onto the filter.

- a. Space out the timing of Inhibitor Solution treatment to ensure that each individual retina has completed the 1-h enzymatic digestion.
- b. After Inhibitor Solution has been added, place the plates back in the incubator for 15 min.

△ **CRITICAL:** Reduce surface tension on the retina by ensuring the Inhibitor Solution completely submerges the retinal tissue. Failure to do so may result in partial or complete detachment of the retina from the membrane.

11. Fill the culture inserts with BSS (2 mL) to dilute the enzyme and inhibitors.
 - a. Add BSS to all the culture inserts before moving to the next step.
 - b. Dry the inside and the bottom of the culture membrane by removing the BSS and blotting the underside with Whatman paper.
 - c. After properly drying, the culture insert membrane becomes firm and taut, and can be placed in a **fresh** set of 6-well plates with 1.5 mL of culture media in each well.
 - d. Place the plates with the culture inserts back in the 37°C incubator until the next day.

Note: As with using Kimwipe, Whatman paper does not require sterilization prior to use. However, practice proper antiseptic technique when handling fresh Whatman paper.

Retinal ganglion cell transplantation and co-culture

⌚ Timing: 2 h for RGC transplantation; 1 week for the culture

This section describes the technique of transplanting retinal ganglion cells on organotypic retinal explants, as well as the proper maintenance of the coculture.

12. On the morning **after** explantation, replace 750 μL of the media in each well (underneath the membrane) with fresh media.

Note: This should be done several hours before RGC transplantation to allow the culture media to equilibrate inside the incubator.

13. Dilute donor RGCs to the desired concentration in Retinal Explant Culture Media.
 - a. Pipette 3 μL of the cell suspension onto each recipient retina using the technique detailed above in [step-by-step method details](#) step 8. We have found that 2×10^4 RGCs per retina works well for downstream quantitative applications.
 - b. Place the culture plates back in the incubator.

Note: Optimize the total number of donor cells transplanted based on individual donor source and cell survival. Limit the transplant volume to 3 μL , as larger volumes may result in the cell suspension rolling off the retinal tissue, giving inaccurate survival counts and enabling RGCs to access the subretinal space.

Note: When deriving donor RGCs through differentiation of pluripotent cells, we recommend using the lowest passage numbers possible. We typically differentiate RGCs from stem cells that have been passaged fewer than 10–15 times.

- c. Exchange retinal explant culture media every other day after RGC transplantation. Media is exchanged by replacing half of the volume (750 μL) in each well with fresh culture media.
14. Using the cells from the same preparation as [step-by-step method details](#) step 13, separately culture 8×10^3 RGCs in 150 μL of Retinal Explant Culture Media per well in the precoated 96-well plate from the **“before you begin”** section. Place the culture plate back in the incubator.
 - a. Allow the cells to settle onto the plate overnight (12 h).
 - b. On the following day, obtain microscopy images of the cell culture focusing on endogenous tdTomato expression using an epifluorescent microscope. Alternatively, cells can be quantified using a vital dye such as Calcein. Count the number of cells in each well to calculate survival rate.
 - c. Image the cell culture daily to examine RGC viability longitudinally.

Retinal tissue immunofluorescent staining

⌚ Timing: 4–7 days for immunohistochemistry

This section describes the process of tissue fixation and immunohistochemistry of the cultured explants.

15. Fix the retinal explants directly in the culture inserts by transferring inserts to new 6-well plates containing 3 mL per well of 4% cold PFA and adding 5 mL of 4% cold PFA directly into the culture insert.

Note: Add PFA to the culture insert slowly to avoid detaching the retinal explants. Maintain at 4°C for 1 h.

16. Wash the explants by removing the PFA and replacing with 5 mL of PBS per well, and then remove the PBS. Fixed retinal explants can be stored in PBS at 4°C for 1–2 weeks prior to downstream histologic applications.
17. Remove PBS from the culture inserts and cut the explants attached to the underlying membrane out of the culture insert by creating a circumferential incision in the membrane around the retinal tissue using a crescent blade.

Note: This step is best performed under a dissecting microscope. Take care to leave a margin on the membrane such that the membrane containing the explant can be picked up with forceps and transferred without touching the retina.

18. For flatmount immunofluorescence and microscopy, transfer the retinas attached to the culture membrane into a prelabeled plate and submerge in PBS.
 - a. Immunofluorescent staining can be performed in 48-well plate, which minimizes the amount of antibody required.
 - b. Alternatively, retinal explants at this step can be transferred to OCT and frozen for cryosectioning per standard protocols.
19. To label donor RGCs, perform wholemount immunofluorescent staining for RFP.
 - a. Wash the retinas with PBS for 10 min while rocking at 80 revolutions per minute.
 - b. Block and permeabilize the tissues with 500 μ L of blocking buffer (0.3% Triton X-100 and 10% normal goat serum in PBS) for 1 h at room temperature (20°C) while rocking.
 - c. Remove blocking buffer and immerse the retinas in 500 μ L of primary antibodies diluted in blocking buffer for 2–5 days at 4°C while rocking. The length of primary antibody incubation should be titrated for specific antibodies.
 - d. Wash the retinas in PBS for 15 min while rocking; repeat 3 times.
 - e. Incubate the retinas in 500 μ L of secondary antibodies in diluted blocking buffer overnight (12 h) at 4°C while rocking.
 - f. Wash the retinas in PBS for 15 min while rocking; repeat 3 times.
 - g. Counterstain the nuclei with DAPI in PBS for 15 min.
 - h. Wash the retinas with PBS for 15 min.
 - i. Mount the retinas on the membranes onto glass slides.
 - j. Add mounting medium (Dako, Denmark) and coverslip over the retinas. Keep the slides in the dark to dry until imaging. When dry, store in the dark at 4°C prior to imaging.
20. Perform low magnification wholemount confocal tiled imaging on each retina. See detailed procedure on confocal microscopy.
21. Perform high resolution stacked confocal images for three-dimensional volumetric analysis. See detailed procedure on confocal microscopy.
22. Perform computational volumetric and topographic analyses on donor cell survival and integration. See detailed procedure the [quantification and statistical analysis](#) section.

Confocal microscopy

⌚ Timing: 1–3 days

This section describes the methods for capturing images on a Zeiss 880 confocal microscope. Other microscope models may have different settings.

23. Wholemout tiled confocal imaging.

Note: The superficial surface of the mounted retinal tissue is not necessarily parallel to the horizontal plane and tends to be slanted and irregular. Thus, capturing a wholemount retinal surface requires imaging tiled z-stacks.

- a. Use low magnification objectives (10× or 20×) to survey and scan across the retinal surface.
- b. Manually focus the objective and identify the most superficial area on the entire retina. Set this vertical position as the top of the z-stack.
- c. Manually focus the objective and identify the deepest region of interest on the entire retina. Set this vertical position as the bottom of the z-stack.
- d. Set the laser power and gain for optimal exposure. We recommend keeping the acquisition settings for master gain and digital gain constant at 650 and 1, respectively, while adjusting the laser power. Using the staining protocol above, a laser power between 1-3 would yield the optimal exposure.
- e. Center the microscope view at the optic nerve head and acquire tiled z stacks.

Note: The tile dimensions sufficient to capture the entire retina are typically 6 × 6 tiles for the 10× objective at 1.0× digital magnification and 11 × 11 tiles for the 20× objective at 1.0× digital magnification. A 512 × 512 pixel dimension for each tile provides sufficient resolution to quantify donor RGC cell bodies and visualize neurites. The z-stack interval can be set to 2.5–3.5 μm, since lower magnification objectives have larger depth of field and depth resolution is not a focus of this imaging modality.

24. Detailed analysis of individual neurons demonstrating neurite integration with high-resolution and high-magnification confocal imaging (Zhang et al., 2021b).

- a. Identify a region or a cell of interest with a 40× or 63× objective.
- b. Set the top and bottom slices of the z-stack to capture the entire donor RGC from the soma to the deepest neurite branch. The bottom slice should extend through the outer nuclear layer to capture the entire host neural retina for characterization of laminar stratification.
- c. Acquire fluorescent channels of interest. Our examples include acquisition of at least the tdTomato and DAPI signals.

Note: Tiling may be required to capture the entire neurite extensions.

Note: Acquire high image resolution images to enhance downstream 3D rendering and analyses. Optimize image quality by increasing line averages and decreasing scanning speed. Pixel dimensions of 2048 × 2048 are preferred.

EXPECTED OUTCOMES

The main outcomes of interest for *ex vivo* RGC transplantation studies using this protocol include donor cell survival and localization within the host retinal tissue. This protocol can be used to visualize donor soma and neurites, and the data gathered allow for the derivation of numerous quantitative metrics, which include but are not limited to: donor cell survival (number and rate), topographical distribution (nearest neighbor distance, density recovery profile, Ripley's functions), donor cell

localization in each retinal layer, donor neurite localization in each retinal layer, donor neurite morphology (dendritic field area, number of branches, Sholl analysis), and donor cell identity (immunohistochemistry for donor-specific cytoplasmic markers, synaptic markers, and neurite markers).

QUANTIFICATION AND STATISTICAL ANALYSIS

Endogenous retinal neurons form mosaic arrangements across the retina to ensure uniform sampling of the visual field. A variety of statistical measures can quantify the spatial patterns of such mosaics. These metrics include nearest neighbor distance (NND), density recovery profile (DRP), and Ripley's L-function, and have been elegantly summarized in a recent review (Keeley et al., 2020).

The NND measures the distance between a reference neuron and its closest neighboring neuron. The frequency distribution constructed from all NND in a population informs the relative spacing among neurons. A narrower peak in the frequency distribution indicates higher uniformity. The spatial uniformity is quantitatively expressed by the regularity index (RI), which is the ratio of mean NND and the standard deviation of NND (Wässle and Riemann, 1978; Cook, 1996). Higher RI correlates with increased regularity and suggests non-random organization (Hutsler and Chalupa, 1994). The RI of randomly arranged points is approximately 1.9; retinal neurons within a sub-type typically have an RI between 3 and 8, indicating mosaic organization (Eglen, 2012).

The density recovery profile (DRP) is a spatial metric that describes densities over large distances and detects cellular clustering (Rodieck, 1991). The distances from a reference cell to every other cell in the sample is measured, and the process is repeated with each cell taken as the reference cell. The resulting measurements are combined and binned at specific intervals and graphed as a histogram. The characteristic DRP of a mosaic pattern shows low cell density at close by distances and a density plateau at a threshold distance (indicating spacing regularity). Low density around the reference cell indicates a zone of exclusion that is expected with regular mosaicism between neighboring cells of a similar subtype (Bleckert et al., 2014). In contrast, a clustered pattern exhibits high densities at nearby distances, followed by an exponential decrease in density with increasing eccentricity from the reference point.

Ripley's L-function determines whether a population distribution deviates from spatial homogeneity, and categorizes the pattern as dispersed, clustered, or random (Ripley, 1988). Complete spatial randomness is calculated using a random Poisson distribution (Besag, 1977). L-function values greater than the expected distribution under complete spatial randomness indicates clustering; L-function values lower than the expected distribution suggests dispersion.

Applying these objective spatial analytic methods to RGC transplantation studies informs donor RGC topographic distribution and facilitates comparative analyses across experimental studies. Building topographic maps of donor RGCs provides crucial information regarding transplantation efficiency and assessing progress in achieving retinotopic coverage on host tissues.

1. Quantify donor cell survival from the tiled wholemount images.

Note: In the method described below, donor RGCs are discriminated based on overlapping tiled confocal images of both intracellular (tdTomato) and nuclear (human nuclei antigen/HuNu) immunofluorescent markers. In our human stem cell derived donor RGCs, tdTomato is expressed in the RGC-specific *BRN3B* locus. Its expression is specific to differentiated RGCs (Figure 3A). We additionally immunolabel for a human-specific nuclear marker (HuNu, Figure 3B), and overlap the two immunofluorescent signals to accurately account for clustered donor cells (Figures 3C and 3C') and rule out potential material transfer as a theoretical confound which could lead to artifactual identification of host neurons as donor-derived (Pearson et al., 2016; Santos-Ferreira et al., 2016; Singh et al., 2016). In the example, the HuNu

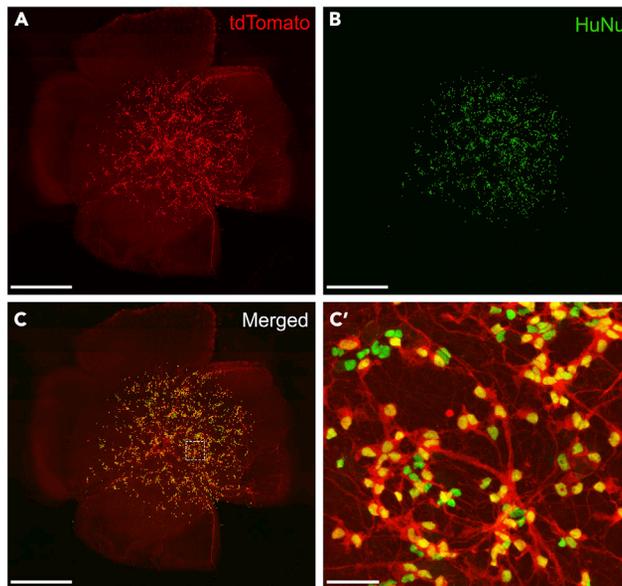


Figure 3. Immunohistochemistry of wholemount tiled confocal images of the retina

(A) Immunolabeling for tdTomato.

(B) Immunolabeling for human nuclei (HuNu).

(C and C') (C) Merged image to discriminate individual cells in high density regions (C'). Scale bars, 1 mm in (A–C), 50 μm in (C').

immunolabel is also necessary to exclude $\text{HuNu}^+\text{RFP}^-$ cells that are human stem cell derived but undifferentiated.

- a. Save each tiled z-stack acquired from [step-by-step method details](#) step 23 as an individual TIFF file.
 - b. Merge all the z-stacks into a single image using Photoshop or ImageJ.
 - c. Use the user-preferred manual, semi-automated, or automated method for quantifying donor cell survival and record in [Table 1](#).
2. Compute the nearest neighbor distance (NND) of donor cells ([Figure 4](#)).

Note: Use ImageJ to scale the wholemount image in microns (Analyze -> Set Scale...). Failure to set scale will result in coordinates and distances reported in pixels rather than micrometers.

- a. Generate spatial positions of donor cells across the wholemount tiled confocal image either manually with the “multi-point tool” or using semi-automatic particle detection methods in ImageJ ([Figure 4A](#)).
- b. Save the detected points as an .ROI file (File -> Save As -> Selection...).
- c. Measure the X,Y coordinates in ImageJ (Analyze -> Measure) ([Figure 4B](#)).
- d. Use the NND plugin (Plugin -> Analyze -> NND) ([Mao, 2016](#)) to obtain the NND for each measured point ([Figure 4C](#)).
- e. Copy the NND values into an Excel spreadsheet ([Figure 4D](#)).
- f. Create a Pivot Table from these values. Summarize the data by “Count” and show the data as “% of Column Total” ([Figure 4E](#)).
- g. Group the values in the newly created Pivot Table by setting the Starting point at “0” and bin size at “10” ([Figure 4F](#)).

Table 1. Example of data on RGC survival and integration

Explant ID	Treatment	Dissection notes	RGC survival	RGCs with dendrites in the neural retina
#001	Pronase	Good dissection	1965	15

- h. The grouped NND data for each retina (Figure 4G) can then be plotted as a histogram showing binned NND on the X-axis and frequencies of each NND size on the Y-axis (Figure 4H).
3. Density recovery profile (DRP).
- Copy the X and Y coordinates from the Results window from step 2b into Excel and save as a .CSV file.
 - Use the following codes in R to analyze the DRP for the positional values in the .CSV file.

```
> install.packages("sjedrp") # If not already
installed
> require(sjedrp)
> data = read.csv(`/users/destination/Results.csv`,
sep=',', header=FALSE)
> x = data[,1]
> y = data[,2]
> drp = autodrp(x, y, nbins=20, r=10, a=NULL)
> plot(drp, scale=1e6)
```

- Install the R package "sjedrp" and run the scripts.
 - Retrieve the data file by replacing the file name and location with the user defined values.
 - Define the x and y values by the coordinates in columns 1 and 2 of the .CSV file, respectively.
 - Define the DRP parameters. "nbins" sets the number of bins analyzed; "r" sets the size of the bin in microns.
 - DRP histogram (Figure 5) plots the X-axis in microns and Y-axis in cells/mm².
4. Ripley's L function and Density plot (Figures 6 and 7).
- Load the Spatstat package in R.
 - Use the following codes to analyze clustering functions for the .CSV file.

```
> install.packages("spatstat") # If not already
installed
> library(spatstat)
> data = read.csv(`/users/destination/Results.csv`,
sep=',', header=FALSE) > Lfn <- ppp(data[,1], data[,2], c(0, 5000), c(0, 5000))
> env.Lfn <- envelope(Lfn, fun=Lest, nsim=99,
rank=1, global=TRUE)
> plot(env.Lfn, xlim=c(0,100))
```

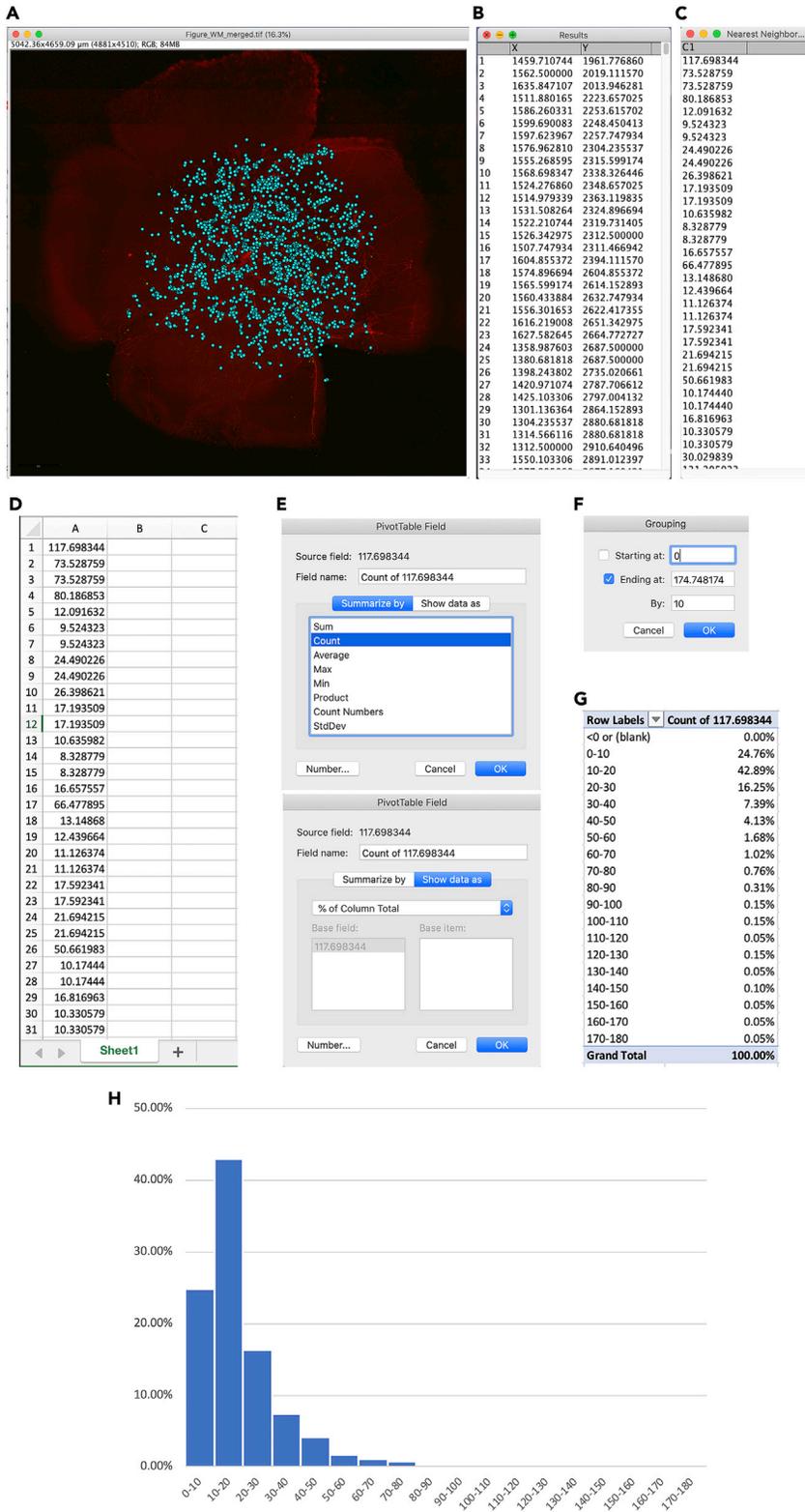


Figure 4. Derivation of NND from donor RGC topography

(A) Spatial position of donor RGCs marked on wholemount recipient retina.

(B) ImageJ readout of donor RGC coordinates.

Figure 4. Continued

- (C) ImageJ readout of NND derived from donor RGC coordinates.
- (D) Excel copy of the NND values.
- (E) Excel Pivot Table "Field" settings.
- (F) Excel Pivot Table "Grouping" settings.
- (G) Excel Pivot Table showing NND values binned at 10 μm intervals and the frequency of each bin.
- (H) Histogram displaying the grouped data from the Pivot Table.

- c. Use the following codes to generate a density heatmap of the sample population from the .CSV file.

```
> plot(Lfn, cex=0.5)
> plot(density(Lfn, 50))
```

- 5. Quantify neurite integration using IMARIS (Figure 8).
 - a. Load high-magnification, high-resolution confocal z-stacks (step-by-step method details step 24) in IMARIS (Oxford Instruments, Zurich, Switzerland).
 - b. Use the "Filament" tool (Figure 9A) to semi-manually trace the entire neurite arbor from an integrated cell of interest.

Note: Only display the channel corresponding to the donor cell fluorescent label (i.e., red/tdTomato channel in our example; Figures 8A and 8A') while tracing.

- c. To quantify neurite length, first toggle the "Mouse Selects" option to "Segment" and click on the neurite of interest (indicated by the arrow in Figure 9B). Then select the "Statistics" tab and choose "Dendrite Length" from the menu option (Figure 9C).
- d. The DAPI channel marks three distinct nucleated retinal layers (RGCL, INL, ONL). The IPL is the non-nucleated layer between the RGCL and the INL. Segment each neurite at retinal layer junctions demarcated by the DAPI labeling and measure the total neurite length within each retinal layer.

Note: This is best achieved by rotating the three-dimensional z-stack to an orthogonal view, such that the vertical profile is projected on the screen (Figure 9C).

Note: While the goal of RGC replacement is to form mature dendrites restricted to the IPL, ectopic neurite growth into the INL or ONL is not uncommon in experimental settings. Hence, documentation of the relative neurite distribution within each retinal layer becomes important toward optimizing donor neurite lamination.

- e. Use the "Point" option under "Mouse Selects" (Figure 9D) to choose the point in the neurite that lies at the junction of two retinal layers (arrow in Figure 9D). Delete this point to segregate neurite segments within a retinal layer.
- f. Toggle back to the "Segment" option in "Mouse Selects" menu and select on the intralaminar neurite segment (Figure 9E). Use the same method as that in step 5b to measure the intralaminar neurite length. Record the sum of all neurite lengths from each retinal layer in Table 2.
- g. Tally the number of intralaminar neurite segments in each retinal layer and record the values in Table 2.
- h. To derive additional measures from the entire neurite tracing, select "Filament" option under "Mouse Selects", and choose the menu options "Number of Branch Points" or "Number of Terminal Points" in the "Statistics" tab. These metrics provide further quantitative description of the overall neurite complexity.

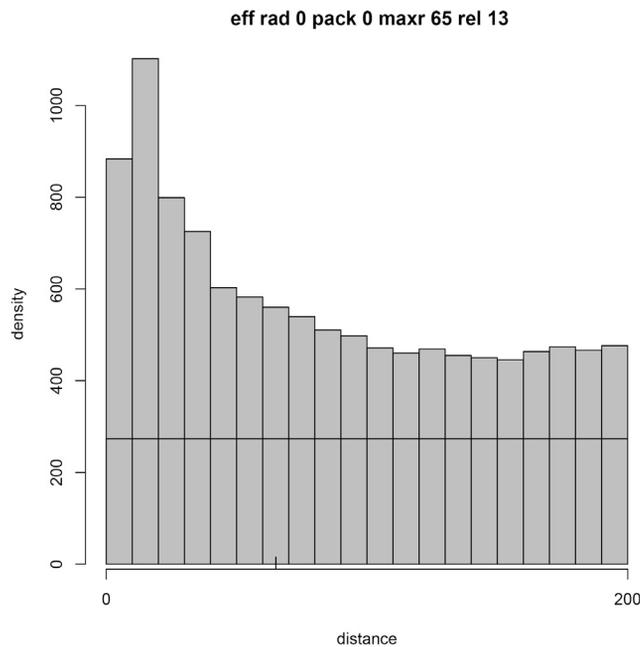


Figure 5. DRP derived from the sample data

The peak at near distances followed by an exponential decay in density indicates clustering within this sample population. X-axis shows distance in μm ; Y-axis shows density in cells/mm^2 .

6. Quantify neurite density and perform Sholl analysis using ImageJ (Figure 10).
 - a. Save the neurite tracing from step 5b as a flatmount projection in a .TIFF file. Open the .TIFF file with ImageJ. Ensure the image is properly scaled.
 - b. Use the default settings in the Sholl analysis plugin (Ferreira et al., 2014) to determine the number of neurite intersections at increasing eccentricities from the soma (Figure 10A).
 - c. The plugin automatically generates a numeric table (Figure 10B) and a histogram (Figure 10C) of the Sholl analysis.

Note: The steps outlined above perform the Sholl analysis on a 2-dimensional projection of the neurite tracing. A 3-dimensional Sholl analysis may be performed in IMARIS as an alternative approach.

LIMITATIONS

The greatest obstacle associated with *ex vivo* RGC transplantation using organotypic retinal explant is maintaining tissue health and normal physiologic function throughout the culture period. Despite optimizing the culture conditions, organotypic retinal explant tissues undergo progressive neurodegeneration, and typically cannot be cultured for more than 1–2 weeks, though adding growth factors or neuroprotective agents may prolong survival. This significantly constrains the utility of assessing long-term (>2 weeks) donor engraftment and functional connectivity with the recipient. Therefore, while this protocol allows for the visualization of donor cellular dynamics in the short-term, the influences of *ex vivo* environmental factors compared to an *in vivo* counterpart may reveal differences in donor cell behavior.

TROUBLESHOOTING

Problem 1

Retinal tissue appears grossly unhealthy at early culture time points, and photoreceptor survival is especially poor (step-by-step method details steps 12 and 13).

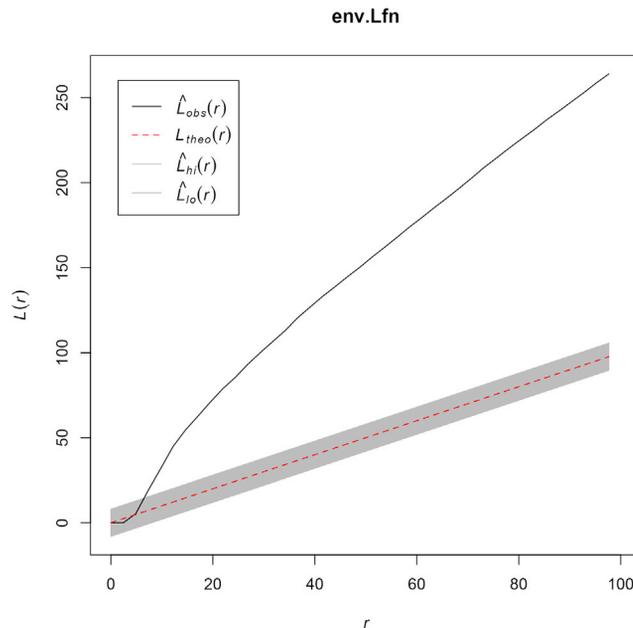


Figure 6. L-function derived from the sample data

The L-function of the sample population is plotted as a black line, $L_{obs}(r)$. The L-function of the expected distribution under complete spatial randomness, $L_{theo}(r)$, is indicated by the red dotted line with 95% confidence interval in gray. The observed L-function in this example lies above the expected values under complete spatial randomness and indicates clustering of donor RGCs. r (radius from cell, μm).

Potential solution

Degenerative retinal phenotypes may be unexpectedly observed before or after enucleation due to previously unrecognized mutations in the *Crb1* gene (the so called retinal degeneration (rd)-8 mutation) in some strains bred on a C57BL/6N background (Mattapallil et al., 2012). It is important to exclude such animals from the experiment. If degenerative phenotypes are observed in multiple littermates, breeders may carry recessive mutations and should be replaced by new breeders. In addition, handling the retinal tissue too roughly during dissection or taking too long with dissection can also undermine host tissue survival.

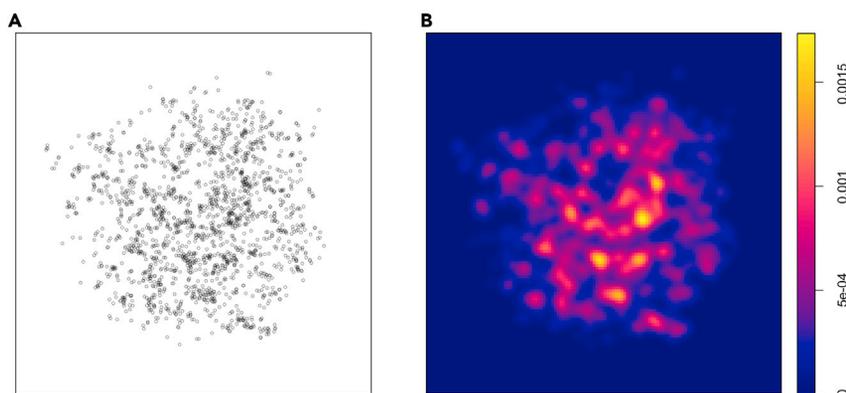


Figure 7. Ripley's L function

(A and B) Sample data illustrated by coordinates (A) as well as by local density (B), where yellow and purple correspond to high and low densities, respectively.

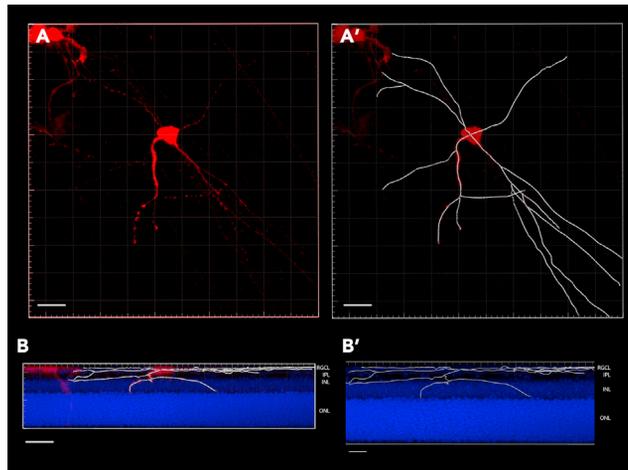


Figure 8. IMARIS three-dimensional rendering of high-resolution confocal z stacks

(A and A') Z plane projection of an integrated tdTomato⁺ donor RGC (A) and its neurite tracing (A').

(B and B') Profile view of the confocal z stacks showing an overlay of tdTomato (red), DAPI (blue), and neurite tracing (B). Neurite tracing overlaid with DAPI (B') enables neurite length quantification within each retinal layer (RGCL, IPL, INL, ONL). Scale bars: 20 μm (A, A', and B); 10 μm (B').

Problem 2

Organotypic retinal explants can sometimes detach from the culture insert at various stages of the culture ([step-by-step method details](#) steps 10–13).

Potential solution

The retinal explant may detach from the underlying culture insert as a result of insufficient membrane adherence. Blotting the undersurface of the membrane to create adhesion is critical prior to introduction fluid onto the retinal explant. Explant tissue detachment can also be exacerbated by enzymatic digestion. Verify the enzyme concentration and limit the digestion to 1 h. Pipetting Inhibitor Solution or BSS onto the explant surface can also cause tissue disruption, thus avoid vigorous pipetting when working with explant tissues. When the explant is partially detached, carefully flatten the lifted edge with a bent vascular probe and blot the membrane undersurface dry before proceeding onto the next step. If the entire explant tissue is detached, ensure the inner retina faces up before reattaching the tissue by blotting the membrane.

Problem 3

Organotypic retinal explants can become unhealthy or die during culturing ([step-by-step method details](#) steps 10–13).

Potential solution

Explants may exhibit poor survival during the culture period for several reasons, including non-physiologic pH of the culture media, irregular temperature regulation, and prolonged or traumatic enzymatic digestion. The Retinal Explant Media is buffered by bicarbonate and requires incubation in 5% CO₂ to maintain physiologic pH. Accordingly, limit the amount of time explants are outside of the incubator to prevent media alkalization and exchange media at least every other day to prevent acidification. Timing Pronase treatment to 1 h is critical, as prolonged enzymatic digestion can cause tissue damage.

Problem 4

Organotypic retinal explants can become contaminated during culturing ([step-by-step method details](#) steps 10–13).

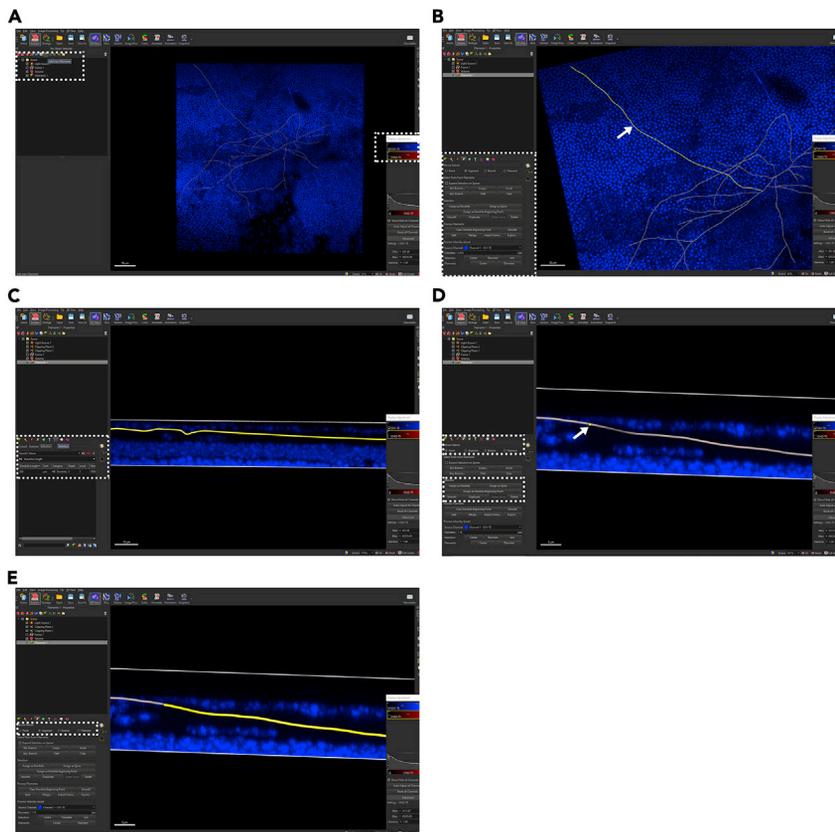


Figure 9. Donor RGC neurite tracing

- (A) Traced neurite filaments with the overlaid DAPI labeling of host retinal cells.
 (B) Select a single neurite segment (arrow).
 (C) Profile view of a selected neurite segment and its measured length.
 (D) Select a single point (arrow) on the neurite filament at the border of adjacent retinal layers. Delete the selected point to separate the neurite into components in each retinal layer.
 (E) Select the neurite component within retinal layers and measure the segment length within a specific retinal layer.

Potential solution

Despite the fact that supplemental antibiotics are added to the culture media, maintaining sterility is critical, especially for primary tissue cultures. This includes sterilizing surgical instruments, dissection hood, dissection microscope, and filtering buffer solutions. Fungal contamination is the most common (and we typically do not include antimycotics in the culture media), and easily spreads among culture inserts and sometimes culture plates. Contamination compromises experimental data integrity. If found, abandon the plate immediately and restart the experiment, sterilize all equipment, and decontaminate the incubator.

Problem 5

Difficulty tracing neurites in IMARIS ([step-by-step method details](#) step 24; Analysis step 5).

Table 2. Example of data from one integrated RGCs

Dendritic area	Neurite density	Total neurite length	Neurite length in RGCL	Neurite length in IPL	Neurite length in INL	Neurite length in ONL	Total neurite segments	Neurite segments in RGCL	Neurite segments in IPL	Neurite segments in INL	Neurite segments in ONL
894.3 μm^2	82,809 $\mu\text{m}/\text{mm}^2$	1,342 μm	1,034 μm	180 μm	308 μm	0 μm	23	20	5	3	0

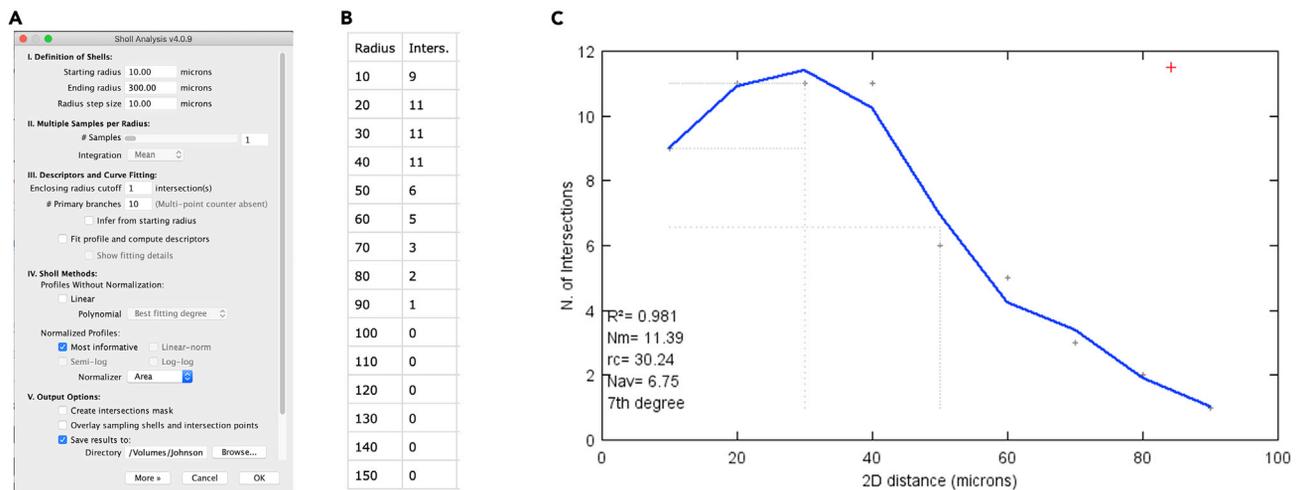


Figure 10. Sholl analysis of donor RGC dendritic arbors

(A) Perform Sholl analysis using the default settings in the ImageJ Plugin.

(B) Numeric printout of the number of neurite intersections at increasing eccentricities from the cell body.

(C) A graphical demonstration of the Sholl analysis for the example neuron.

Potential solution

The automatic filament detection in IMARIS relies on excellent image quality, which is dependent on optimized immunohistochemistry and fluorescent image acquisition. Avoid overexposure and reduce background noise when imaging the tissues. Adjustments to acquisition settings, such as increasing pixel resolution to 2048 × 2048, increasing averaging number to 2 or 4, and decreasing acquisition speed can improve the final image quality. However, be mindful of the balance between higher image quality and increased acquisition time and risk of photobleaching.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Thomas V Johnson (johnson@jhmi.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The data used in this study are available from the lead author upon request. The codes used in analyses are available from cited sources.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101328>.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.V.J.; methodology, K.Y.Z. and T.V.J.; software, K.Y.Z.; validation, K.Y.Z.; formal analysis, K.Y.Z. and T.V.J.; writing – original draft, K.Y.Z. and T.V.J.; writing – review & editing, K.Y.Z. and T.V.J.; funding acquisition, T.V.J.; resources, T.V.J.; supervision, T.V.J.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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