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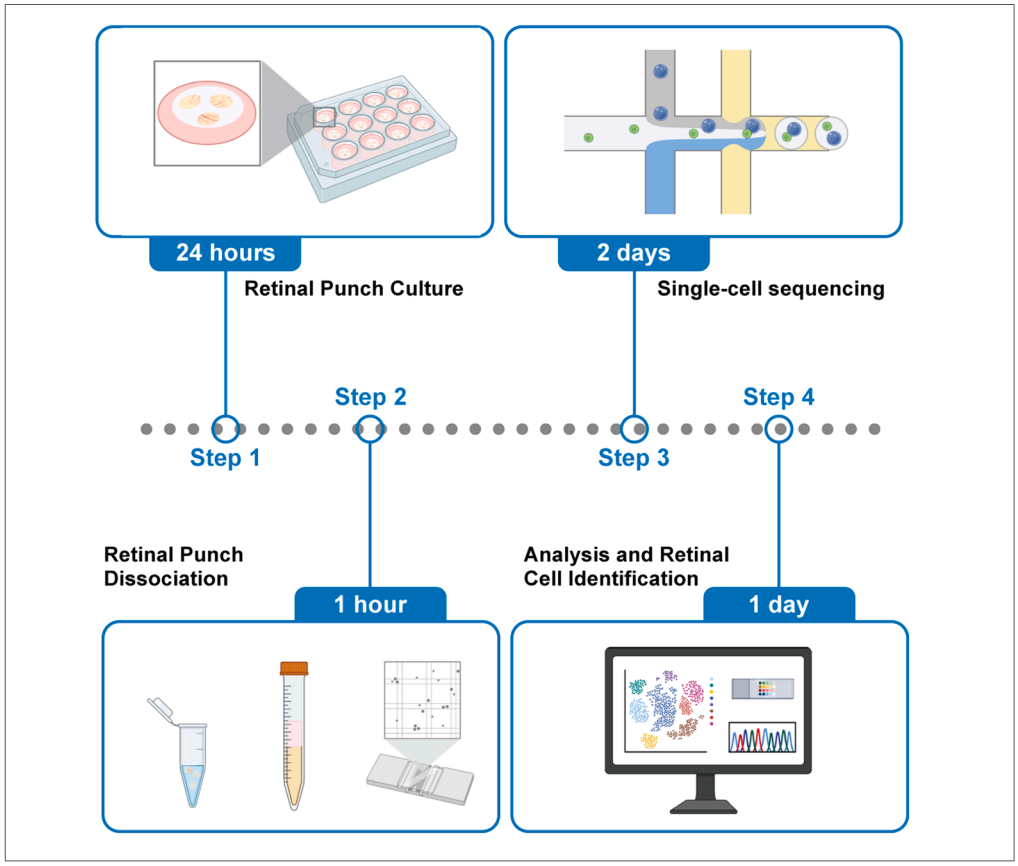
Protocol for retinal stress culture and single-cell analysis



The retina is a dynamic neural tissue that lines the posterior of the eye cup and transfers visual inputs from the world to our brain for processing. However, exposure to stress, injury, and disease can disrupt this important function. Here, we present a protocol for the dissection and culture of retina in various physiologically relevant stress conditions. Furthermore, we describe how to assess the retina for stress and cell-type-specific responses.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for physiological retinal stress conditions

Adaptable protocol for retinal culture and downstream analysis

Guidance on single-cell analysis and retinal cell type identification

Norrie et al., STAR Protocols 7, 104271
March 20, 2026
https://doi.org/10.1016/j.xpro.2025.104271

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Protocol for retinal stress culture and single-cell analysis

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https://doi.org/10.1016/j.xpro.2025.104271

SUMMARY

The retina is a dynamic neural tissue that lines the posterior of the eye cup and transfers visual inputs from the world to our brain for processing. However, exposure to stress, injury, and disease can disrupt this important function. Here, we present a protocol for the dissection and culture of retina in various physiologically relevant stress conditions. Furthermore, we describe how to assess the retina for stress and cell-type-specific responses.

For complete details on the use and execution of this protocol, please refer to Norrie et al.[1]

BEFORE YOU BEGIN

The retina is exposed to daily stressors which can disrupt the precise dynamic interplay between the retinal cell types. Each retinal cell type has a specific function within the tissue and a unique chromatin architecture that enables each cell type to respond to stressors differently. The protocol below describes how to isolate and culture retina in stress conditions to determine cell type specific gene expression changes. While 15 unique physiologically relevant stress conditions are discussed in this protocol other culture conditions can be adapted to this protocol. These stresses were chosen to try to mimic conditions that retina could encounter including neural depolarization (70 μM ouabain/80 mM KCl), excitotoxicity (1 mM glutamate), alkalosis (pH 7.7) and acidosis (pH 7.15), hypoxia (2.3% O_2), hyperoxia (40% O_2), hyperthermia (40°C), and hypothermia (37°C).[1] Additional stressors include methanol poisoning (8 μM formic acid), gram-negative bacterial infection (LPS), inflammation (IFN-γ), ocular candidiasis (*Candida albicans*), neuroretinitis (*Bartonella henselae*), hyperglycemia in diabetes (20 mM glucose).[1]

Innovation

Retinal stress can occur in several different ways, including cellular stress, injury, and disease, all of which can all cause significant damage to this critical tissue. This protocol enables investigation into all types of retinal stress, as well as the ability to interrogate the response of each cell type individually. By following the guided analysis and retinal cell type calling using the curated retinal cell type gene list in this protocol, each retinal cell type can be identified in an unbiased manner. Once identified, cell-type and stress-specific changes can be examined, further explored, and validated. Using this protocol, we recently reported a unique cellular program within the Muller glia that signaled with the immune system.[1] Furthermore, this protocol can be easily adapted to new and varied retinal stress culture conditions.

⚠ CRITICAL: This protocol explains how to complete a retinal stress culture and test the stress levels via RNA extraction and qPCR to ensure a proper culture. Only after confirmation of stress gene expression is it suggested to repeat the retinal stress cultures for dissociation and single-cell sequencing.

1. Determine which stressor(s) that will be tested.

   Note: 15 unique stressors are described in this protocol (see materials and equipment) each needing their own reagents or incubator set ups. All stressors are cultured individually and do not rely on other cultures. It is also not necessary to do all cultures at the same time, but it is recommended to run always a control culture each time. Complete the number of cultures you are comfortable preforming at one time.

2. Obtain the reagents and identify the incubator with the proper environments for each stress.
   a. Temperature conditions require a separate incubator capable of incubating at 33°C or 40°C.
   b. Hypo and hyperoxia conditions require a separate incubator capable of adjusting the O_2 levels to 2% or 40%.
   c. *B. Henselae* cultures and *C. Albicans* require pre-culture before retinal culture.
3. Based on how many stress conditions and controls will be cultured, determine the number of mice needed for the experiment. It is recommended that each condition get 4 or more retinal punches, and it is possible to obtain 4 1.5 mm punches from each retina.

Institutional permissions

All animal procedures and protocols were approved by the St. Jude Laboratory Animal Care and Use Committee under protocol number 393-100500. All studies conform to federal and local regulatory standards. Mice were housed on ventilated racks on a standard 12 h light–dark cycle. Before conducting any murine experiments get all necessary approval and training from the institution where the experiments will be conducted.

Prepare retinal explant media

Timing: 30 min to 2 h

This section describes how to prepare the retinal explant media (REM) and 4% BSA/REM. REM is the base media for all stress conditions and used as the media in which the retina is dissected.

4. Prepare 250 mL Retinal Explant Media (REM) in a sterile hood by combining ingredients in the top of a bottle top vacuum filter system.

Retinal Explant Media (REM)		
Reagent	Final concentration	Amount
Hams F-12; DMEM	N/A	220 mL
FBS	10%	25 mL
HEPES	1%	2.5 mL
Insulin	0.05%	125 µL
Penicillin-Streptomycin-Glutamine	1%	2.5 mL

5. Filter REM through the vacuum system.
6. Prepare 5 mL 4% BSA/REM per sample.
   a. No additional filtering necessary.
7. Store extra REM at 4°C for 1 month.
8. Store extra 4% BSA/REM at 4°C for 1 month.

   Note: BSA will go into solution in the REM best at 4°C.

Preparation of *C. albicans* and *B. henselae*

Timing: 2 days

Here we describe steps for creating *C. albicans* and *B. henselae* agar plates and broth. The agar plates can be streaked up to a month in advance. Furthermore, individual colonies will need to be picked and incubated the day before retinal culture to all time for growth.

Optional: These steps are only necessary if using *B. henselae* or *C. albicans* as a retinal stress.

9. Create agar plates and culture broths needed according to the recipes below.

Media Recipes

Media	Stressor	Amount broth or agar	Water
Tryptic Soy Agar	*B. henselae*	40 g	1000 mL
Tryptic Soy Broth	*B. henselae*	30 g	1000 mL
YM Agar	*C. albicans*	41 g	1000 mL
YM Broth	*C. albicans*	21 g	1000 mL

10. Autoclave the broth and agar at 121°C for 15 minutes.
    a. Cool slightly then pour agar to make plates.
    b. Allow plates to solidify and dry at least 12 hours.
    c. Store plates upside-down at 4°C.
    d. Store broth at 4°C.
11. Streak *B. henselae* or *C. albicans* stabs on dry agar (Tryptic Soy and YM respectively).
12. Incubate *B. henselae* at 37°C 16–24 hours or until visible colonies form.
13. Incubate *C. albicans* at 20°C–22°C for 2 hours then 33°C for 16–24 hours until visible colonies form.

Note: Colony formations should take approximately 24 h.

14. Store plates with colonies at 4°C for up to 1 month.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Bartonella henselae	ATCC	49793
Candida albicans	ATCC	18804
Chemicals, peptides, and recombinant proteins		
IFNy	Sigma	SRP3058
Glucose	Sigma	G6152
KCl	Sigma	P5405
Glutamate	Sigma	G8540
LPS	Sigma	L2630
Formic acid	MP Biomedicals	151162
Ouabain	Sigma	O3125
KCl	Sigma	P5405

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
HCl	Sigma	H9892
NaOH	Fluka	72068
Tryptic Soy Agar	BD	BD 236950
Tryptic Soy Broth	BD	BD 211825
YM Agar	BD	BD 271210
YM Broth	BD	BD 271120
DMEM/F12	Gibco	21041–025
FBS	Biowest	S1620
HEPES	Sigma	H0887
Insulin	Thermo Fisher Scientific	12585014
Penicillin-Streptomycin-Glutamine	Gibco	10378016
BSA	Sigma	A7906-50g
Trizol	Invitrogen	15596026
Glycogen	Roche	10901393001
EtOH	Fisher	BP2818-4
Chloroform	Invitrogen	15593031
Isopropanol	Fisher	A416-2
Papain	Worthington	LS003119
L-Cysteine	Sigma	C7352-100G
0.5 M EDTA	Gibco	15575–038
DNase	Sigma	D4627-40KU
Critical commercial assays		
Chromium Next GEM Single Cell 3' Kit v.3.1	10× Genomics	PN-1000268
RNA-to-cDNA Kit	Applied Biosystems	437406
SyBR Green Master Mix(2×)	Applied Biosystems	4472908
D1000 High Sensitivity Reagents	Agilent	5067–5585
D1000 High Sensitivity Screentape	Agilent	5067–5584
TruSeq Stranded Total RNA Library Prep Kit	Illumina	20020597
Deposited data		
RNA-sequencing data	This paper	GSE264571
Single Cell Data	This paper	GSE272074
Oligonucleotides		
Cxcl2_F AGTGAACTGCGCTGTCAATG Cxcl2_R TTCAGGGTCAAGGCAAACTT	PMC2707360	Cxcl2
Il6_F CCGGAGAGGAGACTTCACAG Il6_R TTCTGCAAGTGCATCATCGT	This paper	Il6
Ccl5_F CTGCTGCTTTGCCTACCTCT Ccl5_R ACACACTTGGCGGTTCCTT	PMC5501689	Ccl5
Gapdh_F CTCCACTCACGGCAAATTCA Gapdh_R CGCTCCTGGAAGATGGTGAT	This paper	Gapdh
Software and algorithms		
R version 4.4.1	The R Project	http://r-project.org
Seurat_5.3.0	Stuart et al.[2]	https://doi.org/10.1016/j.cell.2019.05.031
dittoSeq_1.16.0	Bunis et al.[3]	https://doi.org/10.18129/B9.bioc.dittoSeq
ggplot2_3.5.2	Wickman[4]	https://doi.org/10.32614/CRAN.package.ggplot2
Cellranger	10× Genomics	https://www.10xgenomics.com/
Other		
Vacuum FilterSystem	Corning	430767
12-well plate	Corning	3512
1.5 mm Biopsy punch	Integra	33-31AP/25
16 g needle	BD	305197
40 μM Cell Strainer	Falcon	352340
6 cm Culture dish	Falcon	353002
Siliconized tubes	Fisherbrand	02-681-320
Forceps Dumont #55	FST	11255–20

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Forceps Dumont #3	FST	11293–00
Nuclepore 1.0 μM membrane	Whatman	11410
scRNA-seq Analysis	This paper	https://github.com/jackienorrie/retinalstress

MATERIALS AND EQUIPMENT

In these steps control and stress media is prepared using the table below. Only prepared desired stress media. A single culture well requires 2 mL of media.

Sample Preparations				
Sample	Temp (°C)	Oxygen (%)	Treatment	Stress media recipes (10 mL)
B. Henselae	37	20	100 CFU/mL	1000 CFU + 10 mL REM
33°C	33	20	Incubator set to 33°C	REM
40°C	40	20	Incubator set to 40°C	REM
C. Albicans	37	20	100 CFU/mL	1000 CFU + 10 mL REM
control culture	37	20	None	REM
Glucose	37	20	20 mM glucose	36 mg glucose + 10 mL REM
Glutamate	37	20	1 mM glutamic acid	1.47 mg glutamic acid + 10 mL REM
IFN	37	20	100 U/mL IFNγ	10 μL IFNγ + 10 mL REM
KCl	37	20	80 mM KCl	59.6 mg + 10 mL REM
LPS	37	20	0.2 μg/mL LPS	2 uL (1 mg/mL LPS Stock) + 10 mL REM
MeOH	37	20	8 mM formic acid	3.68 mg + 10 mL REM
2% O2	37	2.3	Incubator set to 2.3% O_2	REM
40% O2	37	40	Incubator set to 40% O_2	REM
Ouabain	37	20	70 mM ouabain	409 mg + 10 mL REM

Optional: If using *B. henselae* or *C. albicans*, the day before the experiment, pick single colonies and incubate in a fresh liquid culture (described in step 9) 16–24 h until turbid and calculate using CFUs using a Veristar.

⚠ CRITICAL: *B. henselae* requires BLS2 safety considerations.

STEP-BY-STEP METHOD DETAILS

Prepare culture dishes

Timing: 10 min

In these steps, 12 well plate(s) are prepared with media and Whatman paper for pre-incubation in preparation for retinal punch culture.

1. Prepare necessary media as explained above and aliquot 2 mL per well of a 12 well plate for each well needed.

⚠ CRITICAL: The number of retinal punches needed per sample will be determined by the number of cells that can be obtained after dissociation. Plan for 3–4 punches per well and approximately 3 retinal punches for qPCR and 6 retinal punches per condition for one single cell run. If there is not enough material for qPCR or single cell more punches can be used.

Note: Conditions can be combined on a single 12 well plate unless samples need to go into separate incubators.

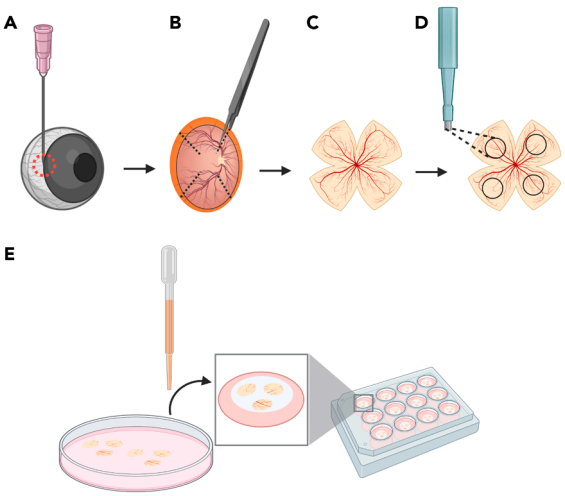

Figure 1. Retinal Dissection for Retinal Punch Culture

(A) Pierce the eye with a needle at the edge of the cornea and sclera. Remove the retina and (B) using forceps make 4 cuts from the edge of the retina halfway to the middle.

(C) Splay the retina out so there are 4 equal sections visible and (D) using a 1.5 mm biopsy punch, punch 4 holes in the retina.

(E) Once the punches are created use a transfer pipette to move 3–4 punches to the membrane of each well of a prepared 12 well plate. Created in BioRender.

2. Using forceps carefully remove the blue paper from each side of the Whatman paper.
   a. Identify the glossy side.
   b. Place the Whatman paper on the surface of the media glossy side up.
3. Put the 12 well dish(es) in the incubator at 37°C until the dissections are complete.

Retinal dissections and retinal punch cultures

Timing: 30 min per condition

Here, we describe the steps in a retinal dissection to prepare and create retinal punches and begin a retinal punch culture.

4. Euthanize mice according to IAUCUC and institutional protocols and policies.
5. Enucleate the eyes with forceps (Dumont #3).

Note: The forceps used are listed, but others can be substituted if preferred.

6. Place eye in 6 cm dish filled halfway with warmed REM under dissection scope.
7. While holding the eye steady with forceps, use a 16 g needle to pierce the eye at the edge of the cornea and sclera, below the limbus so as not to pierce the retina (Figure 1A).
8. Place one blade of each of 2 forceps (Dumont #3 and #55) in the hole created by the needle, pinch down gently with one forcep and hold the sclera steady and with the second forcep grab and pull the cornea away from the sclera.

Note: The cornea will pull away from the sclera with minimal force.

9. Discard the cornea and remove the lens from the eyecup in the sclera.
10. Hold on the posterior of the retina by the optic nerve and use forceps to gentle pull and tease the retina from the eyecup.
11. Clean any remaining tissue from the retina, specifically from the inner area of the retinal cup, and orient the retina ganglion layer up (photoreceptor layer down).

Note: RPE does not have to be removed.

12. Create 4 cuts using the thin Dumont #55 forceps from the outer perimeter of the retina halfway to the center (Figure 1B).
13. Fan out the retina (Figure 1C) so there are 4 approximately equal areas.
14. Use a 1.5 mm biopsy punch to create 4 retinal punches per retina, one in each of the 4 sections created (Figure 1D).
15. Remove the 12 well dish(es) containing the control and stress media from the incubator.
16. Use a transfer pipette to carefully add 3 retinal punches to each Whatman paper with minimal amounts of media so the paper doesn't sink (Figure 1E).

Note: It does not matter what orientation the retina is in.

17. Remove excess liquid.
18. Add 50 µL of media from the 2 mL in the well (to ensure the retina gets stress media) to cover the retinal punches.
19. Return to the incubator for 24 hours.
20. Repeat the addition of 50 µL of media from the well once between 8–16 hours later so the retina does not dry out. troubleshooting 1.
21. At the end of the 24 hour incubation proceed to RNA extraction with trizol if completing qPCR to test stress conditions, or dissociation and preparation of single-cell suspension if conditions have been tested and cultures are ready for single cell sequencing.

RNA extraction with Trizol

Timing: 4 h

In this step RNA is extracted from cultured retinal punches. Complete this step only on the trial cultures to see if the incubation caused significant stress, and that high quality RNA can be obtained before moving forward with single cell sequencing.

⚠ CRITICAL: Keep everything on ice during Trizol extraction to prevent RNA degradation. Limit the time that the dissociated retinal punches are sitting in Trizol on ice. Proceed to extraction as quickly as possible.

22. Remove at least 3 punches from 12 well plate after 24-hour incubation using a transfer pipette into a 1.5 mL siliconized Eppendorf tube.
23. Remove excess media and add 800 µL Trizol.
24. Pipette up and down until tissue is completely dissociated.
25. Add 0.2 mL of chloroform, secure the cap and vortex for 15 seconds.
26. Incubate at RT for 2–3 minutes.
27. Centrifuge for 15 min at 12000 g at 4°C.
28. Carefully remove the tube and tilt at a 45-degree angle without disturbing the liquid interface.
29. Pipette the clear upper aqueous phase into a new 1.5 mL tube.

⚠ CRITICAL: Do not pipette any pink liquid or white interphase material. If you do, pipette everything back into the tube and repeat the centrifugation step.

30. Add 500 uL isopropanol and 1 µL RNAse free glycogen to the aqueous phase.
31. Incubate for 10 min at 20°C–22°C.
32. Centrifuge for 10 min at 12000 g at 4°C.
33. Carefully remove and discard the supernatant.
34. Rinse the pellet with 1 mL 75% EtOH.
35. Centrifuge for 5 min at 7500 g.
36. Repeat steps 32–34 for a total of 2 washes.

37. After removing the EtOH, quick spin the pellet and remove as much excess EtOH as possible.
38. Dry pellet until it begins to turn clear.
39. Resuspend the pellet in 10 μL H2O by pipetting up and down.
40. Quantify RNA concentration using 1 μL on a nanodrop, troubleshooting 2.
41. Store RNA at −20°C and avoid excessive freeze/thaw cycles.

qPCR for markers of retinal stress

Timing: 3 h

In these steps, qPCR is used to detect known markers of retinal stress to ensure the retina had been effectively stressed in the culture.

42. Create cDNA from 150 ng of RNA using the using a High-Capacity RNA-to-cDNA Kit.

RNA-to-cDNA reaction master mix	
Reagent	Amount
2× RT Buffer Mix	10 μL
20× RT Enzyme Mix	2 uL
RNA	150 ng
Nuclease-Free H2O	to 20 μL

PCR conditions	
Temperature	Time
37°C	60 minutes
95°C	5 minutes
4°C	Hold

43. Set up qPCR Reaction using SYBR Select Master Mix.

qPCR SYBR Master Mix	
Reagent	Amount
SyBR Green Master Mix (2×)	5 μL
cDNA	1.5 μL
Primers (50 nM)	3 μL
Nuclease-Free H2O	10.5 μL

44. Run the following above master mix in at least duplicate qPCR reactions in a QuantStudio 3 or equivalent that has a Fast run mode using the following conditions.

qPCR cycling conditions		
Temperature	Time	Cycles
95°C	2:00	1
95°C	00:01	x40
60°C	00:30	

(Continued on next page)

Continued

Temperature	Time	Cycles
95°C	00:01	Melting Curve x1
60°C	00:20	
95°C	00:01	

45. Calculate the fold change using the ddCt method with the following steps.
    a. Calculate the average Ct of technical replicates.
    b. dCT = Average Ct Stress Gene – Average Ct GAPDH.
    c. ddCt = dCT treated sample – dCT untreated sample.
    d. Fold change = 2^{-ddCt}.
46. Fold changes greater than log 2-fold change is considered a significant stress, troubleshooting 3.

Dissociation and preparation of single-cell suspension

Timing: 2 h

In this section retinal punches will be dissociated and prepared for single cell sequencing. The same tissue used for RNA extraction and qPCR cannot be used for single cell sequencing, therefore a separate culture is needed for this step. It is recommended that only 4 conditions be processed at a time to limit the time from dissociation to 10× GEM (Gel Bead-In Emulsion) creation.

47. Thaw DNase I aliquot.
48. Obtain 4% BSA in REM prepared earlier.
    a. Aliquot 5 mL in a 15 mL conical per sample.
    b. Store on ice.
49. Prepare papain buffer 1 mM L-cysteine and 0.5 mM EDTA in PBS (−/−).
    a. To make 50 mL of papain buffer add 6 mg of L-cysteine and 50 μL of 0.5 M EDTA.
    b. Store at RT for up to 6 months.
50. Prepare activated papain buffer.
    a. Scaling as needed, add 40 U papain to 400 μL papain buffer from step 48 per sample.
    b. Incubate at 37°C for ∼10 minutes but not longer than 20 minutes as it will lose effectiveness.
51. Using a transfer pipette, move the retina punches to a 1.5 mL eppindorph (one condition per tube).
    a. Remove excess REM from the tube.
    b. Add 400 μL activated papain buffer to the tube (Figure 2A).
    c. Aggressively flick tube until punches have observable tears/damage.
    d. Incubate at 37°C.
    e. After 2–3 minutes continue to aggressively flick until retina punches no longer quickly sink to the bottom of the tube.
    f. Add 40 μL DNAse I.
    g. Continue to incubate and 37°C and aggressively flick ever 2–3 minutes until the majority of the retina punches float (Figure 2A).

Note: The dissociation is complete as soon as the punches float. At this point the cells have been dissociated but the extracellular matrix may still be visible as it floats at the top of the tube. Additional time dissociation will be detrimental to the cells.

52. Add a 40 uM cell strainer to the top of a 50 mL conical (Figure 2A).
    a. Add 1 mL PBS −/− to the dissociated retina for 1.4 mL total (400 μL activated papain + 1 mL PBS).

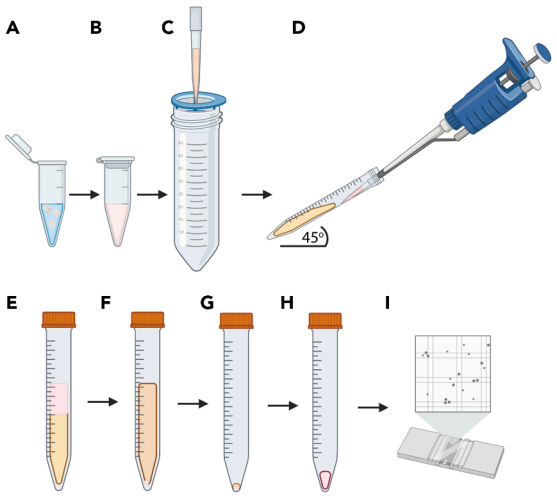

Figure 2. Retinal Dissociation for Single Cell Sequencing

(A) Add retinal punches to 400 μL prewarmed papain buffer containing 40 U of papain.
(B) Alternate between aggressively flicking the tube and incubating at 37°C until the tissue is dissociated and floats. Add DNase.
(C) Add 1 mL PBS to the dissociated tissue and strain through a 40 μM filter. Rinse with 2 mL PBS.
(D) Layer filtered dissociated tissue onto 5 mL of 4% BSA slowly at an angle of 45 degrees.
(E) The dissociated cells should form a layer on top of the BSA cushion.
(F) Centrifuge for 10 minutes at 500 g at 4°C.
(G) Remove supernatant and (H) resuspend in 100 μL of REM.
(I) Count cells. Created in BioRender.

b. Quickly push the dissociated retinal through the 40 uM filter.
c. Rinse filter with 1 mL PBS −/−.

53. Obtain a 15 mL conical with 4% BSA prepared (Figure 2B).
    a. Tilt the conical at a greater than 45 angle (Figure 2C).
    b. Slowly layer the filtered dissociated cells on top of the BSA cushion (Figure 2D).
    c. Carefully tilt the conical back up and observe a layer of dissociated cell suspension on top of the BSA cushion (Figure 2E).
    d. Cap and store the conical on ice until all samples are prepared.
54. Spin dissociated cell suspension and BSA cushion at 500 g for 10 minutes (Figure 2F).
55. Remove supernatant without disturbing the pellet (Figure 2G).
    a. Leave about 30 uL of supernatant above the pellet.
56. Gently flick pellet to resuspend the cells in the remaining 30 uL of supernatant.
57. Add 100 uL REM to the resuspend pellet (Figure 2H).
58. Count cells using a hemocytometer, troubleshooting 4 (Figure 2I).
    a. If the concentration is between 400–2000 cells/μL continue to single cell.
    b. If the concentration is over 2000 cells/μL dilute the cells in REM to a concentration of ~1000 cells/μL and recount to ensure a concentration between 400–2000 cells/μL

Single-cell sequencing

Timing: 2 days

Here, we describe the steps to complete single cell sequencing according to 10x genomics manufacturing specifications.

Note: The link provided is for the most recent version of the 3′ gene expression kit, newer protocols may become available.

59. Based on the 10× genomics Cell Suspension Volume Calculator Table add the appropriate amount of the cell suspension and water to the Master Mix.
60. Load the 10× genomics Chip and insert the chip into the 10× Chromium Controller according to the protocol.
61. Run the 10× Chromium Controller which will create the GEMs in the 10× genomics chip.

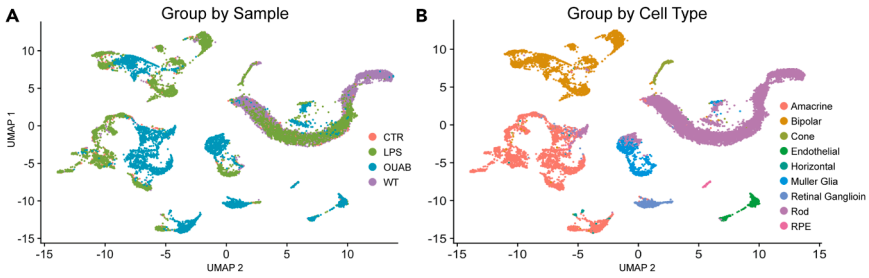

Figure 3. scRNAseq UMAP of integrated retina

(A) UMAP of 4 integrated scRNAseq retina samples. WT is uncultured. CTR is a control 24-hour culture in normal REM. LPS is a 24-hour retinal culture in REM + 0.2 mg/mL LPS. OUAB is a 24-hour retinal culture in REM + 70 μM Ouabain. (B) UMAP of 4 integrated scRNAseq retinal samples that have been labeled by expected cell type using a gene list for each retinal cell types.

62. After GEM creation, carefully transfer the GEMs to an 8-strip tube and complete the RT reaction as specified in the protocol.
63. Proceed with the 10× genomics protocol including GEM-RT Cleanup, cDNA Amplification, Fragmentation, Ligation, Sample Index PCR.
64. After completion of the protocol, run a D1000 high sensitivity tape station.
    a. Prepare the ladder with 2 μL Ladder and 2 μL buffer.
    b. Prepare samples with 2 μL buffer, 1 μl EB buffer, and 1 μL sample.
    c. Run tapestation according to manufacture specifications.
65. Submit samples for sequencing based on 10× genomics run parameters.

Single-cell analysis

Timing: 1 day

Here we describe steps for single cell analysis of retina including running the fastq files generated from sequencing through cell ranger then integrating and calling cell types using Seurat in R. The time will be variable by hardware available.

66. If necessary, run the fastq files through cell ranger count.

```
>cellranger count --id=Stress_WT

>--fastqs=/pathto/fastq

>--transcriptome=/pathto/Reference/refdata-gex-GRCh38-2024-A
```

Note: The code integrates a wildtype, control culture, and 2 stress cultures from Norrie et al. which are available on GEO: 272074.

67. Using the code below or in supplemental information, load the necessary libraries into R.

```
>library(Seurat) # v5.3.0 Core single-cell RNA-seq analysis package

>library(ggplot2) # v3.5.2 General data visualization

>library(dittoSeq) # v1.16.0 Visualization tailored for single-cell data
```

68. Set random seed for reproducibility of results.

```
>set.seed(1234)
```

69. Read in 10× Data from Cellranger output.
    a. Read in the raw count matrix from the 10× genomics output directory.
    b. Create a Seurat Object.
    c. Assign custom metadata to the Seurat object such as "Group" or "orig.ident".

```
>#---- Wild Type sample ----
>WT.data <- Read10X(data.dir = "DYE2519")
>WT <- CreateSeuratObject(counts = WT.data)
>WT$Group = "WT"
>WT$orig.ident = "WT"
>#---- Control sample ----
>CTR.data <- Read10X(data.dir = "DYE1660")
>CTR <- CreateSeuratObject(counts = CTR.data)
>CTR$Group = "CTR"
>CTR$orig.ident = "CTR"
>#---- Stress sample: LPS-treated ----
>LPS.data <- Read10X(data.dir = "DYE1942")
>LPS <- CreateSeuratObject(counts = LPS.data)
>LPS$Group = "Stress"
>LPS$orig.ident = "LPS"
>#---- Stress sample: Ouabain-treated ----
>OUAB.data <- Read10X(data.dir = "DYE1943")
>OUAB <- CreateSeuratObject(counts = OUAB.data)
>OUAB$Group = "Stress"
>OUAB$orig.ident = "OUAB"
```

Note: Replace "DYE####" paths with the directory containing each sample's 10× Genomics output.

70. Combine Seurat objects into a list for batch processing.

```
>object.list <- list(WT, CTR, LPS, OUAB)
>names(object.list) <- c("WT", "CTR", "LPS", "OUAB")
```

Note: Add or remove samples from both this section and "object.list" as needed.

71. Complete initial data filtering and processing.
    a. Calculate mitochondrial gene percentage (percent.mt).
    b. Subset cells with more than 300 detected genes and less than 15% mitochondrial reads.
    c. Normalize the data (log-normalization).
    d. Identify top 5000 variable genes.

```
>object.list <- lapply(X = object.list, FUN = function(x) {

>x[["percent.mt"]] <- PercentageFeatureSet(x, pattern = "^mt-")

>x <- subset(x, subset = nFeature_RNA > 300 & percent.mt <15)

>x <- NormalizeData(x)

>x <- FindVariableFeatures(x, selection.method = "vst", nfeatures = >5000)

>})
```

72. Integrate Data with RPCA.
    a. Select features for integration.
    b. Scale and run PCA on each dataset using integration features.
    c. Identify integration anchors using WT as the reference dataset.
    d. Integrate datasets into a single Seurat object.

```
>features <- SelectIntegrationFeatures(object.list, nfeatures = 5000)

>object.list <- lapply(X = object.list, FUN = function(x) {

> x <- ScaleData(x, features = features, verbose = FALSE)

> x <- RunPCA(x, features = features, verbose = FALSE)

>})

>stress.anchors <- FindIntegrationAnchors(object.list, anchor.features >= features,
reduction = "rpca", reference = c(1))

>stress.integrated <- IntegrateData(anchorset = stress.anchors)

>DefaultAssay(stress.integrated) <- "integrated"
```

Note: c(1) refers to WT as reference, if additional wild type data is added, add sample number to "reference = c(1,2,…)"

73. Complete dimensionality reduction and clustering.
    a. Scale integrated data.
    b. Run PCA.
    c. Construct nearest neighbor graphs.
    d. Perform Louvain clutering.
    e. Run UMAP for visualization.

f. Visualize UMAP (Figure 3).

```
>stress.integrated <- ScaleData(stress.integrated, verbose = FALSE)

>stress.integrated <- RunPCA(stress.integrated, npcs = 30, verbose = FALSE)

>stress.integrated <- FindNeighbors(stress.integrated, dims = 1:10)

>stress.integrated <- FindClusters(stress.integrated, resolution = 0.5)

>stress.integrated <- RunUMAP(stress.integrated, dims = 1:15)

>DimPlot(stress.integrated)
```

74. Use gene lists for cell type identification.
    a. Choose color palette for cell types.
    b. Define cell marker genes.
    c. Isolate the marker genes into a dataset.
    d. Calculate a module score.
    e. Plot spatial distribution (Feature plot) and expression distribution (VlnPlot) of each cell type.

```
> cell_score_colors = c("rod"="#A5D0E8", "bipolar"="#7CBC38", >"cone"="#F9D973", "muller-
glia"="#ED723F",  "amacrine"="#ED79B9",  >"retinalganglion"="#BD63B7",  "horizontal"=
"#827057", "rpe"="#01066E", >"endothelial"="#367C5E")

>Rod.Genes <- c('Rho', 'Rp1', 'Cnga1', 'Nrl', 'Pde6b', 'Nr2e3', >'Guca1b', 'Slc24a1',
'Pde6a', 'Samd11', 'Reep6', 'Rs1', 'Rdh12', >'AI847159', 'Pex5l', 'Nxnl1', 'Sgk1', 'Vtn',
'Aipl1', 'Casz1', 'Grk1', >'Hmgb2', 'Fam161a', 'Gm48551', 'Pdzph1', 'Hk2', 'Nxnl2',
'1-Mar', >'Prom1', 'Rabgef1', 'Faim', 'Fscn2', 'Susd3', 'Kcnj14', 'Wdr17', >'Drd4',
'Ccdc126', 'Cabp4', 'Lbhd1', 'Grtp1', 'Abca4', 'Slc4a7', >'Mak', 'Mpp4', 'Rgs9', 'Pla2g7',
'D1Ertd622e', 'Pla2r1', 'Tnfsf12', >'Plekhf2', 'Glmn')

>Rod.Genes <- Rod.Genes[Rod.Genes %in% getGenes(stress.integrated)]

>stress.integrated <- AddModuleScore(object = stress.integrated, >features = list(Rod.
Genes), name = 'rod_score', ctrl = 80)

>FeaturePlot(stress.integrated, features = "rod_score1", label = TRUE) >+ theme(aspect.
ratio = 1)

>VlnPlot(stress.integrated, features = "rod_score1", pt.size = 0) + >theme(aspect.ratio = 1)
```

75. Repeat 72b-e with all retinal cell types.

```
> Bipolar.Genes = c('Gng13', 'Pcp2', 'Trnp1', 'Trpm1', 'Scgn', >'Gm4792', 'Gsg1', 'Lrtm1',
'Cabp5', 'Isl1', 'Cacna2d3', 'Scg2', >'Neurod4', 'Kcnma1', 'B3galt2', 'BC030499', 'Grm6',
'Ntng1', 'Cadps', >'Ndnf', 'Vsx2', 'Qpct', 'Pcp4', 'Nrn1l', 'C130073E24Rik', 'Ptprd',
>'Ptprz1', 'Sebox', 'Gabra1', 'Car10', 'Gabrr2', 'Prox1', 'Samsn1', >'Lhx4', 'Fam135b',
'Gabrr1', 'Car8', 'Frmd3', 'Celf3', 'Gpr179', >'Asap2', 'Tmem215', 'Grik1', 'Gnb3',
'Slit2', 'Thsd7a', 'Zfhx4', >'Auts2', 'Tnnt1', 'Cntn4')

>Bipolar.Genes <- Bipolar.Genes[Bipolar.Genes %in% >getGenes(stress.integrated)]

>stress.integrated <- AddModuleScore(object = stress.integrated, >features = list(Bipolar.
Genes), name = 'bipolar_score', ctrl = 80)
```

```
>FeaturePlot(stress.integrated, features = "bipolar_score1", label = >TRUE) + theme(aspect.
ratio = 1)

>VlnPlot(stress.integrated, features = "bipolar_score1", pt.size = 0) >+ theme(aspect.
ratio = 1)

>Cone.Genes = c('Opn1sw', 'Pde6h', 'Arr3', 'Opn1mw', 'Gngt2', >'Gnat2', 'Pde6c', 'Kcne2',
'Cngb3', 'Mylk', 'Ccdc136', 'Hopx', >'Osgep', 'Gzmm', 'Pcdh15', 'Olfm1', 'Ndufaf5',
'Adrb1', 'Pgc', >'Acbd6', 'Eif3b', 'Cd59a', 'Ppm1j', 'Scg3', 'Qsox1', 'Fzd10', >'Pygm',
'Ckmt1', 'Thrb', 'Hspb6', 'Nrcam', 'Mgst3', 'Tshz2', >'Slc24a2', 'Fam19a3', 'Gulo',
'Mpp6', 'Dixdc1', 'Mfge8', >'Sptssa', 'Casp7', 'Gsdme', 'Agr2', 'Slc8a3', 'Elovl2',
>'Gm42697', 'Jchain', 'Ankrd33', 'Jam3', 'Camk2b')

>Cone.Genes <- Cone.Genes[Cone.Genes %in% >getGenes(stress.integrated)]

>stress.integrated <- AddModuleScore(object = stress.integrated, >features = list(Cone.
Genes), name = 'cone_score', ctrl = 80)

>FeaturePlot(stress.integrated, features = "cone_score1", label >= TRUE) + theme(aspect.
ratio = 1)

>VlnPlot(stress.integrated, features = "cone_score1", pt.size = >0) + theme(aspect.
ratio = 1)

>Muller.Glia.Genes = c('Apoe', 'Glul', 'Clu', 'Rlbp1', 'Ptgds', >'Spc25', 'Fxyd1',
'Slc1a3', 'Cp', 'Sparc', 'Cd9', 'Prdx6', >'Car14', 'Dkk3', 'Hes1', 'Dapl1', 'Ptn', 'Abca8a',
'Cryab', >'Trf', 'Espn', 'Fos', 'Gnai2', 'Timp3', 'Kdr', 'Cox4i2', >'Gpr37', 'Aqp4', 'Pdpn',
'Vim', 'Sox9', 'Plpp3', 'Crym', >'Zfp36l1', 'Jun', 'Gng5', 'Rbp1', 'Tsc22d4', 'Pmepa1',
'Id3', >'Mlc1', 'Spon1', 'Fxyd6', 'Slmap', 'Ndrg2', 'Hopx', 'Gpm6b', >'S100a16', 'Slitrk2',
'S100a1')

>Muller.Glia.Genes <- Muller.Glia.Genes[Muller.Glia.Genes %in% >getGenes(stress.integrated)]

>stress.integrated <- AddModuleScore(object = stress.integrated, >features = list(Muller.
Glia.Genes), name = 'mullerglia_score', >ctrl = 80)

>FeaturePlot(stress.integrated, features = "mullerglia_score1", >label = TRUE) + theme(aspect.
ratio = 1)

>VlnPlot(stress.integrated, features = "mullerglia_score1", >pt.size = 0) + theme(aspect.
ratio = 1)

> Amacrine.Genes = c('Snhg11', 'Cartpt', 'Pax6', 'Atp1b1', 'Basp1', >'Tfap2b', 'C1ql1',
'Slc6a9', 'Slc32a1', 'Nhlh2', 'Elavl3', 'Slc6a1', >'Rph3a', 'Tkt', 'Gad1', 'Ebf1',
'Nrxn2', 'Ly6h', 'Synpr', 'Calb2', >'Nrxn1', 'Tac1', 'Nrsn1', 'Lamp5', 'Cxcl14', 'Hpca',
'Lsamp', 'Ndrg4', >'Dlgap1', 'Tmx4', 'Six3', 'Ldhb', 'Lhfp', 'Crabp1', 'Stmn1', 'Marcks',
>'Ptprf', 'Insig1', 'Cplx2', 'Tagln3', 'Dync1i1', 'Cdk14', 'Necab1', >'Resp18', 'Gpm6a',
'Chd3os', 'Cabp1', 'Pnmal2', 'Gria3', 'C1ql2')

>Amacrine.Genes <- Amacrine.Genes[Amacrine.Genes %in% >getGenes(stress.integrated)]

>stress.integrated <- AddModuleScore(object = stress.integrated, >features = list(Amacrine.
Genes), name = 'amacrine_score', ctrl = 80)

>FeaturePlot(stress.integrated, features = "amacrine_score1", label = >TRUE) + theme(aspect.
ratio = 1)

>VlnPlot(stress.integrated, features = "amacrine_score1", pt.size = 0) >+ theme(aspect.
ratio = 1)

>Retinal.Ganglion.Genes = c('Calb2', 'Sncg', 'Nefl', 'Nrn1', 'Stmn2', >'Uchl1', 'Thy1',
'Rbpms', 'Pou4f1', 'Fxyd7', 'Rbpms2', 'Tusc5', >'Nrgn', 'Ebf1', 'Slc17a6', 'S100a10',
'Atp1b1', 'Tppp3', 'Nefm', >'Olfm1', 'Gap43', 'Cdk14', 'Tmem163', 'Pcdh10', 'Scn1a',
'Nell2', >'Clec2l', 'Mt3', 'Epha5', 'Scn2a', 'Ina', 'Tubb3', 'Snca', 'Emc10', >'Resp18',
'Atp2b2', 'Nrxn1', 'Srgap1', 'A2ml1', '2900055J20Rik', >'Myt1l', 'Spock2', 'Alcam',
'Fgf12', 'Apbb2', 'Fabp5', 'Scn1b', >'Cend1', 'Rbfox3', 'Nsg2')
```

```
>Retinal.Ganglion.Genes <- >Retinal.Ganglion.Genes[Retinal.Ganglion.Genes %in% >getGenes
(stress.integrated)]

>stress.integrated <- AddModuleScore(object = stress.integrated, >features = list(Retinal.
Ganglion.Genes), name = >'retinalganglion_score', ctrl = 80)

>FeaturePlot(stress.integrated, features = "retinalganglion_score1", >label = TRUE) + theme
(aspect.ratio = 1)

>VlnPlot(stress.integrated, features = "retinalganglion_score1", >pt.size = 0) + theme(aspect.
ratio = 1)

>Horizontal.Genes = c('Calb1', 'Tpm3', 'Sept4', 'Slc4a3', 'Adgrb1', >'Atp1b1', 'Onecut2',
'Tnr', 'Vim', 'Lhx1', 'Ppp1r1a', 'Ndrg1', 'Nebl', >'Gng2', 'Ndrg4', 'Lhx1os', 'Adra2a',
'Rgs3', 'Utrn', 'Lmo1', 'Pcsk6', >'Sept8', 'F2r', 'C1ql1', 'Cdc42ep4', 'Slc12a2',
'Akap12', 'Tfap2b', >'Dock4', 'Stmn2', 'Slc4a5', 'Tmem132a', 'Pde3a', 'Lrrc4', 'Lsamp',
>'Wfdc10', 'Fam126a', 'Tmod1', 'Megf11', 'Arhgap15', 'Syt2', 'Uchl1', >'Rbfox2', 'Akain1',
'Itgb5', 'Rcan2', 'Pcbd1', 'Myo16', 'Tubb4a', >'Cdh4')

>Horizontal.Genes <- Horizontal.Genes[Horizontal.Genes %in% >getGenes(stress.integrated)]

>stress.integrated <- AddModuleScore(object = stress.integrated, >features = list(Horizontal.
Genes), name = 'horizontal_score', ctrl = 80)

>FeaturePlot(stress.integrated, features = "horizontal_score1", label = >TRUE) + theme(aspect.
ratio = 1)

>VlnPlot(stress.integrated, features = "horizontal_score1", pt.size = 0) >+ theme(aspect.
ratio = 1)

>RPE.Genes = c('Ctss', 'C1qb', 'C1qa', 'C1qc', 'Hexb', 'Tyrobp', >'Ccl12', 'Fcer1g',
'Cx3cr1', 'B2m', 'Ccl4', 'Ly86', 'Ccl3', 'Trem2', >'Csf1r', 'Selplg', 'Laptm5', 'Junb',
'Rgs10', 'Cd52', 'Ccl2', 'Egr1', >'Jun', 'Cd9', 'Aif1', 'Zfp36', 'Cyba', 'Ctsz', 'Lyz2',
'P2ry12', >'Gpr34', 'Siglech', 'Sparc', 'Mafb', 'Tmem119', 'Selenop', 'Rhob', >'Npc2',
'Sat1', 'Olfml3', 'Kctd12', 'Grn', 'Ly6e', 'Ifi27l2a', >'Coro1a', 'Sh3bgrl3', 'Klf2',
'Unc93b1', 'Lyn', 'Ctsh')

>RPE.Genes <- RPE.Genes[RPE.Genes %in% getGenes(stress.integrated)]

>stress.integrated <- AddModuleScore(object = stress.integrated, >features = list(RPE.
Genes), name = 'RPE_score', ctrl = 80)

>FeaturePlot(stress.integrated, features = "RPE_score1", label = TRUE) + >theme(aspect.
ratio = 1)

>VlnPlot(stress.integrated, features = "RPE_score1", pt.size = 0) + >theme(aspect.ratio = 1)

>Endothelial.Genes = c('Ly6c1', 'Cldn5', 'Pltp', 'Cxcl12', 'Ly6a', >'Igfbp7', 'Itm2a',
'Slco1a4', 'Rgs5', 'Flt1', 'Mgp', 'Ifitm3', >'Spock2', 'Crip1', 'Ramp2', 'Egfl7', 'Clec2d',
'Klf2', 'Ptprb', >'Sparcl1', 'Ly6e', 'Acta2', 'Abcb1a', 'Slc7a5', 'Id3', 'Tagln',
>'Slc9a3r2', 'Tm4sf1', 'Myl9', 'Id1', 'Crip2', 'Slc2a1', 'Hspb1', 'Fn1', >'Sparc',
'Epas1', 'Adgrl4', 'Abhd2', 'Ctla2a', 'Fxyd5', 'Slco1c1', >'Selenop', 'Pdgfb', 'Adgrf5',
'Ahnak', 'Abcg2', 'Rgcc', 'Pglyrp1', >'Gng11', 'Gpcpd1')

>Endothelial.Genes <- Endothelial.Genes[Endothelial.Genes %in% >getGenes(stress.integrated)]

>stress.integrated <- AddModuleScore(object = stress.integrated, >features = list(Endothe-
lial.Genes), name = 'endothelial_score', ctrl = >80)

>FeaturePlot(stress.integrated, features = "endothelial_score1", label = >TRUE) + theme
(aspect.ratio = 1)

>VlnPlot(stress.integrated, features = "endothelial_score1", pt.size = >0) + theme(aspect.
ratio = 1)
```

76. Assign best cell identity.
    a. Create a dataframe of all module scores.
    b. For each cell select the cell type with the highest score.
    c. Clean up naming.
    d. Store cell type as new metadata named 'cell.ident'.
    e. Visualize cell identities in a UMAP.

```
> All.cell.scores <- data.frame(stress.integrated$rod_score1, >stress.integrated$bipolar_score1, stress.integrated$cone_score1, >stress.integrated$mullerglia_score1, stress.integrated$amacrine_score1, >stress.integrated$retinalganglion_score1, stress.integrated$horizontal_score1, >stress.integrated$RPE_score1, stress.integrated$endothelial_score1)

>best_cell_score <- colnames(All.cell.scores)[max.col(All.cell.scores, ties.method = "first")]

>best_cell_score <- sub("stress.integrated.", "", best_cell_score)

>best_cell_score <- sub("_score1", "", best_cell_score)

>stress.integrated$cell.ident <- best_cell_score

>DimPlot(stress.integrated, split.by="Group", group.by = "cell.ident", cols=cell_score_colors)
```

77. Export, save, and visualize the results.
    a. Save counts per cell type.
    b. Save proportion of each cell type.
    c. Create a bar plot of percentages of cells in each cell type.
    d. Save the R file.
    e. Plot the data. (Figure 3, troubleshooting 5).

```
> write.csv(table(stress.integrated$cell.ident, stress.integrated$Group), file = "stress_Int_1.csv")

>write.csv(prop.table(table(stress.integrated$cell.ident, stress.integrated$Group), margin = 2) *100, file = "stress_Int_prop1.csv")

>cell.percentage.data <- data.frame(prop.table(table(stress.integrated$cell.ident, stress.integrated$Group), margin = 2) *100)

ggplot(aes(y=Freq, x=Var2, fill=Var1), data = cell.percentage.data) + geom_bar(stat = 'identity') + coord_flip() + scale_fill_manual('Cell types', values = cell_score_colors) + theme_classic()

>saveRDS(stress.integrated, file = "RetinaStress.rds")

>DimPlot(stress.integrated, group.by = "cell.ident")
```

EXPECTED OUTCOMES

In this protocol mouse retinal punches are cultured for 24 hours in the presence of a retinal stress. A minimum of 3 1.5 mm retinal punches is needed for RNA extraction. Because 3 retinal punches is a small amount of tissue and the tissue may be significantly stressed, RNA concentrations after Trizol RNA extraction will be low, approximately 25 ng/μL in 10 μL. After performing the qPCR analysis using cDNA made from 150 ng of RNA, it is not uncommon for an uncultured retina to not amplify with any of the stress primers. Control culture retina should have very high Ct values (>30). However, all

stress cultured retina should have a large fold change increase in Cxcl2, Ccl2, and/or Il6. A combination of high-quality RNA and increased expression of stress genes indicate an effective stress culture, and the same culture can be repeated, and the retinal punches can be used for single-cell sequencing. Dissociation of 6 retinal punches should yield at least 100,000 cells with a viability of 95% after BSA cushion.

Single cell sequencing analysis on retina should result in very distinct retinal cell clusters, with rods being the most abundant. Horizontal cells and retinal ganglion cells may not be detected. Additionally, it is normal to see rods have a very low number of genes per cell as they are highly specialized and have a low diversity of gene expression.

LIMITATIONS

Retinal stress culture conditions were chosen due to their physiological relevance to the retina. Therefore, we expect the results to be subtle and have still observed both cell type and stress specific responses. While stresses beyond the 15 we have published can be perfectly suited for this culture system some stresses may not be able to be adapted to a culture system.

For our study we tested multiple time points and chose 24 hours as an optimal time for culture with seeing an acute stress response without seeing many secondary effects. Control cultures are essential for determining a culture response and a stress specific response. It is possible to culture for more or less than 24 hours, but at those timepoints Il6, Cxcl2, and Ccl5 may no longer be reliable markers.

Another important limitation to this study is the lack of immune cells in a retinal culture. After completing an in-vivo stress exposure we found that the upregulation of cytokines in this system does signal for immune infiltration, which will not happen in-vitro.

TROUBLESHOOTING

Problem 1

Retinal punches dry during incubation.

Potential solution

We have found addition of 50 μL of media to the top of 3 retinal punches should be sufficient for 16 hours of culture and should be replenished once during the first 8–16 hours of cultures, if there is insufficient humidity in an incubator a culture could need up to 100 μL of media every 8 hours. Cultures and incubators should be carefully monitored until the correct volume is determined and humidity is maintained.

Problem 2

Low yield or quality of RNA after trizol RNA extraction.

Potential solution

Both low RNA yield and quality after Trizol extraction may be simply due to not enough retinal punches being used for extraction. Adding more cultured punches may be necessary to increase the yield in order to have enough RNA for making cDNA. However, ensure that the samples are always on ice when in trizol and limit the time in trizol by adding the trizol, dissociating the retina and freezing the sample until the experiment is ready to proceed.

Contamination with phenol is also a major issue as phenol is hard to remove and can significantly change qPCR and RNA-sequencing results. This is caused by contamination of the phenol layer during the phenol-chloroform phase separation. It is important to prioritize an uncontaminated sample over retrieving more RNA.

Problem 3

qPCR shows no increase in cytokines in stress conditions.

Potential solution

It is normal for uncultured retinal samples to not have detectable levels of Il6, Ccl5, or Cxcl2. Control retina may not have detectable levels of the cytokines either, which can be normal with well cultured retinal punches. These two results are normal and do not require further action. If stressed retina do not have significantly more cytokine expression than control, there are two possibilities. One possibility is that the control retina has a much higher level of stress than it should and culture media, incubators, and dissection technique should be assessed to determine where the extra stress occurred. This is likely if the control culture has a low Ct value (>30) for the cytokines. The other possibility is that the stress condition was not significantly stressed, which is likely if the stress cultures have a Ct above 35. If the stress is from the list of 15 stressors provided in this protocol, ensure proper preparation of the media. Make sure the stressor has been added correctly, and the media is mixed before adding it to the wells, so the stressor is evenly distributed. Additionally, make sure to remove as much REM from the transfer of the punches to the Whatman paper as possible and add 50 μl from the well mixed stress media in the well. If the stress is not from the protocol, it may be necessary test multiple amounts of stressor or to do a time course to see when the peak acute stress and expression of cytokines is occurring.

Problem 4

Low yield or survival of cells from dissociation.

Potential solution

Timing of retinal dissociation can vary greatly depending on the papain, the papain buffer, and the force of flicking; therefore, it is very important to use the tissue as a guide and not a specific time. Over-dissociated retina will become viscus and may be undetectable, but if this happens it will get stuck in the filter and significantly impact the yield. If this is happening, make sure to add DNase as soon as the punches no longer sink quickly to the bottom of the tube and begin the process of filtering as soon as a majority of the punches float. There can also be diminishing returns on adding more punches so no more than 2 full retinal or 10 punches should be dissociated in one tube of papain.

Problem 5

High ambient RNA in single cell sequencing data.

Potential solution

Retinal cells are incredibly delicate and even the gentlest dissociation can lead to bursting cells which can result in ambient RNA during single cell sequencing. The first solution is to try to complete the gentlest and quickest retinal dissociation with BSA cushion, see above. However, once the single cell sequencing has occurred it may be necessary to use a bioinformatics tool such as SoupX to filter our ambient RNA. Rods tend to be the most significant source of ambient RNA, but when running the retinal cell type code, most of the other cell types have such significant expression of cell type specific genes that it is still possible to determine cell types even with the ambient RNA.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michael A. Dyer (michael.dyer@stjude.org).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Jackie L. Norrie (jackie.norrie@stjude.org).

Materials availability

This study does not generate new reagents.

Data and code availability

- The datasets in this study are available on GEO: 272074.
- The code from this study is fully listed in this article and as supplemental material. The full code from Norrie et al. is available at https://zenodo.org/records/14279513.

ACKNOWLEDGMENTS

J.L.N. received funding from the NIH (CA225442). M.A.D. received grant funding from the National Institutes of Health (CA245508 and EY030180). The project was also supported by NIH grant CA21765 and American Lebanese Syrian Associated Charities.

AUTHOR CONTRIBUTIONS

J.L.N. designed, conducted, and analyzed this study and wrote the manuscript. C.R. analyzed the experiments and edited the manuscript. M.A.D. designed and funded the study and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2025.104271.

REFERENCES

1. Norrie, J.L., Lupo, M.S., Little, D.R., Shirinifard, A., Mishra, A., Zhang, Q., Geiger, N., Putnam, D., Djekidel, N., Ramirez, C., et al. (2025). Latent epigenetic programs in Müller glia contribute to stress and disease response in the retina. Dev. Cell *60*, 1199–1216.e7. https://doi.org/10.1016/j.devcel.2024.12.014.

2. Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W.M., Hao, Y., Stoeckius, M., Smibert, P., and Satija, R. (2019). Comprehensive Integration of Single-Cell Data. Cell *177*, 1888–1902.e21. https://doi.org/10.1016/j.cell.2019.05.031.

3. Bunis, D.G., Andrews, J., Fragiadakis, G.K., Burt, T.D., and Sirota, M. (2021). dittoSeq: universal user-friendly single-cell and bulk RNA sequencing visualization toolkit. Bioinformatics *36*, 5535–5536. https://doi.org/10.1093/bioinformatics/btaa1011.

4. Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis (Springer-Verlag). https://ggplot2.tidyverse.org.