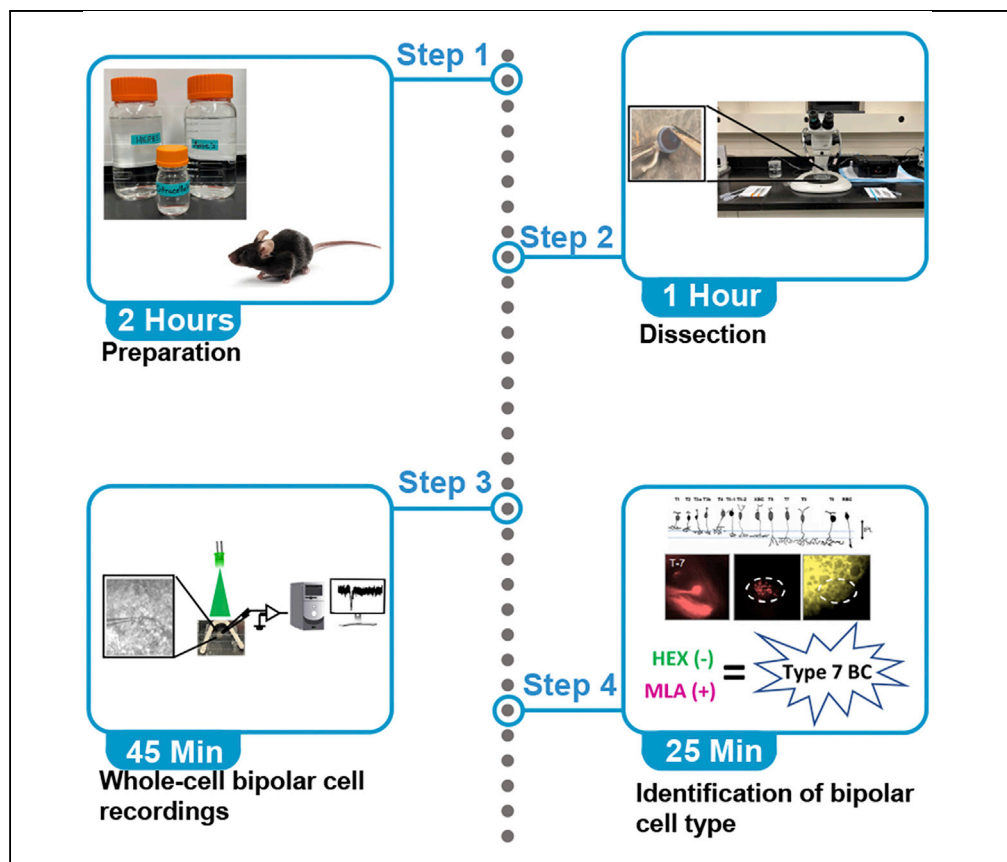


## Protocol

# Patch clamp recording from bipolar cells in the wholemount mouse retina



Bipolar cells are the second-order neurons in the retina that are less accessible for investigating their synaptic responses. Here, we present a protocol to conduct patch clamp recordings from bipolar cells in the wholemount retina from Ai32 mutant mice. We detail whole-cell patch-clamp recording from bipolar cells to examine their light-evoked responses to optogenetic stimulation, followed by imaging terminals of recorded cells to determine bipolar cell type. We describe light stimulus information to activate channelrhodopsin-2 (ChR2).

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

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**Highlights**  
Detailed protocol for bipolar cell patch clamp recordings in wholemount mouse retina

Bipolar cell subtype identification in live retinal tissue

Detailed light stimulus information for channelrhodopsin (ChR2) activation

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## Protocol

## Patch clamp recording from bipolar cells in the wholemount mouse retina

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<https://doi.org/10.1016/j.xpro.2022.101482>**SUMMARY**

Bipolar cells are the second-order neurons in the retina that are less accessible for investigating their synaptic responses. Here, we present a protocol to conduct patch clamp recordings from bipolar cells in the wholemount retina from Ai32 mutant mice. We detail whole-cell patch-clamp recording from bipolar cells to examine their light-evoked responses to optogenetic stimulation, followed by imaging terminals of recorded cells to determine bipolar cell type. We describe light stimulus information to activate channelrhodopsin-2 (ChR2).

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

**BEFORE YOU BEGIN****Institutional permissions**

All animal procedures were approved by the Institutional Animal Care and Use Committee at Wayne State University (protocol no. 17-11-0399). When crafting the protocol, every precaution was taken to ensure it follows all regulations and guidelines for proper animal handling. Other laboratories wishing to replicate these experiments will need to attain the proper approval from their institution's regulatory body.

**Breeding mice**

⌚ Timing: 4–6 weeks

1. Breed ChAT-Cre and Ai32 mice to generate ChAT-Cre Ai32 mice.
  - a. Place a ChAT-Cre mouse (Jackson Labs stock #031661) and an Ai32(RCL-ChR2(H134R)/EYFP) mouse (Jackson Labs stock #024109) in a cage for breeding. Genotype the mice if needed. If breeders are both homozygous, all the offspring will express channelrhodopsin-2 (ChR2) tagged by YFP in their starburst amacrine cells (SACs) and there is no need for genotyping.
  - b. Once a litter is born, wait for 3–4 weeks when the retinal networks are fully developed before performing patch clamp recordings.
  - c. ChAT-Cre × Ai32 mice at the age of 1–3 months old will be feasible for bipolar cell recordings in the wholemount retinal preparations.



### Solution preparation (HEPES Ringer, Ames, and intracellular)

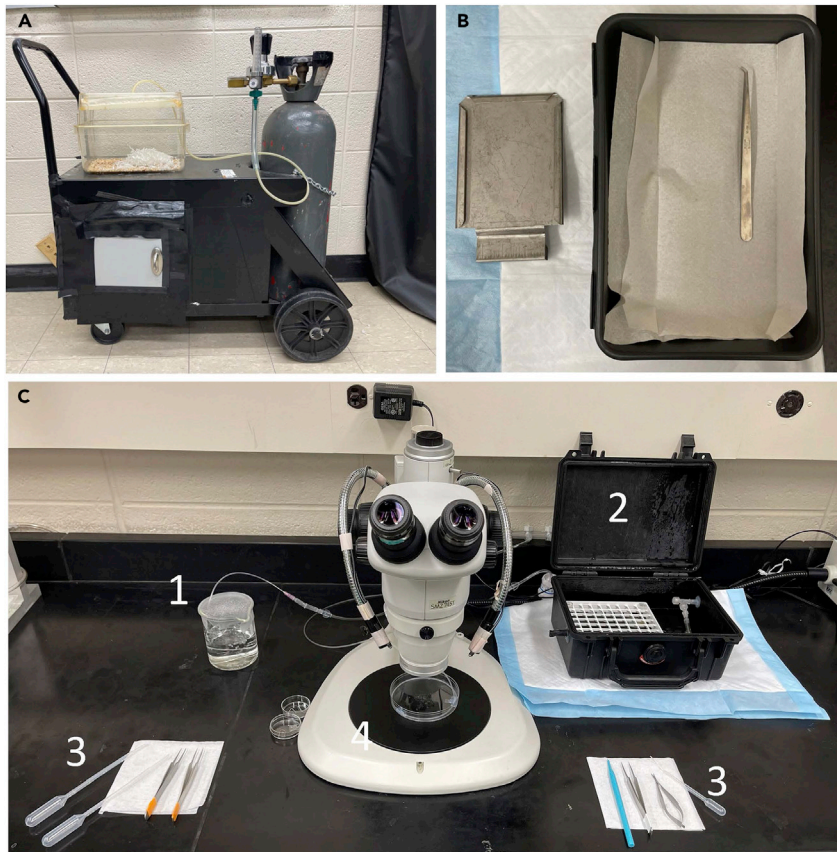
⌚ Timing: 3 h

2. Make HEPES-buffered solution (refer to [materials and equipment](#)).
  - a. Pour ~900 mL double-distilled Milli-Q water (ddH<sub>2</sub>O) in a 1 L glass beaker. Weigh and add each chemical to the water. Mix them by stirring.
  - b. Add all chemicals except CaCl<sub>2</sub>, which should be added after pH is adjusted to > 7.0 with sodium hydroxide (1 M NaOH).
  - c. After all chemicals are added, adjust the pH to 7.4 with NaOH. Then, increase the volume to 1 L using a volumetric flask.
  - d. The solution can be stored in a 4°C refrigerator out of direct light for up to one week.
3. Make Ames' solution on the day of experiment.
  - a. Weigh out 8.8 grams of Ames media powder into ~900 mL of ddH<sub>2</sub>O. Weigh out and add 1.9 grams of NaHCO<sub>3</sub> into the solution.
  - b. Place the beaker on a magnetic stirrer. Start bubbling the solution with a mixed gas of 95%O<sub>2</sub>/5%CO<sub>2</sub> and turn on the magnetic stirring heater. Warm up the solution until it becomes ~30 degrees Celsius while bubbling.
  - c. Measure the pH and add 1 M NaHCO<sub>3</sub> solution dropwise, using a 1 mL transfer pipette, until the pH reads a stable 7.40.
  - d. Use a 1 L volumetric flask and add enough ddH<sub>2</sub>O to make a final volume of 1 L.
  - e. From that 1 L, remove ~300 mL to use for pharmacological Ames'.
    - i. Using a graduated cylinder volume out 100 mL.
    - ii. Remove 85 µL of Ames' solution from the graduated cylinder.
  - f. Add glutamate receptors blockers to the graduated cylinder.
    - i. Add 25 µL of mGluR6 agonist L-AP4. This is used at 1:4000 dilution of a 40 µM stock solution to make a 10 µM working concentration.
    - ii. Add 10 µL of ACET. This is used at 1:10,000 dilution of a 10 mM stock solution to make a 1 µM working concentration.
    - iii. Add 100 µL of GYKI153655. This is used a 1:1000 dilution of a 50 mM stock solution to make a 50 µM working concentration.
    - iv. Cover the graduated cylinder with parafilm and shake thoroughly to homogenize the drugs into the solution.
  - g. Make two sets Ames' solution with glutamate receptor blockers, each containing one of two acetylcholine blockers.
    - i. Take 50 mL of the Ames' made in step 3f. Split this it into two 25 mL portions in separate beakers.
    - ii. For one beaker remove 2.5 µL of solution and replace it with the same volume of α7-nAChR blocker methyllycaconitine (MLA). This is used at a 1:10,000 dilution of a 100 mM stock MLA solution to make a 100 nM working concentration.
    - iii. For the second, beaker remove 25 µL of solution replace it with the same volume of non α7-nAChR blocker hexamethonium (HEX) solution. This is used at a 1:1000 dilution of a 100 mM stock HEX solution to make a 100 µM working concentration.

**Note:** If you need to keep the solution for a few days, keep the solution in a 4°C refrigerator.

4. Make 50 mL of intracellular solution for whole cell recording by referring to [materials and equipment](#).
  - a. Add all chemicals, except for ATP and GTP, to ddH<sub>2</sub>O in a 50 mL beaker. Adjust the pH to 7.20 with 1 M cesium hydroxide (CsOH).

**△ CRITICAL:** Take out the ATP and GTP bottles from a -20°C freezer right before adding to the solution. Make sure to return the bottles back to the freezer following use.



**Figure 1. Dissection preparation for the mouse retina**

(A) A dark box for dark adaptation of mice. A CO<sub>2</sub> tank is attached to a cage lid for euthanasia of a mouse before dissection.

(B) The curved forceps and dish used for enucleation of the eyeballs after euthanasia. Included is a cage card holder used for the cervical dislocation to assure euthanasia.

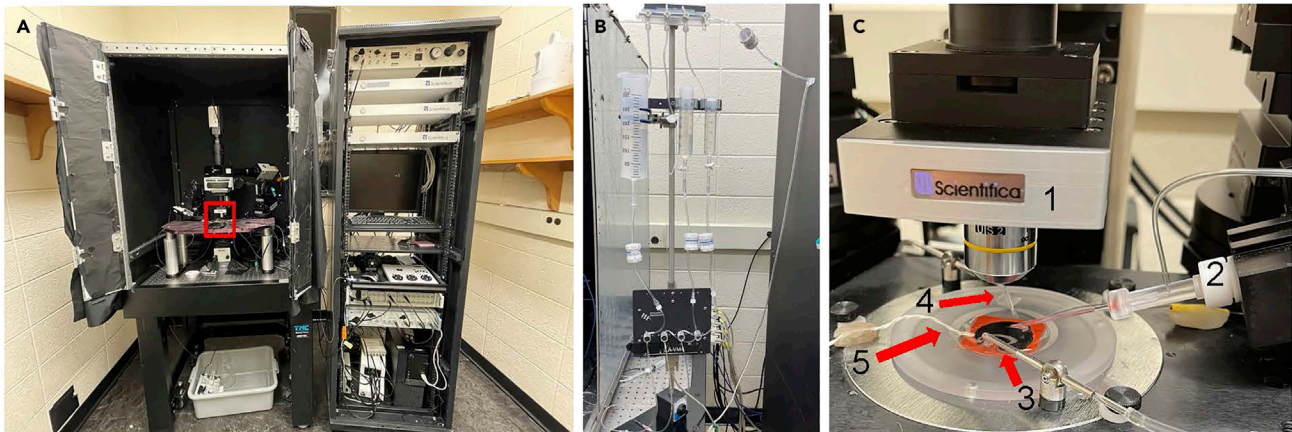
(C) The dissection station used for this protocol. 1) Bubbling HEPES solution used in dissection. 2) An oxygenated dark box used to store the retinal tissue during the experiment. 3) Dissection tools necessary for this protocol. 4) The dissection chamber used for isolation of the retinal tissue.

- b. Add the ATP and GTP, then adjust the pH with CsOH to 7.20. Subsequently, increase the volume by adding ddH<sub>2</sub>O to 50 mL using a volumetric flask.
- c. Aliquot the solution into 500  $\mu$ L tubes and store in a  $-80^{\circ}$ C deep freezer.
- d. On the day of experiment, take one tube and thaw.
  - i. Pipette 2.5  $\mu$ L of sulforhodamine stock solution (1%) into the aliquot of 500  $\mu$ L to stain recording bipolar cells. Sulforhodamine working concentration is 0.0005%.
  - ii. Keep the tube on ice the whole day while experimenting.

### Mouse dark adaptation

⌚ Timing: 1–12 h

5. Dark adapt a mouse for 1 h to 12 h overnight.
  - a. Place a cage with one ChAT-Cre Ai32 mouse in the dark box (Figure 1A). Supply enough food and water for the overnight housing.



**Figure 2. The patch clamp electrophysiology rig**

(A) An image of the full patch clamp recording microscope with the rack mount housing all necessary hardware external to the microscope.

(B) The gravity perfusion setup using an ALA MV4 to control solution channels during the whole-cell recordings.

(C) A magnified view of the patch clamp recording chamber including: 1) The head of recording microscope and a 10× objective 2) A pipette holder connecting to an amplifier head with a microelectrode. 3) A perfusion inlet for fresh oxygenated and warmed Ames' solution during the experiment 4) A suction outlet to remove old Ames' solution. 5) A thermo probe to measure the bath temperature. This communicates with the in-line heater attached to the perfusion tube just prior to the outlet to control the bath temperature.

### Preparation for mouse eye dissection

⌚ Timing: 30 min

6. Setup the dissection station.
  - a. Pour 100 mL of HEPES Buffered Solution into a 250 mL glass beaker. Bubble 100% oxygen gas into both the HEPES dissecting solution and the dark box for storage of retinal tissue (Figure 1C\_1 and 2).
  - b. Rinse dissection tools in 100% ethanol, let dry, and place near microscope (Figure 1C\_3).
  - c. Cut a plastic coverslip into 4 square pieces and apply vacuum grease, making two rails near the edge of each piece and place near the microscope (Figure 3K). After the retinal tissue is cut, it will be placed on top of this coverslip.

### Whole-cell recording setup

⌚ Timing: 30 min

7. Prepare and bubble Ames' solution with a mixed gas of 95%O<sub>2</sub>/5%CO<sub>2</sub>.
  - a. This will include a control Ames' solution and a subset of Ames' solution with a cocktail of glutamate receptor blockers included and two with glutamate receptor blockers in addition to either MLA or HEX.
  - b. Oxygenate the Ames' solution for more than 15 min before recording. Run Ames' solution through the lines right before placement of tissue on microscope.
8. Turn on camera, rack mounted controllers, and computer (Figure 2A).
  - a. Open the recording software listed in the [key resources table](#).
  - b. Turn on both manipulator control boxes. Evaluate the manipulators and stage to ensure they are working properly.
9. Prepare multiple recording pipettes.
  - a. These are pulled with the Sutter P-1000 automatic pipette puller.
  - b. The pipette resistance should be 8–12 MΩ for bipolar cell recordings.



**KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, peptides, and recombinant proteins</b>		
Ames' Media	MilliporeSigma	Cat#A1420-10X1L
L-AP4	Tocris	Cat#0103 Cas#23052-81-5
ACET	Tocris	Cat#2728 Cas#936095-50-0
GYKI53655 hydrochloride	Tocris	Cat#2555 Cas#143692-48-2
Methyllycaconitine Citrate	Tocris	Cat#1029 Cas#351344-10-0
Hexamethonium Bromide	Tocris	Cat#4111 Cas#55-97-0
NaCl	Sigma	Cat#S9888 Cas#7647-14-5
KCl	Sigma	Cat#P5405 Cas#7447-40-7
MgCl <sub>2</sub>	Sigma	Cat#M1028 Cas#7786-30-3
CaCl <sub>2</sub>	Sigma	Cat#C1016 Cas#10043-52-4
TEA-Cl	Sigma	Cat#113042 Cas#56-34-8
HEPES	Sigma	Cat#H3375 Cas#7365-45-9
D-Glucose	Sigma	Cat#G8270 Cas#50-99-7
CsMeSO <sub>3</sub>	Sigma	Cat#C1426 Cas#2550-61-0
KMeSO <sub>3</sub>	Sigma	Cat#83000 Cas#2386-56-3
EGTA	Sigma	Cat#E0396 Cas#67-42-5
ATP-Mg	Sigma	Cat# A9187 Cas#74804-12-9
GTP-Na	Sigma	Cat# G8877 Cas#36051-31-7
Na-OH	Sigma	Cat#306576 Cas#1310-73-2
Cs-OH	Sigma	Cat#232041 Cas#21351-79-1
NaHCO <sub>3</sub>	Sigma	Cat#S6014 Cas#144-55-8
Sulforhodamine B, acid form	Sigma	Cat#341738 Cas#2609-88-3
<b>Experimental models: Organisms/strains</b>		
C57BL/6J Adult mice of both sexes	Jackson Laboratory	RRID: IMSR_JAX:000664
B6.129S- <i>Chat</i> <sup>tm1<sup>cre</sup>Lowl</sup> /MwarJ Homozygous Adult Mice of Both Sexes	Jackson Laboratory	RRID: IMSR_JAX:031661
B6.Cg-Gt(ROSA)26Sor <sup>tm32(CAG-COP4*H134R/EYFP)Hze/J</sup> Homozygous Adult Mice of Both Sexes	Jackson Laboratory	RRID: IMSR_JAX:024109
<b>Software and algorithms</b>		
pClamp10.7	Molecular Devices	<a href="https://www.moleculardevices.com/">https://www.moleculardevices.com/</a>
SigmaPlot14.5	Systat Software	<a href="https://www.systatsoftware.com/">https://www.systatsoftware.com/</a>
GraphPad Prism 9	GraphPad	<a href="https://www.graphpad.com/">https://www.graphpad.com/</a>
Adobe Photoshop	Adobe	<a href="https://www.adobe.com/">https://www.adobe.com/</a>
Microsoft Excel	Microsoft	<a href="https://www.microsoft.com/">https://www.microsoft.com/</a>
Microsoft PowerPoint	Microsoft	<a href="https://www.microsoft.com/">https://www.microsoft.com/</a>

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
QCapture	QImaging	N/A
Lin Lab2	Scientifica	N/A
<b>Other</b>		
#55 Dumont Forceps	Fine Science Tools	Cat#11255-20
#5 Dumont Forceps	Fine Science Tools	Cat#11254-20
#5/45 Dumont Forceps	Fine Science Tools	Cat#11251-35
Vannas 8.5 cm scissors	World Precision Instruments	Item#500086
Surgical Knife	Surgistar	Lot#201117
Transfer Pipettes	Fisher Scientific	Lot#13-711-9CM
Glass Pipettes	World Precision Instruments	Lot#2402324
35 mm Cell Culture Dish	Fisher Scientific	Cat# 08-772-4A
140 mm Petri Dish	Fisher Scientific	Cat# 08-757-100
Plastic Coverslip	Electron Microscopy Sciences	Cat#50-356-009
Platinum Wire	Sigma	CAS#7440-064
Molecular Devices 1440A Digitizer	Molecular Devices	N/A
Axopatch 700B Amplifier	Molecular Devices	N/A
Slicescope Pro 2000 Microscope	Scientifica	N/A
Scientifica PatchStar Micromanipulator	Scientifica	N/A
Axon Instruments Pipette Head Stages	Molecular Devices	Cat# CV-7B
Pipette Holder	Molecular Devices	Cat# 1-HL-U
Accumet AE150 pH Meter	Fisher Scientific	Cat# S30247
50 mL Beaker	Fisher Scientific	Cat# FB10050
100 mL Beaker	Fisher Scientific	Cat# FB100100
250 mL Beaker	Fisher Scientific	Cat# FB100250
1000 mL Beaker	Fisher Scientific	Cat# FB1001000
100 mL Graduated Cylinder	Fisher Scientific	Cat# 08-550E
1 L Volumetric Flask	Fisher Scientific	Cat# 10-210-1F
Stereo Microscope	Nikon	SKU: SMZ745T
Dark Box	Pelican	SKU: 1450-0000-110
Peristaltic Pump	Fisher Scientific	Cat# 13-876-1
ALA Heated Perfusion Tube	ALA Scientific Instruments	Cat# HPT-2A
ALA Thermistor Probe	ALA Scientific Instruments	Cat#TS-1
Pipette Puller	Sutter Instruments	Cat# P1000
Gravity Fed Perfusion System	ALA Scientific Instruments	Cat# ALA VC4-4PG

**MATERIALS AND EQUIPMENT**

**HEPES solution**

Reagent	Final concentration	Amount
Sodium Chloride (NaCl) MW: 58.44	115 mM	6.720 g
Potassium Chloride (KCl) MW: 74.55	2.5 mM	0.1864 g
Magnesium Chloride (MgCl <sub>2</sub> ) MW: 95.21	1 mM	1 mL
Glucose (C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> ) MW: 180.16	28 mM	5.0445 g
HEPES (C <sub>8</sub> H <sub>18</sub> N <sub>2</sub> O <sub>4</sub> S) MW: 280.30	10 mM	5.0445
Calcium Chloride (CaCl <sub>2</sub> ) MW: 110.98	2.5 mM	0.2775 g
<b>Total</b>	<b>n/a</b>	<b>1 L</b>

△ **CRITICAL:** Adjust the pH to 7.40 at room temperature by adding 1 M sodium hydroxide (NaOH). Before adding the calcium chloride, the pH should be 7.0 or above to allow for the calcium chloride to dissolve and not precipitate. The total osmolarity of the solution is 283.5 mOsm on calculation. Store the solution at 4°C for up to one week.

Intracellular solution		
Reagent	Final concentration (mM)	Amount
Cesium Methanesulfonate (CH <sub>3</sub> CsO <sub>3</sub> S) MW: 228.00	110	0.726 g
Tetraethylammonium chloride (C <sub>8</sub> H <sub>20</sub> ClN) MW: 165.2	10	82.9 mg
Magnesium Chloride (MgCl <sub>2</sub> ) MW: 95.21	1	55 μL
EGTA (C <sub>14</sub> H <sub>24</sub> N <sub>2</sub> O <sub>10</sub> ) MW: 380.35	4	76 mg
HEPES (C <sub>8</sub> H <sub>18</sub> N <sub>2</sub> O <sub>4</sub> S) MW: 280.30	10	119.2 mg
ATP-Mg MW: 507.18	4	101 mg
GTP-Mg MW: 523.18	0.5	13.1 mg
<b>Total</b>	n/a	<b>50 mL</b>

△ **CRITICAL:** Adjust the pH to 7.20 by adding CsOH solution. The total osmolarity of the solution is 264 mOsm on calculation. Immediately after the solution is formulated, divide the solution into 500 μL aliquots and store at −80°C. The solution can be stored at this temperature for several months.

**Alternatives:** For current clamp recordings, replace cesium methanesulfonate with potassium methanesulfonate at the same concentration. Also, remove tetraethylammonium chloride (TEA-Cl) and replace with K-Cl (10 mM) to keep the [Cl<sup>-</sup>] concentration and osmolarity.

## STEP-BY-STEP METHOD DETAILS

### Dissection

⌚ **Timing:** 2 h

This step explains how to dissect wholemount retinal tissue.

1. Retinal tissue dissection.
  - a. In dark conditions, euthanize a mouse with CO<sub>2</sub> gas (Figure 1A). Start the CO<sub>2</sub> at a flow rate that will displace 30%–70% of the cage volume of air per minute. Once the mouse stops moving, increase the CO<sub>2</sub> flow rate to its maximum.
  - b. One minute after the mouse stops breathing, move the mouse body to a tray for the enucleation (Figure 1B). Pinch the mouse tail to confirm its unresponsiveness to pain stimulation and perform a cervical dislocation using hemostats. Then, enucleate the eyes using the #5/45 Dumont forceps and place the eyes in a 35 mm dish filled with oxygenated HEPES solution.
  - c. Transfer the dish with eyes into the oxygenated dark box (Figure 1C\_2).

△ **CRITICAL:** Remove the eyes, place them in the oxygenated dark box, and start dissection directly after euthanasia.

- d. Place one eye in the dissection chamber under a stereo microscope, filled with HEPES solution and is continuously oxygenated (Figures 1C\_4 and 3A).
- e. Make a cut on the cornea using a small surgical knife. Then, cut the cornea down to the sclera using a pair of Vannas scissors (Figures 3B and 3C).
- f. Make a circumferential cut around the cornea to remove the iris and cornea (Figure 3D).

△ **CRITICAL:** Cut should be made on the sclera just above the extraocular muscle attachment to separate the anterior segment from the posterior segment of the eye.

- g. Remove the lens from the eyecup using #55 forceps. Then, pour fresh oxygenated HEPES solution into the eyecup using a 1 mL transfer pipette (Figures 3E and 3F).





**Figure 3. The retinal dissection in the HEPES solution-filled chamber**

- (A) An enucleated eye held in place with #5 forceps.  
 (B) Initial corneal cut with a surgical knife.  
 (C) Inserting a blade of scissors into the incision, cut the cornea toward the sclera.  
 (D) Cut out the cornea and the edge of sclera by a circumferential cut.  
 (E) Removing the lens with forceps.  
 (F) A small transfer pipette blowing fresh solution into the eyecup.  
 (G) Detach the retina from the sclera using two sets of forceps. One to hold the sclera of the eyeball and the other to carefully peel off the retina from the pigment epithelium.  
 (H) A piece of isolated retina in the dissecting dish.  
 (I) Cut the retinal tissue into quarters.  
 (J) A piece of quarter retina.  
 (K) A horseshoe net and a coverslip with two grease rails prior to mounting of the retina.  
 (L) A wholemount retinal preparation is mounted between the coverslip and the horseshoe net, ready for patch clamp recordings. Scale bars for panels A-J represent 5 mm.

h. Place this eye back in the dark box. Remove the other eye and complete steps e-f on the new eye. Carry out the rest of the dissection on this more recently removed eye.

**△ CRITICAL:** The previous step allows for the experimenter to have one eye that will be fully dissected, and the eyecup in the box will be a healthy retina if anything goes wrong in the rest of the dissection or after during experimentation.

- i. Remove the transparent vitreous humor from the surface of the retina using sharp forceps. [Troubleshooting 7](#).
- i. The vitreous is sticky and tightly attached to the retina. Using the sharp forceps, remove the vitreous in one location at a time by carefully grabbing near the surface of the retina and pulling it away. In the mouse the vitreous humor is fiber-like and can be carefully removed through grabbing carefully with a set of 55 Dumont forceps and pulling it out of the eyecup. Do not poke the surface of the retina. The vitreous is typically attached to the eye cup near the ora serrata, which can be targeted initially to efficiently remove the vitreous.

- ii. The ventral and dorsal retinas can be distinguished by the orientation of the optic nerve head relative to the choroidal fissure (Wei et al., 2010). Make a small cut on the edge of the retina to mark the side of the retina you would keep or discard for the experiment.
  - j. Detach the retina from the sclera using two pairs of forceps; hold the sclera with one and scrape off the retinal tissue by the other (Figure 3G).
  - k. Isolate the retina from the eyecup by pinching the optic nerve head with forceps.
  - l. Cut and trim the retina into four sections that will be mounted for patch clamp electrophysiological recordings (Figures 3I and 3J).
2. Mounting the retinal tissue.
- a. Using a large transfer pipette, transfer one piece of tissue on to a coverslip with grease rails in a 35 mm dish with HEPES solution.
  - b. Position the tissue between the rails with the ganglion cell side up (the ganglion cell side is the inside of the retinal slab curvature). Grab the horseshoe net using the #5 forceps and place the net over the tissue. The tissue is now immobilized.
  - c. Place the dish in the oxygenated dark box. Repeat same steps for other pieces of the tissue.

### Establishing the whole cell patch clamp

⌚ Timing: 30 min

This major step displays how to achieve the whole cell recordings from bipolar cells in the whole-mount retina.

3. Take a wholemount retina from the dark box and place it in the recording chamber on the patch clamp microscope stage. Immediately, start the Ames' solution perfusion at a rate of 3–5 mL per minute using a gravity fed perfusion setup (Figure 2B). Set the temperature to 33 degrees Celsius.

**Note:** This should be performed in dim red light or dark conditions.

- a. Place a 3 M KCl agar bridge onto a reference wire for the micro pipette before mounting the retinal tissue. Alternatively, you can use a permanent silver chloride wire.

**Note:** Please see Hellmer and Ichinose (2018) for a full protocol on agar bridge formation.

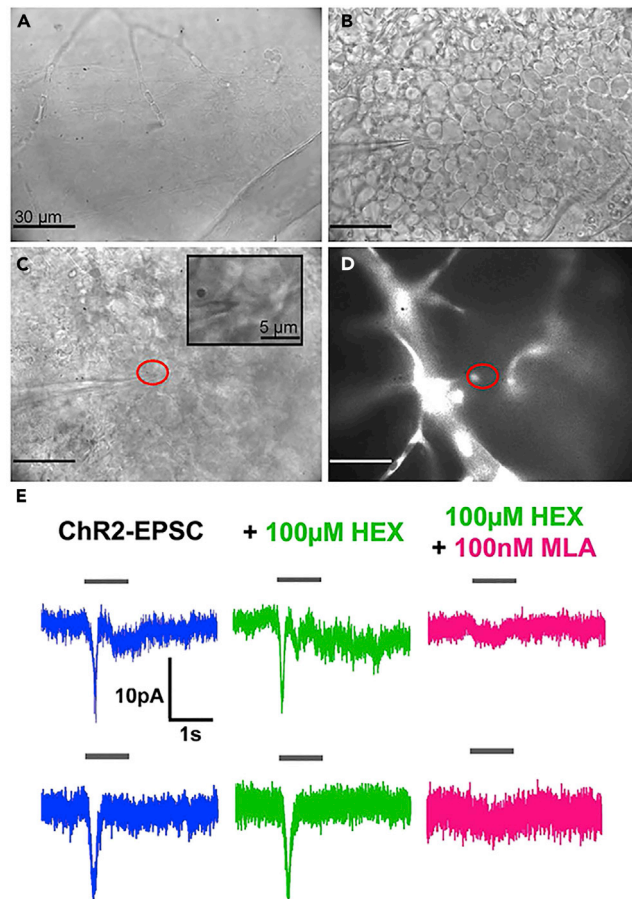
4. Using the 10× objective, place the retinal tissue in the viewing area. Put a recording pipette in the pipette holder on a micromanipulator (Figure 2B).
- a. Advance the tip of the pipette in the center of the viewing area.
  - b. Slowly move it down into the Ames' solution.

**Note:** The tip of the pipette should be close to the tissue but stay above the focal plane of the retina; then, change the objective to 60×.

5. Under the 60× objective, slowly move the lens down until you find the pipette tip. Then, lower the objective focus and the pipette tip in a back and forth manner repeatedly, until the tip is approximately 200 μm above the tissue. Then, apply a positive pressure into the pipette.
- a. Inflate with the 1 mL of air into the pipette for wholemount tissue recordings.

**△ CRITICAL:** Applying the positive pressure into the pipette prevents the tip of the pipette clogging from the tissue and any remaining vitreous.

- b. At this point, set the pipette current to zero by the Axoclamp 700B commander.
- c. Start the Membrane Test program on the Clampex to measure the pipette resistance. The initial position is the Bath mode.



**Figure 4. Whole cell recordings of bipolar cells**

(A) The surface of the retina under a 60× objective DIC imaging prior to puncture with a microelectrode.  
 (B) The ganglion cell layer is exposed after puncturing of the ILM with a microelectrode and applying positive pressure.  
 (C) An image of the bipolar cells layer with a microelectrode.  
 (D) After physiological recording, an image of the bipolar cell was viewed under sulforhodamine imaging. The image shows a soma with the electrode. The vasculature is sometimes labeled by sulforhodamine in the whole mount retina.  
 (E) Example recordings of ChR2-evoked EPSC responses in a bipolar cell under control, HEX, and MLA solution. The recordings show a cell that had MLA sensitive ACh currents and were not sensitive to HEX. Scale bars for main panels A-D are 30 μm. The inset for panel C scale bar is 5 μm.

6. Advance the pipette tip to the tissue. Penetrate the inner limiting membrane (ILM) by the pipette (Figures 4A and 4B). You should see that the positive air separates the membrane and ganglion cells, and each cell shows defined edges (Figure 4B). [Troubleshooting 5](#).

**△ CRITICAL:** If the pipette does not separate the membrane and ganglion cells, advance the pipette further horizontally and slightly deeper. If it is still not achieved, there should be an issue with either pipette pressure leak, or tissue tension due to a loose or older net not creating good tension on the surface of the tissue for penetration.

7. Advance the pipette by an oblique approach into the inner nuclear layer (INL) (Figure 4C).

**Note:** This is identified as a planar region of somas located ~50 μm below the GCL. The distance between the GCL and INL may vary from retina to retina.

a. Advance through a large layer with no visible somas, which is IPL.

b. Following the IPL, there is another region with visible somas, which is the INL.

△ **CRITICAL:** In the INL, the first layer is amacrine cells with medium sized somas, whereas deeper in the INL, smaller somas are evident. The latter is bipolar cells.

c. In the bipolar cell region, target a cell where clearly the edge is visible. Position the pipette on the cell soma and advance slowly until a slight dimple on the cell membrane is visible (Figure 4C). Release the positive pressure.

△ **CRITICAL:** If successful, you should see the pipette resistance on the Membrane Test slowly increase to 100 MΩ. If the resistance does not increase, apply a small amount of negative pressure to the pipette.

8. Once the pipette resistance has reached above 100 MΩ, switch the Membrane Test program from the Bath mode (Holding potential, 0 mV) to the Cell Attached mode (-70 mV), which applies the negative current to the pipette. Then, wait until the resistance becomes above 1 GΩ. This is the “giga-seal”.

9. After the giga-seal is achieved, apply a small amount of negative pressure (less than 1 mL) to the pipette to rupture the membrane within the pipette.

a. Use a 50–100 μs zap to rupture the membrane if negative pressure is not sufficient.

**Note:** The cell can be safely zapped two or three times before the experimenter could try a new cell.

b. When successful, the membrane current shows wider transient current at the onset of the pulse. This is the whole cell patch clamp configuration. [Troubleshooting 1](#).

c. Once the membrane ruptures, switch the Membrane Test to the Cell mode.

d. Wait for ~3 min for your intracellular solution to replace the cytoplasm of the cell and stabilize the recording.

△ **CRITICAL:** The baseline current should be around -20 to -50 pA at the holding potential of -70 mV. If the baseline current becomes more negative than -100 pA, this is the sign of leak current due to a poor seal, and would cause inaccurate recordings. Apply a small negative pressure to the pipette. If it does not improve the baseline current, remove the pipette, and try recording another cell.

e. After this, you are ready to start recordings from the bipolar cell with either a light or electrical stimulus.

**Note:** Check the sulforhodamine image of your bipolar cell after whole cell configuration is established. The soma should be brightly labeled with the fluorescent dye after a successful break-in during step 9 (Figure 4D).

**Note:** In addition, use an area of the retina where you penetrated the tissue earlier to put in a second pipette. This decreases the chances of going through another cells or vasculature that clogs the pipette before reaching the bipolar cell layer.

### Recording ChR2 light responses from bipolar cells

⌚ Timing: 30 min

This step demonstrates how to record light-evoked responses and apply the pharmacological agents of this experiment.

10. Record the light-evoked postsynaptic currents (L-EPSCs) in response to a step of light in control Ames' solution. In voltage-clamp mode, the tissue is illuminated with background green light. Then, use a  $\sim 150$   $\mu\text{m}$  spot of light and increase the light level for one second. [Troubleshooting 2](#).
  - a. The CoolLED was used for the light stimulus, using a 500 nm (green) LED. The light intensity is controlled by changing the current through the analog waveform function in Clampex.
  - b. The light level we used was  $1.0 \times 10^5$  photons/ $\mu\text{m}^2/\text{s}$  for the background,  $1.0 \times 10^7$  photons/ $\mu\text{m}^2/\text{s}$  for the initial spot light stimulation, and  $1.56 \times 10^{10}$  photons/ $\mu\text{m}^2/\text{s}$  for the ChR2 stimulation.
  - c. The bipolar cell would respond either ON, OFF, or no response.

**△ CRITICAL:** If no light response is present, examine the morphology of the cell and move on to recording another cell in the tissue.

11. If the cell responds to a step of light, record several L-EPSCs. Then, start perfusion of the second Ames' solution containing the glutamate receptor blockers. Several minutes after the blocker perfusion, run the same stimulation protocol in Clampex to record L-EPSCs. [Troubleshooting 4](#).
  - a. This time you should see no L-EPSCs because glutamate receptors are blocked and the light level is not high enough to activate ChR2.
  - b. If light response remains, wait another few minutes and record L-EPSCs. The response should disappear at this light level.
12. Increase the stimulus light level to activate ChR2 and record L-EPSCs. The response should be evoked through the ChR2 activation.
  - a. The ChR2 requires a bright light stimulus. In our measurement, the threshold for ChR2 was  $10^7$  photons/ $\mu\text{m}^2/\text{s}$ .
  - b. For the optogenetic recordings, use a 100 ms duration spot of light stimulus.
13. Record the ChR2-evoked EPSCs in the absence or presence of the nicotinic acetylcholine antagonists, MLA and HEX ([Figure 4E](#)).
  - a. After several L-EPSCs recordings in the control Ames' solution, MLA or HEX is added to perfusing Ames' solution with glutamate receptor blockers. The order of MLA and HEX is random, on a cell-to-cell basis. Perfuse the first cholinergic blocker solution for over 5 min, then record several L-EPSCs.
    - i. If the first blocker did not affect the L-EPSCs, apply the second cholinergic blocker in addition to the first drug.
  - b. If there is a change in the response after the first blocker application, wash out the drug for 5 min with Ames' solution, and then solely apply the second cholinergic blocker.
  - c. After 5 min of the second cholinergic blocker perfusion, record the ChR2-evoked EPSCs.
  - d. Once this is complete, wash out the drug solution with regular Ames' solution and see if normal photoreceptor light response is present.

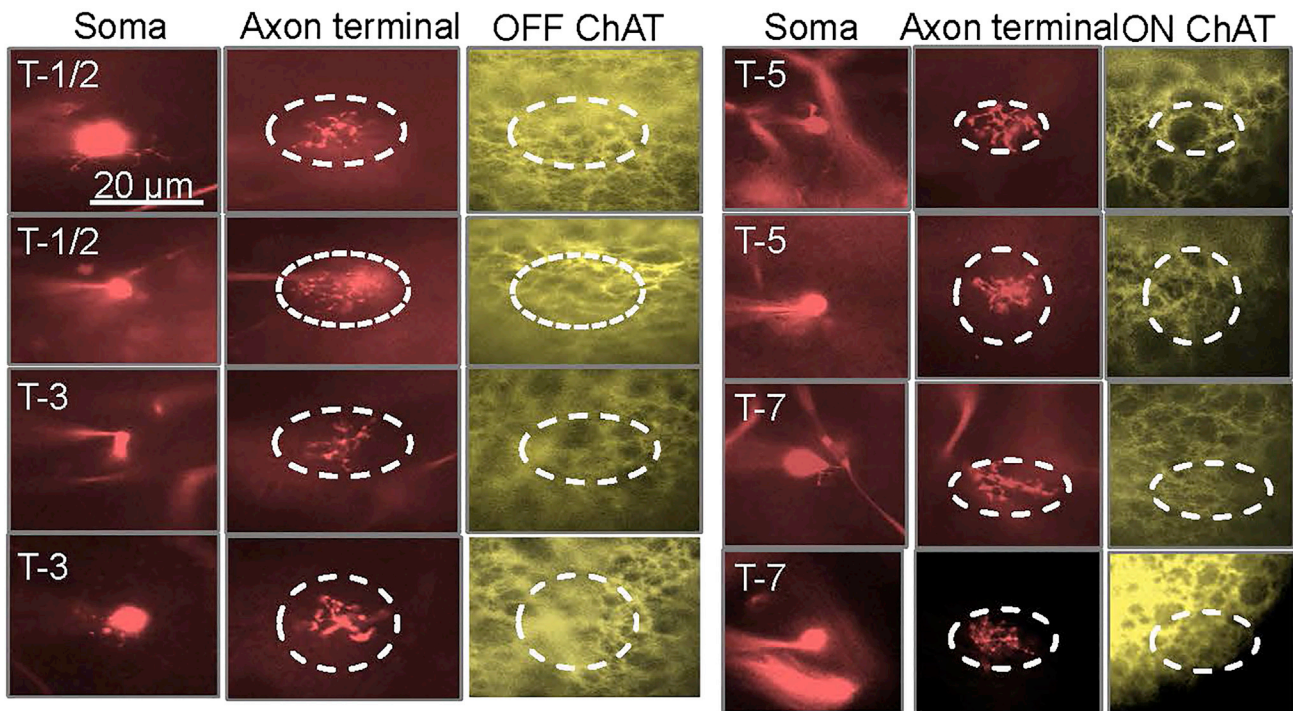
### Imaging terminals of recorded cell to determine bipolar cell type

⌚ Timing: 30 min

This step describes the method for identification of bipolar cell types in live tissue immediately after physiological recordings.

14. Image your cell using epifluorescence for sulforhodamine and ChR2-EYFP fluorescence.
  - a. Visualize the sulforhodamine in the recording pipette and the soma of the recorded cell. Use the rhodamine (red) fluorescence cube and the bright LED light ( $\sim 560$  nm).





**Figure 5. Identification of bipolar cell types**

Representative images of different bipolar cell types are shown under sulforhodamine and YFP fluorescence. The soma with dendrites verifies that the recorded cell was a bipolar cell. Axon terminal ramification in relation to ChAT band allows cell type identification. Panels are multiple cells from Type 1 or 2, Type 3, Type 5, and Type 7 cone bipolar cells.

- b. Identify bipolar cells. After viewing the pipette and the connected soma, view deeper into the tissue to find the dendrites of the cell. The dendrites should go down to the OPL and have some branches. If there are no dendrites, you are likely recording from an amacrine cell.
    - i. Identify bipolar cell axon terminals. View the terminals of the bipolar cell by searching toward the surface of the tissue. A single axon shaft emerges from the soma, which spreads out to the axon terminals in the IPL (Figure 5).
15. Refer to the ON and OFF ChAT bands to identify the bipolar cell type. [Troubleshooting 3](#).

**Note:** The Ai32 ChAT-Cre mouse contains ChR2 tagged with enhanced yellow fluorescent protein (EYFP). Therefore, both ON and OFF SACs are labeled with YFP throughout the soma and processes (Hellmer et al., 2021).

- a. Observe the YFP expression with a GFP filter cube via 500 nm LED light illumination with the CoolLED.

**Note:** The ON ChAT band is closer to the GCL in the IPL. The OFF ChAT band is deeper in the IPL, closer to the INL.

- b. Compare the location of the axon terminals ramification and the ON and OFF ChAT bands (Figure 5). [Troubleshooting 6](#).
  - i. The terminal ramifies outer to OFF ChAT band: type 1 or 2.
  - ii. The terminal ramifies slightly inner to OFF ChAT band: type 3.
  - iii. The terminal ramifies both inner and outer of OFF ChAT band: type 4.
  - iv. The terminal ramifies on the ON ChAT band: type 5.
  - v. The terminal ramifies slightly inner to ON ChAT band: type 7.



- vi. The terminal ramifies from outer to inner ON ChAT band: type 6.
  - vii. The terminal ramifies completely inner to ON ChAT band: type 8 or 9.
  - viii. The terminal ramifies inner to ON ChAT band with the beads shape: rod bipolar cells.
16. Capture images of the YFP at the same depth as that of the terminals.
- a. Have a figure of the bipolar cell type morphology and stratifications at your recording station for easier identification (Wassle et al., 2009).

## EXPECTED OUTCOMES

A subset of bipolar cells exhibit ChR2-evoked L-EPSCs, which are mediated through the nicotinic acetylcholine receptors (nAChRs). We previously found that a subset of bipolar cells express  $\alpha 7$ -nAChRs (Hall et al., 2019). We confirmed that feedback inputs from starburst amacrine cells evoked L-EPSCs in bipolar cells in a type-dependent manner (Hellmer et al., 2021). In type 1/2 and 7 bipolar cells, MLA-sensitive,  $\alpha 7$ -nAChRs-mediated ChR2-evoked EPSCs are recorded. In contrast, in other types of bipolar cells, either no ChR2-evoked EPSCs or HEX-sensitive, non- $\alpha 7$ -nAChRs mediated ChR2-evoked EPSCs are recorded (Figure 4E).

## LIMITATIONS

This protocol displays how to record from bipolar cells that are deep in the wholemount retinal tissue. To achieve this goal, it requires specific technologies and training. Bipolar cells are approximately 70–100  $\mu\text{m}$  deep from the surface of the inter-limiting membrane. For our microscope, there are specific mechanics involved to allow viewing these cells deep in the thick tissue. The differential interference contrast (DIC) with infrared light source and an infrared filter is necessary. Also, a long-working distance, high quality 60 $\times$  objective for patch clamp recording (e.g., UPLFLN 60 $\times$ ) is critical. Furthermore, a high-quality CCD or CMOS camera is important. Without these, it will be difficult to properly observe bipolar cells. If green fluorescent protein (GFP) mouse lines are available for targeting bipolar cells, bipolar cell recordings would be easily achieved with a 2-photon or confocal microscope. Although patch clamp recordings from cells in the deep tissue is a challenge, it is achievable with help of recent technologies.

## TROUBLESHOOTING

### Problem 1

Unable to achieve giga-seal or whole cell configuration (step 9b).

### Potential solution

The viability of the cell is a crucial factor to whole-cell clamp success. Only freshly harvested retinas with no obvious abnormalities (retinal thin or thickness, obvious scarring on the tissue, ect.) should be used. When looking at the tissue under 60 $\times$  objective, the cell somas should appear smooth and cells healthily round. Condensed nuclei are a key sign of an unhealthy tissue. Check the pH of the Ames' solution and keep a high perfusion rate. Also, the pipette resistance is a critical point to check. The goal of the resistance is 8–12 M $\Omega$  for the bipolar cell recording in the tissue. If a pipette tip is too large or too fine, it will impede forming a good seal or to break into the cell. Furthermore, it is important to ensure the osmolarity of your intracellular solution is  $\sim 90\%$  of the Ames' solution as a rule of thumb. Finally, the basic skills for patch clamp recording are required. If you are still in the process of acquiring the skills, it would be better to practice with ganglion cells that are large and lay shallower in the tissue.

### Problem 2

Electrical noise preventing L-EPSC recording from bipolar cells (step 10).

### Potential solution

The noise issue usually occurs with the reference wire problems. Check the agar bridge and the integrity of the reference wire. Check the chloride on the reference wire. Use a piece of sandpaper

and remove any salt buildup and old chloride coating. Stick the wire in a bleach solution for over 30 min to properly coat the wire. Other noise sources may include objects on the patch clamp rig that are not grounded (patch syringe, suction tube, the cage, etc.). Turn off any electrical object in the room not essential for experimenting to check if any of them are causing the noise.

### Problem 3

Unable to see the fluorescence of either the sulforhodamine-labeled bipolar cells or YFP-labeled ChAT-bands (step 15).

### Potential solution

There can be multiple causes: The sensitivity of the CCD camera. Increase the sensitivity by adjusting the exposure time and gain settings. Also, check if the filter cube is correct. Finally, make sure any neutral density filters are not limiting the light intensity.

### Problem 4

There is no ChR2-evoked light response after the application of glutamate receptor blockers (step 11).

### Potential solution

First, check the light level of stimulus for ChR2 activation. We used green light at a level of  $1.0 \times 10^{10}$  photons/ $\mu\text{m}^2/\text{s}$  (Hellmer et al., 2021). The peak wavelength of ChR2 is 470 nm. Depending on the wavelength available, brighter activation might be required (Nagel et al., 2003).

Second, keep in mind that not all the bipolar cells receive cholinergic inputs from SACs. If a cell does not have a response to ChR2, check the morphology of the cell and go to a next bipolar cell recording.

### Problem 5

Unable to penetrate the inter-limiting membrane and visualize the bipolar cell somas (step 6).

### Potential solution

The positive pressure in the recording pipette may not be high enough. The bipolar cell recording pipettes at 8–12 M $\Omega$  are small and easily clogged. If the pipette is clogged, shown by increased pipette resistance and a loss of the clean square wave in Membrane Test, you need to change to a new pipette.

The pressure in a pipette may be leaking out. To check this possibility, inflate 1 mL of air into the pipette and hold it for a minute. Then, release the pressure back into the syringe and see how much air comes back into the syringe. If the air leaks out, check the tubing and connections to find the leak site and replace that piece.

### Problem 6

The bipolar cell terminal staining is not clear (step 15b).

### Potential solution

For complete staining the whole cell configuration should be maintained for more than 10 min. Sometimes tissue is not mounted flat and terminals are not easily located in relation to the ChAT bands. Always focus on the level near the axon shafts and terminals to determine the bipolar cell type.

### Problem 7

The tissue is damaged from the dissection (step 1i).

### Potential solution

The retina can be easily damaged during the dissection if the experimenter is new to the technique. The retinal dissection is a difficult procedure, and requires rigorous practice. Some tips for improvement include using high quality tools, especially for the vitreous removal, grabbing the retina at the far edges, and not deforming the eyeball when removing the anterior segment. Another tip is to keep the second eye for backup in case of any accidental nicking or improper grabbing of the tissue. Furthermore, make sure tools are cleaned after use every day.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tomomi Ichinose ([tichinos@med.wayne.edu](mailto:tichinos@med.wayne.edu)).

#### Materials availability

The study did not generate new unique reagents.

#### Data and code availability

This protocol did not generate new datasets or codes.

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### AUTHOR CONTRIBUTIONS

C.B.H. and A.S. conducted experiments and data analysis. J.M.B. performed partial experiments and data analysis. J.M.B. and T.I. wrote the manuscript. T.I. supervised the project and revised the manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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