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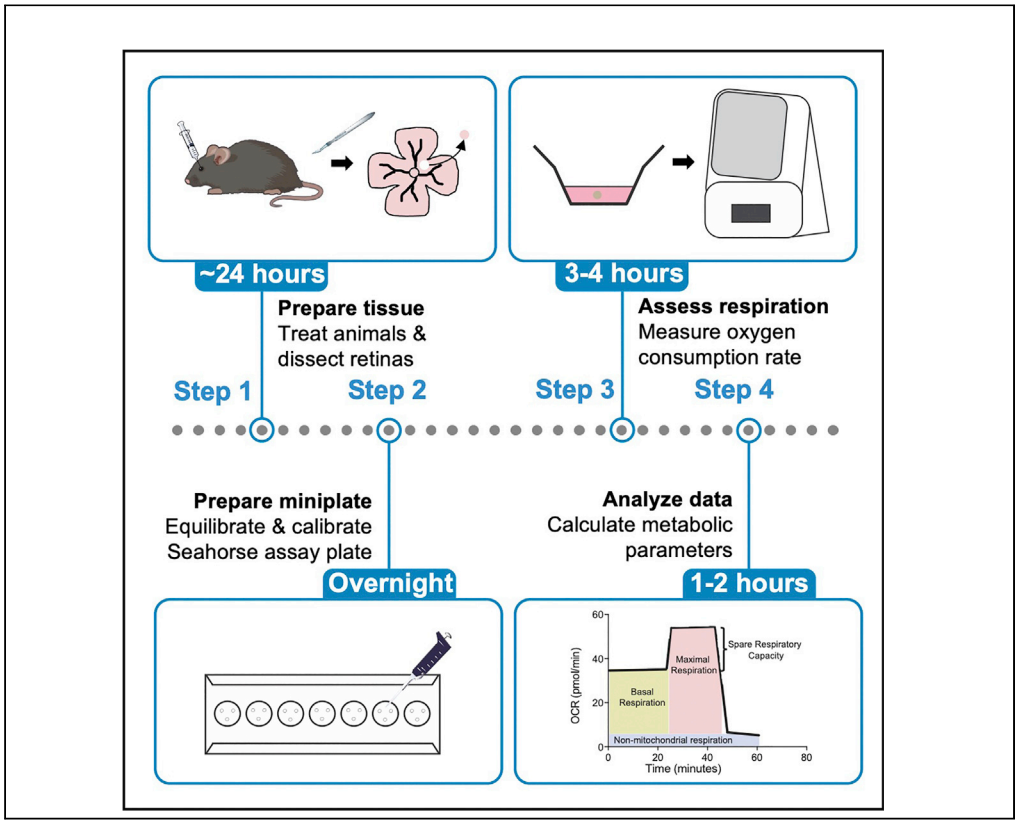
Protocol

Measurement of mitochondrial respiration in the murine retina using a Seahorse extracellular flux analyzer



Mitochondrial metabolism is a critical mechanism that is deregulated in numerous retinal diseases. Here, we elaborate a protocol to quantify oxygen consumption rate as a measure of mitochondrial respiration directly from mouse retinal tissue pieces. Our procedure combines the use of Seahorse extracellular flux technology and *ex vivo* retinal tissue isolation and is robustly reproducible under different treatment conditions. This protocol allows direct assessment of mitochondrial function in response to drug treatments or genetic manipulation in mouse models.

Trupti Shetty,
Bomina Park,
Timothy W. Corson

trups.shetty@gmail.com (T.S.)
tcorson@iu.edu (T.W.C.)

Highlights

Assessment of mitochondrial respiration is valuable for retinal disease studies

Ex vivo mouse retinal tissue punches can be used for Seahorse extracellular flux analysis

Retinal mitochondrial function can be assessed in response to drugs or genetic changes

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Measurement of mitochondrial respiration in the murine retina using a Seahorse extracellular flux analyzer

Trupti Shetty,[1,2,4,5,*] Bomina Park,[1,2] and Timothy W. Corson[1,2,3,6,*]

[1]Eugene and Marilyn Glick Eye Institute, Department of Ophthalmology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[2]Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[3]Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[4]Present address: Neurobiology, Neurodegeneration & Repair Laboratory, National Eye Institute, National Institutes of Health, Bethesda, MD 20892, USA

[5]Technical contact

[6]Lead contact

*Correspondence: trups.shetty@gmail.com (T.S.), tcorson@iu.edu (T.W.C.)
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SUMMARY

Mitochondrial metabolism is a critical mechanism that is deregulated in numerous retinal diseases. Here, we elaborate a protocol to quantify oxygen consumption rate as a measure of mitochondrial respiration directly from mouse retinal tissue pieces. Our procedure combines the use of Seahorse extracellular flux technology and *ex vivo* retinal tissue isolation and is robustly reproducible under different treatment conditions. This protocol allows direct assessment of mitochondrial function in response to drug treatments or genetic manipulation in mouse models.

For complete details on the use and execution of this protocol, please refer to Shetty et al. (2020), Sardar Pasha et al. (2021), Kooragayala et al. (2015), and Joyal et al. (2016).

BEFORE YOU BEGIN

Animals are bred or dosed with the appropriate treatments and assay cartridges are prepared overnight for use the following day. Although intravitreal injections are described here, animals can be treated with systemic or other agents, or genetically modified mouse strains can be used, depending on the experimental goal. For studies with genetically modified mice, congenic animals (and ideally littermates) should be used.

Animal treatment and cartridge preparation – day 1

Timing: ~16–24 h (depending on treatment duration)

An overview of the procedure is provided in Figure 1.

1. For drug studies, intravitreal treatment with inhibitor/drug of choice
   a. Prepare compounds, either vehicle or inhibitor of choice for intravitreal (IVT) injections by dissolving compound in phosphate-buffered saline solution to a final concentration of no more than 0.5% DMSO. For vehicle control, use diluent solution without compound of interest.
   b. Anesthetize C57BL/6J wild-type, healthy mice of both sexes, 8 weeks of age by intraperitoneal (i.p.) injections of 90 mg/kg ketamine hydrochloride and 5 mg/kg xylazine mixture. 4.5 μL/g of the cocktail below is injected (i.e., 90 μL for a 20 g mouse).

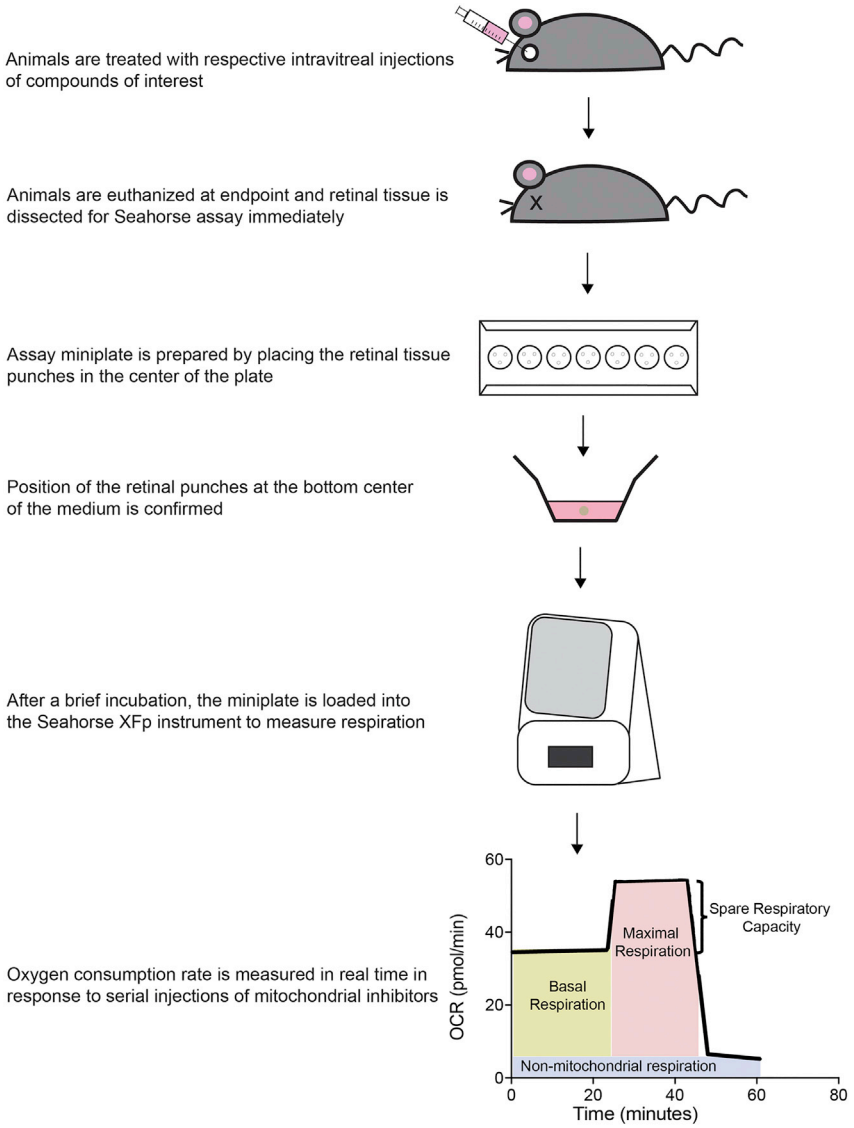

Figure 1. Overview of the procedure
The key steps are indicated

c. Use one drop of ophthalmic tetracaine hydrochloride (0.5%) as a topical anesthetic for the eyes. Following dilation of the pupils using one drop of 1% tropicamide and one drop of 2.5% phenylephrine eye drops to allow a view of the vitreous chamber under the dissecting microscope, make a small incision using an insulin syringe needle, at the nasal-temporal ora serrata (approximately 4 mm below the iris) to gain access to the posterior cavity consisting of the vitreous chamber.
d. Make use of high precision, sterile Hamilton syringes (0.5–5 μL volume) with a sharp tip for injections. Inject vehicle (0.5 μL) with or without compound of interest intravitreally at the angle of about 45–60° towards the plane of injection avoiding any damage to the lens or retina.
e. Monitor the anterior chamber for a slight perturbation immediately after injection to confirm a successful intravitreal administration. Any efflux of compound solution into the scleral pocket should be considered a failed injection.

Note: Include at least six animals per treatment condition for biological replicates and at least two retinal punches from each individual eye for technical replicates, keeping either right or left eye consistent for all animal groups.

2. Hydration of Seahorse sensor cartridges
   a. Hydrate the appropriate number of sensor cartridges corresponding to the assay miniplates that will be used on the day of the assay. Each individual cartridge is compatible with one XFp miniplate.
   b. Separate the white utility plate from the top sensor cartridge (green plate) provided in the XFp FluxPak by placing the cartridge upside down on the bench. Load 200 µL of calibrant solution (provided by the manufacturer) in each well of the bottom utility plate, placing back the sensor cartridge on top followed by incubating the cartridges overnight in a non-CO_2 incubator at 37°C.

Note: Do not incubate the probes for longer than 16 h to avoid salt build-up from the calibrant solution. Troubleshooting 1

MATERIALS AND EQUIPMENT

Anesthesia cocktail	Final concentration (mg/mL)	Amount
Ketamine (100 mg/mL)	20	1 mL
Xylazine (20 mg/mL)	1.11	0.278 mL
Sterile saline	n/a	3.722 mL
Total	**n/a**	**5 mL**

Store at room temperature for several months or until expiry date of any component, whichever comes first.

⚠ CRITICAL: ETC inhibitors FCCP, rotenone, and antimycin A are toxic to humans and can be absorbed by inhalation or ingestion. Personal protective equipment and a fume hood should be used while handling and reconstitution of the powders.

⚠ CRITICAL: Ketamine is a controlled substance and should only be used in accordance with local laws.

Alternatives: This method was originally established using the Seahorse XF24 analyzer instrument and has been successfully adapted to the Seahorse XF96 and is described here using the Seahorse XFp, as described in the key resources table. Intravitreal injections can be performed by other methods such as injection pumps or under surgical microscopes. Other anesthetic regimens may be used.

STEP-BY-STEP METHOD DETAILS

Quantification of oxygen consumption – day 2

Timing: ~3–4 h per run

The following steps accomplish isolation of the retinal tissue from treated animals and setting up of the Seahorse assay for determination of oxygen consumption rates of individual retinal punches.

Note: This protocol is an optimization of previously published protocols (Kooragayala et al., 2015; Joyal et al., 2016; Shetty et al., 2020; Sardar Pasha et al., 2021).

Note: There are no pause points in this protocol given the nature of the experiments. The whole process needs to be done without breaks due to the fragility of the tissue.

1. Preparation of assay medium
   a. Prepare the assay medium for the retinal punches immediately prior to use by supplementing the Seahorse DMEM (which contains 5 mM HEPES) with 12 mM glucose and 2 mM L-glutamine (Sodium pyruvate between 0.12 - 0.5 mM can be included as an additional fuel source and buffering agent).

Reagent	Final concentration	Amount for 20 mL of medium
Seahorse DMEM	-	19.604 mL
D-glucose (2.5 M solution)	12 mM	96 µL
L-glutamine (200 mM solution)	2 mM	200 µL
Sodium pyruvate (100 mM solution) (optional)	0.5 mM	100 µL

   b. Place 180 µL of freshly prepared assay medium in each well.

2. Isolation of retinal tissue (Figure 2)
   a. Euthanize animals using CO_2 asphyxiation followed by cervical dislocation, or other appropriate methods as approved by institutional guidelines.
   b. Immediately enucleate eyes by placing a pair of forceps directly under the eye socket and plucking gently from the optic nerve head; proceed immediately with dissection of the posterior cup.
   c. Separate the cornea and lens from the posterior segment of the eye. Pull the scleral layer away from the neural retina gently and isolate the retinal cup devoid of retinal pigment epithelium layer.
   d. Flatten the retinal cup into a "flower" with four incisions from the periphery towards the optic nerve head. Cut disks of 1 mm diameter using a biopsy puncher (e.g., Miltex) carefully without disintegrating individual retinal layers. Isolate retinal punches from regions adjacent to the optic nerve head in all eyes to maintain homogeneity among the different retinal layers. Troubleshooting 2

⚠ CRITICAL: Only use intact punched disks for the experiment; discard any pieces that appear sheared or fragmented. Troubleshooting 3

   e. Place each disk in one well with the ganglion cell layer facing upward. For experimental design, a maximum of three different conditions can be set up in a single XFp miniplate using two punches per condition (see Figure 1 for diagram). Always use duplicates to account for technical errors and variability.
   f. Incubate retinal punches in assay medium for ~ 45 min to 1 h in a room air tissue culture incubator at 37°C to equilibrate CO_2 levels between tissue and medium.

⚠ CRITICAL: Do not skip step 2f: it is critical for CO_2 levels to equalize between the tissue and the assay medium prior to assay.

Note: If placement of retinal discs on the bottom of the miniplate proves difficult, tissue adhesive (e.g., Cell-Tak, Corning Life Sciences, Tewksbury, MA, USA) can be coated on the plates before placing the tissue. Such adhesives can introduce random folding of the tissue pieces. Care should be taken to avoid interference with sensor probes while using the adsorption method to incorporate Cell-Tak.

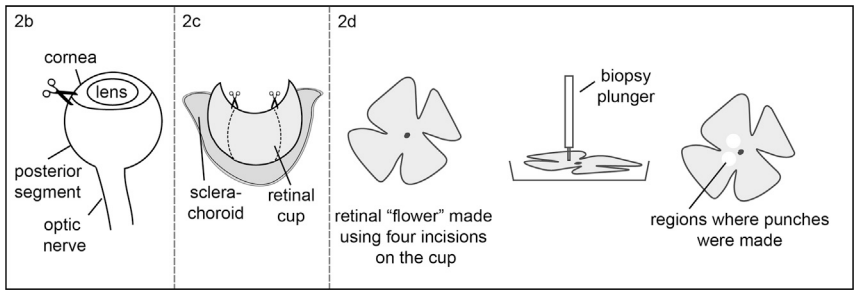

Figure 2. Retinal dissection and punch preparation

Layout of the retinal dissection corresponding to step 2 in the protocol is shown. Incision marks are indicated with a pictorial scissors icon in steps 2b and 2c. After separating the sclera-choroid-RPE layer, the retinal cup is isolated, and four incisions are made. In step 2d, the resulting retinal "flower" is then used to isolate 1 mm sized punches using a biopsy puncher. Regions adjacent to the optic nerve are chosen as displayed.

3. Preparation and loading of injection compounds in sensor cartridge
   a. Resuspend FCCP and rotenone/antimycin mixture provided in the Seahorse XF Cell Mito Stress test kit in the assay medium as directed by the manufacturer.
   b. Use 0.5 μM final well concentration of FCCP and inject the respective volume (22 μL) in port B of the sensor cartridge.
   c. Similarly, use 0.5 μM final well concentration of rotenone/antimycin A mixture and inject the compound in port C of the cartridge.

Note: Skip the manufacturer's recommended oligomycin injection due to high toxicity (Koor-agayala et al., 2015) observed for use with retinal punches.

4. Run the assay on the Seahorse XFp instrument. Troubleshooting 4
   a. Set up the assay program in the instrument or by creating a program template in Wave software by selecting a cycle of mix (3 min), wait (2 min), and measure (5 min) conditions.
   b. Take five measurements of basal respiration, followed by four measurements after the addition of the mitochondrial uncoupler FCCP, and four final measurements following the administration of rotenone/antimycin A mixture.
   c. Place the loaded sensor cartridge and allow calibration of the plate (approximately 20 min) followed by OCR measurements as designed in step 4b.

⚠ CRITICAL: Calibration of the hydrated sensor cartridges takes approximately 20 mins on the XFp (time may vary depending on the type of Seahorse equipment). This should be considered while incubating the retinal tissue in the room air incubator for 1 h to avoid over-incubating the tissue and causing nutrient deprivation.

⚠ CRITICAL: Do not reduce the number of cycles of measurements after FCCP addition (for maximal respiration output). If needed, the number of cycles can be increased to capture the OCR spike expected after FCCP injection. Troubleshooting 5

Optional: To reduce total run time of the assay, measurements can be reduced to four and three for basal respiration and rotenone/antimycin A injections respectively.

EXPECTED OUTCOMES

In this section, five example individual retinal punches B-F (taken from both eyes of a normal, untreated animal) are included with the corresponding OCR curves to show the range of expected OCR reads in a typical assay. A control well lacking tissue is also shown. Data were collected with Wave software and exported to GraphPad Prism for graphical presentation. OCR readings in real-

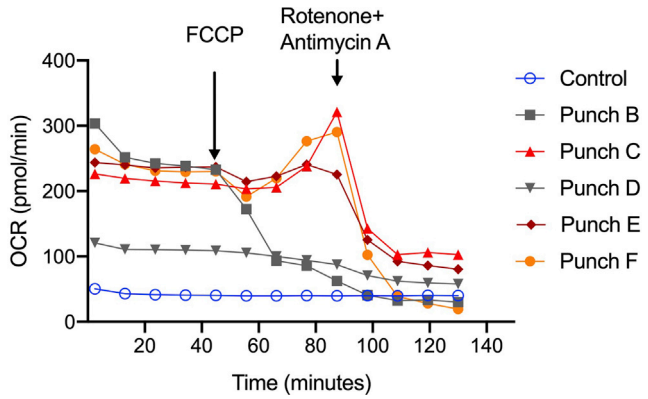

Figure 3. Output of retinal oxygen consumption rate

Plot showing individual oxygen consumption rates (OCR) of five individual retinal punches (taken from both eyes of a normal, untreated animal) plus no tissue control, with indicated arrows showing time points at which FCCP and rotenone/antimycin are injected during the assay

time can be seen along with spikes or dips in the OCR traces in response to FCCP, rotenone, and antimycin (Figure 3).

From the above example, data from retinal punches B and D can be excluded. Both the punches show a decline in OCR curve and have no activity post FCCP and rotenone injections (Figure 3), suggesting a damaged and poorly viable disk of retinal tissue. Responses from such disks should not be considered for analysis. The remaining punches show a response to both FCCP and rotenone+-antimycin A and can be used for calculation of respiratory parameters using Wave software (Figure 4). The variability in OCR parameters between punches highlights the importance of performing multiple technical (and biological) replicates to obtain robust data.

QUANTIFICATION AND STATISTICAL ANALYSIS

Table 1 shows raw values from the individual retinal punches B (unusable) and C (viable) displayed in Figures 3 and 4 that can be further processed for data analysis as detailed below. Troubleshooting 6

1. Using Wave software, the following parameters can be determined from the experimental set-up detailed in this protocol (see Figure 1 for a graphical representation).

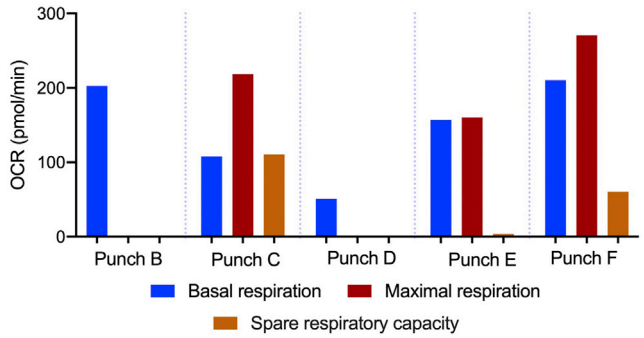

Figure 4. Quantification of mitochondrial respiration parameters

Parameters of basal and maximal respiration along with spare respiratory capacity for five individual punches (taken from both eyes of a normal, untreated animal) are graphed using the raw values from the Seahorse instrument (see Figure 1 for a graphical representation of how these parameters are calculated)

Table 1. Oxygen consumption rate readings

Time (min)	Group name: Punch B	Group name: Punch C
2.47	303.43	226.35
13.07	251.89	219.48
23.69	242.37	215.54
34.30	238.35	212.49
44.92	232.91	210.75
55.61	172.61	203.45
66.22	93.51	205.76
76.84	85.75	238.26
87.46	62.63	321.21
98.16	40.96	142.71
108.74	32.64	102.89
119.36	33.49	106.33
129.99	30.23	102.88

Raw values measured at indicated time points corresponding to the oxygen consumption rate (OCR) (in pmol/min) of two example individual retinal punches from the same animal, one unusable (B) and one viable (C).

a. Non-mitochondrial respiration: Take the minimum OCR measurement after addition of rotenone/antimycin A.
b. Basal respiration: Calculate by subtracting non-mitochondrial respiration from the highest OCR measurement right before FCCP injection.
c. Maximal respiration: Calculate by subtracting non-mitochondrial respiration from the highest OCR measurement after FCCP injection.
d. Spare respiratory capacity: Calculate by subtracting basal respiration from maximal respiration.

LIMITATIONS

The Seahorse XFp instrument only allows for six tissue readings at a time, restricting the number of technical replicates and treatment groups that can be included in single assay, although in our hands plate-to-plate variability is manageable. Other Seahorse instruments offer more sample number flexibility and thus the possibility of additional technical and/or biological replicates in a single assay for enhanced robustness. Additionally, the reproducibility of this assay relies on equal input tissue amounts. A robust biochemical assay to normalize such small tissue fragments is lacking. The use of a biopsy punch maximizes the uniformity of tissue pieces to mitigate this source of variability. ETC inhibitors like FCCP can cause damage to the retinal tissue during the assay, especially if the tissue is already sensitive (maybe due to a pre-treatment), or if higher concentrations of FCCP than the recommended are used to ensure an OCR spike. Such samples should be excluded. Finally, the predominance of photoreceptor mitochondrial activity in the retinal tissue can make it difficult to differentiate OCR contributions of other cell types or otherwise deconvolute the signal from different cells. This should be considered in interpreting results.

TROUBLESHOOTING

Problem 1

Over-hydration of the cartridge sensors with the XF Calibrant solution can cause salt buildup from the solution. This can result in an inaccurate reading and interfere with media buffering, evidenced by errors in the calibration step, large error bars, or inconsistent OCR readings between injections. (before you begin step 2)

Potential solution

XF readings are very sensitive to any changes in pH and users must always account for changes in buffering capacity. Incubate the cartridge in calibrant solution for hydration only for a minimum of

2 h and not exceeding 16 h. If a delay in procuring retinal tissue is anticipated on the day of the assay, the cartridges can be hydrated in pure distilled water and moved to calibrant solution the following day, for at least 2 h, before proceeding with the assay.

Problem 2

Certain treatment conditions of the retina may induce retinal detachment or cause loss of photoreceptors leading to thinning of the retinal layers. In such cases, 1 mm size of the punches may not result in sufficient OCR output and/or may have low response to the ETC inhibitors. (step 2d)

Potential solution

In case of expected damage to the retinal layers owing to the mouse model or the treatment condition, retinal punch size may be increased to 1.5 mm. However, beyond this size retinal punches may begin curling and render them difficult for the assay.

Problem 3

Biopsy punches may cause tears in the retinal tissue which may introduce retinal detachments and loss of cells. (step 2d)

Potential solution

Retinal dissections for isolating punches using a biopsy punch result in consistent tissue size and may reduce overall variability in replicates. To minimize tissue damage, the punch should be held at a 90° angle to the retinal tissue. The punch should be made with a gentle jab, and enough force to pop out the disk-shaped tissue piece. Some practice may be required in this step, to master the technique without causing tissue damage and to obtain viable punches.

Problem 4

The force of injection of the ETC inhibitors can flip or tear the retinal punches in the XFp miniplate wells. (step 4)

Potential solution

Damaged retinal tissue pieces will give a poor and/or flat OCR curve which will need to be excluded from analysis. Having sufficient technical replicates can minimize such issues. If the problem persists with multiple punches in a single assay plate, use of tissue adhesive like Cell-Tak might be necessary.

Problem 5

Some retinal preparations may not respond to FCCP in the template set-up in this protocol and may not give a high enough OCR value before the addition of rotenone-antimycin mixture. (step 5)

Potential solution

Increase the number of cycles of measurement after FCCP addition above the recommended five measurements. However, this may prolong the assay time and retinal exposure to FCCP causing a failure to respond to the rotenone step, interfering with non-mitochondrial OCR readings.

Problem 6

Normalization of the OCR readings using biochemical assays like BCA Protein assay or cytochrome *c* release can be difficult owing to the size of the tissue. (quantification and statistical analysis step 1)

Potential solution

Keeping the size of the punches consistent can minimize the need for secondary normalization. If optimization experiments do not yield consistent results between punches, normalization against total protein concentration can be attempted. Tissue punch size can be increased (see problem 2 above) and multiple punches can be pooled for protein estimation as demonstrated in Jassim et al., 2019.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Timothy W. Corson (tcorson@iu.edu).

Material availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all datasets generated or analyzed during this study.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Seahorse XF DMEM Medium, pH 7.4	Agilent	Cat#103575-100
D-Glucose 45% solution	Sigma	Cat#G8769
L-Glutamine	Sigma	Cat#G8540
Ketamine hydrochloride (100 mg/mL) (KETASET)	Zoetis	NADA #043-304
Xylazine (20 mg/mL)	Akorn	NDC 59399-110-20
0.5% Tetracaine hydrochloride ophthalmic	Bausch + Lomb	NDC 24208-920-64
1% Tropicamide	Alcon	NDC 61314-354-01
2.5% Phenylephrine	Akorn	NDC 17478-201-15
Critical commercial assays		
Seahorse XFp FluxPak	Agilent	Cat#103022-100
Seahorse XF Cell Mito Stress Test kit	Agilent	Cat#103010-100
Experimental models: Organisms/strains		
Mouse C57BL/6J	Jackson Laboratory	JAX: 000664
Software and algorithms		
Wave 2.4	Agilent	N/A
GraphPad Prism v.9.0	GraphPad	N/A
Other		
Seahorse XFp	Agilent	N/A
Stereo dissecting microscope	Nikon	Model#SMZ1500
Disposable biopsy punch, 1 mm	Miltex	Cat#33-31AA
Dissection tools: curved spring scissors, forceps	Fine Science Tools	Various
Tissue culture incubator (without CO_2)	Thermo	Model#3533
5 µL Glass microliter syringe #75	Hamilton	Cat#87930
33G beveled needles, 0.5-inch length	Hamilton	Cat#7803-05
U-100 insulin syringes with 28G permanent needle	BD	Cat#329461

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AUTHOR CONTRIBUTIONS

Conceptualization, T.S. and T.W.C.; methodology, T.S.; investigation, T.S. and B.P.; writing – original draft, T.S.; writing – review and editing, T.S., B.P., and T.W.C.; funding acquisition, T.W.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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