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# Measurement of energy metabolism in explanted retinal tissue using extracellular flux analysis

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**TITLE:**

# Measurement of Energy Metabolism in Explanted Retinal Tissue Using Extracellular Flux Analysis

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**KEYWORDS:**

Retina, oxygen consumption rate, extracellular acidification rate, oxidative phosphorylation, glycolysis, mitochondria, metabolism

**SUMMARY:**

This technique describes real time recording of oxygen consumption and extracellular acidification rates in explanted mouse retinal tissues using an extracellular flux analyzer.

**ABSTRACT:**

High acuity vision is a heavily energy-consuming process, and the retina has developed several unique adaptations to precisely meet such demands while maintaining transparency of the visual axis. Perturbations to this delicate balance cause blinding illnesses, such as diabetic retinopathy. Therefore, the understanding of energy metabolism changes in the retina during disease is imperative to the development of rational therapies for various causes of vison loss. The recent advent of commercially-available extracellular flux analyzers has made the study of retinal energy metabolism more accessible. This protocol describes the use of such an analyzer to measure contributions to retinal energy supply through its two principle arms — oxidative phosphorylation and glycolysis — by quantifying changes in oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) as proxies for these pathways. This technique is

readily performed in explanted retinal tissue, facilitating assessment of responses to multiple pharmacologic agents in a single experiment. Metabolic signatures in retinas from animals lacking rod photoreceptor signaling are compared to wild-type controls using this method. A major limitation in this technique is the lack of ability to discriminate between light-adapted and dark-adapted energy utilization, an important physiologic consideration in retinal tissue.

**INTRODUCTION:**

The retina is among the most energy-demanding tissues in the central nervous system[1]. Like most tissues, it generates adenosine triphosphate (ATP) *via* glycolysis in the cytosol or *via* oxidative phosphorylation in mitochondria. The energetic advantage of oxidative phosphorylation over glycolysis to produce ATP from one molecule of glucose is clear: 36 molecules of ATP generated from the former *vs.* 2 molecules of ATP generated from the latter. Accordingly, retinal neurons primarily depend on mitochondrial respiration for energy supply and this is reflected by their high density of mitochondria[2]. Yet, the retina also relies heavily on glycolytic machinery even when oxygen is abundant. This process of aerobic glycolysis was originally described in cancer cells by Otto Warburg[3], who once noted that the retina was the only post-mitotic tissue capable of this form of metabolism[4]. Since those initial observations, many post-mitotic tissues have been described to engage in varying degrees of glycolysis in addition to oxidative phosphorylation to meet their ATP demands.

Phototransduction, visual pigment recycling, biosynthesis of photoreceptor outer segments, and synaptic activity are all energy demanding processes in photoreceptors, the predominant neuronal subclass in the retina. But the need to actively transport ions against their electrical and concentration gradients is by far the most energetically consuming process in neurons[1]. Photoreceptors are peculiar neurons in the sense that they are depolarized in the absence of stimulation (*i.e.,* in the dark), whereas a light stimulus triggers channel closure and subsequent hyperpolarization. Therefore, in the dark, the retina consumes large quantities of ATP to maintain its depolarization or "dark current" as it is commonly called. From an adaptive standpoint, a major challenge in supplying these vast quantities of ATP is the need for organisms to maintain visual clarity through the optical axis. The inverted retinal architecture seen in modern creatures is the dominant solution, as it keeps the dense capillary network supplying photoreceptors away from the path of light. But this marvel of natural bioengineering places the retina at a precipice in terms of metabolic reserve. Even small insults to retina can potentially disrupt the delicate balance of energy supply to demand, and visual dysfunction or frank blindness may ensue quickly.

Given the unique energetic demands of the neural retina, coupled with its tight restriction of vascular supply, accurate measurement of ATP consumption in the retina and its changes during disease could have profound implications in understanding and treating blinding conditions such as retinitis pigmentosa and diabetic retinopathy. Traditionally, these measurements require costly, custom-designed equipment with most studies emerging from a handful of laboratories entirely dedicated to measurements of metabolic activity[2,5-8]. Techniques include individual assays for specific metabolites, tracer studies using radio-labeled precursors, oxygen consumption recording using Clark electrodes, and metabolomic profiling[9].

With advances in high-throughput technology and increased availability of commercial devices, techniques to record retinal metabolism are increasingly accessible and affordable. The method described here measures both oxidative phosphorylation and glycolysis in retina using explanted tissue and a commercially-available extracellular flux analyzer[9-12]. This analyzer separately records oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR), serving as indirect indicators of oxidative phosphorylation and glycolysis, respectively[13]. These measurements are done by a probe submersed within a microchamber created over the tissue of interest. This adaptation of previously published methods uses a capture plate originally designed for pancreatic Islets of Langerhans to record metabolic activity in small, circular sections of mouse retina. Multiple pharmacologic exposures can be delivered to the tissue during the course of a single recording because the system contains 4 injection ports for each sample well. Using this system with separate protocols optimized for ECAR and OCR recordings, the responses of wild-type retinas can be compared to retinas lacking *transducin* (*Gnat1*$^{-/-}$), a cause of congenital stationary night blindness in humans[14].

**PROTOCOL:**

Protocols followed the Association for Research in Vision and Ophthalmology Statement for the Use of Animals and were approved by Washington University.

**1. Animal Preparation**

1.1. Keep animals in standard housing with a 12 hours dark to 12 hours light cycle. Begin experiments at standardized times to avoid circadian effects, typically in the morning shortly after lights are turned on.

**2. Solution Preparation**

2.1. Prepare base media by dissolving 8.4 mg of powdered media in double-distilled $H_2O$ ($ddH_2O$) and adjust pH to 7.4 with either HCl or NaOH, to a final volume of 1 L. Filter sterilize this solution with a 0.22 µm tissue culture filter.

2.1.1. Add 1 M glucose and 100 mM sodium pyruvate to the media to achieve final concentrations of 5 mM glucose and 1 mM pyruvate.

2.1.2. Place a 50 mL aliquot of the base media in a 37 °C water bath.

2.2. Prepare lysis solution, for tissue quantitation at the end of the flux analysis, by adding tris base to 10 mM, polyethylene glycol tert-octylphenyl ether to 0.2% (v/v), and EDTA to 1 mM, all in $ddH_2O$. Mix until all components completely dissolved and adjust pH to 8.0.

2.3. For the mitochondrial stress protocol, prepare an 11 µM stock of oligomycin, an ATP Synthase inhibitor, dissolved in base media to target a final concentration of 1 µM in the well.

Prepare an 11 µM stock of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), an uncoupling agent, dissolved in base media to target a final concentration of 1 µM in the well. Prepare a cocktail of the electron transport chain inhibitors, rotenone and antimycin A (RAA) by adding rotenone to 11 µM and antimycin A to 22 µM, dissolved in base media to target a final concentration of 1 µM rotenone and 2 µM antimycin A in the well.

2.4. For the glycolysis protocol, prepare a 220 mM stock of glucose dissolved in base media, to target a final concentration of 20 mM in the well. Also prepare a 1.1 M stock of 2-deoxyglucose (2-DG), a competitive inhibitor of glucose and glycolytic antagonist, by dissolving it in base media. This will target a final concentration of 100 mM 2-DG in the well.

## 3. Instrument Calibration

3.1. To calibrate the instrumentation for an extracellular flux assay, add 1 mL of calibration solution to each well of the sensor cartridge (24 wells total) and incubate at 37 °C in a $CO_2$-free incubator overnight (or at least 8 h).

3.2. Load additives for the mitochondrial stress protocol or the glycolysis protocol into each injector port, A through D, on the sensor cartridge, adjusting for volume changes in the well.

**Note:** As an example, a typical assay would include 45 µL of an additive into the first injector port, 49.5 µL into the second, 54.5 µL into the third, and 60 µL into the last, assuming an initial well volume of 450 µL.

3.3. During the first several experiments, load base media into port A to gauge how much tissue movement/artefact occurs after a port injection.

3.4. Allow the loaded sensor plate to incubate at 37 °C in a non-$CO_2$ incubator for at least 60 min.

## 4. Isolation of Fresh Mouse Retinal Tissue

**Note:** This step is adapted from the technique of Dr. Barry Winkler[15].

4.1. After administering deep anesthesia using a standard ketamine/xylazine cocktail, euthanize mice by cervical dislocation.

4.2. Use a pair of medium-sized curved forceps to grasp the posterior globe at the optic nerve and gently apply forward pressure to proptose the eye (**Figure 1A**).

4.2.1. With a clean razor blade, create a limbus to limbus incision across the cornea using a single, deliberate pass of the blade.

4.2.2. Using fine, angled McPherson-style forceps, pinch the posterior globe to express the lens and anterior hyaloid membranes out of the corneal incision. Discard these tissues.

4.2.3. Repeat the pinching maneuver with forceps to express the neural retina.

4.2.4 Using the forceps like a spoon to lift the retina, rather than to grasp the tissue, transfer the retina away from the corneal incision and place directly into warm media in a 3-cm dish or a 6-well tissue culture cluster plate.

4.3. Use two pairs of fine, angled McPherson-style forceps to gently dissect remaining vitreous from the retina. This is best done by grasping the vitreous at the periphery of the retinal cup and pulling towards the center, with a final disinsertion of the vitreous body from the center as a whole. Using the forceps, remove any residual retinal pigment epithelium from the photoreceptor surface of the retina.

4.3.1. Cut a P1000 pipet tip with a razor blade to create a ~4 mm opening to prevent undue trauma to the delicate retinal tissue during transfer maneuvers.

4.3.2. Transfer the isolated retinal tissue into fresh media using a cut P1000 pipet tip.

4.3.3. Cut 1 mm punches of retina around the optic nerve with a 1 mm biopsy punch equipped with a plunger to dislodge the tissue in case it becomes lodged into the bore (**Figure 1B**). Set the retinal punches aside in clean media kept on a 37 °C block or heating pad.

**5. Assay Protocol**

5.1. Transfer individual punches to a 24-well islet capture microplate using a cut P1000 tip, aspirating 450 µL of media along with the tissue.

5.2. Use fine, straight forceps to gently manipulate punches into the center of the microplate. Keep punches orientated in the same direction (*e.g.*, ganglion cell side upwards).

5.2.1. Rest the capture plate on a heating pad or heating block set to 37 °C as these steps are repeated for the remaining samples.

**Note:** For each experiment, set aside 3-4 blank wells with 450 µL of base media to serve as negative controls. In the remaining 20-21 wells, test each biologic replicate in triplicate. Therefore, each 24-well plate will allow for recording from 6-7 different animals or treatment conditions.

5.3. Avoiding air bubbles, gently position the islet capture mesh inserts into each well using forceps and secure the inserts with a metal plunger, or with a cut P1000 pipet tip (**Figure 1C**).

**Note:** Avoid excessive tissue movement during this step to maintain retinal punch position within the center of the sample microchamber. Air bubbles severely distort OCR readings. Although the microchamber has adequate depth to accommodate typical mouse retina (**Figure 1C**), occasionally machining-defects in the mesh inserts may cause tissues to become overly compressed during screen insertion. If this happens, simply make a note of the crushed tissue and exclude that sample from the final analysis.

5.4. Incubate the tissue plate in a 37 °C $CO_2$-free incubator for at least 60 min.

5.5. Program the extracellular flux analyzer using Mix, Wait, Measure, and Repeat commands. As an example, a typical experiment with retinal tissue may include the following: Mix 2 minutes, Wait 2 minutes, and Measure 5 minutes. Repeat the experiment with these three steps between 5-8 times (cycles) for a baseline recording, and after injection of each compound being tested during the run.

5.6. Press the program START button on the extracellular flux analyzer and follow the instructions on the screen to insert the sensor cartridge for calibration.

5.7. At the end of the calibration, follow instructions on the screen to replace the calibrant plate with the plate containing the retinal samples.

5.8. Allow the program to run, as programmed. At the completion of the run, follow instructions on the screen to eject the tissue plate. View the results of the run and store the data file.

5.9. With a bent 20 gauge needle and forceps, remove all mesh inserts from the wells, leaving the retinal punch behind.

5.10. Carefully aspirate the media from the tissue and wash the tissue twice with 0.5 mL of cold phosphate-buffered saline (PBS). Aspirate the PBS after washing.

5.11. Add 100 µL of lysis buffer to each well and pipet up and down to homogenize tissue.

5.12 Quantitate input levels based on total double-stranded DNA (dsDNA) or total protein content.

**Note:** The following is based on a commercially available dsDNA assay.

5.13. Dilute lysed samples 1:1 by adding 100 µL of Tris-EDTA (TE) buffer and transfer 100 µL of the diluted sample to a 96 well plate.

5.13.1. Add 100 µL of detection buffer into each well. After mixing for 2-5 minutes, quantitate fluorescence after excitation at 480 nm and emission at 520 nm, comparing to a standard curve.

5.13.2. Normalize the raw extracellular flux tracing to DNA content within the well.

**Note:** In the results presented here, samples are normalized to 50 ng dsDNA — a typical amount in a 1 mm retinal punch. Therefore, absolute values presented here may be compared to studies presenting data "per retinal punch". Normalize tracings to an internal standard, such as the baseline run at a glucose concentration of 5 mM.

**REPRESENTATIVE RESULTS:**

Using the described techniques (summarized in **Figure 1**), retinal explants from 8 week-old wild type (WT) mice were compared to age- and background-matched *transducin* null mice (*Gnat1*$^{-/-}$). Because *Gnat1*$^{-/-}$ animals lack the machinery to close cyclic-nucleotide gated ion channels in response to light stimuli, their rod photoreceptors remain depolarized even in light[14]. The subsequent need to maintain potassium efflux would create a large ATP demand, resulting in bioenergetic strain. To determine if such shifts in energy demands would increase oxidative phosphorylation or glycolytic flux, tissues from wild type mice and *Gnat1*$^{-/-}$ mice were compared using the extracellular flux analyzer. At baseline, in the presence of 5 mM glucose and 1 mM pyruvate, retinal tissues from wild-type animals have similar rates of acid efflux compared to *Gnat1*$^{-/-}$ mutants. Similar patterns are seen after addition of 20 mM glucose and the glycolytic inhibitor 2-DG (**Figure 2A**). These data may also be mathematically transformed to represent fractional changes from baseline (**Figure 2B**), a format which may allow for better comparison between different experimental interventions.

Absolute OCR at baseline is equivalent between WT and mutant retinal tissue (**Figure 3A**). The addition of 20 mM glucose increases mitochondrial respiration, but no change is observed between groups in terms of absolute quantification or in terms of change from baseline (**Figure 3B**). The addition of 1mM FCCP (an uncoupling agent) to demonstrate maximal mitochondrial respiratory rates does not significantly increase OCR above the level seen with high glucose in WT or *Gnat1*$^{-/-}$ tissues. However, signals from both mice drastically drop after the addition of RAA cocktail.

Ideally, extracellular flux experiments run in the presence of the ATP synthase inhibitor oligomycin aid in identifying sources of proton leak, occurring when movement through the electron transport chain is not associated with ATP production[16]. In retinal explants from 8 week-old C57BL/6J mice, oligomycin treatment robustly lowers OCR, as expected (**Figure 4**). But subsequent addition of FCCP, to find maximal OCR, only nominally increases the OCR to about 60% of baseline, consistent with a prior study[11].

The XF24 analyzer uses submersible probes that make a tight seal within the tissue well, creating a transient microenvironment for measurement of oxygen and acid flux. Positioning of retinal tissue in the well plate (in relation to the sensors) could potentially influence OCR recordings, and lead to unwanted confounders, and some reserachers have advocated placing retinal tissue directly underneath the oxygen sensor with the photoreceptor outer segments oriented toward the sensor[11]. To test the effects of retinal tissue position on OCR

measurements, tissues from young C57BL/6J mice (8 weeks old) were analyzed in two orientations: retinal ganglion cells facing down onto the plate (*i.e.,* photoreceptors placed upright closest to sensor) or facing up toward the sensor. No differences in relative OCR in response to FCCP or RAA were observed, indicating equivalent sensitivity for maximal and minimal mitochondrial respiratory rates in either orientation (**Figure 5A**). In addition, absolute OCR measurements were also equivalent between retinal tissues in either orientation (**Figure 5B**).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Isolation of retinal explant and setup into the extracellular flux analyzer. A.** Isolation of the neural retina *in situ* by applying propulsive force on globe with forceps, incising the cornea, and removing retina after discarding lens and anterior hyaloid membranes. **B.** Preparation of tissue for flux recording with retinal punch creation, placement of individual punches into islet capture microplate, and use of a mesh insert to minimize tissue movement with microchambers (scale bars = 1 mm). **C.** A schematic, derived from manufacturer-provided data, showing the procedure outline and demonstrating that height of the microchamber is adequate to accommodate the murine retina.

**Figure 2: Mitochondrial stress analysis using explanted retina from 8 week-old animals. A.** Averaged tracings with standard error of measurement (SEM), normalized to DNA content of the tissue, from an experiment optimized for oxygen consumption recordings, comparing *transducin* knockout animals (*Gnat1*$^{-/-}$) to wild type controls. **B.** Same experiment with data transformed such that baseline recordings are set as the reference.

**Figure 3: Analysis of glycolytic rate using explanted retina from 8-week old animals. A.** Averaged, DNA content-normalized, tracings from an experiment optimized for acid efflux recordings comparing *transducin* knockout animals (*Gnat1*$^{-/-}$) to wild type controls. **B.** Data from the same experiment normalized to baseline recordings. Traces show mean ± SEM.

**Figure 4: Irreversible effects of oligomycin on retinal respiratory rates.** In acutely prepared retinal explants from 8 week-old C57BL/6J mice, oligomycin treatment reduces the maximal OCR induced by subsequent addition of FCCP. Traces show mean ± SEM.

**Figure 5: Effects of tissue positioning on OCR measurement.** Retinal tissues from 8 week-old C57BL/6J mice were placed in wells with ganglion cells facing toward the sensor (Ganglion Cells Up) or away from the sensor (Ganglion Cells Down). Between groups, similar extracellular flux recordings are observed in terms of (**A**) respiratory capacity relative to baseline or (**B**) in terms of absolute quantification. Traces show mean ± SEM.

**DISCUSSION:**

OCR and ECAR are readily measured in explanted retinal tissue using a bioanalyzer using the described techniques. This method departs from those of other groups in several critical steps. Retinal tissues are isolated through a large corneal incision without enucleating the globe, as originally described by Winkler[15]. This method of retinal isolation allows for a rapid transfer

from the living eye into the tissue capture plate (often within 5 minutes). Tissues are kept at 37 °C throughout the process, which preserves cellular respiration better than when they are placed on ice. Media with minimal additives are used for initial measurements, omitting any buffering agents such as HEPES or sodium bicarbonate, serum, or excess macronutrients, as this allows for more reliable measurements of basal energy metabolism and does not inhibit assessment of ECAR. Retinal punches are best recorded in a 24-well format, rather than in 96-wells, to minimize trauma to the tissue. Retinal tissue is placed in the center of the well, within the tissue microchamber, and secured with a mesh insert. Doing so allows for accurate recording of ECAR and OCR, while preventing excessive tissue movement during the run, and eliminates the need for other reagents such as tissue adhesives. A vehicle injection during the run is recommended, especially when setting up recording experiments for the first time, as this will provide a sense of how well the tissue is secured within the capture plate. OCR recordings using this technique are independent of retinal orientation within the well, but the orientation must be kept consistent among samples within experiments. All measurements are taken only after retinal metabolism has reached steady state following injection of test compounds.

This protocol describes normalization of data to total DNA content, as a proxy for cell number. Such a technique is advantageous because it will account for changes to cell number driven by variation in retinal thickness, punch size, or differences in cellularity between genetically dissimilar samples. Total protein-based normalization is also a reliable method, and has the advantage of minimizing differences in total mitochondrial mass between retinal samples. However, normalization based on protein content in whole tissue may be confounded by changes in extracellular matrix between samples. Normalization of flux recordings to the baseline, as shown in the representative results, is advantageous because it minimizes variability in retinal punch size between replicates and easily allows interpretation of changes due to pharmacologic interventions. However, reporting of raw values allows for better comparison between different experiments and for experiments performed using different flux recording methods.

Results using these techniques are comparable to those reported elsewhere. Though oligomycin — an ATP synthase inhibitor — is typically used to measure proton leak within tissues or cells of interest, this compound has untoward effects on retinal metabolism. In the experiments reported here, a 60% decrease in maximal retinal OCR elicited by FCCP is observed after exposure to oligomycin (**Figure 4**), nearly identical to a previous study[11]. A potential explanation for this finding is irreversible damage or modification to retinal mitochondria by ATP synthase inhibition. Furthermore, as Kooragayala and colleagues first reported[11], retinal mitochondria are likely operating at near maximal rates in basal conditions since an electron transport chain uncoupling agent, FCCP, only increases OCR by about 15% (**Figure 3**, **Figure 5**).

Extracellular flux recordings in explanted retinal tissues demonstrate a large reserve glycolytic capacity, since the addition of 20 mM glucose elicits a two-fold increase in ECAR (**Figure 2**). Because oligomycin, usually used to gauge maximal glycolytic capacity, has deleterious effects in retinal tissue (**Figure 4**), the use of high glucose addition to the recording may serve as better

proxy to gauge glycolytic reserve in the retina. An important caveat preventing the use of ECAR as a pure proxy for glycolytic rate is that $CO_2$ liberated by the citric acid cycle can be a significant source of acid in cultured tissue[17]. Excess pyruvate does not increase ECAR above the levels elicited by high glucose, and glycolytic flux is nearly eliminated in retinal explants using excess molar ratios of 2-DG (**Figure 2**). Since ATP is used to regenerate membrane potential occurring from depolarization events in the dark-adapted retina, animals lacking *transducin* are expected to expend more energy than WT counterparts. However, in explants, differences in retinal energy metabolism between *Gnat1*$^{-/-}$ and controls were not seen in the experiments described here (**Figures 2 and 3**). These results are consistent with those obtained using a custom perfusion apparatus to measure OCR in *Gnat1*$^{-/-}$ animals[18]. Notably, the custom apparatus used by Du and colleagues allowed for measurement of OCR under light-adapted and dark adapted conditions. By doing so, a small but significant decrease in OCR after light exposure is detected in wild-type retinas, but not in *Gnat1*$^{-/-}$ retinas[18]. This illustrates a major limitation of the current technique in measuring ECAR and OCR in explanted retinas – the extracellular flux analyzer used here relies on visible light excitation of fluorophores embedded in its oxygen and pH sensors, eliminating the possibility of performing measurements in dark-adapted tissues. Such measurements are highly relevant to visual physiology and will still require the use of older techniques designed exclusively for the study of retinal metabolism.

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1

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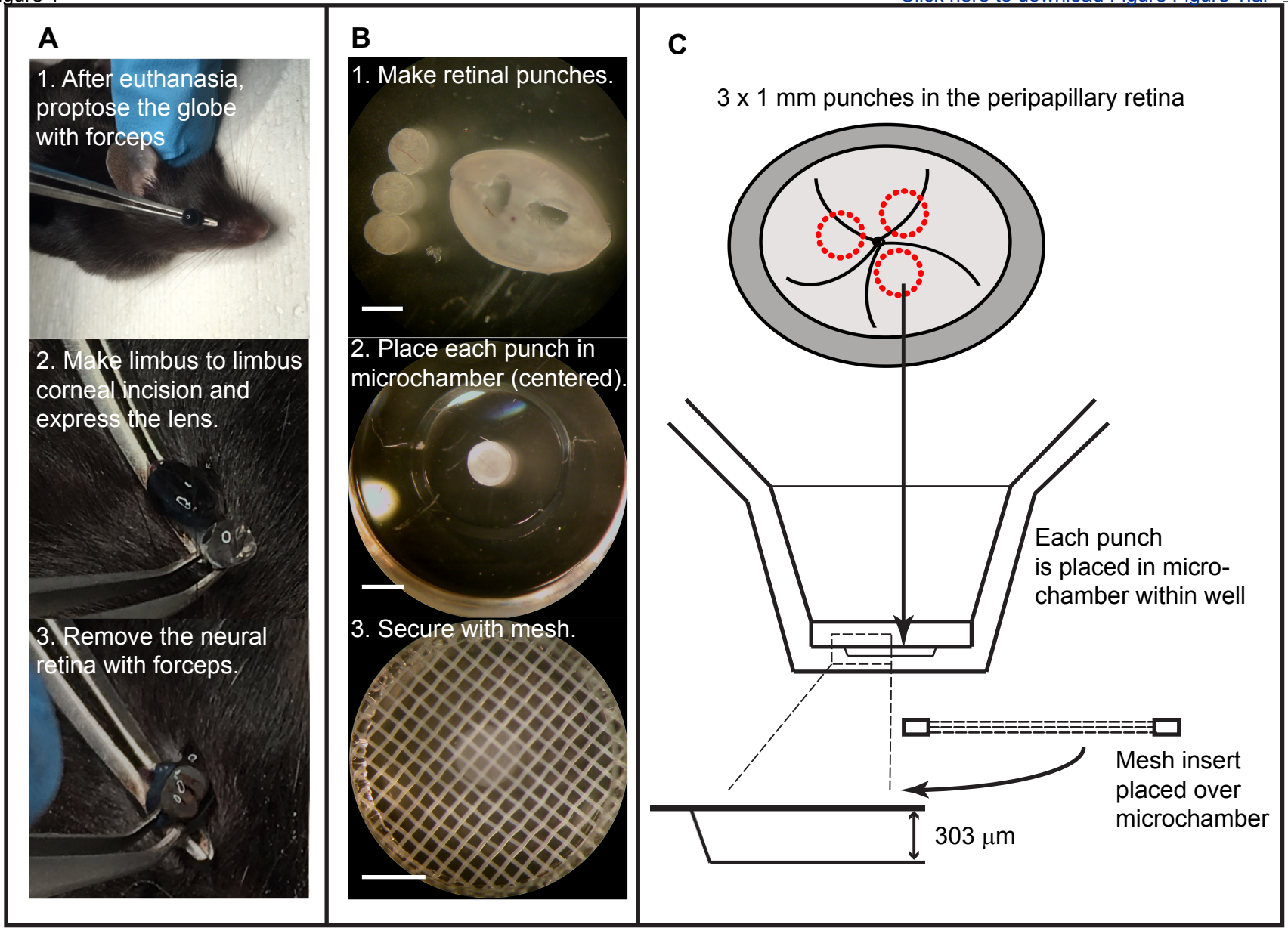

Figure 2

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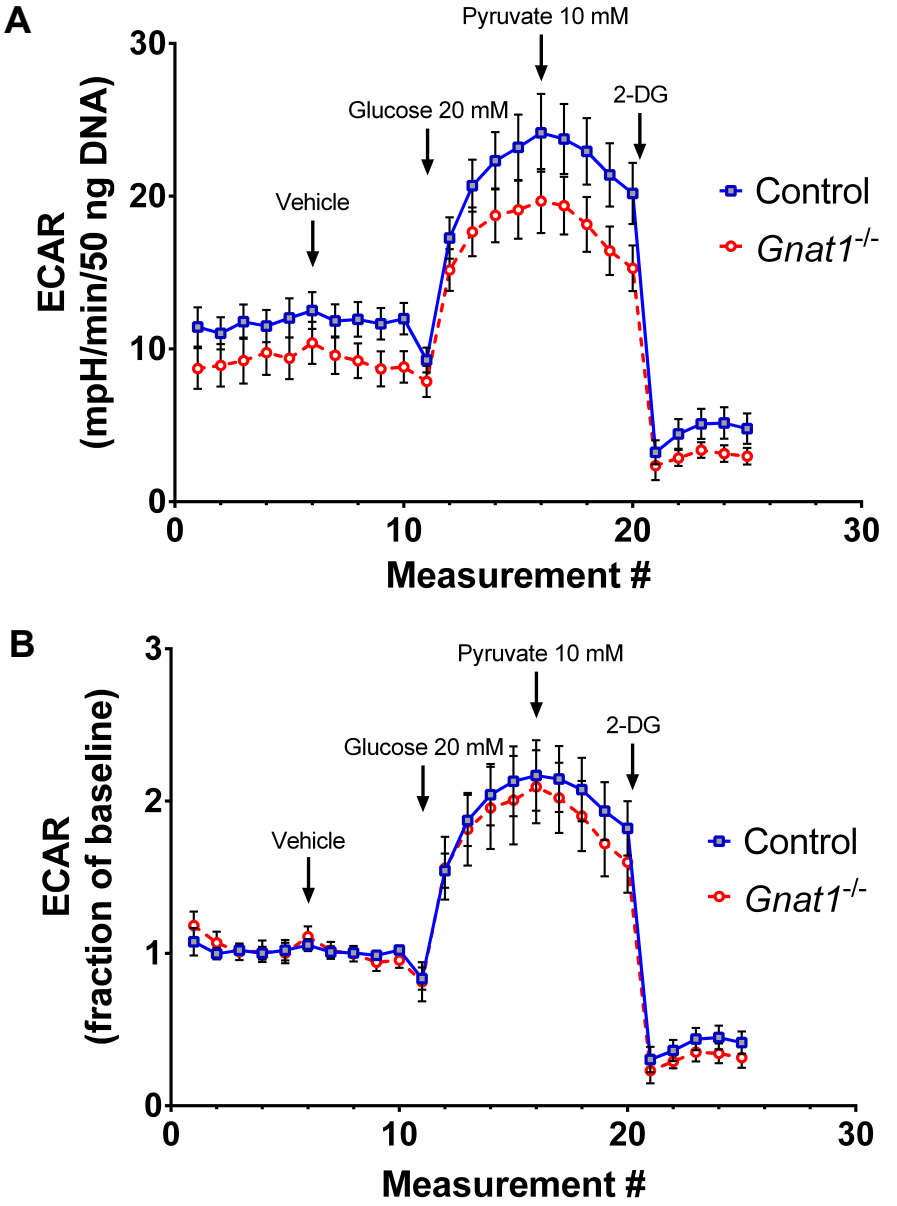

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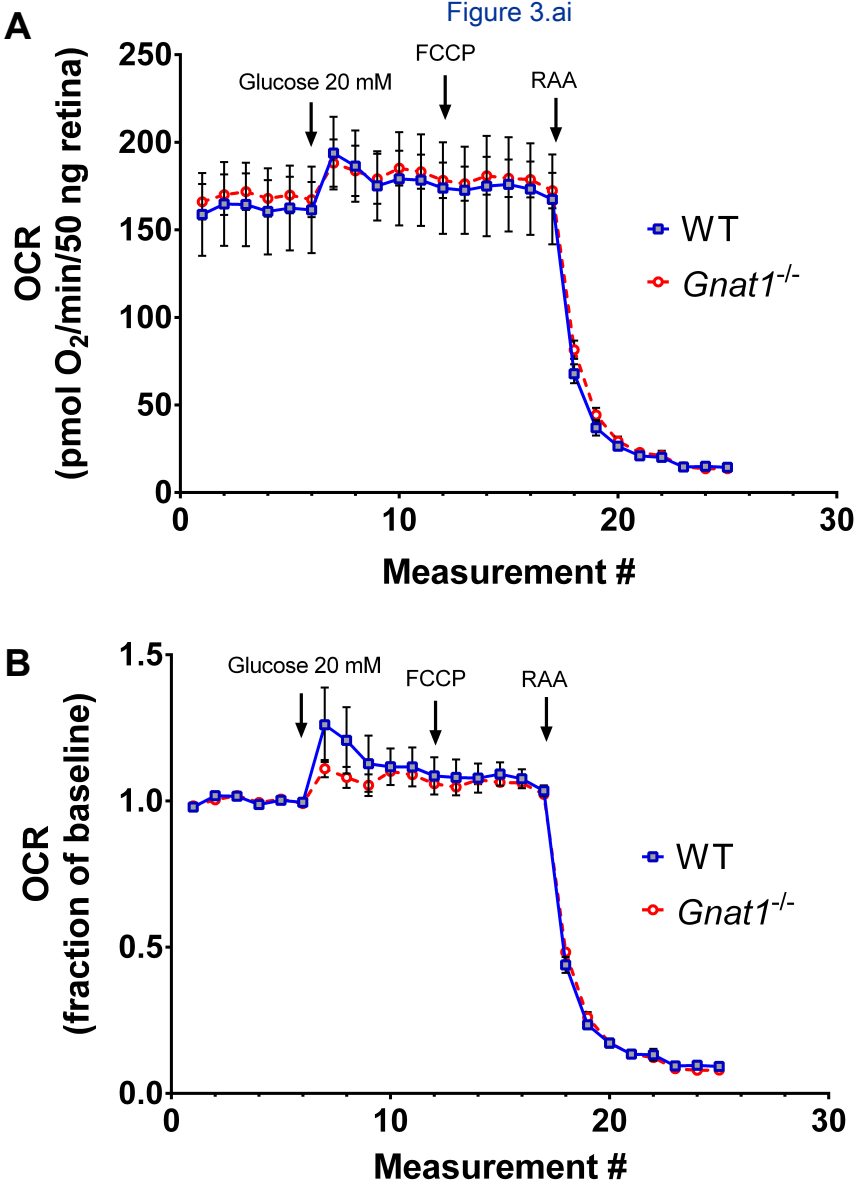

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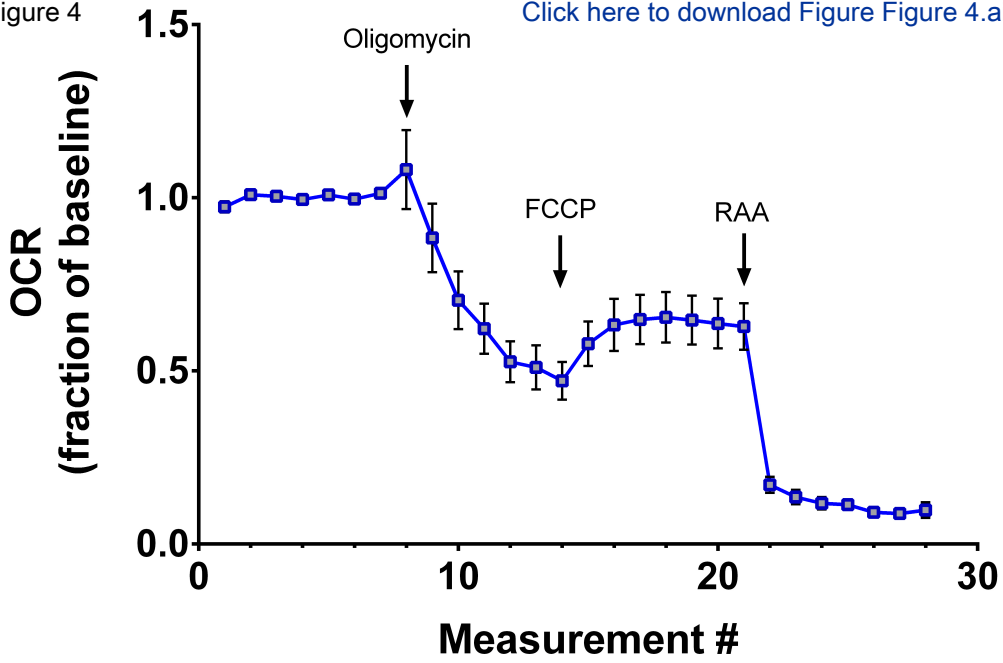

Figure 5

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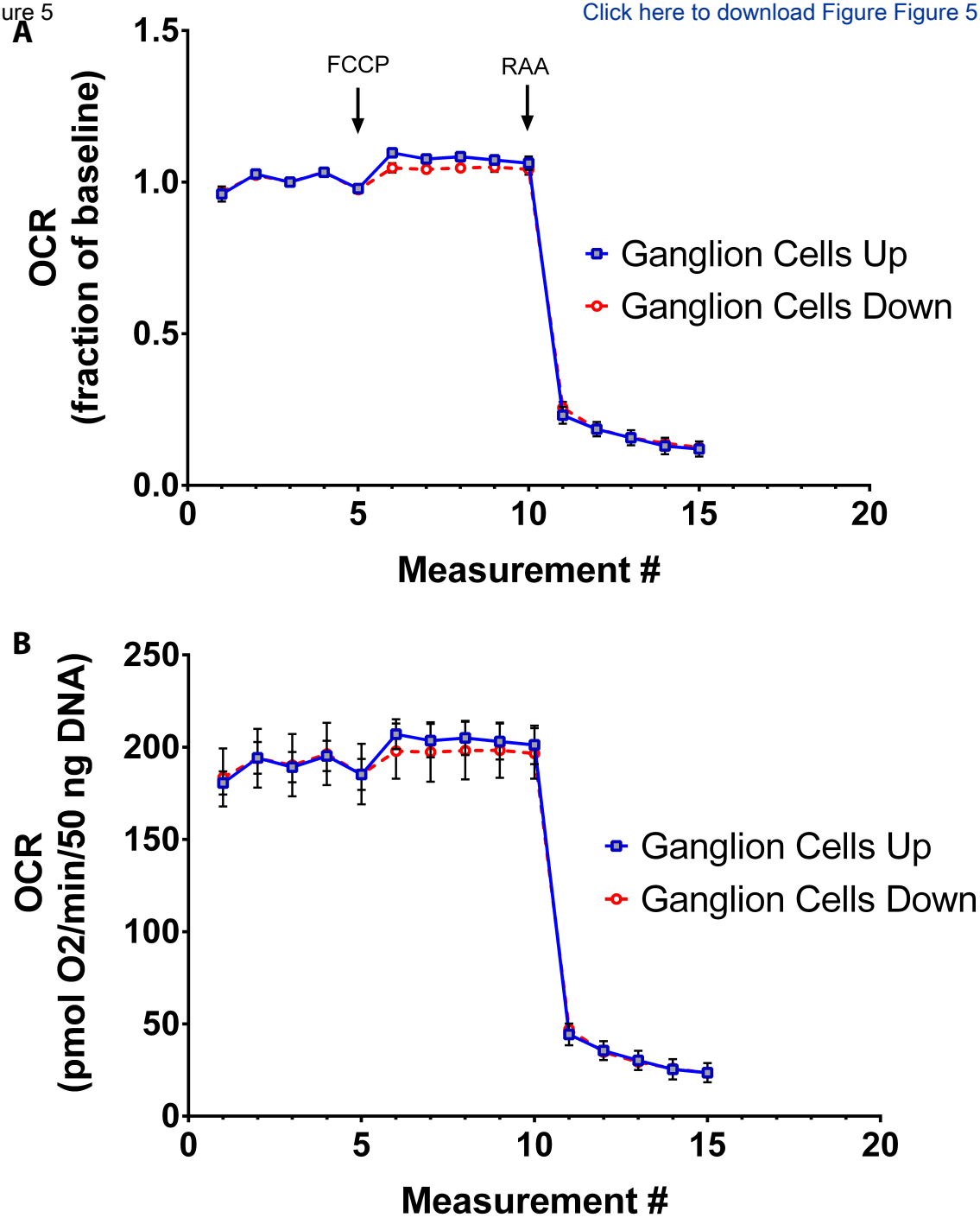

Table of Materials

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| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| Seahorse XF24 Extracellular Flux Analyzer | Agilent, Santa Clara, CA | | |
| Seahorse XF24 Islet Capture FluxPak (includes: Islet Capture Microplate, Sensor Cartridge and Calibrant Solution) | Agilent, Santa Clara, CA | 101174-100 | Includes islet capture microplate, sensor cartridge and calibrant solution |
| RPMI 1640 Media (Powdered medium) | Millipore-Sigma | R1383 | RPMI 1640 Media with L-Glutamine and without |
| D-Glucose | Millipore-Sigma | G8270 | 1M D-Glucose filtered, for media preparation |
| Sodium pyruvate | Corning | 25000CI | 100 mM sodium pyruvate |
| Antimycin-A | Millipore-Sigma | A8674 | Mitochondrial stress protocol component |
| FCCP | Millipore-Sigma | C2920 | Mitochondrial stress protocol component |
| Rotenone | Millipore-Sigma | R8875 | Mitochondrial stress protocol component |
| 2-deoxyglucose | Millipore-Sigma | D6134 | Glycolysis protocol component |
| 1 mm skin biopsy punches with plunger | Integra-Miltex | 33-31AA-P/25 | Explanting retinal tissue tool |
| Dumont Mini-Forceps Straight | Fine Science Tools | 11200-10 | Explanting retinal tissue tool |
| Dumont Medical #5/45 Forceps- Angled 45 degrees | Fine Science Tools | 11253-25 | Explanting retinal tissue tool |
| Dumont #7 Forceps - Curved | Fine Science Tools | 11271-30 | Explanting retinal tissue tool |
| Quant-iT Picogreen dsDNA Assay Kit | Fisher Scientific | P7589 | Loading normalization assay |
| Trizma base (Tris base) | Millipore-Sigma | T6066 | Component of lysis buffer |
| Triton X-100 (polyethylene glycol tert-octylphenyl ether) | Millipore-Sigma | X100 | Component of lysis buffer |
| 0.5M EDTA pH 8.0 | Ambion | AM9262 | Component of lysis buffer |
| C57BL/6J mice | Jackson Laboratories | Strain 000664 | Animals |
| *Gnat1* $^{-/-}$ and background-matched *Gnat1* $^{+/+}$ | Vladimir Kefalov, PhD; Washington University Sch | | Animals |

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| | |
|---|---|
| Title of Article: | Measurement of energy metabolism in explanted retinal tissue using extracellular flux analysis. |
| Author(s): | Jeffrey R. Millman, Teresa Doggett, Christina Oberlin, Sheng Zhang, Clay F. Semenkovich and Rithwick Rajagopal |

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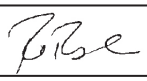
| Signature: |  |
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Washington University in St. Louis
SCHOOL OF MEDICINE

*Rithwick Rajagopal, M.D., Ph.D.*
Assistant Professor
Department of Ophthalmology and Visual Sciences

August 5, 2018

Editorial Office
*Journal of Visualized Experiments*

Re: Manuscript entitled “Measurement of energy metabolism in explanted retinal tissue using extracellular flux analysis.”

Dear Colleagues,

Thank you for the valuable feedback on our manuscript. We have addressed all concerns from the three peer reviewers as well as the editorial comments. The point-by-point rebuttal is pasted below.

I appreciate your consideration of this work for publication in *Journal of Visualized Experiments*.

Sincerely,

Rithwick Rajagopal

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POINT-BY-POINT REBUTTAL

| Comment | Rebuttal | Location in Revised Document |
|---|---|---|
| Editorial Comments | | |
| 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. | Thank you. Completed. | |
| 2. Figures 2-5: Please define error bars in the figure legend. | Completed. | Figure legends 2,3,4,5 |
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| 5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations. | Completed. The protocol section was revised to match this formatting. | |
| 6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). | Completed. | Introduction and discussion was revised to remove personal pronouns. |
| 7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” | The term “should be” was identified in several instances within the discussion and all instances were revised. | Discussion, lines 3017 and 317. |

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| "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion. | Imperative tense is used in the revision. | |
| 8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. | These points have been addressed in a revised protocol that incorporates several editorial comments. | Protocol, lines 103-224. |
| 9. Lines 98-100: Please move materials information to the Table of Materials. | Completed. | Materials supplement. |
| 10. Line 106: Please specify which powder. What is used to adjust the pH? Please spell out ddH2O. | Both points have been addressed. | Protocol, lines 112-113 |
| 11. Lines 109 and 111: These steps as written are unclear. Please revise. For instance, adding 1 M stock of what? | These points have been addressed in a revised protocol that incorporates several editorial comments. | Protocol, lines 115-117 |
| 12. Line 120: What is used to adjust the pH? | HCl or NaOH. Text Added | Protocol, line 112 |
| 13. Lines 125-136: Please write the text in the imperative tense in complete sentences. | These points have been addressed in a revised protocol that incorporates several editorial comments. | Protocol, 123-133 |
| 14. Please do not include Note as a separate step. | Completed and revised. | Protocol, line 140-142, 180-183, 186-191 |
| 15. After you have made all the recommended changes to your protocol (listed above), | Completed. | Protocol, highlighted text. |

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| please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.<br>16. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia. | | |
| 17. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted. | Completed. | Protocol, highlighted text. |
| 18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:<br>a) Critical steps within the protocol<br>b) Any modifications and troubleshooting of the technique<br>c) Any limitations of the technique<br>d) The significance with respect to existing methods<br>e) Any future applications of the technique | The discussion section was modified to incorporate these changes and the changes suggested by reviewer 3. | Discussion, lines 227-264. |
| 19. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, | Completed | References |

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| F.I.<br>Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. | | |
| 20. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references. | Completed | References |
| REVIEWER 1 | | |
| 1. Line 106 "Dissolve powder …" . Which powder ? | The protocol revised to clarify this ambiguity. | The protocol now reads: “2.1. Prepare base media by dissolving 8.4 mg of powdered media in double-distilled H20, and adjust pH to 7.4 with either HCl or NaOH, to final volume of 1 L. Filter sterilize this solution with a 0.22 µm tissue culture filter.” |
| 2. Line 111 What is the stock concentration pyruvate? | Corrected | The new protocol reads: “Add 1 M glucose and 100 mM sodium pyruvate to the media to achieve final concentration of 5 mM glucose and 1 mM pyruvate.” |
| 3. The first letter of catalog number for 2-deoxyglucose is illegible. | This has been corrected as well. | The new materials list has the catalog number from Sigma: " D6134" |
| REVIEWER 2 | | |
| Though the authors explains their technique very well and but the fact that their method used, doesn't provide<br>any extra added information about how the Gnat1 gene would be responsible in retinal energy metabolism. | This is an excellent point. We agree that the negative results for the Gnat1 tissues do not provide any additional insight into this gene on retinal bioenergetics. However, the contrast between our measurements and those made by Du et al (JBC 2016) using a custom-made perfusion apparatus point out an important limitation in the use of commercially-available extracellular flux analyzers: that light-induced changes in retinal energy metabolism cannot be reliably recorded. For this reason, we would like to leave the results included – in order to highlight the limitation of this protocol. However the manuscript was modified to provide additional details about why one would | See answer to comment below. |

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| | expect a change in bioenergetic demand in these animals. | |
| Minor concern is that authors should include more of description about Gnat1 gene and how this protein is associated with retinal metabolism. | We agree, and have expanded our description of this gene in the Representative Results section. | These sentences were added/modified in the Results section:<br>“Because Gnat1-/- animals lack the machinery to close cyclic-nucleotide gated ion channels in response to light stimuli, their rod photoreceptors remain depolarized even in light[14]. The subsequent need to maintain potassium efflux would create a large ATP demand, resulting in bioenergetic strain. To determine if such shifts in energy demands would increase oxidative phosphorylation or glycolytic flux, tissues from wild type mice and Gnat1-/- mice were compared using the extracellular flux analyzer.” |
| REVIEWER 3 | | |
| There are no major concerns with the manuscript, and only minor concerns with regards to data analysis and interpretation exist and are listed below. Many of these comments are addressed in "Analysis and interpretation of microplate-based oxygen consumption and pH data" by Divakaruni...and Jastroch (PMID:25416364). | The paper suggested by this reviewer is highly relevant to the content of this manuscript and to the overall goal of the protocol we describe. The descriptions of the caveats of extracellular flux recording using XF Analyzers is exceptional and was enlightening to read. We have added this reference to our bibliography and have used it as a guide to describe limitations of our own technique. | |
| (1) Lines 46 & 71: Any references to energy generation or consumption should be avoided, and replaced with energy transduction or ATP generation/consumption. | This is an important distinction, and we appreciate the correction. In both instances, phrases were modified as suggested. | Introduction |
| (2) Line 54: Although it was previously noted that the retina was the only post-mitotic tissue capable of aerobic | This point is also well-taken, and we have made the suggested addendum. | The following phrase was added to the introduction, immediately following the sentence highlighted by this reviewer: |

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| glycolysis, a comment should be made regarding how it is now clear most all tissues sit on a continuum of oxidative phosphorylation and glycolysis, even many post-mitotic cells & tissues. | | "Since those initial observations, many post-mitotic tissues have been described to engage in varying degrees of glycolysis in additional to oxidative phosphorylation to meet their ATP demands." |
| (3) Line 245: It is unclear why the authors choose to normalize to total DNA content over protein. A discussion about the relative merits and drawbacks of each normalization method should be addressed. | We have now added discussion around this choice of normalization to the manuscript. | In the discussion: "This protocol describes normalization of data to total DNA content, as a proxy for cell number. Such a technique is advantageous because it will account for changes to cell number driven by variation in retinal thickness, punch size, or differences in cellularity between genetically dissimilar samples. However, total protein-based normalization is also a reliable method, and has the advantage of minimizing differences in total mitochondrial mass between retinal samples. |
| (4) Line 263: The drawbacks of measuring the fractional changes from "baselined" data between groups should be mentioned in addition to the benefits | These points were also added to the discussion. | To the discussion, the following was added: "Normalization of flux recordings to the baseline, as shown in the representative results, is advantageous because it minimizes variability in retinal punch size between replicates and easily allows interpretation of changes due to pharmacologic interventions. However, reporting of raw values allows for better comparison between different experiments and for experiments performed using different flux recording methods." |
| (5) Line 314: It is not stated whether the authors have titrated the FCCP concentration to arrive at 1 uM. It is possible that a rate close to the basal rate of metabolism can be achieved with a different concentration of FCCP | Interestingly, we and others have attempted titration of FCCP to high doses, and used other uncouplers (such as BAM15) without any observable change in retinal OCR. These findings (including titration of the uncoupler) are extensively discussed in Kooragayala et | |

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| after oligomycin injection? Additionally, if the maximal respiratory rate cannot be measured in the presence of oligomycin, the authors should note that addition of FCCP without ATP synthase inhibition may create an unsustainable ATP demand from mitochondrial hydrolysis of ATP by the ATP synthase. | al. IOVS 2013, which is a reference in our bibliography. | |
| (6) Line 330: It is well established that in 3D cultures CO2 evolved from the TCA cycle can be a dominant source of medium acidification (addressed in PMID:25416364). This caveat should be addressed in the discussion of ECAR measurements in retinal explants. | We have addressed this important caveat in the revised version of the discussion.<br><br>The reference suggested by this reviewer was also added, as mentioned before. | To the discussion, the following lines were included: "An important caveat preventing the use of ECAR as a pure proxy for glycolytic rate is that CO2 liberated by the citric acid cycle can be a significant source of acid in cultured tissue17." |
| (7) The oligomycin-independent respiratory rate in Figure 4 does not appear to reach steady-state (likely due to<br>the time required for penetration of the drug into the core of the explant). It should be noted that all measurements should be made at steady-state whenever possible. | We agree that this representative figure appears not to allow oligomycin-independent respiration to reach steady state. However, our group has observed an oligomycin-dependent mitochondrial toxicity that appears to be specific to retinal mitochondria. This is similar to observations of Dr. Anand Swaroop's group at the National Eye Institute (Kooragayala et al, IOVS, 2013). Therefore, the lack of drop to steady state may be due to progressive toxicity, rather than tissue penetrance. Nevertheless, the point about allowing compounds to reach steady-state is well-taken and has been amended to the revision. | To the discussion:<br>"All measurements are taken only after retinal metabolism has reached steady-state following injection of test compounds." |