Journal of Visualized Experiments

Determination of Mitochondrial Respiration and Glycolysis in Ex Vivo Retinal Tissue Samples

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62914R1
Full Title:	Determination of Mitochondrial Respiration and Glycolysis in Ex Vivo Retinal Tissue Samples
Corresponding Author:	Ke Jiang National Institutes of Health Bethesda, MD UNITED STATES
Corresponding Author's Institution:	National Institutes of Health
Corresponding Author E-Mail:	coco.jiang@nih.gov
Order of Authors:	Ke Jiang
	Jacob Nellissery
	Anand Swaroop
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Neuroscience
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Bethesda, Maryland, USA
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	None
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release

TITLE:

Determination of Mitochondrial Respiration and Glycolysis in *Ex Vivo* Retinal Tissue Samples

AUTHORS AND AFFILIATIONS:

Ke Jiang[1], Jacob Nellissery[1] and Anand Swaroop[1]

[1]Neurobiology, Neurodegeneration & Repair Laboratory, National Eye Institute, National Institutes of Health, Bethesda, Maryland, USA.

Corresponding authors:

Ke Jiang (coco.jiang@nih.gov)

Anand Swaroop (swaroopa@nei.nih.gov)

Email Addresses of Co-Authors:

Ke Jiang (coco.jiang@nih.gov)

Jacob Nellissery (nellisseryj@nei.nih.gov)

Anand Swaroop (swaroopa@nei.nih.gov)

SUMMARY:

Described here is a detailed protocol for performing mitochondrial stress assay and glycolytic rate assay in *ex vivo* retinal tissue samples using a commercial bioanalyzer.

ABSTRACT:

Mitochondrial respiration is a critical energy-generating pathway in all cells, especially retinal photoreceptors that possess a highly active metabolism. In addition, photoreceptors also exhibit high aerobic glycolysis like cancer cells. Precise measurements of these metabolic activities can provide valuable insights into cellular homeostasis under physiological conditions and in disease states. High throughput microplate-based assays have been developed to measure mitochondrial respiration and various metabolic activities in live cells. However, a vast majority of these are developed for cultured cells and have not been optimized for intact tissue samples and for application *ex vivo*. Described here is a detailed step-by-step protocol, using microplate-based fluorescence technology, to directly measure oxygen consumption rate (OCR) as an indicator of mitochondrial respiration, as well as extracellular acidification rate (ECAR) as an indicator of glycolysis, in intact *ex vivo* retinal tissue. This method has been used to successfully assess metabolic activities in adult mouse retina and demonstrate its application in investigating cellular mechanisms of aging and disease.

INTRODUCTION:

Mitochondria are essential organelle that regulates cellular metabolism, signaling, homeostasis, and apoptosis by coordinating multiple crucial physiological processes[1]. Mitochondria serve as the powerhouse in the cell to generate adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) and provide energy that supports almost all cellular events. The majority of cellular oxygen is metabolized in mitochondria, where it serves as the final electron acceptor in the electron transport chain (ETC) during aerobic respiration. Low amounts of ATP

can also be produced from glycolysis in the cytosol, where glucose is converted to pyruvate, which can be further converted to lactate or be transported into mitochondria and oxidized to acetyl-CoA, a substrate in the tricarboxylic acid cycle (TCA cycle).

The retina is one of the most metabolically active tissues in mammals[2], displaying high levels of mitochondrial respiration and extremely high oxygen consumption[3]. The rod and cone photoreceptors contain a high density of mitochondria[4], and OXPHOS generates most ATP in the retina[5]. In addition, the retina also relies heavily on aerobic glycolysis[6,7] by converting glucose to lactate[5]. Mitochondrial defects are associated with various neurodegenerative diseases[8,9]; and with its unique high energy demands, the retina is especially vulnerable to metabolic defects, including those affecting mitochondrial OXPHOS[4] and glycolysis[10]. Mitochondrial dysfunction and defects in glycolysis are implicated in retinal[11,12] and macular[13] degenerative diseases, age-related macular degeneration[10,14-16], and diabetic retinopathy[17,18]. Therefore, accurate measurements of mitochondrial respiration and glycolysis can provide important parameters for assessing the integrity and health of the retina.

Mitochondrial respiration can be measured through the determination of oxygen consumption rate (OCR). Given that the conversion of glucose to pyruvate and subsequently to lactate results in extrusion of protons into and acidification of the extracellular environment, measurements of the extracellular acidification rate (ECAR) provide an indication of glycolysis flux. As the retina is composed of multiple cell types with intimate relationships and active synergy, including the exchange of substrates[6], it is imperative to analyze mitochondrial function and metabolism in the context of whole retinal tissue with intact lamination and circuitry. For the past several decades, the Clark type O_2 electrodes and other oxygen microelectrodes have been used to measure oxygen consumption in the retina[19-21]. These oxygen electrodes have major limitations in sensitivity, requirement of the large sample volume, and the need for continuous stirring of suspending sample, which usually leads to the disruption of cellular and tissue context. The protocol described here was developed using a microplate-based, fluorescence technique to measure mitochondrial energy metabolism in freshly dissected *ex vivo* mouse retina tissue. It allows mid-throughput real-time measurements of both OCR and ECAR simultaneously using a small sample (1 mm punch) of *ex vivo* retinal tissue while avoiding the need for suspension and continuous stirring.

Demonstrated here is the experimental procedure for mitochondrial stress assay and glycolytic rate assay on freshly dissected retinal punch disks. This protocol allows the measurement of mitochondria-related metabolic activities in an *ex vivo* tissue context. Different from the assays performed using cultured cells, the readings obtained here reflect combined energy metabolism at the tissue level and are influenced by interactions between the different cell types within the tissue. The protocol is modified from a previously published version[22,23] to adapt to the new generation of the Agilent Seahorse extracellular flux 24-wells (XFe24) analyzer with Islet Capture plate. The assay medium, injection compound concentrations, and assay run protocol have also been optimized for retinal tissue. A detailed step-by-step protocol is given for the preparation of retinal punch disks. More information on the program setup and data analysis can be obtained from the manufacturer's user guide[24-26].

PROTOCOL:

All mouse protocols were approved by the Animal Care and Use Committee of the National Eye Institute (NEI ASP# 650). Mice were housed in 12 h light-dark conditions and cared for by following the recommendations of the Guide for the Care and Use of Laboratory Animals, the Institute of Laboratory Animal Resources, and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

1. Hydrating sensor cartridge and preparation of the assay medium

1.1. The day before the experiment, add 1 mL of the calibration medium to each well of the utility plate. Place the Hydro-Booster cover on the top and lower the sensor cartridge through the opening on the cover. Check to ensure that the sensor is submerged in the calibration medium. Incubate the sensor cartridge overnight in a CO_2-free incubator at 37 ˚C to activate the fluorophores.

NOTE: To prevent evaporation, the incubator is humidified by keeping a tray of water inside, and the sensor cartridge cassette is wrapped with clear plastic wrap.

1.2. Prepare the assay medium by reconstituting the Seahorse DMEM medium with the addition of glucose, pyruvate, and glutamine to the desired concentrations. In the assays reported in this article, the final concentration of substrates in the assay medium are: 6 mM of glucose, 0.12 mM of pyruvate, and 0.5 mM of glutamine. For each assay plate, 40 mL of the assay medium is prepared fresh on the day of the experiment.

1.3. Set up the assay program in the analyzer following the manufacturer's instruction[26]. In the assay demonstrated here, the protocol is set as follows: 5 cycles of measurements for baseline, then inject port A, followed by 4 cycles of measurements, then inject port B and 4 measurements. Each cycle is composed of mix (3 min), wait (2 min) and measure (3 min).

2. Coating mesh inserts of islet capture microplate

2.1. Prepare the coating mix by combining 20 µL of the cell attachment medium (e.g., Cell-Tak) with 171 µL of 0.1 M sodium bicarbonate and 9 µL of 1 M NaOH.

2.2. Open the lid of the cassette containing mesh inserts. Pipette 8 µL of the coating mix to each mesh inserts. Use a pipette tip to gently smear/spread the droplet around to distribute the coating mix equally throughout the mesh insert.

2.3. Close the cassette and allow the mesh inserts to incubate at room temperature for at least 25 min for adsorption.

2.4. Wash the mesh insert by pipetting 4 mL of the assay medium directly onto the mesh inserts. Gently shake the cassette to ensure all mesh inserts are washed with the assay medium.

2.5. Keep the mesh insert aside. It is ready to use.

3. Preparing injection compounds

3.1. Take out stock aliquots of Bam15 (10 mM), Rotenone (10 mM), Antimycin A (10 mM) and 2-DG (500 mM) from -80 °C freezer and thaw at room temperature.

NOTE: The 2-DG stock is ready to use. The other drugs need to be diluted to working stock.

3.2. Warm up 10 mL of the assay medium in a 37 °C water bath.

3.3. Dilute 10 mM Bam15 stock to 50 µM working stock using a two-step dilution procedure: mix 20 µL of 10 mM stock with 20 µL of DMSO to get 5 mM intermediate stock. Then mix 10 µL of the above 5 mM intermediate stock with 990 µL of pre-warmed assay medium to get the final 50 µM working stock.

3.4. Dilute and combine 10 mM Rotenone and 10 mM Antimycin A stock to 10 µM Rotenone/Antimycin A (Rot/AA) working stock by two steps of dilutions: mix 10 µL each of 10 mM Rotenone and 10 mM Antimycin A stock with 80 µL of DMSO to get 1 mM Rot/AA intermediate stock. Then mix 10 µL of the above 1 mM intermediate stock with 990 µL of pre-warmed assay medium to get the final 10 µM Rot/AA working stock.

3.5. Freshly prepare the above-mentioned working stocks of injection compounds on the day of the experiment and set them aside at room temperature until loading into injection ports of the sensor cartridge.

4. Retinal dissection and retinal punch preparation

4.1. Euthanize a mouse by CO_2 asphyxiation following AVMA Guidelines on Euthanasia[27].

NOTE: Do not leave the animal in a CO_2 chamber longer than the time needed for euthanasia.

4.2. Enucleate eyes and place into ice-old 1x PBS buffer in a Petri-dish and then under a dissection microscope.

4.3. Carefully remove, by cutting with microscissors, the extra rectus muscles attached outside the eyeball and cut off the optic nerve.

4.4. Use a 30 G needle to punch a hole at the edge of the cornea; this serves as the insertion site for the microscissors. Then, use a fine dissection microscissors to make a circular cut along the edge of the cornea, separating it from the posterior eye cup.

4.5. Use sharp dissection forceps to remove the cornea, lens, and the vitreous humor away from the eye cup.

4.6. Use fine dissection microscissors to make several small cuts on the scleral layer at the rim of eye cup. Avoid cutting the retina layer. Use two sharp dissection forceps to hold on to the scleral tissue at each side of the cut and very carefully pull on the scleral layer to remove it from the neural retina.

4.6.1. Repeat this around the eye cup until all sclera is removed and an intact retinal cup is obtained.

4.7. Use dissection microscissors and make radial cuts on the retinal cup to flatten it and generate several distinct sections.

NOTE: Depending on the person's dissection skills and experience in handling fresh retinal tissue, the retinal cup can be cut to generate 3 to 5 distinct sections.

4.8. Use 1 mm diameter biopsy puncher to cut one retinal disk from each section of the flattened retinal cup.

NOTE: Care should be taken to get the retinal disks punched at equal distance from the optic nerve head.

4.9. Use forceps to transfer the pre-coated mesh inserts into the dissecting petri-dish. With the help of two superfine eyelash brushes, place the retinal punch disk onto the mesh insert. The retinal punch disk is placed at the center of the mesh insert with ganglion cell layer side down touching the mesh and photoreceptor layer facing up.

NOTE: Frequently, some RPE cells remain attached to the photoreceptors, and the pigmentation of these cells can be used as an indicator of the retinal punch disk orientation.

5. Loading the sensor cartridge injection ports and calibration

5.1. Take the hydrated sensor cartridge plate cassette out of the 37 ˚C incubator. Remove the Hydro-Booster cover and place the sensor cartridge back on the utility plate.

5.2. Load the desired volume of injection compound solutions into appropriate ports. Hold the pipette tip at 45˚ angle. Insert the pipette tip halfway into an injection port with the bevel of the tip against the opposite wall of the injection port and gently load the compound into each port. Avoid introducing air bubbles.

5.3. Refer to the instrument user guide for the volume of the compound loaded in each injection port for a specific assay. In the experiments presented in this paper, 68 µL of 50 µM Bam15 working stock (for mitochondrial stress assay) or 68 µL of 10 µM Rot/AA working stock

(for glycolytic rate assay) is loaded into port A; 75 µL of 10 µM Rot/AA working stock (for mitochondrial stress assay) or 75 µL of 500 mM 2-DG working stock (for glycolytic rate assay) is loaded into port B.

5.4. Load all wells of the plate including background correction wells and blank wells to ensure proper injection. Load the respective compound solution in each port for the background correction wells. Assay medium can be substituted, instead of the compound solution, in each of the ports of the bank wells.

5.5. Place the loaded sensor cartridge plate into the analyzer machine to start calibration prior to the assay run. After the calibration is over, the program will automatically pause, waiting for the replacement of the utility plate with the islet capture plate containing retinal punches.

6. Loading the islet capture plate and start assay run

6.1. Add 607 µL of the assay medium to each well of the islet capture plate

6.2. Use forceps to grab the rim of the mesh insert containing retinal punch disks on top and take it out from the Petri-dish. Lightly tap the bottom of the mesh insert on an absorbing wipe tissue to remove extra liquid and put it into the well of the islet capture plate. Repeat this step until all mesh inserts with retinal punches are placed into the islet capture plate. Fill background correction wells and blank wells with empty mesh inserts.

6.3. Use two Graefe forceps to carefully and gently press the rim of each mesh insert and make sure that these are securely inserted at the bottom of the islet capture plate.

6.4. Place the loaded Islet Capture plate into a 37 ˚C incubator for 5 min to warm up.

6.5. Eject the utility plate after the calibration is complete and replace it with an islet capture plate containing retinal punches.

6.6. Resume the assay run.

7. Run termination and data storage

7.1. After the run is complete, eject the sensor cartridge and islet capture plate containing retinal punches. The data is automatically saved as .asyr file.

7.2. Use the associated data analysis software to view and analyze the data following the manufacturer's user guide[26].

7.3. Use the **Export** function to export .xslx file of the data, which can be viewed and analyzed using spreadsheet software.

8. Saving the retinal punch sample

8.1. After the assay, take out the plate from the machine, remove the sensor cartridge and gently remove the assay medium from each well using a pipette.

8.2. Apply the cover back on and seal the sides of the plate with the parafilm strip.

8.3. Store at -80 ˚C.

8.4. For normalization, quantify the total DNA or protein content of the punch in each well.

9. Data analysis

9.1. Mitochondrial stress assay

NOTE: The measured OCR value (totalOCR) represents total oxygen consumption by the tissue. After Bam15 (uncoupler) injection, OCR increases from the basal level ($totalOCR_{basal}$) to the maximum level ($totalOCR_{max}$) and goes down following the Rot/AA injection. The residual OCR value after Rot/AA injection ($totalOCR_{Rot/AA}$) represents non-mitochondrial oxygen consumption.

9.1.1. Calculate mitochondria-related oxygen consumption as:

$$mitoOCR\ (pmol\ O_2/min) = totalOCR\ (pmol\ O_2/min) - totalOCR_{Rot/AA}\ (pmol\ O_2/min)$$

(Eq. 1)[28]

9.1.2. Calculate the mitochondrial reserve capacity (MRC) as:

$$MRC = \frac{mitoOCR_{max}\ (pmol\ O^2/min) - mitoOCR_{basal}\ (pmol\ O^2/min)}{mitoOCR_{max}\ (pmol\ O_2/min)} \times 100\%$$

(Eq. 2)[29]

NOTE: The last reading among the 5 measurements before Bam15 injection is taken as the "basal" value (for $totalOCR_{basal}$ and $mitoOCR_{basal}$). The highest reading among the 4 measurements following Bam15 injection is used as "max" value (for $totalOCR_{max}$ and $mitoOCR_{max}$). The lowest reading among the 4 measurements following the Rot/AA injection is used as $totalOCR_{Rot/AA}$.

9.2. Glycolytic rate assay

NOTE: The measured ECAR value (totalECAR) represents the total acidification of the medium by the tissue's metabolic activity. In general, acidification of the extracellular micro-environment results mainly by extrusion of the glycolytic product, lactate. Catabolism of substrates in mitochondrial TCA cycle results in the production of CO_2, which also acidifies the extracellular medium through hydration to bicarbonate.

9.2.1 Substract mitochondrial contributed medium acidification (mitoECAR) from totalECAR to obtain the glycoECAR.

$$glycoECAR\ (mpH/min) = totalECAR\ (mpH/min) - mitoECAR\ (mpH/min)$$ (Eq. 3)[28]

NOTE: Mitochondrial respiration and TCA cycle are strongly coupled processes. Production of CO_2 from mitochondria is a function of the rate of OXPHOS, which is measurable by mitoOCR.

9.2.2. Calculate the mitoECAR as:

$$mitoECAR\ (mpH/min) = \frac{mitoOCR\ (pmol\ O_2/min)\ \times\ CCF\ (pmol\ H^+/pmol\ O_2)}{BF\ (mmol\ H^+/L/pH)\ \times\ Vol_{microchamber}\ (\mu L)\ \times\ Kvol}$$

(Eq. 4)[28]

where, the CCF (CO_2 Contribution Factor) is an empirically calculated ratio value, representing the amount of H^+ contribution from CO_2-mediated acidification vs each O_2 consumption from OXPHOS. CCF for this system is pre-determined to be 0.60[28]. Accurate measurement of medium acidification is determined by the buffer capacity of the medium, the sensitivity of instrument pH sensor, and the effective measurement chamber capacity. Here, the BF (Buffer Factor) is a parameter of the *in situ* experimental buffer capacity, representing the amount of H^+ or OH^- added to the effective measurement chamber to change the pH level by 1 unit. When customized assay medium is used, the BF can be determined by titrating known amounts of acid into the assay medium following the Buffer Factor protocol[30]. The Seahorse DMEM medium pH 7.4 used in this protocol has a pre-determined BF of 2.60 mmol H^+/L/pH. The islet capture plate used in this protocol has a $Vol_{microchamber}$ = 16.6 µL[31]. The volume scaling factor, Kvol, is an empirically determined constant. Kvol value is not available for the islet capture plate but can be calculated from the value of the microplate[28], accounting for the volume difference in their microchambers, to be 0.41.

NOTE: Injection of the Rot/AA shuts down mitochondrial respiration and forces the tissue to switch to glycolysis for ATP production, leading to higher lactate extrusion and an increase in ECAR measurement. Glycolysis is ceased with 2-DG injection, and the residual ECAR measurement reveals non-glycolytic and non-mitochondrial acidification of medium.

9.2.3. Calculate the glycolytic reserve capacity (GRC) as:

$$GRC = \frac{glycoECAR_{max}\ (mpH/min) - glycoECAR_{basal}\ (mpH/min)}{glycoECAR_{max}\ (mpH/min) - glycoECAR_{2-DG}\ (mpH/min)}\ \times\ 100\%$$

(Eq. 5)[32]

where, the last reading among the 5 measurements before Rot/AA injection is taken as the "basal" value ($glycoECAR_{basal}$). The highest reading among the 4 measurements following Rot/AA injection is used as "max" value ($glycoECAR_{max}$). The lowest reading among the 4 measurements following 2-DG injection is used as $glycoECAR_{2\text{-}DG}$.

9.3. Normalization

NOTE: Normalization is essential when comparing the readings from retinal tissues of different age groups or between wild-type and pathological/degenerative samples, which might differ in cell numbers.

9.3.1. Use commerically available kits to assess the DNA content in each retinal punch disk[33,34].

9.3.2. Alternatively, use RIPA buffer to extract total protein from the retinal punch and used for normalization.

NOTE: The surface area of an adult mouse retina has been previously determined to be around 20 mm^2, and each retina contains ~6.5 million cells[35]. Hence, each 1 mm diameter retinal punch is ~1/25 of a single retina and contains ~260K cells. One can refer to these numbers when comparing the data from a retinal punch to those from other tissue samples or cultured cells,

REPRESENTATIVE RESULTS

The data reported here are representative mitochondrial stress assay showing OCR trace (**Figure 1**) and glycolytic rate assay showing ECAR trace (**Figure 2**), which were performed using freshly dissected 1 mm retinal punch disks from 4 months old transgenic *Nrl-L-EGFP* mice[36] (C57B/L6 background). These mice express GFP specifically in rod photoreceptors without altering normal retinal development, histology, and physiology and have been widely used as wild-type controls in retinal research. Two *Nrl-L-GFP* mouse littermates were used in the assays presented here. GFP expressed in the *Nrl-L-GFP* mice does not interfere with the measurements of OCR and ECAR in this protocol. Five retinal punches were taken from each retina. Half of the 20 retinal punches were used for mitochondrial stress assay, and the other half used for glycolytic rate assay. Seahorse XF DMEM medium, pH 7.4 (constituted with 6 mM glucose, 0.12 mM pyruvate, and 0.5 mM glutamine) and Seahorse XFe24 Islet Capture plates were used in the experiments. The representative data presented here were obtained using the same 1 mm diameter puncher but was not normalized with respect to the DNA/protein content.

In mitochondrial stress assay, the uncoupler Bam15[37] was injected after establishing the OCR baseline, leading to enhanced OCR to the maximal level. Rotenone and Antimycin A were injected to inhibit mitochondria respiration at complex I and complex III, respectively, resulting in OCR to drop to the minimal level (**Figure 1**). The difference between the maximal level of OCR and the last measurement of the basal OCR level reflects mitochondrial reserve capacity (MRC). The MRC is calculated to be 19.2%±3.4% using Eq. 2, consistent with previously measured MRC values in retinas of ~3 months old *Nrl-L-EGFP* mice using the previous generation Seahorse XF24 analyzer[22,38].

In the glycolytic rate assay, Rotenone and Antimycin A were injected after establishing the baseline for the total ECAR. With the production of ATP from OXPHOS halted, the tissue is forced to rely on glycolysis for energy, and an increase in the extracellular release of lactate drives ECAR

to the maximal level. Glycolysis is ceased by injection of 2-DG, which competes with glucose for hexokinase binding, causing ECAR to drop to the minimal level (**Figure 2**). Mitochondria contributed ECAR (mitoECAR) can be calculated from the mitoOCR value (Eq. 4). Glycolysis contributed ECAR glycoECAR is calculated and plotted by subtracting mitoECAR from totalECAR. The difference between maximal level of glycoECAR and the last measurement of glycoECAR basal level reflects the glycolysis reserve capacity (GRC). Here, the GRC is calculated to be 35.7% ± 3.4% using Eq. 5.

As a highly glycolytic tissue, lactate production from the retina accounts for a major source of extracellular acidification, as revealed by the small difference of glycoECAR from the totalECAR. Interestingly, ECAR measurement does not plateau immediately following the Rot/AA injection but drops after the second measurement. The retinal punch disk is an intact *ex vivo* system composed of different cell types, including the Müller glia cells, which are known to receive lactate (glycolysis end product) released from the photoreceptors[6]. Hence, a drop in ECAR measurement following the Rot/AA injection is likely explained by increased removal of lactate from the intercellular space, slowing down/preventing its release into the medium.

FIGURE LEGENDS

Figure 1: Mitochondrial stress assay. The plotted graph shows OCR trace from 1 mm retinal punch disks in Seahorse XF DMEM buffer, supplemented with 6 mM of glucose, 0.12 mM of pyruvate and 0.5 mM of glutamine. Each data point represents the average of measurements from 10 wells. Error bar = standard error. MRC is calculated to be 19.2%±3.4%.

Figure 2: Glycolytic rate assay. The plotted graph shows the measured OCR trace, ECAR trace (totalECAR), and the calculated glycolysis contributed ECAR (glycoECAR) from 1 mm retinal punch disks in Seahorse XF DMEM buffer supplemented with 6 mM of glucose, 0.12 mM of pyruvate, and 0.5 mM of glutamine. Each data point represents the average of measurements from 10 wells. Error bar = standard error. GRC is calculated to be 35.7%±3.4%

DISCUSSION

Provided here are detailed instructions for performing microplate-based assays of mitochondrial respiration and glycolysis activity using *ex vivo*, freshly dissected retinal punch disks. The protocol has been optimized to: 1) ensure the use of a suitable assay medium for *ex vivo* retinal tissue; 2) employ proper size of retinal punch disks to obtain OCR and ECAR readings that fall within the machine's optimal detecting range; 3) coating mesh inserts to enhance the adhesiveness of retinal punch for stable reading during the measuring cycle; 4) use of optimal concentration of each injected drug compounds; and 5) ensure altered cycle length to reach a plateau of mitochondrial states at each step. The reagents and protocol have been modified from a previously published version[23] to adapt to the new generation Seahorse XFe24 machine. Instead of the Ames' buffer used in the previous protocol[23], a basic Seahorse DMEM medium is used here to allow the custom constitution of fuel source by adding glucose, glutamine, and pyruvate separately. This also makes it possible to perform various assays where a specific fuel substrate is supplied or deprived from the medium. In the assays presented here, the medium was constituted to the same concentration of glucose (6 mM), glutamine (0.5 mM), and pyruvate

(0.12 mM) as in Ames' buffer, which are proven suitable for retinal tissue. Another advantage of this medium (with 5 mM HEPES) over the Ames' buffer (with 22.6 mM $NaHCO_3$) is its low buffer capacity, which ensures sensitive and accurate measurement of ECAR[28].

Both mitochondrial stress and glycolytic rate assays can be performed following the protocol described here with high precision, as evidenced by the tight standard error values between replicating wells. However, it is worthwhile to note the factors that can contribute to data variability. Avoid cell death in retinal tissue. The entire dissection process should be performed in ice-cold 1x PBS, and the process from enucleation of eyes to putting Islet Capture plate containing the retinal punch into the machine should not exceed 2 hours. Caution should be taken during the dissection of the retina cup to avoid any damage to the retinal tissue, and punches should not be taken from areas damaged by dissection. New, sharp biopsy puncher should be used in each experiment, and change the puncher when the edge is dull or bent to ensure consistency and accuracy in cutting retinal punches at 1 mm diameter. Try to get the retinal disks punched at equidistant from the optic nerve head to avoid regional variations (center versus peripheral). After the assay, check each well for any sign of the retinal punch being detached from the mesh insert. When a retinal punch has poor adhesion on mesh insert or detaches during measurement, the distance from senser probe to the tissue will change, affecting the readings. Omit the data from such wells with detached retinal punch.

Measurement of the real-time mitochondrial metabolism in intact retinal tissue has broad applications and can provide useful information for various studies. These assays have been used to measure mitochondrial respiration in retinal tissues from mice of different genetic backgrounds to reveal their intrinsic difference in mitochondrial activity[39,40]. It was also used to study changes in mitochondrial energy metabolism during aging of the retina[38]. By providing different fuel substrates and utilizing various inhibitors targeting different metabolic pathways, it provides insights to the preference of the cell/tissue on certain fuel sources[22,38]. Furthermore, comparison on OCR and MRC between wild-type mouse and mouse models of inherited retinal degeneration can provide evidence of mitochondrial defects in degenerating retina[22].

There are limitations of this technique. The Islet Capture plate used in these assays only contains 24 wells; hence, it is only able to provide mid-throughput analysis. The data quality from this method is contingent upon the quality of retinal punch disks and viability of cells. Also, retinal dissection and retinal punch disks preparation is a time-consuming process, rendering it less feasible to high-throughput analysis on live *ex vivo* retinal tissues even when 96-well plates are available. Compared to a monolayer of cultured cells, penetration of drug compound into the retinal tissue also affects data readout. In addition, the measured OCR and ECAR values represent the total performance of the entire tissue, which is composed by many different cell types; hence, one needs to consider the relationship and interactions among different neuronal and glial cells in the retina while interpreting the data. Specific experimental designs should be implemented by tailoring to each project. It is recommended that one includes 3 to 5 retinal punches (from same eye or same mouse) as technical replicates and use samples from 3 or more mice as biological replicates.

ACKNOWLEDGMENTS:

This work is supported by the Intramural Research Program of the National Eye Institute (ZIAEY000450 and ZIAEY000546).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Nunnari, J., Suomalainen, A. Mitochondria: In sickness and in health. *Cell.* **148** (6), 1145-1159, (2012).

2 Wong-Riley, M. T. Energy metabolism of the visual system. *Eye Brain.* **2** 99-116, (2010).

3 Yu, D. Y., Cringle, S. J. Oxygen distribution and consumption within the retina in vascularised and avascular retinas and in animal models of retinal disease. *Progress in Retina and Eye Research.* **20** (2), 175-208 (2001).

4 Barot, M., Gokulgandhi, M. R., Mitra, A. K. Mitochondrial dysfunction in retinal diseases. *Current Eye Research.* **36** (12), 1069-1077 (2011).

5 Joyal, J. S., Gantner, M. L., Smith, L. E. H. Retinal energy demands control vascular supply of the retina in development and disease: The role of neuronal lipid and glucose metabolism. *Progress in Retina and Eye Research.* **64** 131-156 (2018).

6 Hurley, J. B., Lindsay, K. J., Du, J. Glucose, lactate, and shuttling of metabolites in vertebrate retinas. *Journal of Neuroscience Research.* **93** (7), 1079-1092 (2015).

7 Haydinger, C. D., Kittipassorn, T., Peet, D. J. Power to see-Drivers of aerobic glycolysis in the mammalian retina: A review. *Clinical and Experimental Ophthalmology.* **48** (8), 1057-1071 (2020).

8 Wright, A. F. et al. Lifespan and mitochondrial control of neurodegeneration. *Nature Genetics.* **36** (11), 1153-1158 (2004).

9 Bossy-Wetzel, E., Schwarzenbacher, R., Lipton, S. A. Molecular pathways to neurodegeneration. *Nature Medicine.* **10 Suppl** S2-9 (2004).

10 Leveillard, T., Philp, N. J., Sennlaub, F. Is retinal metabolic dysfunction at the center of the pathogenesis of age-related macular degeneration? *International Journal of Molecular Sciences.* **20** (3), (2019).

11 Vlachantoni, D. et al. Evidence of severe mitochondrial oxidative stress and a protective effect of low oxygen in mouse models of inherited photoreceptor degeneration. *Human Molecular Genetics.* **20** (2), 322-335 (2011).

12 Grenell, A. et al. Loss of MPC1 reprograms retinal metabolism to impair visual function. *Proceedings of the National Academy of Science U. S. A.* **116** (9), 3530-3535 (2019).

13 Wright, A. F., Chakarova, C. F., Abd El-Aziz, M. M., Bhattacharya, S. S. Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait. *Nature Reviews in Genetics.* **11** (4), 273-284 (2010).

14 Jarrett, S. G., Boulton, M. E. Consequences of oxidative stress in age-related macular degeneration. *Molecular Aspects of Medicine.* **33** (4), 399-417 (2012).

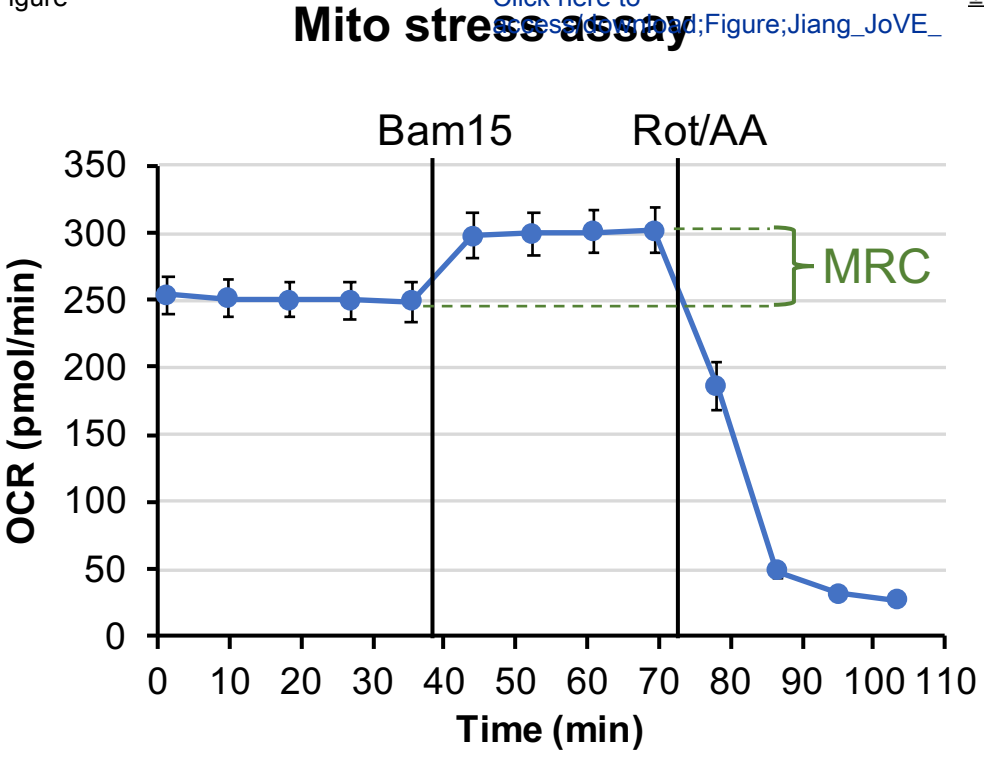
15 Rozing, M. P. et al. Age-related macular degeneration: A two-level model hypothesis. *Progress in Retina Eye Research.* **76**, 100825 (2020).

16 Yokosako, K. et al. Glycolysis in patients with age-related macular degeneration. *Open Ophthalmology Journal.* **8**, 39-47 (2014).
17 Bek, T. Mitochondrial dysfunction and diabetic retinopathy. *Mitochondrion.* **36**, 4-6 (2017).
18 Yumnamcha, T., Guerra, M., Singh, L. P., Ibrahim, A. S. Metabolic dysregulation and neurovascular dysfunction in diabetic retinopathy. *Antioxidants (Basel).* **9** (12), (2020).
19 Futterman, S., Kinoshita, J. H. Metabolism of the retina. I. Respiration of cattle retina. *Journal of Biological Chemistry.* **234** (4), 723-726 (1959).
20 Linsenmeier, R. A. Effects of light and darkness on oxygen distribution and consumption in the cat retina. *Journal of General Physiology.* **88** (4), 521-542 (1986).
21 Medrano, C. J., Fox, D. A. Oxygen consumption in the rat outer and inner retina: light- and pharmacologically-induced inhibition. *Experiments in Eye Research.* **61** (3), 273-284, (1995).
22 Kooragayala, K. et al. Quantification of oxygen consumption in retina ex vivo demonstrates limited reserve capacity of photoreceptor mitochondria. *Investigative Ophthalmology and Visual Science.* **56** (13), 8428-8436 (2015).
23 Adlakha, Y. K., Swaroop, A. Determination of mitochondrial oxygen consumption in the retina ex vivo: applications for retinal disease. *Methods in Molecular Biology.* **1753**, 167-177 (2018).
24 Agilent Mitocondrial stress test user guide https://www.agilent.com/cs/library/usermanuals/public/XF_Cell_Mito_Stress_Test_Kit_User_Guide.pdf.
25 Agilent Glycolytic rate assay user guide https://www.agilent.com/cs/library/usermanuals/public/103344-400.pdf.
26 Agilent wave 2.6 user guide https://www.agilent.com/cs/library/usermanuals/public/S7894-10000_Rev_C_Wave_2_6_User_Guide.pdf.
27 AVMA Guidelines for the Euthanasia of Animals https://www.avma.org/sites/default/files/2020-01/2020-Euthanasia-Final-1-17-20.pdf
28 Improving Quantification of Cellular Glycolytic Rate Using Agilent Seahorse XF Technology https://www.agilent.com/cs/library/whitepaper/public/whitepaper-improve-quantification-of-cellular-glycolytic-rate-cell-analysis-5991-7894en-agilent.pdf
29 Report Generator User Guide Agilent Seahorse XF Cell Mito Stress Test https://www.agilent.com/cs/library/usermanuals/public/Report_Generator_User_Guide_Seahorse_XF_Cell_Mito_Stress_Test_Single_File.pdf
30 Agilent Seahorse XF Buffer Factor Protocol https://www.agilent.com/cs/library/usermanuals/public/usermanual-xf-buffer-factor-protocol-cell-analysis-S7888-10010en-agilent.pdf
31 Agilent sensor cartridges and cell culture microplates https://www.agilent.com/cs/library/brochures/5991-8657EN_seahorse_plastics_brochure.pdf
32 Agilent Seahorse XF Glycolysis Stress Test Kit User Guide https://www.agilent.com/cs/library/usermanuals/public/XF_Glycolysis_Stress_Test_Kit_User_Guide.pdf

33 Fan, Y. Y. et al. A bioassay to measure energy metabolism in mouse colonic crypts, organoids, and sorted stem cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology.* **309** (1), G1-9 (2015).
34 Huang, L. et al. Ductal pancreatic cancer modeling and drug screening using human pluripotent stem cell- and patient-derived tumor organoids. *Nature Medicine.* **21** (11), 1364-1371 (2015).
35 Jeon, C. J., Strettoi, E., Masland, R. H. The major cell populations of the mouse retina. *Journal of Neuroscience.* **18** (21), 8936-8946 (1998).
36 Akimoto, M. et al. Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. *Proceedings of the National Academy of Science U. S. A.* **103** (10), 3890-3895, (2006).
37 Kenwood, B. M. et al. Identification of a novel mitochondrial uncoupler that does not depolarize the plasma membrane. *Molecular Metabolism.* **3** (2), 114-123 (2014).
38 Corso-Diaz, X. et al. Genome-wide profiling identifies DNA methylation signatures of aging in rod photoreceptors associated with alterations in energy metabolism. *Cell Reports.* **31** (3), 107525 (2020).
39 Berkowitz, B. A. et al. Mitochondrial respiration in outer retina contributes to light-evoked increase in hydration in vivo. *Investigative Ophthalmology and Visual Science.* **59** (15), 5957-5964 (2018).
40 Joyal, J. S. et al. Retinal lipid and glucose metabolism dictates angiogenesis through the lipid sensor Ffar1. *Nature Medicine.* **22** (4), 439-445 (2016).

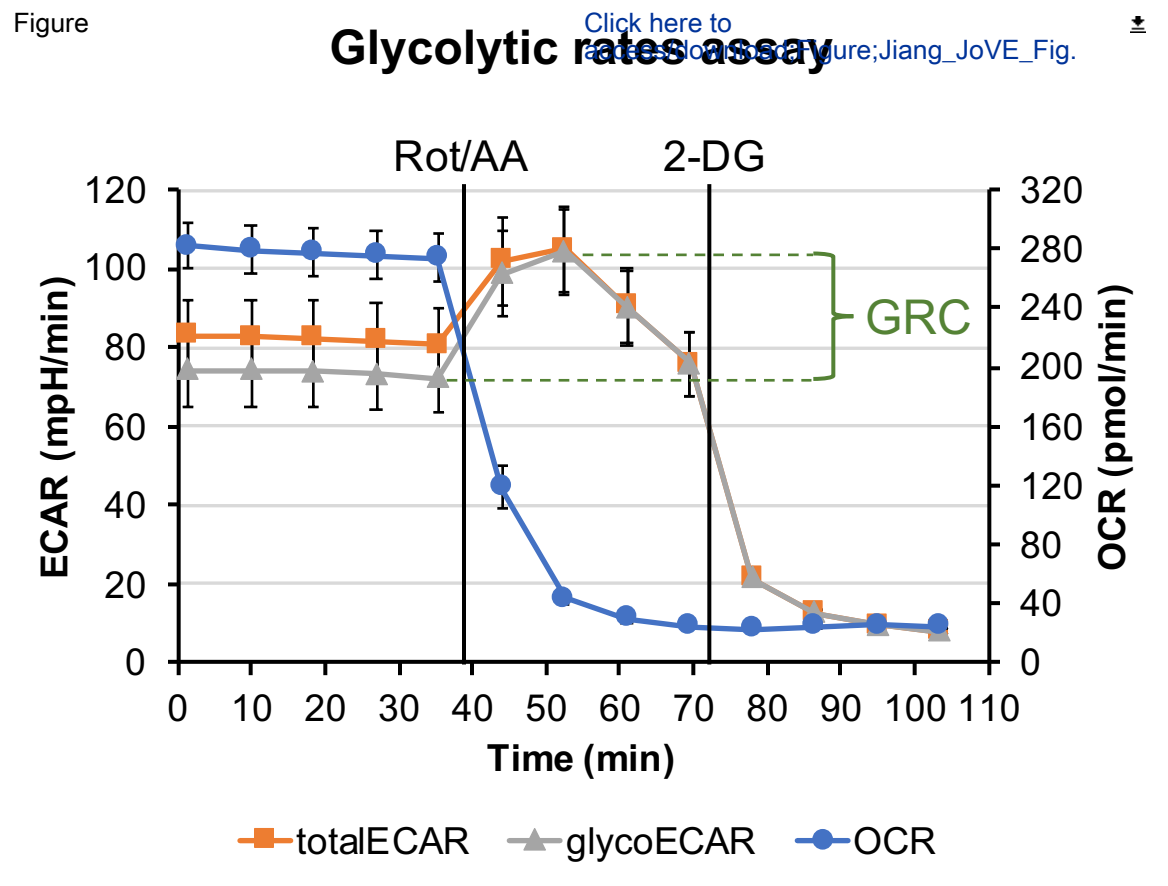
Figure

Click here to Access/Download;Figure;Jiang_JoVE_



Figure

Click here to



Response to Editorial Board Member and Reviewers' Comments

We would like to thank the editorial board member and reviewers for their suggestions. We have altered the text and added more details to address reviewers' comments. A detailed response to the editorial board member and reviewers' comments is as follows:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. *Done*

2. Please provide an institutional email address for each author.

Ke Jiang: coco.jiang@nih.gov
Jacob Nellissery: nellisseryj@nei.nih.gov
Anand Swaroop: swaroopa@nei.nih.gov

3. Please revise the title to "Determination of Mitochondrial Respiration and Glycolysis in Ex Vivo Retinal Tissue Samples". *Fixed*

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Checked and revised.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Seahorse XFe24, Agilent's Wave 2.6, Cell-Tak, Kimtech, Islet Capture, etc.

The term "Seahorse XFe24", Wave 2.6 program" "Islet Capture plate" and "Cell-Tak" are necessary because they identify which type of machine and equipment are used for the particular assay. Without these terms, the reader will not be able to choose the correct product suitable for experiments using ex vivo tissue. Also, there is no generic term for these reagents. Kimtech has been removed from the text as any absorbing tissue wipe is suitable.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "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." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Checked and corrected, as needed.

7. Being a video-based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:
a) Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution. *Done*
b) Please specify the euthanasia method. How is it performed? *Done*

c) Please do not highlight any steps describing euthanasia. *Done*

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. 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. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) 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. *Done*

9. Line 134-135: Please specify how is the rectus muscle removed. What is used for removing the muscle?

Micoscissors were used to cut off the rectus muscles. Details are now added.

10. Line 202-206/215-250: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Please move the discussion about the protocol to the Discussion.

The text originally from line 202-206/215-250, now between line 270-275/291-297, are not part of the protocol but represent the descriptive text within instructions for data analysis. These texts are necessary to define different measurements obtained and calculated from the raw data.

11. Please include a one-line space between each protocol step and then highlight up to 3 pages 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. *Done*

12. Please do not use the &-sign or the word "and" when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

We followed instructions and used the JoVE EndNote style in EndNote to generate the reference list.

13. Figure 2: The bracket for the parenthesis is missing in the right Y-axis. Please insert. *Fixed*

Reviewers' comments:

Reviewer #1:

This is an overall well written and detailed methods manuscript providing a step-by-step protocol for the assessment of mitochondrial and glycolytic flux surrogates in an ex vivo retinal tissue model. This will be very useful for the extracellular flux community interested in system level metabolic analysis of cells in tissue context. However, the authors should convey in a much clearly way that the obtained readings reflect the combined energy metabolism and the interactions of the many cell types present in retinal tissues. This is an extremely important point, but is only buried in the last part of the discussion. The authors should touch upon this

key point already in the introduction. Another point of concern is the lack of normalization at the end of the assay. How do the authors address differences in thickness of the retina resulting in differential tissue (protein) content? This may not be relevant for the analysis of wild-type tissue but certainly for the assessment of pathological tissues. The other question I have is how the authors infer the basal respiration in the mitochondrial stress assay without the application of oligomycin.

We are grateful to the reviewer for his/her encouraging comments and recognizing the importance of our study.

We thank the reviewer for the valuable suggestion and have expand the last paragraph of Introduction to indicate that this protocol is measuring metabolic activity at tissue level and reflecting the sum contribution of many different cell types.

We agree with the reviewer that normalization is an important step especially if pathological tissues are involved. We have added a section for normalization in the data analysis section. Either total DNA content or total protein content can be used to normalize the raw data. The CyQuant Cell Proliferation Assay Kit was frequently used to quantify total DNA content and normalize seahorse data when dealing with samples of different mass (PMID: 25977509 and PMID:26501191). We have added these details in the protocol.

The "basal OCR" refers to the measurement of respiration at the physiological stage of tissue before any compound drug is added. Oligomycin is not used in either of the two assays presented in this protocol.

Reviewer #2:

Manuscript Summary:

The manuscript by Jiang and colleagues provides a detailed protocol for performing analysis of mitochondrial respiration and glycolysis in retinal tissue samples. Overall, the manuscript was organized and well written, except for a few grammatical errors and odd sentence structure. The information is valuable for expanding analytical protocols beyond cultured cells. With the plethora of transgenic mice being used in vision research, these techniques will provide another means to compare the effect of knocking out specific proteins on retinal metabolism. A few suggestions for improving the clarity or quality of the information are provided below.

We thank the reviewer for his/her comments. We are delighted that the reviewer appreciated the relevance of our study.

Major Concerns:

1. The table of reagents and supplies was very helpful. However, more detail needs to be added for the dissection forceps. Several specific types are listed in the protocol and should be identified in the table.

We thank the reviewer for this suggestion. As the choice of specific forceps can frequently depends on the preference of the person performing the dissection, we did not want to specify which forceps need to be used for a specific step. However, upon reviewer's suggestion, we have added catalog numbers for all the forceps and microscissors we used in the reagents and material list.

2. Pictures should accompany the dissection method, especially concerning the dissection of the retina, removal of the sclera, and placement of the punches in the free floating retina. A picture of each forcep that is used would also be helpful since use of the correct tool is critical for successful dissection. (These pictures could accompany section 4.) Same comment for the microscissors and trephine.

As a video strip will be produced and accompany this protocol focusing on the retinal dissection steps, we did not think there is a need to provide pictures for the dissection procedure.

3. It seems that the plate preparation should occur prior to starting the mouse dissection. The order of preparation should be emphasized in the description.

Coating the mesh inserts with Cell-Tak and the dilutions of compound drugs should be performed prior to the retinal dissection. However, loading drug into cartridge plate ports usually takes less than 5 minutes. To prevent unnecessary evaporation and resulted volume change, we prefer to perform this step after the retinal dissection and retinal punch preparation is completed since the above steps usually takes approx. 1.5 hr. Calibration of sensor cartridge takes around 15~20 minutes, and we use that time for loading retinal punch-containing mesh insert into the Islet Capture plates. The procedures are listed in the protocol in the order as performed in a typical experiment.

4. Orientation of the retinal punch- are there tips to knowing which side is the ganglion cell layer? Or is this based on the orientation after dissection? If so, this should be noted as a caution.

Frequently, one will notice pigmented cells from RPE still attached to the photoreceptors on the retinal punch disk. This can be used as an indication of the retinal punch orientation. We have added more instructions in the text.

5. It would be good to include an average measurement of total protein in each punch. This would allow for comparison of respiration with other systems, such as cultured cells.

The retinal punch sticks to the insert mesh and is hard to retrieve after the experiment. We have not measured the total protein content from a single retinal punch. We have previously determined the total surface area of a single adult mouse retina is ~20mm^2 and the 1mm diameter retinal punch disk accounts ~1/25 of one retina. Each mouse retina is known to contain ~6.5 million cells. Hence, we estimate each retinal punch containing 260K cells. We have added this information to the text as a reference.

6. There is a bit of confusion about the representative results. The description says the data are from 2 mice, but there are 10 wells. Were both eyes used? Directions say that a punch is taken from each of the 4 sections. A bit of clarification is needed. Along these same lines, it would be good to mention the number of technical replicates (punches per mouse) that are needed for good reproducible data. I would guess that the number of required biological replicates would be user determined, correct? This type of information would be good to include to help guide a new investigator in setting up their experimental protocol.

In general, one adult mouse retina has enough surface area to provide 5 punches. However, depending on the personnel's dissection skills and experience in handling freshly dissected retinal tissue, the number of quality punches one can get from a single adult retina can range from 3 to 5. Hence, we set the protocol at a mid-level (4 punches taken from 4 sections of a

single retina) so that most people can perform the study. In the experiment presented as a representative result, 4 eyes from 2 mice were used, and 5 punches were taken from each retina to make a total of 20 punches. We have modified the text to clarify this.

We recommend including 3 to 5 technical replicates from the same eye or the same mouse and take samples from 3 or more mice as biological replicates. Specific experimental designs can be tailored to requirements of each project. We have added further instructions regarding technical and biological replicates in the discussion.

7. Does GFP in the mouse photoreceptors interfere with the fluorescence measured in the Seahorse plate?

No. The GFP protein expressed within the cell will not affect the fluorescence measured during data recording because these will be out of focus for the detecting probe. We have modified the text to indicate this point.

8. Most of the description of the representative results was easy to follow. However, there is still some confusion on how the mitoECAR is determined. At what point in the trace is the mitoOCR value used? An example calculation for the GRC and the MRC would also be good to add so that the reader can see where the values were generated.

We thank the reviewer for raising this point. We have added more details in data analysis instruction to better define each component in the equations. mitoOCR is calculated by subtracting the residual OCR reading (totalOCR$_{Rot/AA}$) from totalOCR. mitoECAR is calculated from mitoOCR using equation 4.

9. The last paragraph in Results explaining the drop in ECAR was interesting. Did you check if perhaps the buffer had maxed out its capacity?

We have checked the pH values throughout glycolytic rate assay. The DMEM assay medium has a starting pH around 7.40~7.50. During glycolytic rate assay measurement cycle, the pH reading drops to 7.00~7.20 depending on the amount of glycolysis released lactate. However, the pH is restored back to their original range ~7.40 in between each measurement cycle (during mixing and waiting period). Hence, the buffer capacity is not likely to be maxed out.

10. Discussion- The paragraph (lines 329-344) on factors contributing to data variation was particularly helpful. The final paragraph on limitation was also valuable.

We thank the reviewer for this comment.

Minor Concerns:

- Line 43- high levels *Fixed*
- Line 44 contain a high….no comma after mitochondria *Fixed*
- Line 79-80 (and elsewhere) use the actual company name of the test (the company has many different mito tests now) *Fixed*
- Line 93- no comma after assay *Fixed*

- Line 105- "and smear around" use the pipette tip to do this?
  Yes. Specific details have been added to the text.

- For the various reagents, indicate if they can be used later ("can be stored at 4 degrees for one week") or if they need to be prepared fresh each day.
  Details have been added in the text for preparation of reagents. Assay medium, Cell-Tak coating mix and various drug dilutions are prepared fresh on the day of experiment and not reused.

- Line 131- does the CO2 asphyxiation alter retina/activity of metabolism?
  Mice should not be left in CO_2 chamber longer than the necessary time needed for euthanasia. More details have been added in the text. However, we did not assess if prolonged exposure to CO_2 alters seahorse results of the retinal tissue.

- Are the equations provided in the manuscript developed by the authors or are they from the company instructions?
  The equations are provided by Agilent. Reference sources have been added for each equation.

- Line 161- Avoid introducing air bubbles. How should the pipetting be done? Do you tap the plate at the end? More details here would be good.
  More details have been added to the text. It is not recommended to tap the plate after loading the injection ports as the vibration might cause leaking of drugs from the ports.

- Line 265- what is the benefit of Bam15 versus FCCP (what is typically used in Seahorse assays)?
  FCCP is one of the most widely used protonophore uncoupler. However, FCCP was reported to have off-target activity leading to plasma membrane depolarization, mitochondrial inhibition, and cytotoxicity. Bam15 is relatively newly identified uncoupler. Compared to FCCP, Bam15 displays equal or high efficiency of uncoupling and lower cytotoxicity without depolarizing the plasma membrane. Reference for Bam15 has been added to the text.

- Line 360- even if 96-well plates *Fixed*