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Real-Time Analysis of Bioenergetics in Primary Human Retinal Pigment Epithelial Cells Using High-Resolution Respirometry

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Citation

Fitch, T.C., Frank, S.I., Li, Y.K., Saint-Geniez, M., Kim, L.A., Shu, D.Y. Real-Time Analysis of Bioenergetics in Primary Human Retinal Pigment Epithelial Cells Using High-Resolution Respirometry. *J. Vis. Exp.* (192), e64572, doi:10.3791/64572 (2023).

Date Published

February 3, 2023

DOI

10.3791/64572

URL

jove.com/video/64572

Abstract

Metabolic dysfunction of retinal pigment epithelial cells (RPE) is a key pathogenic driver of retinal diseases such as age-related macular degeneration (AMD) and proliferative vitreoretinopathy (PVR). Since RPE are highly metabolically-active cells, alterations in their metabolic status reflect changes in their health and function. High-resolution respirometry allows for real-time kinetic analysis of the two major bioenergetic pathways, glycolysis and mitochondrial oxidative phosphorylation (OXPHOS), through quantification of the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), respectively. The following is an optimized protocol for conducting high-resolution respirometry on primary human retinal pigment epithelial cells (H-RPE). This protocol provides a detailed description of the steps involved in producing bioenergetic profiles of RPE to define their basal and maximal OXPHOS and glycolytic capacities. Exposing H-RPE to different drug injections targeting the mitochondrial and glycolytic machinery results in defined bioenergetic profiles, from which key metabolic parameters can be calculated. This protocol highlights the enhanced response of BAM15 as an uncoupling agent compared to carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) to induce the maximal respiration capacity in RPE. This protocol can be utilized to study the bioenergetic status of RPE under different disease conditions and test the efficacy of novel drugs in restoring the basal metabolic status of RPE.

Introduction

Retinal pigment epithelial cells (RPE) exist as a monolayer of pigmented epithelial cells strategically located between the photoreceptors and fenestrated endothelium of the choriocapillaris. RPE are highly metabolically active with numerous functions, including (1) phagocytosis of shed photoreceptor discs, (2) recycling of visual pigment to

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maintain the visual cycle, (3) transport of nutrients, metabolites, ions, and water, (4) absorption of light, (5) protection against photooxidation, (6) secretion of essential factors to support retinal integrity, and (7) formation of the outer blood-retinal barrier[1]. Degeneration of RPE is associated with metabolic dysfunction and mitochondrial defects, leading to blinding eye diseases such as age-related macular degeneration (AMD) and proliferative vitreoretinopathy (PVR)[2].

Two key bioenergetic pathways include glycolysis, which occurs in the cytoplasm, and oxidative phosphorylation (OXPHOS), which occurs in mitochondria. During glycolysis, one molecule of glucose is converted to two molecules of pyruvate and a net production of two molecules of adenosine triphosphate (ATP). In contrast to glycolysis, OXPHOS produces far higher levels of ATP (~32-38 molecules of ATP per glucose molecule). Notably, OXPHOS consumes oxygen and requires functional mitochondria to occur, whereas glycolysis occurs in the cytoplasm and does not require oxygen.

Prior to the introduction of fluorescence- or phosphorescence-based techniques to examine mitochondrial respiration, oxygen levels were measured in permeabilized cell suspensions in chambers equipped with a Clark-type oxygen electrode[3]. While the Clark electrode is much cheaper than fluorescence-based respirometry and works in non-adherent cells, it is relatively low-throughput, with each respiratory run lasting around 15-20 min and requiring far higher quantities of cells for each sample[3]. Thus, the fluorescence-based respirometry technique has largely replaced the Clark electrode and has become a popular technique in the fields of metabolism and mitochondrial research.

This protocol describes a high-throughput, high-resolution, fluorescence-based respirometry technique that kinetically measures the OXPHOS and glycolytic bioenergetic profiles of living cells. Since the process of OXPHOS consumes oxygen, the bioenergetic profile for OXPHOS is produced by mapping changes in the oxygen consumption rate (OCR) over time[4]. In this technique, two fluorophores are embedded in the sensor cartridge sleeve which is connected to fiber optic bundles that emit light, exciting the fluorophores. Changes in fluorophore emission are measured by highly sensitive fluorescence sensors and transmitted through the fiber optic bundle to be converted to an OCR readout[5]. The fluorophore becomes quenched by oxygen, thus enabling the determination of extracellular oxygen levels in the assay media, known as oxygen flux or OCR. The other fluorophore is a pH sensor probe sensitive to changes in proton efflux, which is converted to a measure of extracellular acidification rate (ECAR). During measurements, the fiber optic bundles with embedded fluorophores are lowered to 200 µm above the cell monolayer, creating a transient micro-chamber that enables rapid, real-time readings. Once a 10% change in oxygen or proton levels is detected, the sensors are lifted upward, allowing a larger volume of media to mix with the transient micro-chamber media, restoring OCR and ECAR values back to baseline. Each sensor cartridge is equipped with four ports to allow for the sequential administration of up to four compounds per well during the assay. Measurements can be collected before and after injection of the compounds in each port, revealing key information about the metabolic status of the cells.

Interrogating these two distinct metabolic pathways can yield important discoveries regarding the metabolic status of RPE following exposure to different pathogenic stimuli, and thus can be used to test the efficacy of drugs in restoring the metabolic integrity of RPE[6,7,8]. The advent of

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high-throughput respirometry and the availability of specific mitochondrial inhibitors have stimulated more research in defining the bioenergetic profiles of RPE and identifying defects in metabolism and mitochondria during disease states[6,7,8,9,10,11,12,13]. High-resolution respirometry has highlighted the key role of metabolic reprogramming of RPE in retinal pathologies such as AMD and PVR. Two key cytokines involved in the pathogenesis of AMD and PVR are transforming growth factor-beta 2 (TGFβ2) and tumor necrosis factor-alpha (TNFα). The induction of epithelial-mesenchymal transition (EMT) by TGFβ2 is accompanied by mitochondrial dysfunction, OXPHOS suppression, and a compensatory increase in glycolytic capacity in RPE[6]. More recently, the pro-inflammatory cytokine, TNFα, has been shown to induce a significant upregulation of basal OXPHOS and reduced glycolysis in H-RPE[7]. The administration of dimethyl fumarate significantly suppressed TNFα-induced inflammation in H-RPE and restored mitochondrial morphology and basal bioenergetic profiles[7]. The divergent metabolic profiles induced by these two growth factors stimulate intriguing mechanistic questions regarding the involvement of metabolic reprogramming in retinal diseases. The following protocol describes the steps for assessing OXPHOS and glycolytic bioenergetic profiles in H-RPE using high-resolution respirometry.

Protocol

1. Plating H-RPE in the cell culture plate

1. Thaw H-RPE in a T25 flask in human RPE medium supplemented with RPE growth medium supplements (4 mM L-glutamine, 25 ng/mL FGF-2, 2% FBS, 30 mg/mL gentamicin, and 15 µg/mL amphotericin).
2. Incubate the cells at 37 °C and 5% CO_2 in a humidified incubator. Refresh the media the next day and wait for the cells to reach at least 80% confluence before passaging at a ratio of 1:3 into a T75 flask.
3. Once confluent, passage the cells using the subculture reagents kit consisting of trypsin/EDTA 0.025% solution, trypsin neutralizing solution, and HEPES buffered saline (pH 7.0-7.6).
    1. Rinse H-RPE gently with 3 mL of HEPES buffered saline. Then add 3 mL of trypsin/EDTA and incubate (37 °C and 5% CO_2) for 5 min (or until the cells have lifted off the base of the flask, as observed under the microscope). Neutralize the trypsin/EDTA with 3 mL of trypsin neutralizing solution.
    2. Centrifuge the cells at 200 x *g* for 3.5 min to form the cell pellet. Carefully aspirate and discard the supernatant.
4. Resuspend the cells in human RPE medium to a final concentration of 20,000 cells per 100 µL per well.
    1. Pipette up and down multiple times to ensure that the cell suspension is homogeneous, using a multichannel pipette for ease and consistency of pipetting into the 96-well cell culture microplate. Rest the pipette tip just below the circular rim at the top of the well for an even, homogeneous cell layer.
    NOTE: No coating is required as the cells adhere well to the microplate.
    2. Be sure to leave the four corner wells empty of cells (100 µL of media only) to serve as the background correction wells (**Figure 1A**).
    NOTE: The microplate is formatted in a typical 96-well plate design; however, the surface area of

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each well is 0.106 cm^2, which is 40% smaller than a standard 96-well plate. At this cell density, the cells should be 100% confluent the next day. It is recommended to perform a cell density optimization experiment first before testing treatments to ensure that basal OCR and ECAR readings are around 50-100 pmol/min and 10-20 mpH/min, respectively.

5. Leave the cell culture plate at room temperature (RT) for 1 h before placing it back into the incubator (5% CO_2, 37 °C, humidified) to help minimize edge effects. Edge effects are changes in media volume in the wells of the peripheral borders of the 96-well plate due to evaporation[14].

   NOTE: The cells will adhere overnight and form a confluent monolayer the next day. H-RPE are matured in the plate for at least 1 month by refreshing half of the growth media every 2-3 days.

6. Examine the cells under the microscope before changing the media to check their morphology and pigmentation level. Ensure that the cells are confluent with a characteristic cobblestone-like morphology and acquire pigmentation over time, as seen in the morphology images in Shu et al.[7].

2. The day before running the assay

1. Ensure that the sensor cartridge is hydrated the day before the assay by filling each well of the utility plate with 200 µL of deionized water.

   NOTE: The lid of the utility plate contains fluorescent biosensors for measuring oxygen and pH levels for each well. The sensors are coupled to fiber-optic waveguides that deliver light at various excitation wavelengths and transmit a fluorescent signal through optical filters to photodetectors.

2. Place the sensor cartridge submerged in water in the utility plate along with ~20 mL of the calibrant (to warm up for use the next day) in a 37 °C humidified oven (without CO_2) overnight.

   NOTE: The calibrant is a proprietary solution designed for sensor cartridge calibration and is likely similar in composition to phosphate-buffered saline (PBS). The minimum time for cartridge hydration is 4 h, but the best results are obtained with overnight hydration.

3. Ensure that the instrument is turned on and start the software to allow the instrument to stabilize to 37 °C overnight (**Figure 2**). Generally, the instrument can be left on even when not in use.

3. Real-time Mito Stress Test using the extracellular flux analyzer

1. On the day of the assay, replace the water in the utility plate with an equal volume of warmed calibrant at least 45 min prior to running the assay.

2. Make the Mito Stress Test assay media using the base medium without phenol red by adding supplements, as shown in **Table 1**. Warm the media to 37 °C, adjust the pH to 7.4, and vacuum filter the media using a tube top filter unit. For running a full plate, make ~25 mL of assay media.

3. Remove the human RPE media and replace it with 180 µL of freshly prepared assay media (step 3.2). Place the cell culture plate in a humidified 37 °C oven (without CO_2) for 1 h before starting the assay.

   NOTE: This is important for de-gassing the cell plate, allowing for CO_2 diffusion. Since the cells are no longer

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in their growth media and no longer in an incubator with CO_2, their viability will deteriorate over time, therefore, care should be taken to conduct the assay as efficiently as possible. Care should also be taken to ensure that there are no bubbles in the cell culture plate; if any are present, pop them with a pipette or needle.

4. Each sensor cartridge has four reagent delivery ports per well for the injection of test compounds into the wells of the cell culture plate during the assay (**Figure 1C,D**). Prepare ~3mL of the 10x drug solutions by diluting the drug stocks in the respective assay media according to **Table 2**. For example, load Port A with 25 µM of oligomycin dissolved in the Mito Stress Test assay media, such that when the drug volume is injected into the well by the instrument, the final concentration that each cell is exposed to is 2.5 µM. Pipette 20 µL of the 10x drug stock into Port A, 22 µL into Port B, and 25 µL into Port C, to achieve the specified final drug concentration in each well. For protocols requiring all four drug ports, pipette 28 µL into Port D.

   NOTE: Refer to **Figure 1B** when pipetting the drugs into the drug ports for the proper orientation of Ports A/B/C/D. The notch on the edge of the cartridge should be located in the bottom left corner when loading the drug ports. By pipetting at an angle into each drug port, bubbles can be minimized. If there are any bubbles present, care should be taken to pop them with a pipette or needle.

5. Open the **Templates** tab in the analysis software, select **Mito Stress Test**, and fill out the **Group Definitions**.

   1. Input details on the **Injection Strategy** (in this case, it is pre-inputted as the Mito Stress Test drugs). Input details regarding the different experimental groups in the assay (e.g., **Control or Treatment**). Input details on the **Assay Media** (addition of different supplements and their specific concentrations to the base assay media) and finally add the **Cell Type**.

   2. Click on the next tab, **Plate Map**, where the different groups being examined will be assigned to their specific location on the 96-well plate map. Once this is done, click on the **Protocol** tab to review the instrument protocol for the default **Mito Stress Test** protocol.

      NOTE: The default **Mito Stress Test** template is ready to use but can be modified in any way to suit the experimental design. For example, the default measuring time is 3 min, but this can be modified to 5 min if desired.

6. Click on **Run the Assay** and insert the sensor cartridge that is submerged in the calibrant solution in the utility plate. This process takes around 25 min. In this step, each biosensor is calibrated independently based on the sensor output measured in the calibrant solution of known pH and oxygen concentration.

   1. Once this is calibrated, remove the utility plate and insert the cell culture plate.

      NOTE: The cell culture plate is equilibrated first, after which, the instrument begins mixing the assay media and measuring the OCR and ECAR values. This step takes around 1.5 h and is conducted inside the instrument without any intervention from the user. A baseline reading of OCR and ECAR is first established by mixing the assay media for 3 min and then measuring OCR and ECAR for 3 min. The instrument performs three loops of mixing and measuring.

   2. After the baseline measurement, the instrument automatically injects the Port A drug solution into

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each well. This is followed by three loops of mixing and measuring (3 min each). The same pattern occurs after each subsequent drug injection (Ports B and C).

7. Once the run is complete, remove the cell culture plate and sensor cartridge. For quality control purposes, ensure that all the drug ports in the sensor cartridge have been injected by examining the ports to check that no residual drug remains. Discard the sensor cartridge and utility plate as they are single-use items.

8. Examine the cells in the cell culture microplate under the microscope to ensure that there is still a confluent monolayer of cells. Discard the assay media and replace it with 60 µL of 1x lysis buffer in each well.
   NOTE: The lysis buffer is made from 10x lysis buffer diluted to 1x in deionized water with the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF).

9. Wrap the edges of the plate in Parafilm to prevent evaporation and place it in a -80 °C freezer to aid in cell lysis overnight, before quantification of the protein content using the BCA assay.

10. For data analysis, normalize all data by dividing the OCR and ECAR values by the microgram of protein in each well. Export the Mito Stress Test report generator, which utilizes Excel macros to automatically calculate the Mito Stress Test parameters using the data analysis software.
    NOTE: This can be used to determine basal respiration, maximal respiration, spare respiratory capacity, proton leakage, ATP production, and non-mitochondrial respiration. Definitions of these calculations are listed in **Table 3**.

4. Real-time Glycolytic Stress Test using the extracellular flux analyzer

1. Perform the Glycolytic Stress Test by following the same steps as the Mito Stress Test, except using the different assay media supplements and drug injections shown in **Table 1** and **Table 2**.

2. For data analysis, export the Glycolytic Stress Test report generator, which utilizes Excel macros to automatically calculate the Glycolytic Stress Test parameters from the data analysis software.
   NOTE: This can be used to determine non-glycolytic acidification, glycolysis, glycolytic capacity, and glycolytic reserve. Definitions of these calculations are listed in **Table 3**.

5. BCA protein quantification assay

NOTE: The bicinchonic acid (BCA) protein quantification assay (also known as the Smith assay[15]) is a copper-based colorimetric assay used to determine the total protein content in a sample. Normalizing OCR and ECAR data to the microgram of protein in each well ensures that different amounts of cells/protein in each well do not skew the readings. The mechanism of the BCA assay is based on two chemical reactions. Firstly, the peptide bonds in proteins reduce cupric ions (Cu^{2+}) to cuprous ions (Cu^{+}), which is a temperature-dependent reaction assisted by higher temperatures (37 to 60 °C). If there are more peptide bonds, making the amount of Cu^{2+} proportional to the protein content in the solution[16]. This reaction results in a color change from green to an intense purple solution, with a peak absorbance at 562 nm[16]. The higher the protein content in the sample, the higher the absorbance at this wavelength. The working range of this kit is 20-2,000 µg/mL.

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1. The BCA assay kit contains 1 mL aliquots of bovine serum albumin (BSA) at 2 mg/mL, which serves as the protein concentration reference standard. Prepare a serial dilution in a clear, flat-bottomed 96-well plate by starting from the undiluted 2 mg/mL BSA and subsequently halving the concentration by diluting in deionized water (e.g., 2, 1, 0.5, 0.25, 0.125 mg/mL, etc.). Using a known concentration of protein allows for the calculation of a standard curve, which is used to calculate the protein content of the experimental samples. To improve the accuracy of the calculated protein content measurements, measure absorbance readings of the protein concentration reference standards alongside the experimental samples for each assay.
2. Pipette 25 µL of each BSA serial dilution in duplicate into the 96-well plate.
3. Pipette 25 µL of the 1x lysis buffer in duplicate into the 96-well plate to serve as the blank.
4. Thaw the cell culture plate to room temperature and pipette 25 µL of each cell lysate in duplicate into the 96-well plate.
5. Prepare the working reagent in a 50:1 ratio of BCA kit reagents A:B. Mix thoroughly by vortexing to remove any turbidity in the working reagent, so that it is a homogenous green solution. Add 200 µL of the working reagent to each well.
6. Protect the plate from light by covering the plate in foil and incubate in a 37 °C oven for 20 min.
7. Measure the absorbance at 562 nm in a 96-well plate reader.
8. For data analysis, average all the duplicate values. Subtract the blank absorbance levels determined from the 1x lysis buffer wells from the measurements of all the samples. Determine the standard curve by plotting the absorbance of each BSA standard to its known concentration in µg/mL. Use the linear equation derived from the standard curve to determine the protein concentration of the experimental samples.

Representative Results

The instrument simultaneously measures both OCR and ECAR for each run. For the Mito Stress Test, parameter calculations are based on OCR readings (**Figure 3A**), whereas for the Glycolytic Stress Test, parameter calculations are based on ECAR readings (**Figure 3B**). **Figure 3** shows representative graphs for the Mito Stress Test OCR curve over time (**Figure 3C**) and parameter calculations in the form of bar graphs for H-RPE (**Figure 3D**). The Glycolytic Stress Test is represented as an ECAR curve over time (**Figure 3E**), and parameter calculations are displayed in bar graphs for H-RPE (**Figure 3F**).

Basal respiration provides an understanding of the energetic demands of the cells under basal conditions[17]. The first drug injection, oligomycin (Oligo), is an ATP synthase inhibitor, and thus, any reduction of OCR following the first drug injection is a measure of ATP-linked respiration[18]. Any remaining basal respiration following subsequent drug injections is regarded as a proton leak since it is not coupled to ATP synthesis. Increased proton leak may indicate increased mitochondrial uncoupling, which is regulated by uncoupling proteins that are physiologically present but have also been linked to pathologies such as obesity, cancer, type 2 diabetes, and cardiovascular disease[19]. The second injection is of an uncoupling agent, such as BAM15 or FCCP, to determine the maximal respiration potential of the mitochondria. Uncoupling agents collapse the proton gradient and reduce the proton

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motive force across the mitochondrial inner membrane. The result is an uninhibited flow of electrons through the electron transport chain (ETC), which elevates the rate of oxygen consumption and causes mitochondrial respiration to reach its maximal capacity[20]. Spare respiratory capacity (SRC) is the difference between maximal and basal respiration, indicating the capacity of the cells to respond to changes in energetic demands when challenged, indicating cell fitness. Importantly, for the Mito Stress Test in H-RPE, BAM15 is superior to FCCP in enhancing mitochondrial respiration capacity (**Figure 4A,B**), as maximal respiration and spare respiratory capacity are significantly higher with 10 µM BAM15 compared to 500 nM or 1 µM FCCP. No significant differences were observed between either FCCP dose. The third and final injection of rotenone and antimycin A (Rot/AA) inhibit mitochondrial complexes I and III, respectively, of the ETC, which shuts down mitochondrial respiration; any residual OCR is due to non-mitochondrial sources[21].

During glycolysis, one molecule of glucose is converted to two molecules of lactate in the absence of oxygen. The extrusion of lactate from the cell is accompanied by the efflux of one proton per lactate molecule, hence causing acidification of the extracellular space. The flux of proton production in the media is measured by changes in ECAR. Basal glycolysis levels are established first, after which glucose is injected into the assay media, which is devoid of glucose, to induce glycolysis and thus enhance ECAR levels[22]. Oligomycin is injected to induce the "highest" ECAR as it halts mitochondrial ATP production, thus forcing the cell to derive its ATP through glycolysis. Finally, glycolysis is shut down by adding 2DG, which inhibits hexokinase, the first enzyme of glycolysis[23]. Any remaining ECAR is likely due to other sources of acidification, such as CO_2 production by the TCA cycle during OXPHOS, and is denoted as non-glycolytic ECAR. The glycolytic reserve is calculated as the difference between the "highest" ECAR and the ECAR in the presence of glucose. The glycolytic capacity is the sum of glycolysis and the glycolytic reserve.

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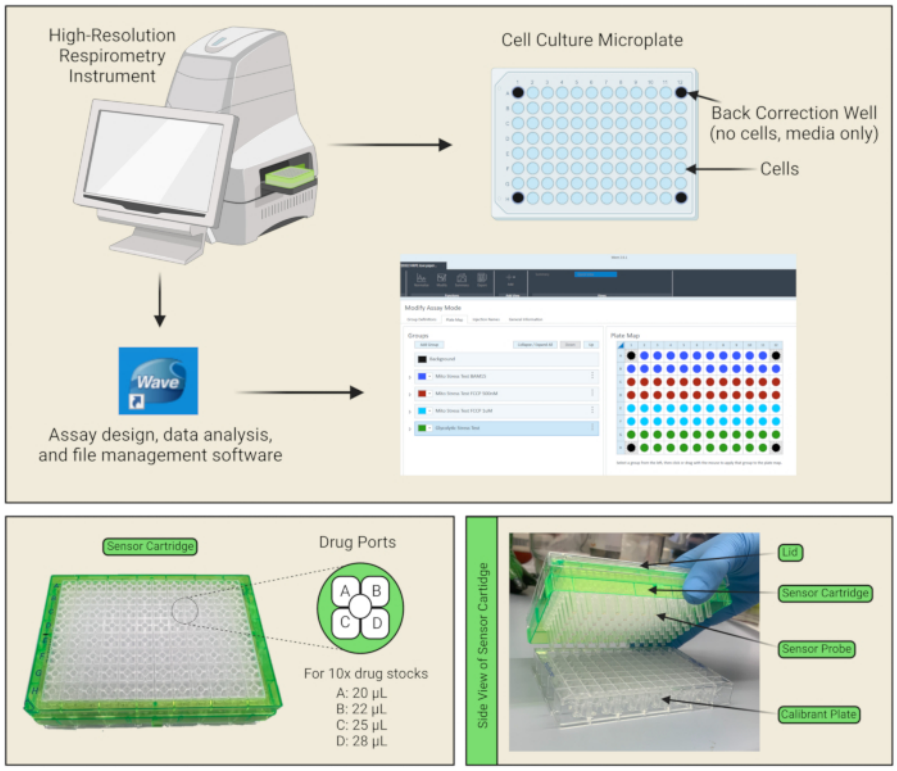

Figure 1: Components of the plate and sensor cartridge. (**A**) The instrument, data analysis software, and protocol setup interface are shown. In the 96-well culture plate layout, the cells are plated in the blue-colored wells and the four corner wells are marked in black, as they serve as the back correction wells which contain media only (and no cells). (**B**) There are four drug ports for each well of the sensor cartridge where drugs A, B, C, and D can be loaded. The volumes to pipette into each port are listed based on the calculations for 10x drug stock preparations. (**C**) The sensor cartridge consists of sensor probes which are placed directly into the calibrant-filled utility plate. Please click here to view a larger version of this figure.

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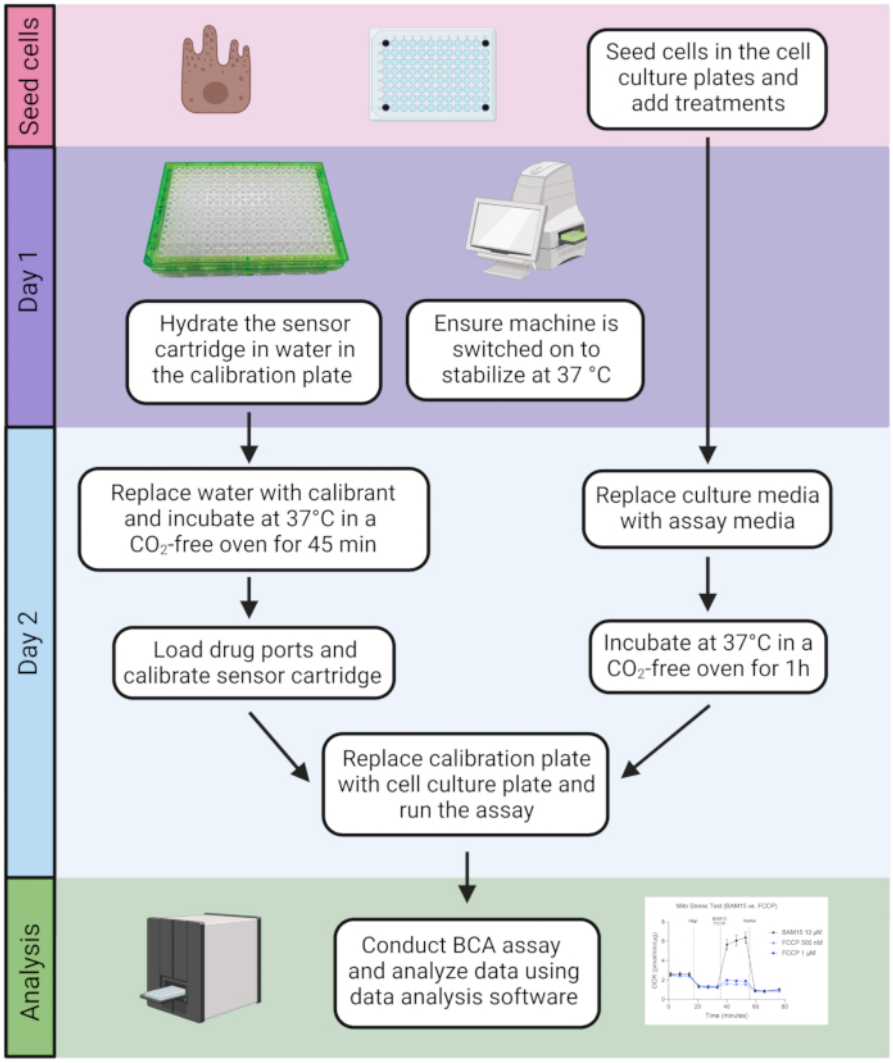

Figure 2: Timeline of the assay. The cells are seeded in the cell culture plate. Once ready, the assay involves a 2 day procedure followed by quantification of the protein content using the BCA assay and subsequent data analysis. Please click here to view a larger version of this figure.

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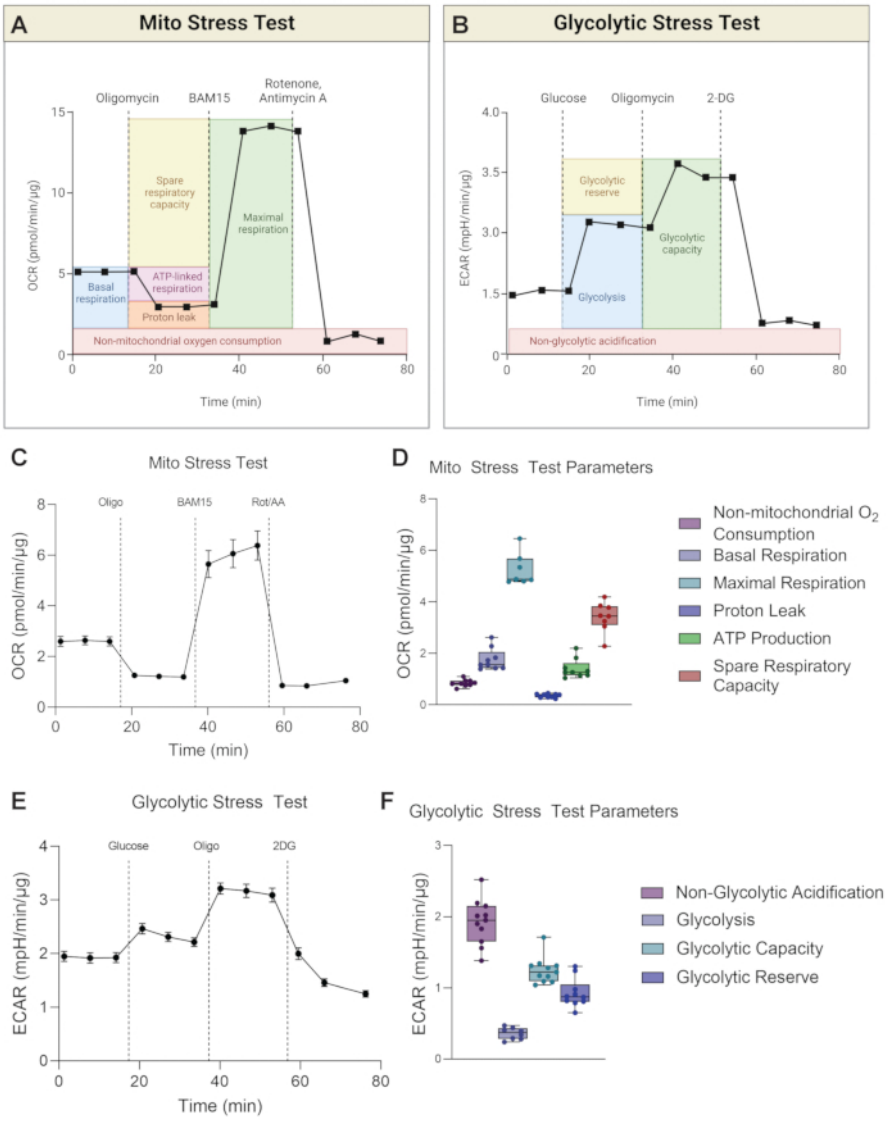

Figure 3: Representative graphs of the Mito Stress Test and Glycolytic Stress Test. Calculations of (**A**) Mito Stress Test and (**B**) Glycolytic Stress Test parameters are depicted in the schematic. (**C**) Representative oxygen consumption (OCR) curve and (**D**) Mito Stress Test parameters for H-RPE are shown. (**E**) Representative extracellular acidification rate (ECAR) curve and (**F**) Glycolytic Stress Test parameters for H-RPE are shown. Error bars are means ± SEM. Please click here to view a larger version of this figure.

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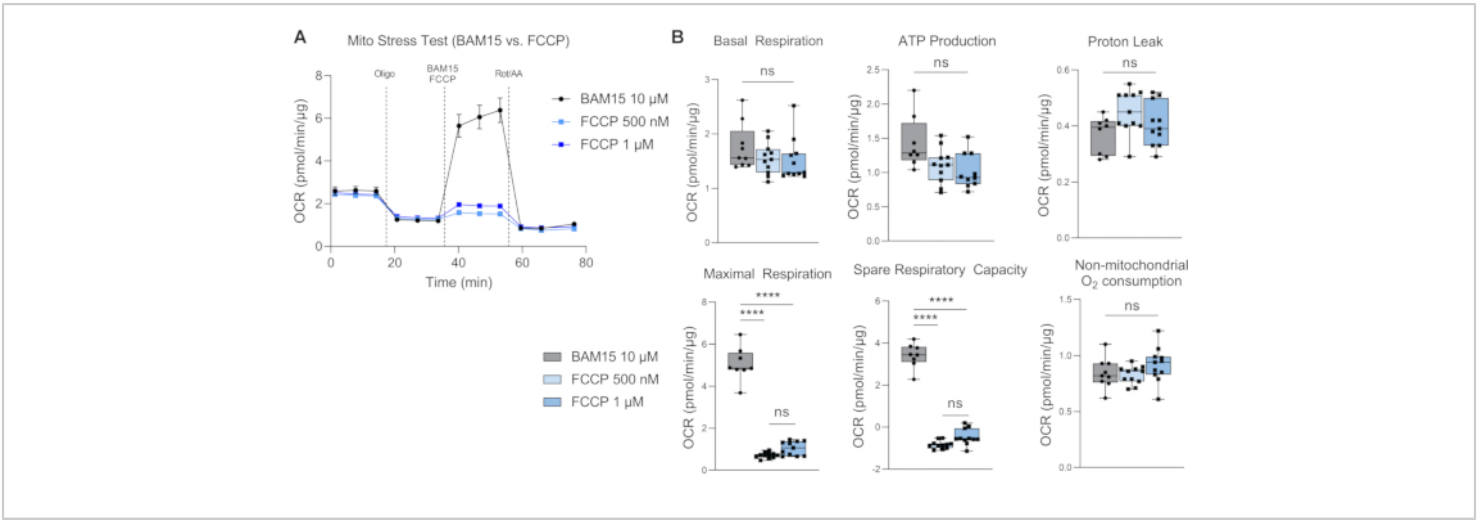

Figure 4: Comparison of BAM15 versus FCCP as an uncoupling agent. Mito Stress Test on H-RPE comparing the efficacy of inducing maximal respiration using 10 µM BAM15, 500 nM FCCP, or 1 µM FCCP, showing the (**A**) oxygen consumption rate (OCR) curve and (**B**) Mito Stress Test parameters. Error bars are means ± SEM. **** $p \leq 0.0001$; ns, not significant. Please click here to view a larger version of this figure.

Test	Glucose (mM)	GlutaMax (mM)	Sodium pyruvate (mM)	HEPES (mM)
Mito Stress Test	25	2	1	1
Glycolytic Stress Test	None	0.5	None	1

Table 1: Concentration of supplements added to the assay media for the Mito and Glycolytic Stress Tests.

Test	Port A	Port B	Port C
Mito Stress Test	Oligomycin 2.5 µM	FCCP 500 nM OR BAM15 10 µM	Rotenone 2 µM AND Antimycin A 2 µM
Glycolytic Stress Test	Glucose 10 mM	Oligomycin 2 µM	2-Deoxyglucose 50 mM

Table 2: Concentrations of drug port injections for the Mito and Glycolytic Stress Tests. It is important to note that these are the final concentrations that the cells are exposed to following injection of the drugs into each well. The drugs should be prepared 10x stronger when loading the drug ports.

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Term	Definition
Calibrant	The calibrant is a solution that is used for calibrating the sensor cartridge. Its formulation is proprietary and has a similar composition to PBS.
Sensor Cartridge	The sensor cartridge contains two fluorophores embedded in the sensor cartridge sleeve which are connected to fiber optic bundles that emit light, exciting the fluorophores. One fluorophore measures oxygen flux and the other measures proton flux. Each sensor cartridge is also equipped with 4 ports to allow for the sequential administration of up to 4 compounds per well during the assay.
Utility Plate	The utility plate (also known as the calibrant plate) is used for calibrating the sensors. The Calibrant solution is placed in the Utility Plate.
Mito Stress Test	The Mito Stress Test is the name for the assay that provides the mitochondrial respiration bioenergetic profile by plotting changes in OCR over time.
Glycolytic Stress Test	The Glycolytic Stress Test is the name of the assay that provides the glycolytic bioenergetic profile by plotting changes in ECAR over time.
Oxygen Consumption Rate (OCR)	Oxygen consumption rate is a measure of oxygen flux (pmol/min) and indicates the mitochondrial metabolic status.
Extracellular Acidification Rate (ECAR)	Extracellular acidification rate is a measure of proton efflux (mpH/min) and indicates the glycolytic metabolic status.
Wave Software	The Wave Software is used for programming the assay and subsequent data analysis
Non-mitochondrial Oxygen Consumption	Minimum rate measurement after Rotenone/antimycin A injection
Basal Respiration	(Last rate measurement before first injection) – (Non-Mitochondrial Respiration Rate)
Maximal Respiration	(Maximum rate measurement after FCCP injection) – (Non-Mitochondrial Respiration)
H^+ (Proton) Leak	(Minimum rate measurement after Oligomycin injection) – (Non-Mitochondrial Respiration
ATP Production	(Last rate measurement before Oligomycin injection) – (Minimum rate measurement after Oligomycin injection)
Spare Respiratory Capacity	(Maximal Respiration) – (Basal Respiration)

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Spare Respiratory Capacity as a %	(Maximal Respiration) / (Basal Respiration) × 100
Coupling Efficiency	ATP Production Rate) / (Basal Respiration Rate) × 100
Glycolysis	(Maximum rate measurement before Oligomycin injection) – (Last rate measurement before Glucose injection)
Glycolytic Capacity	(Maximum rate measurement after Oligomycin injection) – (Last rate measurement before Glucose injection)
Glycolytic Reserve	(Glycolytic Capacity) – (Glycolysis)
Glycolytic Reserve as a %	(Glycolytic Capacity Rate) /(Glycolysis) × 100
Non-glycolytic Acidification	Last rate measurement prior to glucose injection

Table 3: List of definitions of key components of the assay.

Discussion

This optimized protocol for high-resolution respirometry of H-RPE involves the use of BAM15 as the uncoupler instead of the commonly used FCCP. While previous studies on high-resolution respirometry of RPE utilized FCCP[9,24], BAM15 appears to induce a more robust induction of maximal respiration levels in H-RPE compared to FCCP. While both FCCP and BAM15 are safe to use in cells, BAM15 is reported to have fewer side effects in normal cells compared to FCCP or carbonylcyanide-3-chlorophenylhydrazone (CCCP)[25]. Kenwood et al. showed that BAM15 depolarizes mitochondria without impacting plasma membrane potential, thus inducing a sustained maximal mitochondrial respiration rate at low cytotoxicity[26]. FCCP, on the other hand, depolarizes both mitochondria and the plasma membrane and displays higher cytotoxicity[26].

There are several critical steps in the protocol, including ensuring the cells are properly plated in a confluent, even, and homogenous monolayer of RPE in all experimental wells of the microplate. Mature RPE are highly dependent upon OXPHOS for energy generation, and thus the cells should be allowed to mature for at least 1 month to ensure that the RPE generate proper basal and maximal OCR readings during the assay. De-gassing the cell culture plate for 1 h at 37 °C in a humidified oven (without CO_2) before placing it into the instrument is crucial for accurate ECAR readings, as CO_2 can impact the pH of the assay media. It is important to remember to hydrate the sensor cartridge the day before the assay to ensure that it provides reliable OCR and ECAR readings on the day of the assay. Care should be taken to properly reconstitute the drugs to be injected and aliquot the reconstituted drug stocks into smaller volumes for long-term storage to minimize freeze/thaw cycles. It is critical to prepare 10x drug solutions that are diluted in the respective assay

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media (e.g., dilute in Mito Stress Test assay media for the Mito Stress Test) to factor in the dilution from injection of the drug into each well filled with assay media. With each subsequent drug injection there is more media in each well, and thus the volumes of drug being loaded increase with each injection, and care should be taken to follow the volumes specified in the protocol. It is important to perform quality checks on completion of the experiment by examining the sensor cartridge to ensure that no residual drug is evident and observing the cell culture plate under a microscope to ensure that the confluent and homogeneous cell monolayer remains.

Modifications to this protocol include injecting different drugs in the ports and determining how these drugs impact OCR and ECAR readings. One popular modification is to inject an experimental drug of choice as Port A before injecting the usual Mito or Glycolytic Stress Test drugs. This type of protocol provides insights into how an acute injection of the drug of choice impacts the subsequent OXPHOS and glycolytic parameters. Other modifications include examining different cell types; this requires initial troubleshooting of the optimal seeding cell density and optimization of the assay media by ensuring that basal readings range from 50-100 pmol/min for OCR and 10-20 mpH/min for ECAR. The optimal concentrations of the injected drugs need to be determined for each new cell type examined by observing the OCR and ECAR responses to a serial dilution of the drugs.

A key limitation of the protocol is that cell viability diminishes over time, as the cells are not in a CO_2 incubator in their normal growth media, and thus the assay should be completed within 3-4 h to ensure maximal cell viability. Furthermore, exposure to mitochondrial toxins injected into each well can further diminish cell viability over time. Once the assay is completed, the cells must be lysed for evaluation of the protein content to normalize OCR and ECAR readings, and thus the same cells cannot be harvested for subsequent molecular biology assays.

Alternatives to the Seahorse for bioenergetic profiling include the Oroboros Oxygraph 2k (O2k)[27], BaroFuse[28,29], and Resipher (Lucid Scientific)[7]. The Oroboros O2k is a closed two-chamber respirometer that utilizes S1 Clark-type polarographic oxygen electrodes. While the Oroboros O2k produces highly sensitive measurements of real-time metabolic flux, the device is labor-intensive as the operator is required to manually inject each drug[30]. The BaroFuse is a novel multichannel microfluidic perfusion system that uses gas pressure to enable multiple parallel perfusion experiments and is linked to an oxygen detection system to measure OCR. The advantage of this flow culture system is that tissue function and viability is maintained, as opposed to the Seahorse where cell viability diminishes over longer assays. The Resipher utilizes highly sensitive optical oxygen sensors to measure OCR whilst the cells are in a 96-well plate in the incubator, hence enabling continuous OCR measurements over several weeks to months. Notably, these instruments do not measure ECAR, and thus the Seahorse has the advantage of simultaneous exploration of both OXPHOS and glycolysis.

Interrogating real-time bioenergetic profiles of OXPHOS and glycolysis is emerging as a key factor in characterizing RPE health and function. High-resolution respirometry enables an efficient means of comparing the metabolic status of normal and diseased RPE, thus opening new avenues of screening drug efficacy for retinal diseases such as AMD and PVR. Future directions for high-resolution respirometry on RPE include optimizing a protocol to examine bioenergetic profiles for highly polarized RPE monolayers grown on

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transwell filters. Calton et al. (2016) successfully achieved this by cutting a triangular section of the polarized RPE monolayer grown on transwell filters[31]. Further expansion of the methodology includes examining the bioenergetic profiles of induced pluripotent stem cell-derived RPE (iPSC-RPE), isolated from patients with different retinal degenerative conditions[32]. Exploration of how pathogenic cytokines involved in AMD and PVR impact the dynamic nature of RPE metabolism can reveal metabolic vulnerabilities that can inform the identification of novel druggable targets.

Disclosures

The authors have nothing to disclose.

Acknowledgments

This study was supported in part by grants from: the Fight for Sight Leonard & Robert Weintraub Postdoctoral Fellowship (D.Y.S.); BrightFocus Foundation Postdoctoral Fellowship Program in Macular Degeneration Research (M2021010F, D.Y.S.); Department of Defense, Spinal Vision Research Program under Award no. VR180132 (M.S.-G. and L.A.K.); National Eye Institute of the National Institutes of Health under Award no. R01EY027739 (L.A.K.). Acknowledgment is made to the donors of the Macular Degeneration Research M2021010F, a program of the BrightFocus Foundation, for support of this research. Schematics were created with Biorender.com

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