

Long-term Monitoring of Oxygen Consumption Rates in Highly Differentiated and Polarized Retinal Pigment Epithelial Cultures

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Abstract

Mitochondrial metabolism is critical for the normal function of the retinal pigment epithelium (RPE), a monolayer of cells in the retina important for photoreceptor survival. RPE mitochondrial dysfunction is a hallmark of age-related macular degeneration (AMD), the leading cause of irreversible blindness in the developed world, and proliferative vitreoretinopathy (PVR), a blinding complication of retinal detachments. RPE degenerative conditions have been well-modeled by RPE culture systems that are highly differentiated and polarized to mimic *in vivo* RPE. However, monitoring oxygen consumption rates (OCR), a proxy for mitochondrial function, has been difficult in such culture systems because the conditions that promote ideal RPE polarization and differentiation do not allow for easy OCR measurements.

Here, we introduce a novel system, Resipher, to monitor OCR for weeks at a time in well-differentiated RPE cultures while maintaining the RPE on optimal growth substrates and physiologic culture media in a standard cell culture incubator. This system calculates OCR by measuring the oxygen concentration gradient present in the media above cells. We discuss the advantages of this system over other methods for detecting OCR and how to set up the system for measuring OCR in RPE cultures. We cover key tips and tricks for using the system, caution about interpreting the data, and guidelines for troubleshooting unexpected results.

We also provide an online calculator for extrapolating the level of hypoxia, normoxia, or hyperoxia RPE cultures experience based on the oxygen gradient in the media above cells detected by the system. Finally, we review two applications of the system, measuring the metabolic state of RPE cells in a PVR model and understanding how

the RPE metabolically adapts to hypoxia. We anticipate that the use of this system on highly polarized and differentiated RPE cultures will enhance our understanding of RPE mitochondrial metabolism both under physiologic and disease states.

Introduction

The retinal pigment epithelium (RPE) is a monolayer of functionally postmitotic, highly polarized epithelial cells that form a barrier between light-sensitive photoreceptors in the retina and their blood circulation, a capillary bed termed the choriocapillaris. Like the role of glia-supporting neurons, the RPE carries out myriad functions to support photoreceptors, including phagocytosis of photoreceptor outer segments, transport of nutrients and metabolic support for photoreceptors, and secretion of essential growth factors, all critical for maintaining visual function.

Degeneration of the RPE underlies several common degenerative disorders of vision. In age-related macular degeneration (AMD), one of the most common causes of incurable vision loss in the world, the RPE dies, and overlying photoreceptors therefore suffer a secondary degeneration. In proliferative vitreoretinopathy (PVR), the RPE instead exits its normally quiescent postmitotic state, proliferating and dedifferentiating into a mesenchymal state (a so-called epithelial-to-mesenchymal transition [EMT]) with alterations in its metabolism. RPE dedifferentiation causes a loss of RPE support to photoreceptors while also triggering a more fibrotic state. This results in both photoreceptor degeneration and RPE-induced scarring, both of which trigger vision loss^{1,2}.

A major part of the RPE's support to photoreceptors is metabolic, and metabolic dysregulation is a critical factor in numerous retinal diseases, including AMD and PVR. The RPE serves as a regulatory barrier between photoreceptors and their source of oxygen and nutrients,

the choriocapillaris. Thus, what the RPE chooses to metabolize versus what the RPE chooses to pass through from the choriocapillaris to the photoreceptors strongly governs photoreceptor metabolism and survival. Numerous studies have shown that the RPE is heavily dependent on mitochondrial metabolism for its normal health, and that photoreceptors instead rely heavily on glycolysis³. This has introduced the concept of complementary, intertwined metabolic states between photoreceptors and the RPE. Specifically, the RPE reduces its metabolism of preferred photoreceptor metabolic substrates and instead utilizes the byproducts of photoreceptor metabolism combined with the metabolites that photoreceptors do not consume. In diseases such as PVR and AMD, evidence strongly suggests that the RPE becomes more glycolytic and less dependent on mitochondrial metabolism; this shift towards RPE glycolysis may starve photoreceptors of metabolites it needs, triggering degeneration^{4,5,6}. Given how interdependent RPE and photoreceptor metabolism are and how much altered metabolism underlies retinal disease, there is strong interest in modeling and manipulating RPE metabolism for therapeutic purposes.

While studying RPE mitochondrial metabolism *in vivo* is ideal, many aspects of RPE mitochondrial metabolism can only be practically probed in an *in vitro* culture system. Significant progress towards high-fidelity RPE cultures has been made in the past several decades, to the point that the most carefully groomed RPE cultures are now being used for cell

replacement therapy in human clinical trials⁷. To maintain such high-fidelity cultures, the RPE needs to be grown on particular substrates in particular media for months prior to experimentation. With these conditions, RPE cultures are maximally differentiated and polarized, approximating *in vivo* RPE. Unfortunately, there is no equipment currently available that can measure mitochondrial metabolism specifically from the RPE *in vivo*. While oxygen monitoring of the retinal capillary network has been achieved *in vivo* using electron paramagnetic resonance (EPR) oximetry⁸, this is not possible for RPE analysis. Differences between RPE metabolism *in vivo* and *in vitro* are not well-described, but RPE cultures have been shown to have high mitochondrial activity, similar to RPE *in vivo*^{3,9}, suggesting significant insight into RPE mitochondrial metabolism can be gained using high-fidelity RPE cultures.

As all mitochondrial metabolism leads to oxygen consumption, measuring RPE oxygen consumption rates (OCR) is a faithful proxy for mitochondrial metabolism. Measuring OCR in RPE cultures has been difficult, as the conditions that promote maximal RPE polarization and differentiation often preclude long-term accurate OCR measurements with currently available techniques, such as the Seahorse Analyzer. In this methods paper, a novel device, termed the Resipher (hereafter referred to as "the system"), is introduced, which allows continuous measurement of OCR over weeks in RPE grown under conditions that maximally promote polarization and differentiation. The ease with which OCR can be measured by this system in RPE culture conditions that maximally promote RPE differentiation and polarization is unique among existing OCR-measuring devices.

This paper provides tips and tricks for using the system with RPE cultures, followed by a demonstration of two particular applications. First, RPE EMT, mimicking PVR, is triggered by exposure to transforming growth factor-beta (TGFβ)^{1,10,11,12}. The system is used to monitor how RPE metabolism evolves during the EMT process. Second, the role of hypoxia in RPE metabolism is explored using this system. Hypoxia is an important contributor to the pathogenesis of AMD, as the choriocapillaris thins with age^{13,14}. Combining this system with hypoxia chambers allows one to model altered RPE mitochondrial metabolism with the subtle hypoxia that accompanies aging. Finally, an online calculator using Resipher data is introduced to allow one to determine whether RPE cultures are in hypoxic, normoxic, or hyperoxic conditions. Such information is critical for drawing any conclusions about RPE metabolism from *in vitro* RPE culture studies.

Protocol

For protocols to establish human primary or iPSC-RPE cultures, see the following references^{15,16,17,18}. The acquisition and use of human tissue for these protocols were reviewed and permitted by the University of Michigan Institutional Review Board (HUM00105486).

1. General application of the system to RPE culture

1. Plate human induced pluripotent stem cell (iPSC) derived-RPE or primary human RPE cells in system-compatible 96-well plates.
 1. Assuming already-existing mature cultures grown on 24-well cell culture plates, wash the cells with phosphate-buffered saline (PBS) once and then add 500 µL of 0.25% trypsin-EDTA (**Table of Materials**).

Incubate for 10-40 min in a cell culture incubator, checking every 5-10 min until the cells are rounded and almost ready to detach.

2. Gently pipette media over the cells to detach them from the cell culture plastic, followed by transfer to 3x volume of cell culture media (1,500 μ L per 24-well), spinning down in a centrifuge at $250 \times g$ for 5 min at room temperature.

NOTE: The recipe for RPE cell culture media is detailed in **Table 1** and available in previously published references^{9, 15, 16, 17, 18}.

3. Resuspend cells in RPE cell culture media with 15% fetal bovine serum (FBS) and count the cells with a hemocytometer.
4. Seed 74,000 cells for each well of a 96-well plate (generally $225\text{-}300 \times 10^5$ cells/cm²), after coating with highly specialized extracellular matrix coating substrates (**Table of Materials**) following the manufacturer's instructions.

NOTE: Seed cells only on a Resipher-compatible plate (**Table of Materials**) and only in wells corresponding to the probe array on the sensing lid (columns 3, 4, 9, and 10 for the 32-channel lid; see **Table of Materials**). The system's sensing lid is available with 4-, 32-, or 96-channel sensors (see sensor locations in **Figure 1A-D**), and a range of standard cell culture plates are compatible.

5. Since edge wells are prone to evaporation, which dramatically affects oxygen availability, and therefore, OCR readings, avoid seeding cells on all edge wells (Rows A and H of a 96-well plate). Furthermore, add 200 μ L of sterile water in each

of the empty wells of the 96-well plate to reduce evaporation effects.

6. Keep the plate on a stable table surface for 10 min to allow the cells to settle; then return it to the incubator. Change the media after 24-72 h, replacing with standard RPE culture media with 5% FBS. Culture for at least 4 weeks, changing media 2x per week.

NOTE: Cells are ready for experimentation when they are pigmented, cobblestone, and highly compact (**Figure 1E**).

2. Setting up and acquiring data with the system

1. Place the sensing lid on an empty 96-well receiver plate and then place this back in the cell culture incubator. Align and mount the Device on the sensing lid and connect it to the Hub via the provided USB cable. This creates a Resipher "**sandwich**." (**Figure 2A**).
2. Go to the Lucid lab website application (<https://lab.lucidsci.com/>) and click the **New Experiment** button on the right top corner to create a new experiment.
3. Name the **title of the experiment** and input any relevant **experimental notes** for the particular study (e.g., passage number of the iPSC-RPE one is using).
4. Create **well conditions** and **treatment groups**. For example, if testing the effects of different serum concentrations in the media on OCR, select **Serum** in **group name**, then enter media serum concentrations to be used, adding more serum percent values by clicking the **+** button. Assign which wells will receive a particular serum percentage and select a color pattern for those wells. Add more

groups (e.g., a different experimental variable to test) as needed by clicking **add group** button.

5. Define the **plate setup** and select the corresponding **Device** and **plate style**. Click **add plate** button and choose **Device**; select **plate type**. Select **treatment** and click on the corresponding well.
6. Click the **start now** button to start the experiment. Check that the indicator on the website and the LED on the Hub is **solid green**.
7. Allow the system to run for 15-60 minutes to ensure each sensor is properly calibrated and accurately detecting atmospheric oxygen, which should be at a concentration of ~200 μM in a fully humidified cell culture incubator with 5% CO_2 (**Figure 2Bi**).

NOTE: If any of the sensors are more than 20% off from the average of the other sensors in a standard cell culture incubator setup, consider excluding that well in data analysis, as the sensor is faulty (**Figure 2Bii**).

NOTE: If all of the sensors are more than 20% off from the average of the other sensors and from the expected atmospheric O_2 reported as 200 μM in a standard cell culture incubator setup, the sensing lid has been used too many times; replace it with a new sensing lid (**Figure 2Biii**).

NOTE: Obtaining the O_2 readings in the air prior to starting an experiment significantly improves troubleshooting afterward. If a particular well appears as an outlier after an experiment is done and that well had a sensor that was out of calibration, the problem may be with the sensor, not the biological replicate.

8. Remove the Device from the sensing lid (but do not detach the USB cable from the Device). Place the Device upside down in the incubator to allow the motor on the Device to reset. Do not reuse the Device until all the motor sounds have stopped (approximately 20-30 s).

NOTE: It is important to keep the Device connected to the Hub via the USB cable at all times during an experiment, even when the Device is off the sensing lid. This allows the Device to continuously monitor and report on the cell culture incubator environment.

9. Place the plate with the sensing lid back in the cell culture hood, along with a 96-well plate containing the RPE cultures. Change the media in the plate with RPE cultures with the same media and the same volume for all wells to obtain baseline OCR values for each well. Be sure to fill all the wells without RPE with sterile water to help prevent evaporation.

NOTE: In general, we recommend 60-100 μL of RPE culture media per 96-well, based on data demonstrating that lower media volumes lead to rapid nutrient depletion but higher media volumes lead to inadvertent hypoxia⁹.

10. Transfer the sensing lid to the plate containing cell cultures and the standard (non-Resipher) lid on the plate containing cell cultures to the empty 96-well receiver plate to maintain the sterility of the standard lid, which will be needed at various points during experimentation.
11. Place the plate with cell cultures and the sensing lid back in the cell culture incubator. Once again, align and mount the Device on the sensing lid and connect it to the Hub via the provided USB cable (assembling

the "**sandwich**"). Check that the indicators on the website interface and the Hub are all **solid green**.

NOTE: O₂ concentration data will show up right away, while OCR data will only show up after enough O₂ data has been collected, approximately 1 h.

12. Let the Device measure OCR on each well for at least 12-24 h to capture a baseline OCR.

NOTE: Baseline OCR will differ depending on many factors, including the type of media being applied to cells. In **Figure 2C**, standard RPE culture media with different amounts of FBS (0%, 5%, 15%) have slightly different OCR values. Further, cultures with more FBS can sustain mitochondrial activity for longer after media is applied to cells before OCR drops due to depletion of mitochondrial metabolites (right side of curve).

13. Once a baseline OCR is obtained, repeat steps 1.2.8-1.2.12, but change the media on the plate with RPE cultures to one's experimental conditions (typically, $\pm$ a drug or a comparison of different media conditions).

NOTE: Routine media changes can also be handled the same way. Anytime the cell culture incubator is opened, expect significant disruption to OCR readings, as they are dependent on temperature, humidity, CO₂ concentrations, and other factors that are all temporarily disrupted with incubator door opening. During any removal of the Device from the sensing lid, there is no need to pause the experiment online. After the cell culture incubator door is opened or media is changed on the plate being probed, it will generally take 2-4 h to re-establish the oxygen gradient and begin measuring an accurate OCR.

14. After data are obtained, subtract the **baseline** OCR for each well from the OCR after the experimental condition is applied, to determine the **Delta OCR** triggered by the treatment.

15. After the experiment is completed, sterilize and reuse the sensing lid (which can be reused 3-5x, although performance degrades with repeated use). To sterilize the sensing lid, immerse the whole lid in 70% ethanol for 10 min in a cell culture hood, then remove from immersion and rest in cabinet probe side up (avoid touching the delicate probe tips), and let ethanol and water totally evaporate before putting the sensing lid on a new 96-well receiver plate.

3. Take brightfield images to normalize OCR values to cell number.

NOTE: Melanin accumulation in RPE facilitates the normalization of OCR based on a simple count of cell numbers in living cultures using a brightfield microscope.

1. Remove the Device and place it upside down, as in step 1.2.8. In the cell culture hood, swap the sensing lid on the RPE culture plate for a standard 96-well lid.

2. Using a standard inverted microscope, take brightfield images of each well.

NOTE: Keep the area one images consistent between wells (relative location in the well and objective magnification).

3. In the cell culture hood, replace the standard 96-well lid on the RPE culture plate with the sensing lid and place it back in the incubator for further monitoring.
4. Count the cell number in each well with ImageJ or other software.

5. Normalize OCR to cell number in the different experimental groups. The system reports OCR in units of $\text{fmol} \cdot (\text{mm}^2)^{-1} \cdot \text{s}^{-1}$ - the consumption per cross-sectional unit area. Therefore, to normalize the OCR per cell, divide the OCR by cell count per mm^2 (rather than cell count per well). Equivalently, multiply the OCR reported by the system by the cross-sectional area of the well (around 31 mm^2 for a standard 96-well plate) to yield OCR in units of $\text{fmol} \cdot \text{s}^{-1} \cdot \text{well}^{-1}$.

4. Controls for determining RPE bioenergetic parameters.

NOTE: To ensure the system and RPE cultures are responding to mitochondrial manipulations in predictable ways, one can test certain small molecules well-established to target particular steps of mitochondrial oxidative phosphorylation. These tests are analogous to the steps performed in a Mitochondrial Stress Test using the Seahorse Analyzer¹⁰ and provide a bioenergetic profile for RPE cultures.

1. Culture RPE cells in 65 μL of standard RPE culture media with 5% FBS and measure OCR for 24 h to establish a baseline.
2. Aspirate cultured media and add 65 μL of standard RPE culture media with 5% FBS and with mitochondrial uncouplers (3 μM carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone [FCCP] or 500 nM Bam15, **Table of Materials**). Incubate overnight.

NOTE: Mitochondrial uncouplers should significantly increase OCR. Ensure the increased OCR is not at or near the theoretical maximum diffusion-limited OCR of approximately $275 \text{ fmol} \cdot (\text{mm}^2)^{-1} \cdot \text{s}^{-1}$ for 65 μL of media in standard culture conditions. Max

OCR for different commonly used media volumes is available in the Discussion section.

3. After overnight incubation, aspirate the media with mitochondrial uncouplers and replace with 65 μL of standard RPE culture media with 5% FBS. Observe the plate for another 24 h to determine the recovery of RPE OCR after the removal of the drugs.
 4. Calculate the effects of uncouplers on OCR by subtracting the OCR obtained after treatment from the OCR obtained before treatment (**Figure 3**).
- NOTE:** The increase in OCR with the addition of mitochondrial uncouplers signifies the reserve mitochondrial respiratory capacity of the cell¹⁹.
5. To measure other mitochondrial parameters typically captured during the Mitochondrial Stress Test with a Seahorse Analyzer, perform the same steps above with the following compounds (**Figure 4**).

1. Use oligomycin at 1 μM to assess for ATP-linked respiration.
2. Use antimycin A and rotenone at 1 μM to assess for any non-mitochondrial respiration.

NOTE: FCCP, oligomycin, antimycin A, and rotenone can be toxic to RPE. If they induce significant cell death, then OCR measurements will not be accurate. Thus, OCR measurements should be obtained only at a time point where there is no obvious cell death, generally within the first several hours after the drugs are added (**Figure 4**).

2. Measuring changes in mitochondrial metabolism in RPE undergoing EMT

1. Follow the same protocol as outlined in section 1.2.
2. To induce EMT after obtaining baseline OCR measurements, add TGF- β 2 (10 ng/mL) to RPE cultures and monitor OCR over 3 weeks. Refresh media with fresh TGF β 2 every 2-3 days (**Figure 5**).

3. At least 2x a week, obtain brightfield images as in section 1.3 to ensure that the cell count is not changing. If the count does change, normalize the OCR values to the cell number in each well.

NOTE: The same protocol can be used for other inducers of EMT to monitor long-term OCR changes throughout the culture period whilst the cells are in the incubator.

3. Measuring changes in mitochondrial metabolism in hypoxic RPE

NOTE: The application of the system under hypoxic, normoxic, or hyperoxic conditions is the same as section 1.2, except for keeping the "sandwich" in a hypoxia chamber (**Table of Materials**) placed in a standard cell culture incubator.

1. Drill a hole on the lid of the hypoxia chamber to mount the USB type-c cable through and seal it with silicone or putty (**Figure 6A**).
2. Assemble the sensing lid with the cell culture plate in the hood and transfer them into the hypoxia chamber.
3. Set up the "sandwich" (plate, sensing lid, Device) in the hypoxia chamber. Also place in the hypoxia chamber a Petri dish with sterile water to help maintain humidity. Finally, place a portable O₂ sensor in the hypoxia

chamber (**Table of Materials**). Setup is displayed in **Figure 6A**.

4. Seal the chamber and connect it to a gas cylinder with a hypoxic concentration of O₂ and standard cell culture concentration of CO₂ (e.g., 1% O₂ and 5% CO₂).
5. Slowly exchange the air in the hypoxia chamber with the 1% O₂/5% CO₂ air in the cylinder until the oxygen sensor shows the O₂ concentration desired, usually somewhere between 1% and 10%.

NOTE: The portable O₂ sensor requires time to equilibrate, so gas from the cylinder should be added into the hypoxia chamber slowly.

6. Close the inlet and outlet valves of the hypoxia chamber and transfer the whole chamber into the incubator.
7. Set up and start the experiment as in section 1.2.

NOTE: Pre-equilibrating media in the hypoxia chamber prior to adding to cells can decrease the time it takes for the cell monolayer to experience true hypoxia (**Figure 6B**).

4. Calculating the O₂ concentration at the RPE monolayer to determine if cells are in hypoxic, normoxic, or hyperoxic conditions

NOTE: The system measures the O₂ concentration between approximately 1 to 1.5 mm above the bottom of the well by default assuming a standard plate is used with the corresponding recommended sensing lid for monolayer culture (refer to https://lucidsci.com/docs/LucidScientific_Sensing_Lid_Selection_Guide.pdf). While the O₂ concentration at the cell monolayer is not directly measured, data from the system can be used to estimate O₂ concentration at the level of the RPE. Specifically, knowing that an oxygen gradient exists between the top of the media column, where O₂ is available, and the bottom of the

media column, where O_2 is being consumed, Fick's Laws of Diffusion can be combined with the measured OCR rate to extrapolate O_2 levels at the cell monolayer. A calculator for this estimation is provided online: https://lucidsci.com/notes?entry=oxygen_diffusion (and in the form of an open-source interactive notebook at <https://observablehq.com/@lucid/oxygen-diffusion-and-flux-in-cell-culture>, source code of this calculator could be found at <https://github.com/lucidsci/oxygen-diffusion-calculator>).

1. Once the calculator is accessed, enter the **media volume in microliters** (e.g., 100 μ L).
2. Enter the saturated oxygen concentration at the air-liquid interface (the top of the media column above cells). This value is $\sim 185 \mu$ M at standard atmospheric pressure with 5% O_2 and 95% humidity.
NOTE: This can also be determined from O_2 measurements by system probes in media-only wells (with no cells at the bottom of the well).
3. In the **Flux input**, enter the **Flux/OCR** reported by the system.
4. Based on these values, the **steady-state oxygen concentration at each height in the media** column is calculated and shown in the plot (**Figure 7**). The **x-axis** of the plot is the **height in mm above the bottom of the well**. The **y-axis** is the **calculated O_2 concentration at that height** (in μ M).
5. The O_2 concentration at the bottom of the well (where the cells are) is reported in the line below the plot.
NOTE: In general, it is estimated that RPE *in vivo* sees an O_2 concentration of 4-9%. Each 10 μ M of O_2 at standard atmospheric conditions corresponds to approximately 1% O_2 . Thus, normoxia at the level of

cells is approximately 40-90 μ M of O_2 . The higher the media column, the lower the O_2 will be at the level of the RPE monolayer⁹. Thus, if cells appear to be in hypoxic conditions, media volume can be reduced. If cells appear to be in hyperoxic conditions, media volume can be increased.

Representative Results

The "sandwich" setup for the Resipher experiment is demonstrated in **Figure 2A**. Sensing lids with 32 probes corresponding to columns 3, 4, 9, and 10 on the 96-well plate sit between the cell plate and the Device. After connecting to the Hub, the Device activates motors to move the sensing lid up and down, measuring O_2 concentration in the media column at a range of heights above the cell monolayer (typically 1-1.5 mm). The O_2 gradient is therefore continuously measured by recording the O_2 concentrations at these various heights above the monolayer. From gradient measurements and applying Fick's Laws of Diffusion, the Device automatically calculates the oxygen flux from above the probe height range to below it, which is the oxygen consumption rate (OCR) of the cell monolayer. The sensing lid detects O_2 regardless of whether a well is empty (air-only), filled with media but no cells (media-only), or has media overlying a cell monolayer (media + cells). As a quality check for how well the sensors in the sensing lid are working, the lid can first be placed over an empty (air-only) 96-well plate. If each sensor for each well is working, the reported O_2 should be consistent with each other and close to 200 μ M, which is close to the expected O_2 in the atmosphere under standard cell culture incubator conditions. **Figure 2Bi** shows O_2 readings from eight probes in atmospheric air (reported as the concentration in μ M since the default unit conversion assumes the probes are in 37 °C liquid). These

readings are tight ($\pm 5\%$) and close to 200 μM , implying that all eight sensors are functioning well. In **Figure 2Bii**, the same sensing lid as in **Figure 2Bi** is being used, but a different Device is deployed. Here, two faulty Device sensors are picked up based on outlier curves (in red). This suggests the Device is problematic and needs to be replaced (or the wells corresponding to faulty sensors should be left out of the experiment). In **Figure 2Biii**, the same Device as **Figure 2Bi** is used, but two different sensing lids are employed. Lid1 (left side) has high variability among the probes, suggesting that the lid's sensing material is degraded, usually because the lid has been used too many times. Lid2 (right side) is new, demonstrating readings that are tight and clustered around 200 μM . Using the system, one can analyze the effect of serum supplementation in the media on RPE mitochondrial metabolism. As seen in **Figure 2C**, increasing levels of serum supplementation triggered subtly higher RPE OCR levels. Importantly, higher serum supplementation also allowed mitochondrial metabolism to be sustained for a longer period. Even without any serum supplementation, OCR is sustained for approximately 90 h before the exhaustion of mitochondrial metabolic substrates triggers a drop in OCR. Thus, media changes with 100 μL per 96-well every 3.5 days (twice a week) are sufficient, even with serum-free media, to avoid nutrient depletion in mature, highly differentiated, and polarized RPE cultures.

OCR rates can vary significantly from well to well, even in cultures where all wells appear to have the same cell number and morphology. Thus, to isolate whether increases in OCR are attributable to an experimental condition or simply baseline differences in OCR between wells, normalization is necessary. The first step in normalization is ensuring cell counts between wells are consistent, as outlined in protocol section 1.3. If cell counts vary between wells, the OCR

for the well needs to be normalized to the cell number. In general, for highly mature and polarized RPE cultures, cell counts between wells are remarkably consistent⁹. Even in such scenarios, baseline OCR between wells can vary, as demonstrated in **Figure 3A**. Here, baseline OCR, prior to any treatment, varies by as much as 100 $\text{fmol}/\text{mm}^2/\text{sec}$ between wells. By measuring OCR prior to treatment, one can then determine the **Delta OCR** triggered by the experimental treatment and isolate this from any differences in baseline OCR between wells. In this case, the treatments are comparing two mitochondrial uncouplers (FCCP vs. BAM15) with vehicle control. Once baseline OCR is utilized for normalization, the treatment effects specific for the uncouplers versus control become obvious (compare **Figure 3B** vs. **Figure 3C**). Generally, experiments that can alter OCR should be split into at least two phases: initial OCR to establish a baseline OCR and a treatment phase to determine the **Delta OCR**.

To confirm that RPE cultures respond in expected ways to mitochondrial manipulation and establish a bioenergetic profile for RPE, the classic small molecule tools used in the Mitochondrial Stress Test on Agilent's Seahorse Analyzer can be employed with the system. First, spare mitochondrial capacity can be measured utilizing a mitochondrial uncoupler¹⁹. In RPE cultures, the mitochondrial uncoupler BAM15 shows a much more robust and sustained increase in OCR compared to FCCP (**Figure 3C,D**), without inducing toxicity. Other parameters available on the Seahorse Analyzer Mitochondrial Stress Test, including basal respiration, ATP-linked respiration, proton leak, and non-mitochondrial respiratory capacity, can be calculated using the system. Whereas the Seahorse Analyzer relies on injection ports to sequentially deliver toxins that inhibit specific oxidative phosphorylation complexes to infer the

above parameters, Resipher does not have this capability. However, each mitochondrial toxin can be added to separate wells, and different respiratory parameters can be calculated based on fold-change over wells treated with vehicle. In **Figure 4A**, the differences in OCR between wells treated with the mitochondrial uncoupler BAM15, the ATP synthase inhibitor oligomycin, the complex I and III inhibitors antimycin/rotenone, and vehicle control (DMSO) are all compared. As these toxins can induce cell death over longer periods of time and cell death will affect OCR, the OCR readings were taken after only a few hours of treatment. **Figure 4B** shows the differences in OCR between vehicle and wells treated with each mitochondrially active small molecule in bar graph form, extrapolated from the last time points in **Figure 4A**. The OCR values in **Figure 4B** can then be used to calculate bioenergetic parameters for the RPE culture, as seen in **Figure 4C**. Each color-coded value in the bioenergetic profile in **Figure 4C** comes from the same color-coded value in **Figure 4B**. Thus, parameters like ATP-linked respiration, proton leak, maximal mitochondrial respiratory capacity, and non-mitochondrial respiratory capacity can be estimated using the OCR system. A summary of the mode of action of each mitochondrially active small molecule is shown in **Figure 4D**.

Having demonstrated the protocol for this system on RPE cultures and control experiments to assay for RPE bioenergetic profiles, one can explore experimental applications of the system to RPE biology and pathology. In proliferative vitreoretinopathy (PVR), a condition that occurs after retinal detachment or ocular trauma, RPE undergoes a dramatic transformation known as epithelial-to-mesenchymal transition (EMT) whereby the highly regular and cobblestone-like RPE lose their cell-cell adhesions and transdifferentiate into spindle-shaped mesenchymal cells

that are contractile and motile^{1,2}. This triggers tangential contractile forces on the retina that can induce retinal detachment. Classic inducers of EMT in RPE include transforming growth factor-beta (TGF β) and tumor necrosis factor-alpha (TNF α)^{10,11,12}. Of the three mammalian TGF β isoforms, TGF β 2 is the most prominent and potent inducer of EMT in the retina¹, causing a distinct cellular elongation of the characteristically hexagonal RPE cells and increased expression of mesenchymal markers. The EMT response induced by TGF β 2 in RPE is accompanied by a suppression of mitochondrial respiration and a subsequent increase in glycolytic capacity, as previously demonstrated using a Seahorse XFe96¹⁰. While the Seahorse provides real-time bioenergetic profiles, it does not allow for long-term OCR monitoring. Thus, in the first application of the OCR system in this study (**Figure 5**), mitochondrial respiration was tracked over three weeks as EMT was induced with TGF β 2 in RPE, allowing *in vitro* modeling of the metabolic reprogramming that occurs during PVR. Unlike the single time-point analysis of RPE EMT using a Seahorse Analyzer, the metabolic state of RPE undergoing EMT can be tracked for several weeks. It is apparent that repeated exposure of RPE to TGF β 2 causes a continued progressive decline in mitochondrial metabolism. Previous data using the Seahorse XF Analyzer indicated that TGF β 2 significantly reduced maximal respiration capacity at 24h and 72h but did not affect basal OCR levels¹⁰. The unchanged basal OCR with TGF β 2 up to 72 h is corroborated by the OCR system used in this study (**Figure 5**). However, the longer-term data from the OCR system in this study reveals that after 5 days of exposure to TGF β 2, basal OCR levels begin to decrease, with a drop in basal OCR becoming most evident at 20 days. These findings highlight long-term changes in basal OCR that were not captured with prior Seahorse time points.

In a second application, the system can be used to monitor RPE mitochondrial metabolism under hypoxic conditions. As AMD is linked to choriocapillaris thinning and hypoxia^{13,20}, understanding the RPE's adjustments to subtoxic hypoxia should provide insights into AMD pathogenesis. To subject RPE cultures to controlled hypoxia while monitoring for mitochondrial metabolism, the system can be placed in a hypoxia chamber that, in turn, is placed in a cell culture incubator. To facilitate this setup, a small hole is drilled into the lid of a hypoxia chamber, allowing the system's USB cable to reach inside the hypoxia chamber. The hole is sealed with putty or silicone (**Figure 6A**). A portable O₂ sensor can be placed in the hypoxia chamber to monitor atmospheric O₂ levels and ensure the hypoxia chamber seal remains intact. Using this setup, the rate of equilibration between atmospheric O₂ concentration and media O₂ concentration can be determined. In **Figure 6B**, media equilibrated with atmospheric O₂ is placed in individual wells on a 96-well plate, without any cells, and introduced into a hypoxia chamber containing just 5% O₂ (~50 μ M O₂). Over time, the media in each well equilibrates with the new atmospheric concentration. The higher the media column height (more volume), the longer the equilibration takes. With 65 μ L of media in a well of a 96-well plate, equilibrium with atmospheric O₂ takes approximately 5 h. However, equilibrium time takes more than 10 h when the media volume is 100-200 μ L. These results underscore the importance of pre-equilibrating media with the desired atmospheric O₂ concentration before applying the media to cells during a hypoxia experiment.

For hypoxia experiments, it is important to ensure the O₂ levels in the hypoxia chamber remain stable. Keeping one of the edge wells on the 96-well plate free from cells or media will allow one of the sensing lid sensors to continuously monitor atmospheric O₂. In **Figure 6C**, such monitoring

with a single well exposed to air demonstrates that the hypoxia chamber has a slow leak, such that by 30 h, the cells are close to atmospheric O₂ concentrations. As a final control for hypoxia experiments, it is important to determine whether O₂ solubility differs between "fresh" and "conditioned" media. If solubility differs between media initially placed on cells and media that has been on cells for a significant amount of time, then the O₂ gradient in the media column above cells will differ as media "conditions" with time over the cells. This, in turn, affects the calculation of OCR. In **Figure 6D**, a 96-well plate with empty wells ("air"), wells without cells but with fresh media ("new media"), and wells without cells but with media previously incubated over RPE cultures for 48 h ("conditioned media") was first placed in atmospheric O₂ to allow full equilibration. The plate was then placed in a hypoxia chamber at approximately 3-4% O₂ (30-40 μ M) and allowed to equilibrate. Finally, the plate was again returned to atmospheric O₂ concentrations. The O₂ concentration curves for new media and conditioned media are identical, demonstrating that O₂ solubility between new and conditioned media is identical, confirming that changes in media composition over time as nutrients are consumed and byproducts are secreted into the media do not meaningfully alter O₂ solubility and therefore do not inadvertently affect OCR.

As the height of the media column above cells dramatically affects O₂ availability at the RPE monolayer⁹, it is important to determine whether a particular media volume is inducing hypoxia, normoxia, or hyperoxia at the RPE monolayer. Using measurements from the system, combined with an online calculator (https://lucidsci.com/notes?entry=oxygen_diffusion (and in the form of an open-source interactive notebook at <https://observablehq.com/@lucid/oxygen-diffusion-and-flux-in-cell-culture>) - source

code of this calculator at <https://github.com/lucidsci/oxygen-diffusion-calculator>, the concentration of oxygen at the RPE monolayer can be estimated. **Figure 7** demonstrates a

screenshot of the interactive calculator. RPE *in vivo* typically sees an O₂ concentration of 4-9%, translating into an O₂ molar concentration of ~40-90 μM.

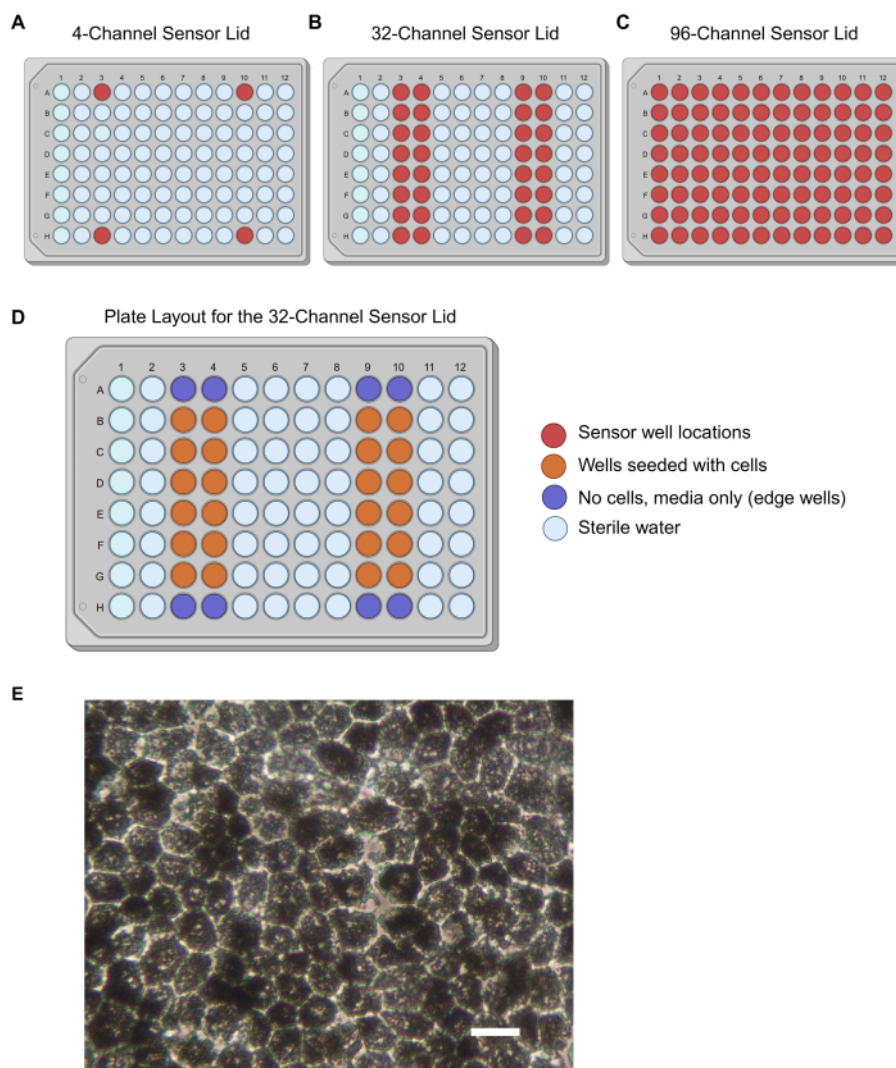


Figure 1: Location of sensors on different sensing lids, plate layout for the 32-channel sensing lid, and RPE morphology. Wells that correspond to the location of the sensors in the different sensing lids for the **(A)** 4-Channel, **(B)** 32-Channel, and **(C)** 96-Channel lid options. **(D)** Recommended plate layout for the 32-channel sensor lid. Due to edge effects, cells should not be seeded in the edge wells and instead, media must be placed in these four wells (purple). Cells are seeded in orange wells. The remaining wells should be filled with sterile water (blue) to prevent evaporation effects. **(E)** Mature RPE culture with hexagonal, tightly packed, pigmented cells. Scale Bar = 20 μm. Abbreviations: RPE = retinal pigment epithelium. [Please click here to view a larger version of this figure.](#)

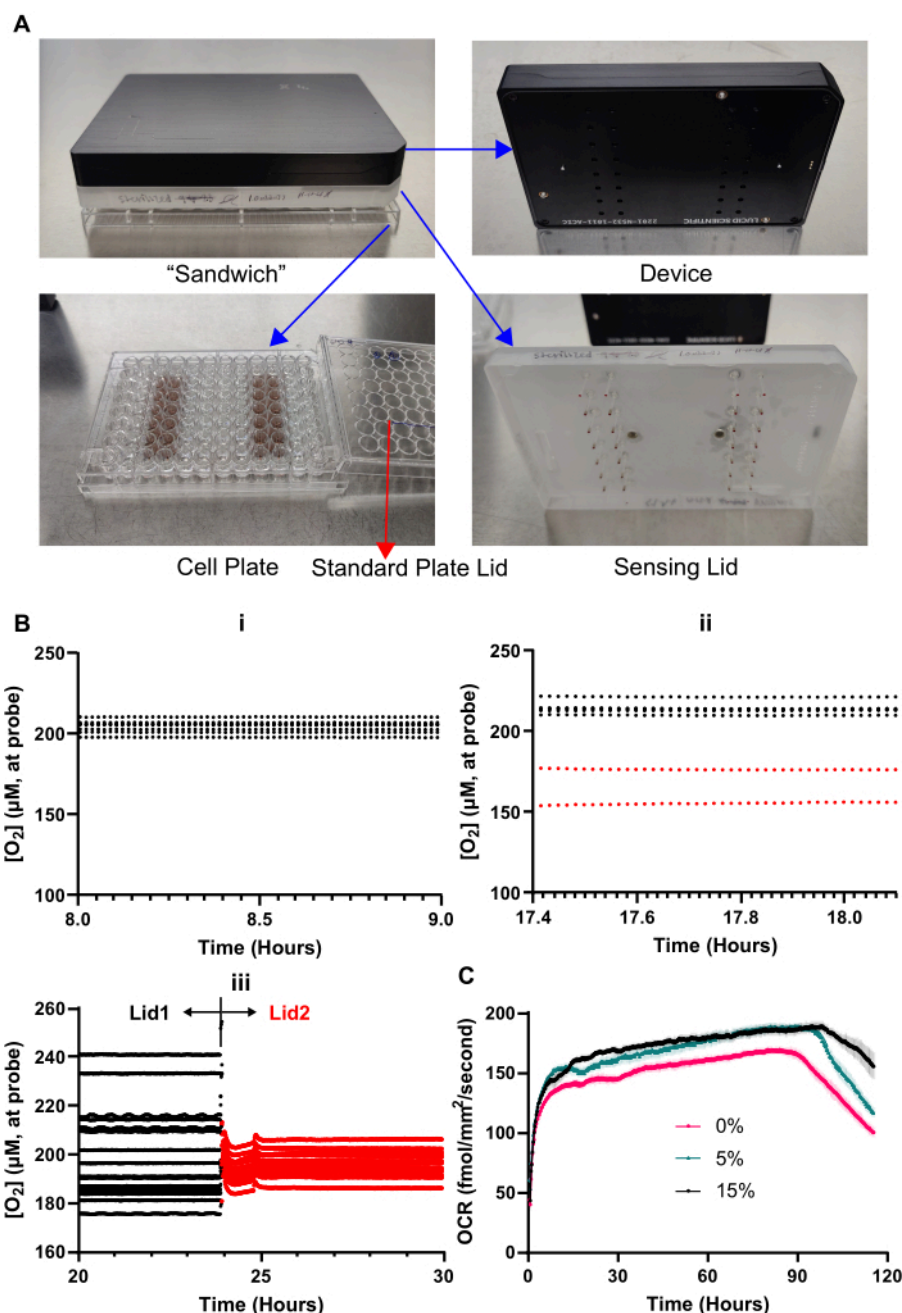


Figure 2: Setup of Resipher system "sandwich" and quality control. (A) The sensing lid replaces a standard 96-well plate lid and has its probes inserted into corresponding wells. The Device in black sits tightly on the sensing lid via magnets. The images on the left side of the figure show the Device and sensing lid in their correct orientation. However, for the photographs on the right side of the figure, the Device and sensing lid have been rotated to their sides to better display their underprofile. (B) Quality control for sensing lids and the Device. (i,ii) The same sensing lid is placed in an empty 96-well receiver plate (air-only), and two different Devices are sequentially placed on top of the sensing lid. (i) The first Device demonstrates

data from eight probes with low variability and O_2 readings $\sim 200 \mu M$, expected for atmospheric O_2 . (ii) The second Device demonstrates data from eight probes in which two probes are reading anomalously (red dots). (iii) One Device was used with an empty 96-well plate (air-only) but with two different sensing lids sequentially tested. Data from 16 probes were plotted, demonstrating wide variability for Lid1 (left side) and low variability for Lid2 (right side). (C) OCR plots of RPE cultures in 100 μL media with different amounts of serum were monitored for 120 h. RPE cells in serum free media (magenta) have lower OCR, with OCR dropping earlier than media with higher amounts of serum. Abbreviations: OCR = oxygen consumption rate; RPE = retinal pigment epithelium. [Please click here to view a larger version of this figure.](#)

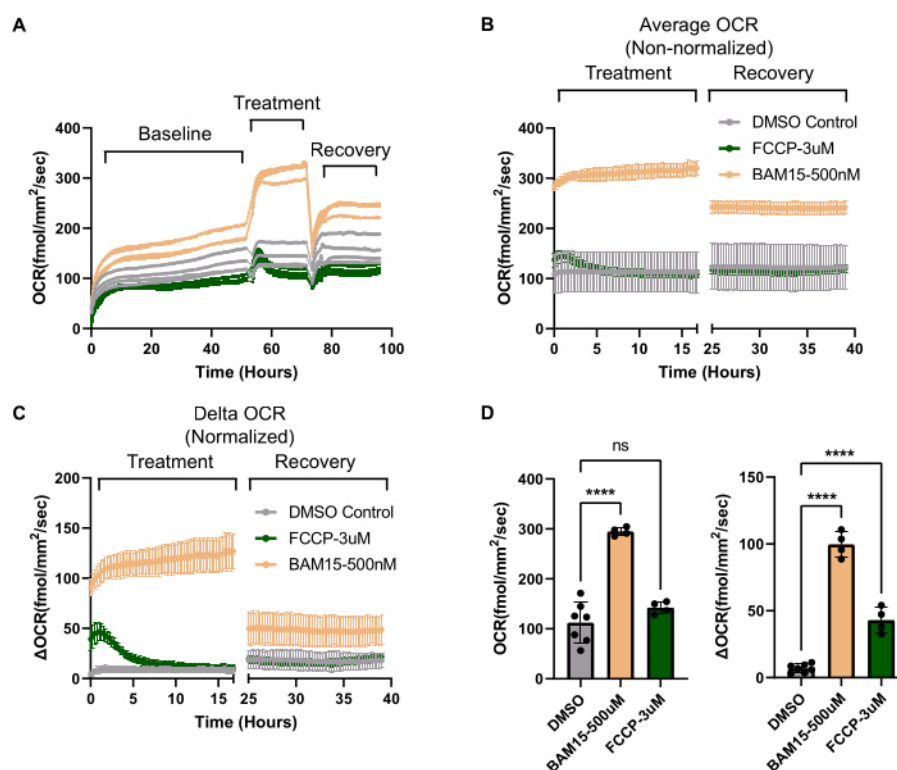


Figure 3: Long-term effects of mitochondrial uncouplers uniquely demonstrated with OCR system. (A) Raw OCR data over time (hours) highlighting the initial OCR (established over 50 h), treatment phase (~20 h after the addition of drugs), and recovery phase (~20 h after drug washout). RPE was treated with two different mitochondrial uncouplers, FCCP (3 μ M, N = 4) or BAM15 (500 nM, N = 4) and compared to DMSO vehicle control (0.6%, N = 7). Each trace represents a single well. (B) Average OCR of all wells for each condition in (A) plotted for the treatment and recovery phases. Without normalization (Y axis is absolute OCR), it is difficult to see the magnitude of the treatment effect with FCCP. (C) When each OCR trace is subtracted from its OCR prior to treatment, a **Delta OCR** can be calculated (plotted on Y axis). This allows one to isolate the effects of the treatment on OCR, despite variability in baseline OCR between wells. Using **Delta OCR**, the effect of FCCP becomes apparent. It also becomes apparent how short-lived the effect of FCCP is on mitochondrial uncoupling, compared to BAM15. Finally, it becomes apparent that in comparison to FCCP, exposure to BAM15, even after the drug is washed off, creates a new "adapted" state of sustained higher OCR. (D) Plots in (B) and (C) are displayed in bar graph form, allowing for direct comparison between non-normalized (left graph, from (B)) and normalized (**Delta OCR**, right graph, from (C)) data. Abbreviations: OCR = oxygen consumption rate; RPE = retinal pigment epithelium; FCCP = carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; DMSO = dimethyl sulfoxide. [Please click here to view a larger version of this figure.](#)

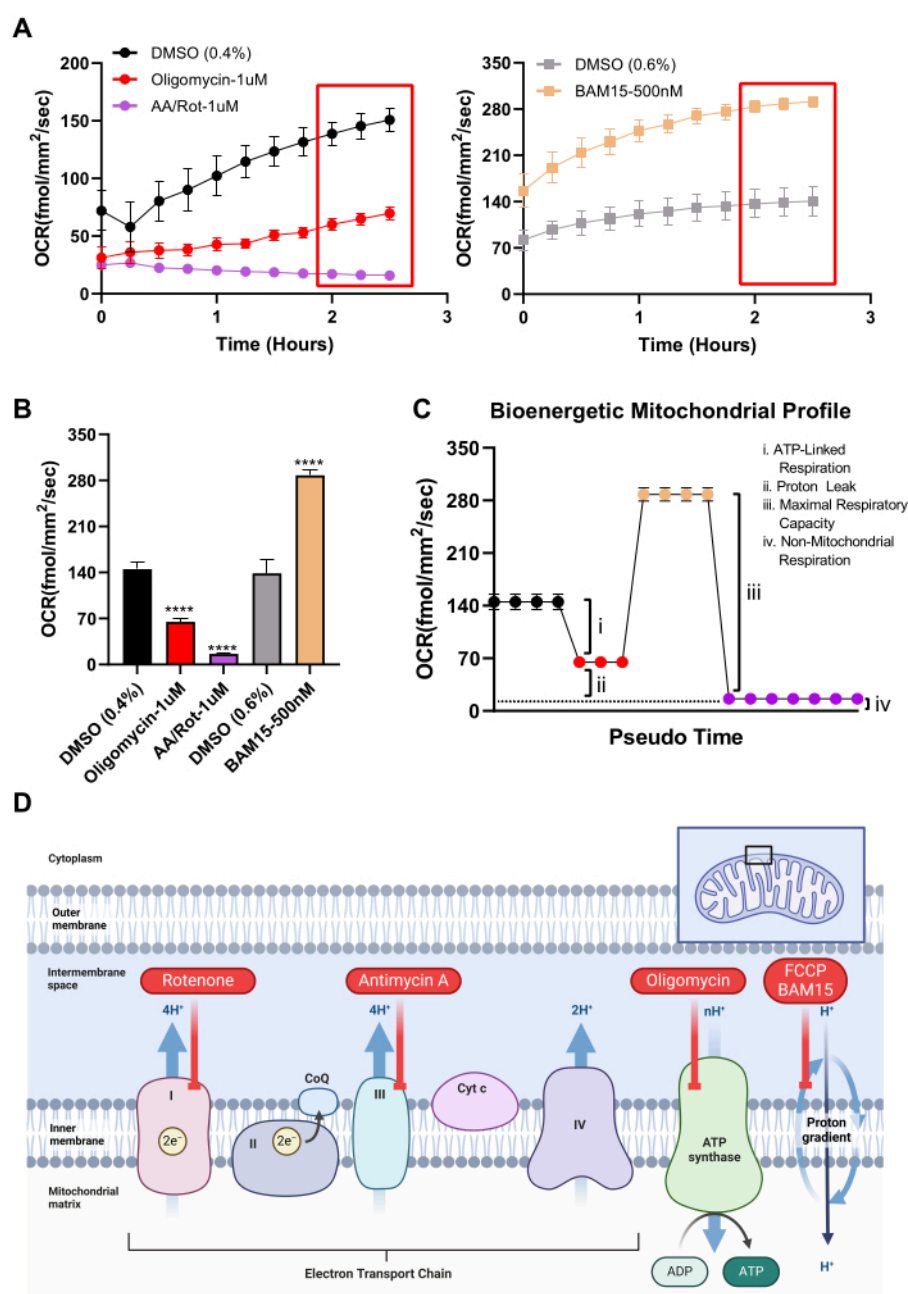


Figure 4: Utilizing OCR system to calculate parameters of mitochondrial bioenergetics. (A) Utilizing the same small molecules that are part of the Seahorse Analyzer Mitochondrial Stress Test, OCR over the first 2.5 h of the treatment phase is depicted for RPE cultures. (Left graph) The ATP-synthase inhibitor oligomycin (N = 3), the complex I and III inhibitors antimycin A/rotenone (N = 7), or vehicle, DMSO 0.4% (N = 4). (Right graph) The mitochondrial uncoupler BAM15 (N = 4) or vehicle DMSO 0.6% (N = 4). **(B)** Average of the final three time points for each treatment group (red boxes in (A)). **(C)** Depiction of a simulated Seahorse Analyzer Mitochondrial Stress Test bioenergetic profile based on Resipher data. The

color-coded data points on the simulated graph correspond to color-coded bar graphs in (B). All respiratory parameters can be calculated using Resipher data and select small molecules. (D) Diagram of the mode of action of the reagents used in **Figure 3** and **Figure 4**. Abbreviations: OCR = oxygen consumption rate; RPE = retinal pigment epithelium; DMSO = dimethyl sulfoxide; AA = Antimycin A; Rot = Rotenone. [Please click here to view a larger version of this figure.](#)

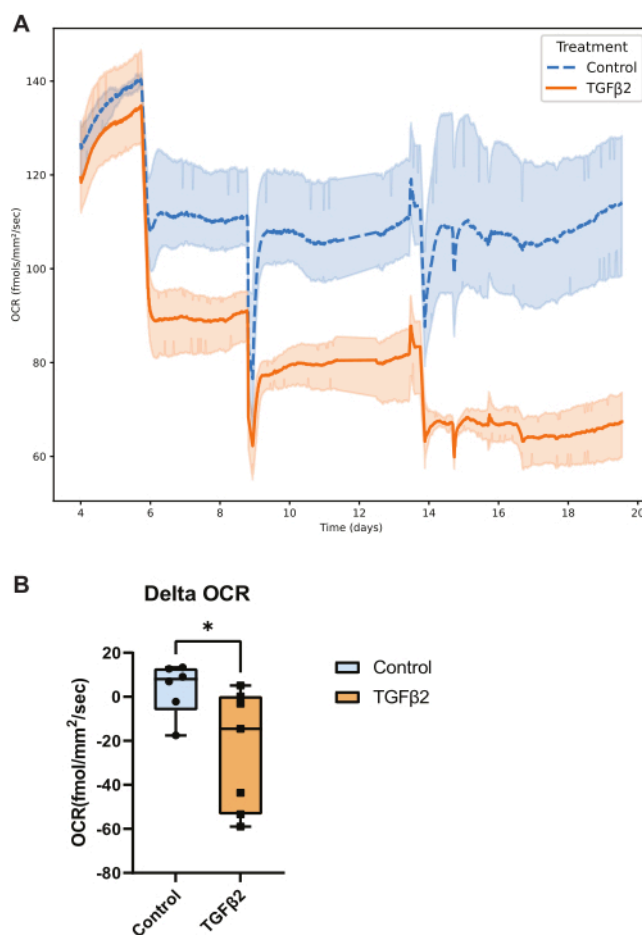


Figure 5: Long-term OCR monitoring of RPE undergoing epithelial-to-mesenchymal transition stimulated by TGFβ2.

Mature primary human RPE cultures were treated with 10 ng/mL TGFβ2 or vehicle control every 2-3 days. (A) Real-time OCR measurements were monitored for 3 weeks with (B) Quantification of Delta OCR over time (Day 19-Day 6). N = 6-7 wells per condition, unpaired t-test, * P < 0.05. Abbreviations: OCR = oxygen consumption rate; RPE = retinal pigment epithelium; TGFβ2 = transforming growth factor beta-2. [Please click here to view a larger version of this figure.](#)

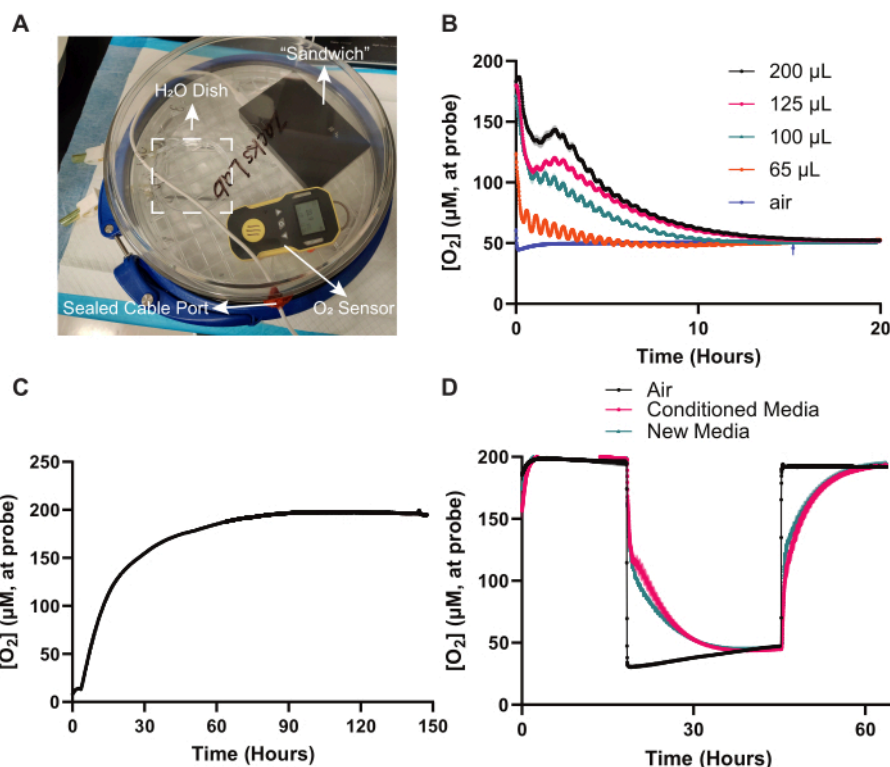


Figure 6: Monitoring RPE mitochondrial response to hypoxia. **(A)** The OCR system "sandwich" was assembled in a hypoxia chamber and the USB cable was connected to the Hub via a hole drilled in the lid and sealed with silicone grease or putty. Also in the hypoxia chamber is a Petri dish of sterile water to maintain humidity and a portable O₂ sensor. The whole system was left in a cell culture incubator after the hypoxia chamber's atmosphere was replaced with a lower O₂ concentration. **(B)** Media takes time to equilibrate to O₂ concentrations above the air-liquid interface. Different volumes of RPE media equilibrated to atmospheric O₂ were added in empty wells (no cells) of a 96-well plate. The hypoxia chamber was then adjusted to 5% (50 μM) O₂. The O₂ concentration in the media of each well was monitored for 20 h. Wells with higher media volumes equilibrated to 5% O₂ more slowly than lower media volumes. N = 8. **(C)** The system can be used to detect air leaks in the hypoxia chamber. The hypoxia chamber was set up with 1% O₂, and the sensing lid was placed over empty wells (air-only). The inlet and outlet ports of the hypoxia chamber were left open, and the hypoxia chamber atmosphere re-equilibrated to atmospheric O₂ levels over ~90 h. **(D)** New media and supernatants ("conditioned media") have similar O₂ solubility. This eliminates the concern of differential O₂ solubility in media as its composition changes while in culture, which would impact the consistency of OCR readings over time. The same volume (100 μL) of fresh media ("new media") or media incubated for 48 h over cells ("conditioned media") were added to wells of a plate without cells. The OCR system "sandwich" was initially subjected to atmospheric O₂ levels, then transferred to the hypoxia chamber (3-5% O₂ or 30-50 μM), then transferred again to atmospheric O₂ levels. O₂ levels in the air were monitored by probes in empty (air-only) wells.

The change in O_2 concentration during each transition between the two media types is identical, showing that O_2 solubility between the two media types is identical. $N = 6$. Abbreviations: OCR = oxygen consumption rate; RPE = retinal pigment epithelium. [Please click here to view a larger version of this figure.](#)

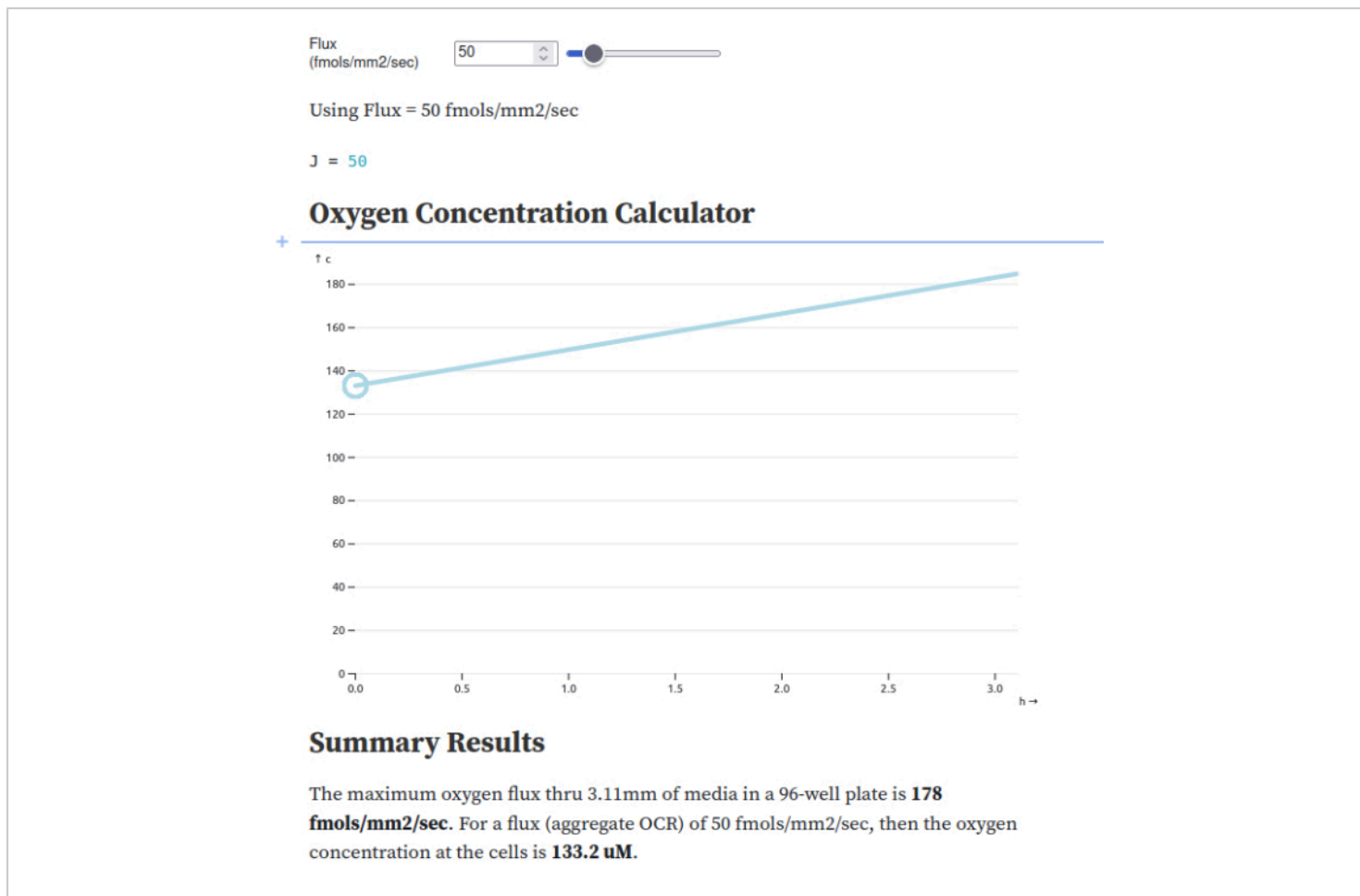


Figure 7: O_2 concentrations at the RPE monolayer determined with an online calculator. Screenshot of the online calculator (<https://observablehq.com/@lucid/oxygen-diffusion-and-flux-in-cell-culture>). See text for details of how the calculator can be used to determine amount of oxygen available at the RPE monolayer. Abbreviations: RPE = retinal pigment epithelium. [Please click here to view a larger version of this figure.](#)

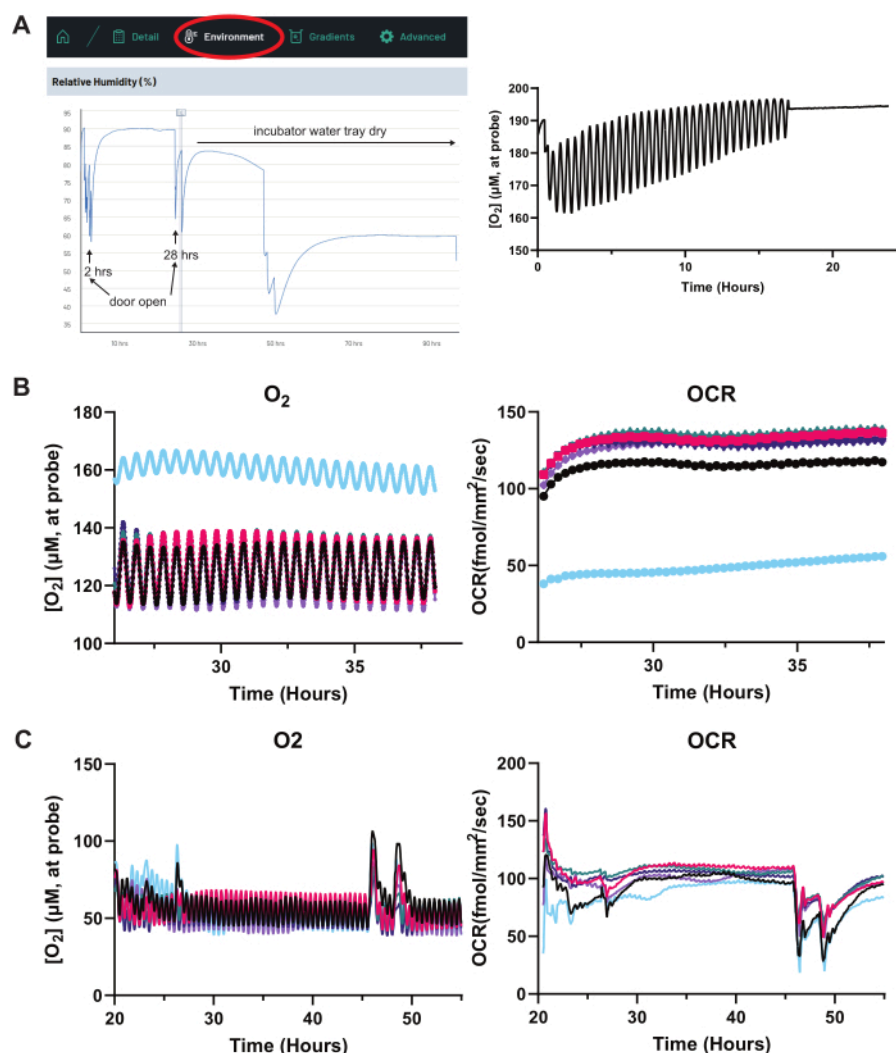


Figure 8: Troubleshooting experiments: evaporation, unhealthy cells, and excessive OCR fluctuations. (A) Excessive evaporation. When there is irregularity in the OCR trace for a well, start by clicking the **Environment** tab on the system's online interface (Left). Here, humidity, temperature, and atmospheric pressure can be monitored. Opening the cell culture incubator will temporarily disrupt humidity (see 2 h and 28 h on the trace). However, starting at 30 h, the humidity trace consistently lowers over time, even without door opening. This suggests the cell culture incubator water tray is dry. Any disruption to humidity or temperature will alter OCR readings. (Right) Low humidity exacerbates evaporation. In the trace on the right, a well undergoing rapid evaporation demonstrates progressively smaller differences in O_2 concentration between the top and bottom of the sensor probe excursion (1 mm to 1.5 mm above the RPE monolayer). This suggests the probe tip is close to the air-media interface. When the O_2 concentration reading "flatlines" at a value close to known atmospheric O_2 concentrations ($\sim 200 \mu M$), this suggests the probe tip is entirely out of the media column and only sampling air. OCR readings are no longer valid and such wells need immediate media change. **(B)** Unhealthy cells. Plotting each individual

well's O_2 and OCR trace enables the identification of an outlier well, usually caused by the cells being unhealthy. Here, the well designated by the blue trace is unhealthy. N = 6 wells. **(C)** Incubator door openings and media change. Changes in atmospheric humidity, temperature, pressure, and CO_2 during incubator door openings, along with any media change, temporarily disrupt the equilibrium O_2 gradient established in the well, causing aberrant O_2 and OCR spikes early and late in these graphs. N = 6 wells. Abbreviations: OCR = oxygen consumption rate. [Please click here to view a larger version of this figure.](#)

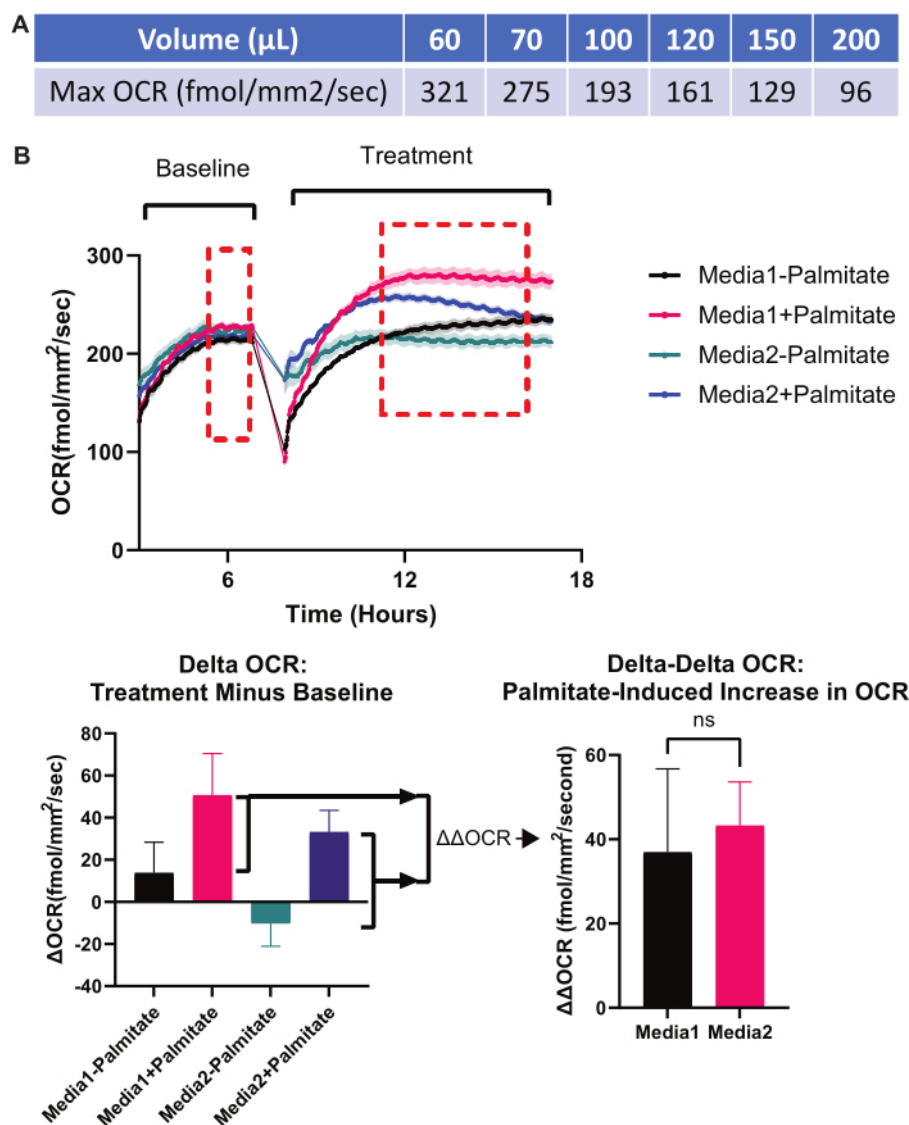


Figure 9: Troubleshooting experiments: media volume and normalization. (A) Media volume limits the maximum possible OCR. Since higher media volumes lead to lower O₂ availability at the RPE monolayer, each media volume in a single well of a 96-well plate has a maximum OCR. Above this OCR, the O₂ at the level of the cells is essentially 0% (anoxic). Thus, if one is close to the maximum theoretical OCR for a given media volume, there is utility in lowering the media volume, so that no OCR "ceiling effect" is seen over the course of the experiment. Maximum theoretical OCR achievable at each media volume (in a standard 96-well plate) is shown. All values were calculated using the online calculator discussed in protocol section 4 and displayed in **Figure 7. (B)** Appropriate normalization. For experiments where one is interested in the effect of one variable on the ability of another variable to induce mitochondrial metabolism, a **Delta-Delta OCR** experimental setup is ideal. In the setup in (B), the ability of two different media to promote β -oxidation of palmitate is tested. If β -oxidation of the fatty acid occurs, there should be a significant increase in mitochondrial OCR. Some media may promote

palmitate β -oxidation better than other media. On the left side of the OCR graph, two different media (Media 1 vs. Media 2) are introduced to parallel wells of RPE cultures. Baseline OCR is obtained. Next palmitate is added to half of the wells containing each media type. This creates four conditions: Media 1 - palmitate, Media 1 + palmitate, Media 2 - palmitate, Media 2 + palmitate. The OCR response to each of these new media conditions is recorded. Next, the OCR values after experimental treatments (average of red dashed box under Treatment phase) are subtracted from the baseline OCR values before treatment (average of red dashed box under Baseline phase), creating **Delta OCR** bar graphs (bottom left). Finally, the ability of Media 1 vs. Media 2 to promote β -oxidation is determined in a **Delta-Delta OCR** bar graph (bottom right). Here, the difference between "Delta OCR of Media 1 + palmitate" and "Delta OCR of Media 1 - palmitate" is calculated and compared to the difference between "Delta OCR of Media 2 + palmitate" and "Delta OCR of Media 2 - palmitate." This type of experimental set-up and normalization isolates the effects of Media 1 vs. Media 2 on promoting β -oxidation; the general structure of this normalization is applicable to any experiment where one is measuring the effect of one variable on the ability of another variable to alter OCR. Abbreviations: OCR = oxygen consumption rate; RPE = retinal pigment epithelium. [Please click here to view a larger version of this figure.](#)

Table 1: The recipe for RPE cell culture media. [Please click here to download this Table.](#)

Discussion

Mitochondrial metabolism of the RPE plays a critical role in the pathogenesis of common blinding retinal diseases, including AMD and PVR. Modeling RPE mitochondrial metabolism *in vitro* allows one to isolate its metabolic state from those of surrounding tissues, along with subjecting the tissue to different disease-simulating insults in a controlled manner. Such *in vitro* modeling of RPE mitochondrial metabolism has been facilitated by the advent of high-fidelity human primary and iPSC-RPE cultures that attain the proper differentiation state and polarity necessary to closely approximate RPE *in vivo*. While monitoring oxygen consumption rate (OCR) in such cultures is an ideal method for monitoring mitochondrial metabolism, the setup for OCR monitoring often precludes growing cultures in the conditions necessary for highly polarized and fully differentiated RPE. Here, we describe Resipher as a novel system for monitoring RPE mitochondrial

metabolism via real-time, long-term assessment of OCR in highly differentiated and polarized RPE cultures.

Other methods have been used to monitor OCR in RPE. The Seahorse XF Analyzer is designed to monitor OCR over short periods of time with acute manipulations of mitochondrial function. However, Seahorse assays require cells to be grown on custom plates, which are not conducive to supporting high RPE polarity and differentiation²¹. Calton, Vollrath, and colleagues adapted the Seahorse for the assessment of highly differentiated RPE cultures grown on microporous supports by cutting the microporous membrane into pieces and placing them in the Seahorse plate, but this method still only works for short-term, end-point monitoring of cultures as the cells do not have access to CO₂ during the assay, the assay can only be done under non-sterile conditions, and cutting the microporous support will induce death and dysfunction to the edge portion of the microporous support over time²². The Barofuse is a pumpless perfusion system in which strips of RPE grown on microporous supports can be loaded into the perfusion system, allowing OCR to be

monitored in real time²³. Similar to the modified setup for the Seahorse by Calton, Vollrath, and colleagues, the major limitations of RPE assessment with Barofuse are the need to cut the microporous supports and the short-term, end-point nature of the assay. The Oxygraph is also designed for fast kinetic changes in OCR in response to metabolic manipulations²⁴. It has not been used for RPE cultures and would suffer the same limitations for assessing highly polarized and differentiated RPE cultures as the Seahorse and Barofuse.

In contrast to these other methods, the OCR system described here allows for monitoring of OCR while RPE is cultured in standard cell culture plates/substrates under standard conditions with standard RPE media in a standard cell culture CO₂ incubator, providing easy and convenient probing of highly differentiated and polarized RPE cultures. In addition to generating data on real-time OCR changes by the system, those same cells can be utilized for additional assays at the end of the OCR monitoring period. This allows for highly flexible experiments where the effect of an intervention on OCR is repeatedly tested over a period of weeks (e.g. - RPE EMT induction in **Figure 5**) or where the long-term recovery from an intervention can be tested (e.g. - recovery from mitochondrial uncoupling in **Figure 3**) or where cascading metabolic changes in response to an initial intervention can be monitored (e.g. - effects of serum on OCR once media nutrients are depleted in **Figure 2**). The OCR system in this paper is particularly convenient for measuring OCR in highly polarized and differentiated RPE cultures. Such cultures are functionally postmitotic, with tight packing and heavy pigmentation. This means each well contains nearly identical cell numbers⁹, thereby obviating the need to normalize OCR to cell number for most experiments. Even when normalizing to cell number is required, the heavy RPE pigmentation and

well-demarcated cell borders allow the normalization to occur with a non-invasive simple cell count based on brightfield imaging. For experiments involving undifferentiated RPE and RPE proliferation, such as the EMT experiments in **Figure 5**, normalizing the per-well OCR to cell number becomes necessary to know what the "per-cell" OCR is.

Outside the retinal field, the system has been employed to study cellular respiration and metabolic activity in various cell types, including pancreatic islet cells²⁵, skeletal muscle²⁶, ovarian cancer²⁷, cardiomyocytes²⁸, and even whole organisms, such as *C. elegans*²⁹. Real-time, long-term monitoring of OCR using the OCR system described here revealed key insights into bioenergetic shifts and mitochondrial dysfunction across these diverse disease models.

The Resipher system does carry limitations. The method for determining OCR is fundamentally different than the Seahorse Analyzer, BaroFuse, or Oxygraph. It requires the establishment of an O₂ gradient to calculate OCR, and the gradient can take hours to establish. Thus, fast kinetic experiments, possible in the other systems, are not possible with this system. Furthermore, unlike the other systems, Resipher does not have any injection ports for the rapid delivery of small molecules or collection of metabolites. Instead, the sensing lid must be removed in a cell culture hood, with drugs being spiked in or media replaced, along with manual collection of metabolites to be analyzed.

To establish and troubleshoot an experiment using the OCR system described in this paper, the following is recommended. First, minimize evaporation. Recall that the O₂ gradient in the media column above the RPE monolayer is highly dependent on the height of the column⁹, and the system calculates OCR based on the O₂ gradient. Thus,

if there is a marked lowering of the media column during the experiment due to evaporation, the O_2 gradient will change, affecting OCR readings. Keeping the water tray for the incubator full is critical, but it is also suggested that water is placed in all the unused wells of the 96-well plate. Further, the wells at the edge of the plate have higher evaporation rates, so these wells are left empty of cells in typical experiments presented above. The humidity sensor on the OCR system can help monitor incubator humidity. In Figure 8A, the drop in humidity during the experiment suggests that the incubator's water tray is empty. As evaporation accelerates in the well, the O_2 concentration readings for the well will climb higher and eventually "flatline" at atmospheric O_2 concentration ($\sim 200 \mu M$) (Figure 8A).

Second, confirm that the OCR system's "sandwich" is appropriately assembled. The metal contact spots on the bottom of the Device and the top of the sensing lid should be clean, without debris to impede full contact. The Device and sensing lid should be attached fully flush and evenly aligned on the 96-well plate without any tilt. The USB cable connecting the Device to the Hub should not be on tension, which can result in torque of the "sandwich" and restriction of proper movement of the Device.

Third, ensure the Device and sensing lid sensors are calibrated. Figure 2B details how to quickly check for proper sensor function at the start of each experiment. Fourth, ensure a particular well is not demonstrating an outlier OCR prior to the experimental manipulation. After ensuring sensors are working, determining baseline OCR values for all wells with cells can help determine if certain wells contain unhealthy cells. Figure 8B shows several wells undergoing baseline OCR assessment, in which the blue-colored well clearly demonstrates a lower OCR (and therefore, high O_2

concentration). This well is an outlier and should be excluded from experimental manipulation.

Fifth, minimize environmental manipulations during active OCR readings. The O_2 gradient is highly sensitive to changes in temperature, humidity, incubator CO_2 levels, and atmospheric pressure. Incubator door opening significantly affects these factors, causing temporary disruptions in OCR readings until the media column's O_2 gradient returns to equilibrium (Figure 8C). Parameters affecting OCR can be monitored in the system's online interface under the "Environment" tab (Figure 8A).

Sixth, determine the optimal media volume for the experiment. While insufficient media volume can create artifacts from excessive evaporation (see the first troubleshooting measure above) or premature exhaustion of nutrients in the media, excessive media volume limits O_2 availability to the RPE monolayer, triggering hypoxia⁹.

Figure 7 is a screenshot of the online calculator available for determining the effect of media volume on O_2 concentration at the RPE monolayer. The maximum OCR possible for a given media volume is displayed in Figure 9A. If the OCR rate for a given experiment using a given volume approximates the rate displayed in Figure 9A, then O_2 concentration at the RPE monolayer approaches anoxia (0%) and a lower media volume is needed. In general, experiments involving mitochondrially intensive processes in RPE culture should have media volumes no greater than 75 μL per 96-well.

Seventh, perform appropriate normalization to isolate the effects of an experimental intervention on OCR. As different wells can have different baseline OCR, it is critical to obtain baseline OCR readings for each well prior to an experimental intervention. Subtract the baseline OCR from the OCR after the experimental intervention to obtain the "Delta

OCR," which demonstrates the effect of the experimental intervention on OCR. The method for obtaining "Delta OCR" is demonstrated in **Figure 3** and **Figure 9B**. If one is interested in the effects of one experimental intervention on the ability of another experimental intervention to induce OCR, an additional normalization step, the "Delta-Delta OCR", is needed. Such an example is illustrated in **Figure 9B**, where the ability of palmitate (one experimental intervention) to induce increased OCR (via induction of β -oxidation) depends on the type of media for the RPE culture (a second experimental intervention).

Finally, minimize the risk of contamination. The system monitors OCR by introducing an oxygen sensor into the media above cells. This increases the risk of infection. Unexpected drops in OCR during the experiment or unexpectedly low OCR at the start of the experiment could be signs of an infection. Plates need to be actively monitored for infection under a cell culture microscope. Cross-contamination between 96-well plates could happen if a sensing lid is reused. To minimize such cross-contamination, one should immerse the whole lid in 70% ethanol for 20 minutes for decontamination after an experiment is done, prior to re-use of the sensing lid in a new experiment (see protocol section 1.2.15).

In conclusion, a protocol is presented for real-time, long-term monitoring of mitochondrial metabolism in high-fidelity RPE cultures via assessment of OCR using a novel system called Resipher. It can be used for numerous applications on highly polarized and differentiated RPE cultures, in particular, probing physiologic metabolic properties of isolated RPE as well as altered RPE metabolism in *in vitro* models of retinal disease, including AMD and PVR.

Disclosures

Richard A. Bryan and Kin Lo are employees of Lucid Scientific, which manufactures the Resipher system.

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