

Method for measuring extracellular flux from intact polarized epithelial monolayers

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Purpose: The Seahorse XFp platform is widely used for metabolic assessment of cultured cells. Current methods require replating of cells into specialized plates. This is problematic for certain cell types, such as primary human fetal RPE (hfRPE) cells, which must be cultured for months to become properly differentiated. Our goal was to overcome this limitation by devising a method for assaying intact cell monolayers with the Seahorse XFp, without the need for replating.

Methods: Primary hfRPE cells were differentiated by prolonged culture on filter inserts. Triangular sections of filters with differentiated cells attached were excised, transferred to XFp cell culture miniplate wells, immobilized at the bottoms, and subjected to mitochondrial stress tests. Replated cells were measured for comparison. Differentiated hfRPE cells were challenged or not with bovine photoreceptor outer segments (POS), and mitochondrial stress tests were performed 3.5 h later, after filter excision and transfer to assay plates.

Results: Differentiated hfRPE cells assayed following filter excision demonstrated increased maximal respiration, increased spare respiration capacity, and increased extracellular acidification rate (ECAR) relative to replated controls. hfRPE cells challenged with POS exhibited increased maximal respiration and spare capacity, with no apparent change in the ECAR, relative to untreated controls.

Conclusions: We have developed a method to reproducibly assay intact, polarized monolayers of hfRPE cells with the Seahorse XFp platform and have shown that the method yields more robust metabolic measurements compared to standard methods and is suitable for assessing the consequences of prolonged perturbations of differentiated cells. We expect our approach to be useful for a variety of studies involving metabolic assessment of adherent cells cultured on filters.

Changes in cellular energy metabolism are central to aspects of cancer biology, embryonic development, stem cell differentiation, and aging [1-5]. The Seahorse XFp Analyzer has accelerated discoveries in cellular energy metabolism by providing a platform scaled for individual laboratories that enables extracellular flux measurements of oxygen and pH from relatively small amounts of biologic material.

Primary human fetal retinal pigment epithelial (hfRPE) cells most closely resemble their *in vivo* counterparts when cultured for months on filter inserts in specialized media that together promote a differentiated, polarized epithelial phenotype [6-8]. Differentiated primary hfRPE cells cultured under these conditions have been used as models to understand the pathogenesis of age-related macular degeneration (AMD), the most common cause of blindness in the developed world [9-11]. Standard methods for assaying such adherent cells with the Seahorse XFp Analyzer require trypsinization and replating into Seahorse cell culture miniplates, often followed by several days of growth. These manipulations likely result in loss of some differentiation characteristics and may confound efforts to assess the consequences of experimental

perturbations performed on polarized cells before replating. Our goal was to devise a method for analyzing adherent cells in the Seahorse XFp Analyzer that avoided regrowth in miniplates. Attainment of this goal would allow application of the instrument to a wider range of cells, including highly differentiated RPE cells.

We describe a method for transferring to a well of an XFp miniplate an intact monolayer of healthy, differentiated hfRPE cells, and immobilizing the monolayer precisely in the small assay area. Our approach allows reproducible extracellular flux measurements to be taken across hours from as few as 40,000 cells, and for at least three independent replicates to be made from the same 12-well filter. Our method is applicable to a variety of perturbations of differentiated RPE cells and could be used to study other adherent cell types compatible with culture on polyester filters.

METHODS

Cell culture: Primary human fetal RPE (hfRPE) cells (Advanced Bioscience Resources, Inc., Alameda, CA) were isolated according to Maminishkis and Miller's methods [8] and plated onto human extracellular matrix-coated Corning 12-well Transwells (#3460, Corning Inc., Corning, NY) in medium (#M4526, MilliporeSigma, St. Louis, MO) with 5% heat inactivated fetal bovine serum (FBS; #100-106,

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Gemini Bio-products, West Sacramento, CA), 1% GlutaMAX (#35050061, Life Technologies Corporation, Carlsbad, CA), 1% N1 Supplement (#N6530, MilliporeSigma), 1% Non-essential Amino Acid Solution (#M7145, MilliporeSigma), 1% Antibiotic-Antimycotic (#15240062, Life Technologies Corporation), 250 µg/ml Taurine (#T0625, MilliporeSigma), 20 ng/ml Hydrocortisone (#H0396, MilliporeSigma), and 13 pg/ml Triiodothyronine (#T5516, MilliporeSigma) [7]. Cells were allowed to differentiate for at least 5 months before the experiments were begun.

Transwell insert plating: Triangular inserts were cut from Corning 12-well Transwells with cultured hFRPE and placed in XFp cell culture miniplate wells (#103022–100, Agilent, Santa Clara, CA) in less than 2 min to prevent drying. For the phagocytosis assay, isolated inserts were plated during calibration of the XFp sensor cartridge (#103022–100, Agilent). To account for background measurements, inserts cut from unseeded Transwells were placed in two wells of each XFp cell culture miniplate.

Phagocytosis assay: Purified bovine photoreceptor outer segments (POS; 500 µl of 1×10^7 POS/ml) in culture medium with 5% FBS, 4 µg/ml human protein S (#HPS, Enzyme Research Laboratories, South Bend, IN), and 1.5 µg/ml human recombinant MFG-E8 (#2767-MF-050, R&D Systems, Inc., Minneapolis, MN) were added to about 500,000 differentiated hFRPE cells in the upper chamber of a 12-well Transwell. Cells with POS were incubated at 37 °C for 1.0 or 3.5 h and then washed three times with phosphate buffered saline (PBS; 1X; 1.06 mM Potassium Dihydrogen Phosphate, 154.11 mM Sodium Chloride, 5.60 mM Disodium Phosphate, pH 7.3-7.5) to remove unbound POS and fixed in 4% paraformaldehyde for 30 min at room temperature. For the 3.5-h time point, cells were transferred to Seahorse assay medium for the last 30 min before fixation. Phagocytosis was quantified with double immunofluorescence labeling of bound and total POS with anti-rhodopsin monoclonal primary antibody rho 4D2 (gift of Robert Molday, University of British Columbia), as previously described [12]. Bound POS were labeled with a goat-anti-mouse Alexa Fluor 594-conjugated secondary antibody (#A-11032, Invitrogen, Carlsbad, CA), and total POS were labeled with a goat anti-mouse Oregon Green 488-conjugated secondary antibody (#O-6380, Invitrogen). Slides were mounted with Fluoromount-G (#0100–01, SouthernBiotech, Birmingham, AL) and visualized with an upright epifluorescence microscope (Axioskop 2 plus, Zeiss, Oberkochen, Germany) using a 20X objective. Six images per time point at 20X magnification were captured and quantified with ImageJ (National Institutes of Health, Bethesda, MD).

Ingested POS were estimated by subtracting the number of cell surface-bound POS from total POS in a given image.

Cellular energetics : We used a Seahorse XFp Analyzer (Agilent) to measure the oxygen consumption rate (OCR), an indicator of mitochondrial respiration, and the extracellular acidification rate (ECAR), an indicator of aerobic glycolysis. Cells were either trypsinized using 0.25% Trypsin-EDTA (#25200056, Life Technologies Corporation) and reseeded into XFp cell culture miniplates at 40,000 cells per well 2 days before the assay, or excised triangular inserts were placed in the XFp culture miniplates. Three replicate miniplate wells (technical triplicates) were used for each condition. XF Assay Medium (#103334–100, Agilent) was supplied with 5.5 mM glucose (#G8769, MilliporeSigma), 1 mM sodium pyruvate (#11360070, Life Technologies Corporation), and 2 mM L-glutamine (#25030081, Life Technologies Corporation). For the phagocytosis cellular flux assay, cells were incubated in phagocytosis medium for 3 h and then transferred to assay medium for approximately 30 min before the mitochondrial stress test. For all other assays, cells were incubated in assay medium for 60 min. The mitochondrial stress test was performed using the following final concentrations of inhibitors: 2.0 µM oligomycin (#O4876, MilliporeSigma), 1.5 µM FCCP (#C2920, MilliporeSigma), and 2.0 µM rotenone/myxothiazol (#R8875, #T5580, MilliporeSigma). After each inhibitor was added to the medium, three sequential measurements were taken. Total protein was isolated from individual wells following completion of the stress test and quantified using a BCA Protein Assay Kit (#23225, Pierce Biotechnology, Waltham, MA). Each well was normalized either to the percent of initial OCR or ECAR or to micrograms of protein. The mitochondrial stress test parameters in mitochondrial stress test medium were calculated according to the manufacturer's suggestions.

Statistical analysis: Statistical analysis was performed using GraphPad Prism. Multiple group comparisons for statistical significance were performed using Bonferroni correction for one-way ANOVA. Results are expressed as mean ± standard deviation (SD).

RESULTS

hFRPE cells differentiated on Transwell filters can be plated as intact monolayers: Our goal was to devise a method for analyzing adherent cells in the Seahorse XFp that avoided regrowth in cell culture miniplates. To do this, we had to overcome three main obstacles: 1) consistently produce uniform inserts with minimal disruption of cell integrity, 2) transfer and place the excised inserts into the Seahorse XFp

miniplate wells, and 3) sufficiently adhere the inserts to the bottom of the miniplate wells.

We used Computer Aided Design and three-dimensional printing to fabricate a triangular template that was sized to fit within the 3 mm² XFp miniplate assay area (Figure 1A). The code used to design and print the template is supplied in Appendix 1 as text suitable for a .stl file. In our initial efforts, we used a wet-cutting approach to ensure that the cells were well hydrated. We used forceps to remove a 12-well Transwell from its plate and inverted it (apical side down) in the well of a 6-well plate filled with medium. The inverted Transwell was then visualized under a microscope (Leica Microsystems, Wetzlar, Germany). The triangular template was placed on the top (basal side) of the Transwell, and the filter was lightly scored around the triangular template with a fine diamond scribe (#54466, Ted Pella, Inc., Redding, CA; Figure 1B,C). The triangular template was removed, and the scribe was used to cut through the filter completely on all three sides, leaving only one corner slightly attached. We detached the

insert by holding the remaining attached corner with a fine #5 forceps and gently pulling it away from the rest of the Transwell. The Transwell was returned to its original plate with medium to prevent drying of the remaining cells. After we had mastered the process and were able to cut, transfer, and position an insert in less than 2 min, we skipped the 6-well step and cut inserts from inverted Transwells placed directly under a microscope. We routinely cut three inserts (and easily up to five) from a single 12-well filter and alternated between experimental and control filters. We also successfully used our approach on smaller Transwells from 24-well plates.

After testing several compounds, including tissue glue, we found that petroleum jelly worked best to adhere isolated inserts to the bottom of the miniplate wells. To prepare the miniplates for insert adherence, a small amount of petroleum jelly was placed in each well using the blunt end of a 4-inch cotton swab. The petroleum jelly was spread into a thin layer over the bottom of the well with a reverse Sinskey ophthalmic hook (#K3-5002, Katena Products, Inc., Denville, NJ; Figure

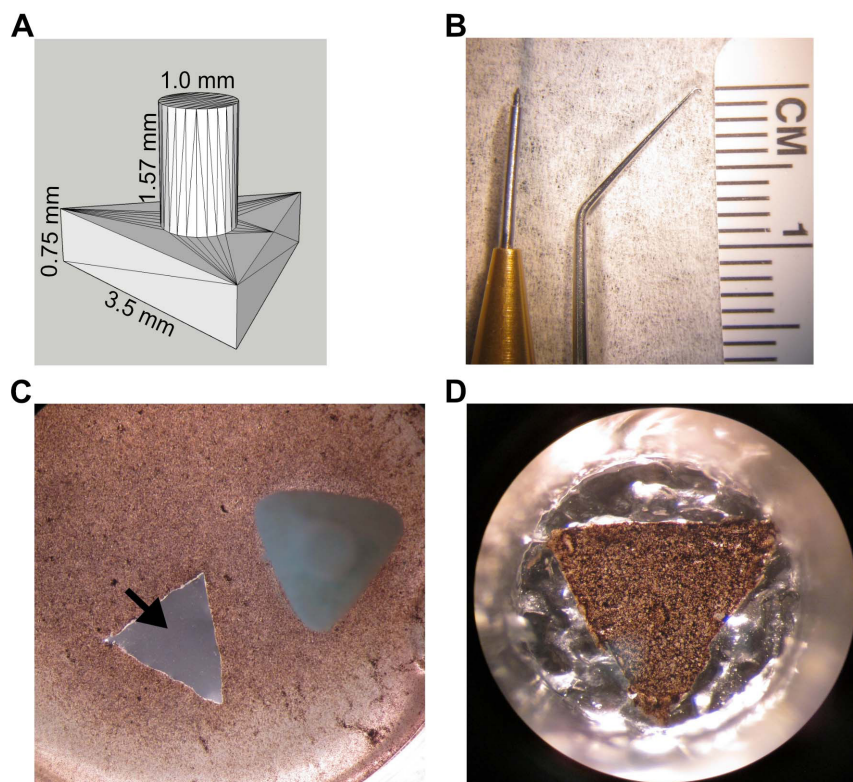


Figure 1. Method for assaying RPE cells cultured on Transwell polyester supports. **A:** Depiction with the dimensions of the triangular template used to guide the insert scoring and cutting. **B:** Image of the fine diamond scribe (left) and a reverse Sinskey ophthalmic hook (right). **C:** Image of a Transwell inverted basal side up and in view with examples of a scored and cutout insert (arrow) and the placement of the template on the Transwell. **D:** Image taken from above of a human fetal RPE insert positioned in a miniplate well.

1B), leaving a slightly raised peak in the middle. Miniplates with petroleum jelly can be prepared several hours before use.

Following excision, an insert was gently placed apical side-up on top of the peak of the petroleum jelly in an XFp miniplate well. The triangular shape of the insert allows for a minimal area touched by the forceps, so that any compromised cells are located along the edge of the insert, away from the probe assay area. The well with the isolated insert was viewed under a microscope, and the position of the insert was adjusted using the ophthalmic hook such that the corners of the triangle fell between the offsets molded on the bottom of the miniplate well. The insert was gently pushed down at the corners with the ophthalmic hook so that the insert lay flat at the bottom of the well (Figure 1D). Extreme care was taken not to scrape off or disturb any of the cells in particular in the probe assay area during positioning. Immediately following placement of the insert, assay medium was gently pipetted into the well. Once each well of a miniplate was seeded with an isolated insert, the plate was equilibrated in a non-CO₂ incubator as usual.

hfrPE maintained as monolayers have stable cellular respiration: Having mastered the cutting, transferring, and plating of inserts in miniplate wells, we assessed the stability of the metabolic parameters over time. We measured OCR and ECAR in Seahorse assay medium over 107 min for three different hfrPE lines. The OCR values in the cells that remained on the inserts were stable for the duration of the test period (OCR_{initial}: 119.8±60.7 versus OCR_{last}: 100.9±55.6 pmol/min; p=0.6361; Figure 2A,C). The ECAR levels also remained consistent throughout the assay (ECAR_{initial}: 123.7±38.5 versus ECAR_{last}: 123.1±24.7 mpH/min; p=0.9891; Figure 2B,C). These results support the use of our method over an extended time.

hfrPE maintained as monolayers have more robust extracellular flux compared to replated cells: The stability of the cellular respiration measurements allowed us to compare metabolic parameters (Figure 3A) between differentiated hfrPE cells preserved as monolayers on inserts, and otherwise identical cells trypsinized and replated by standard methods. Trypsinized and replated cells exhibited levels of basal respiration similar to those of insert monolayers (3.745±0.246 versus 3.481±0.576 pmol/min/μg protein, respectively; p>0.9999; Figure 3B,C). However, a mitochondrial stress test showed that the cells from the inserts were able to increase their maximum respiration more robustly. After the addition of the potent mitochondrial oxidative phosphorylation uncoupler FCCP, the insert cells demonstrated a statistically significant increase in maximal respiration

(9.820±2.037 pmol/min/μg protein) compared to the replated cells (4.634±1.124 pmol/min/μg protein; p<0.0001; Figure 3B,C). This resulted in a statistically significant increase in spare capacity in the intact monolayer (6.339±1.464 pmol/min/μg protein) compared to the replated cells (0.889±1.368 pmol/min/μg protein; p<0.0001; Figure 3B,C). Replated cells had a statistically significantly lower ECAR (p<0.0001; Figure 3D), resulting in a higher OCR/ECAR ratio compared to the cells that remained on the inserts (Figure 3E). The robust metabolic profile of the hfrPE cells maintained on the inserts makes it possible to assess the metabolic consequences of in culture manipulations of differentiated cells.

hfrPE cells have different metabolic requirements during POS phagocytic ingestion: To demonstrate an additional utility of the insert technique and explore the hypothesis that phagocytosis of POS by RPE cells changes their metabolic capacity, we assessed the effect of POS incubation for 3.5 h, a time when substantial numbers of POS have been ingested (Figure 4A). We challenged four different lines of differentiated hfrPE with POS and examined the metabolic demand on the RPE cells using our insert technique. We found that the addition of POS to differentiated hfrPE cells resulted in a modest but statistically significant increase in the maximum OCR (MaxResp_{+POS}: 14.63±2.53 versus MaxResp_{-POS}: 10.36±1.57 pmol/min/μg protein; p=0.0285) and spare capacity (Spare_{+POS}: 3.666±2.054 versus Spare_{-POS}: 9.06±1.412 pmol/min/μg protein; p=0.0146; Figure 4B,C). A mitochondrial stress test did not reveal any differences in the basal ECAR or OCR/ECAR ratio after an oligomycin challenge (Figure 4D,E), suggesting that the addition of POS did not alter the glycolytic balance of the hfrPE cells tested.

DISCUSSION

We have developed a method to reproducibly assay polarized hfrPE cells with the Seahorse XFp platform that does not require trypsinization and replating. Maintaining the integrity of cell–cell and cell–basement membrane connections that form in a monolayer over weeks and months should more accurately reflect conditions in vivo. Our results demonstrate that replating highly differentiated hfrPE cells causes a considerable decrease in maximal and spare respiration capacity, while at the same time decreasing the ECAR. Our experience assessing these parameters for more than 25 hfrPE lines suggests that the lower rates are indicative of less healthy cells. The proportionately larger decrease in the ECAR caused by replating led to an inflation of the OCR/ECAR ratio relative to the differentiated cells assayed on the filter inserts. This suggests that the two modes of energy metabolism may be more balanced in vivo than is apparent

from assaying replated cells, which may be experiencing inadvertent stress.

This approach is suitable for metabolic assessment of RPE cells from a variety of sources, including induced pluripotent stem cell (iPSC)-derived RPE [13] and human adult RPE [14], and could be used to characterize the metabolic consequences of coculture of hfRPE with choroidal endothelial cells [15]. More broadly, this approach should be applicable to any cell type compatible with culture on polyester filters, such as corneal endothelial cells [16] and cells from a variety of non-ocular epithelia [17,18].

Our finding of a modest but statistically significant increase in the maximum and spare respiratory capacity 3.5 h after the addition of POS to hfRPE monolayers highlights

the utility of the method for assessing the metabolic consequences of experimental perturbations. The increased respiration capacity engendered by the addition of POS may reflect the ability of differentiated hfRPE to oxidize POS-derived lipids ingested by phagocytosis [19,20]. This method should also be useful to investigators seeking to obtain energetics measurements from cells subjected to treatments lasting weeks or months, for example, treatments with AMD-relevant stressors. Finally, this method could be adapted for the ex vivo study of tissues. The Seahorse XF24 Analyzer with commercial mesh inserts has been used to assess the ex vivo metabolism of mouse neural retina tissue [21]. We have used knowledge gained from our cell culture studies to obtain preliminary metabolic data from mouse RPE/choroid/

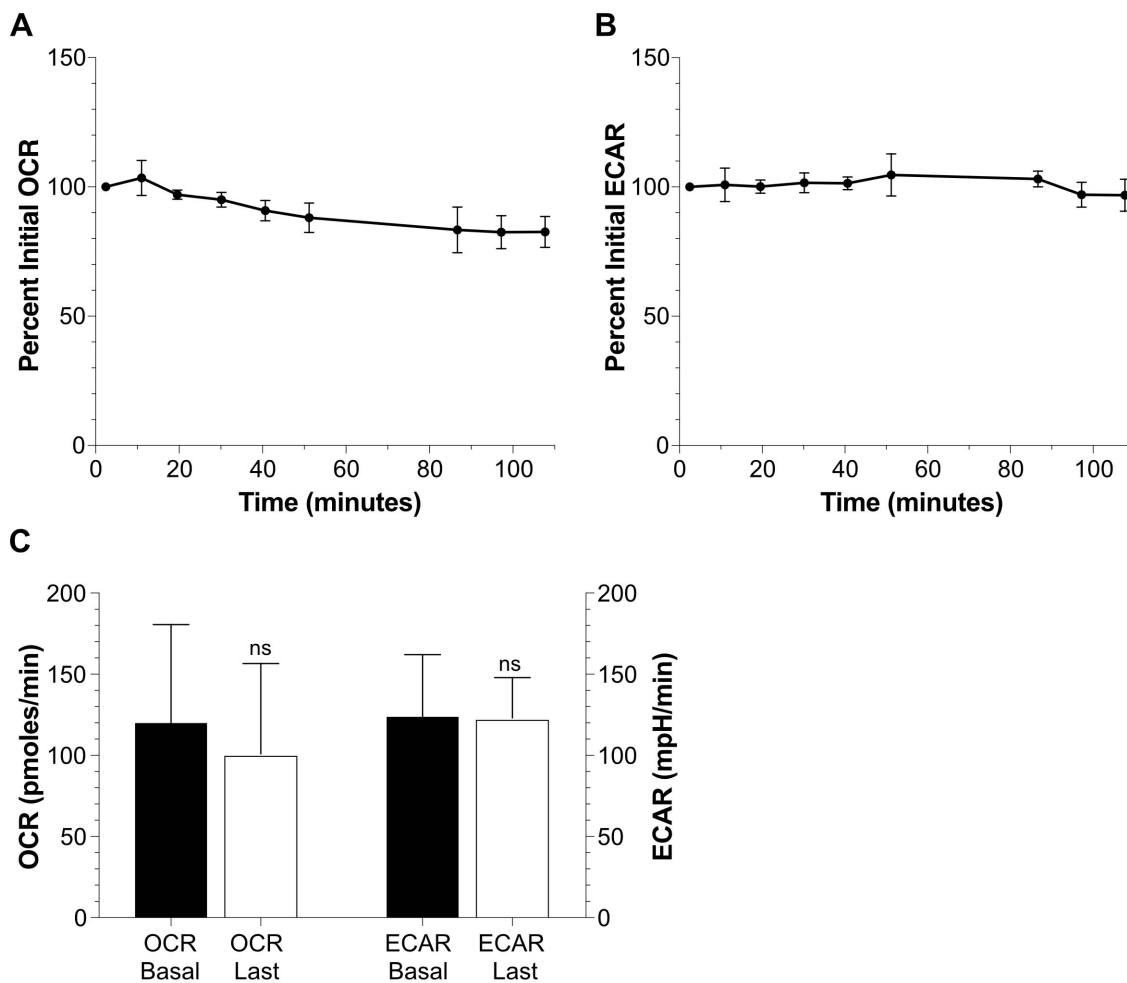


Figure 2. Stability of metabolic measurements from hfRPE cells assayed on polyester inserts. Average percent of the initial (A) oxygen consumption rate (OCR) and (B) extracellular acidification rate (ECAR) values for three different human fetal RPE (hfRPE) lines measured over time; technical triplicates were performed for each line. C: The average initial OCR and ECAR measurements compared to the final measurements obtained for the OCR and the ECAR show no statistically significant change over time. Results are expressed as mean ± standard deviation. ns = not significant.

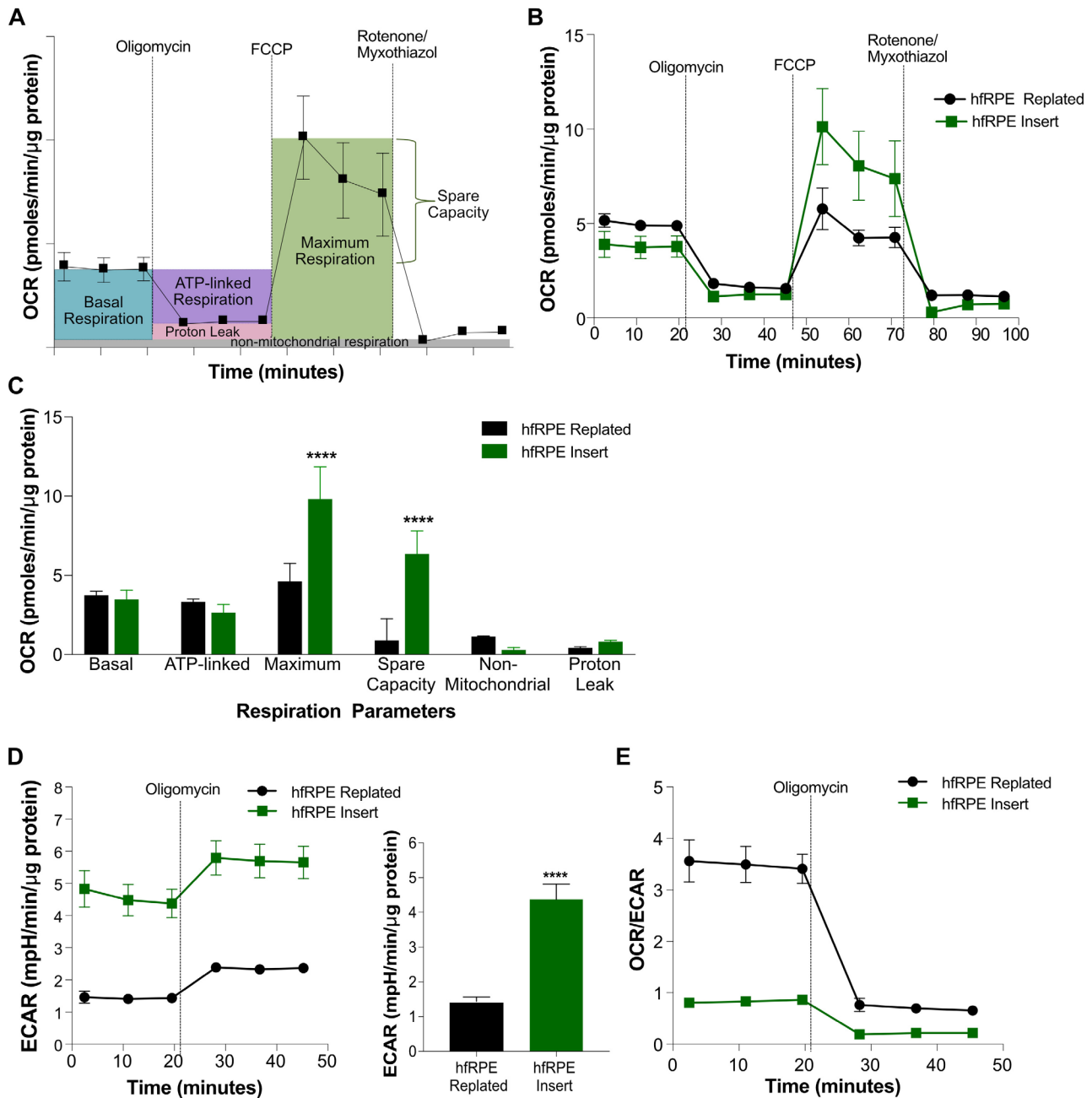


Figure 3. Enhanced respiratory capacity of polarized hfRPE cells compared to replated controls. **A:** Pictorial representation of a mitochondrial stress test and respiration parameters calculated. **B:** Paired wells of a single human fetal RPE (hfRPE) cell line were subjected to mitochondrial stress tests on an XFp Analyzer with two different methods. Triplicate samples of polarized cells excised from a Transwell filter (green) were compared to triplicate samples trypsinized from a Transwell filter and replated in a miniplate (black). **C:** Respiration parameters calculated from the mitochondrial stress test. **D:** Basal extracellular acidification rate (ECAR) values obtained during the mitochondrial stress test revealed a statistically significant difference between replated and insert hfRPE, and differences in the OCR/ECAR ratios (**E**). **** $p < 0.0001$. Results are expressed as mean \pm standard deviation.

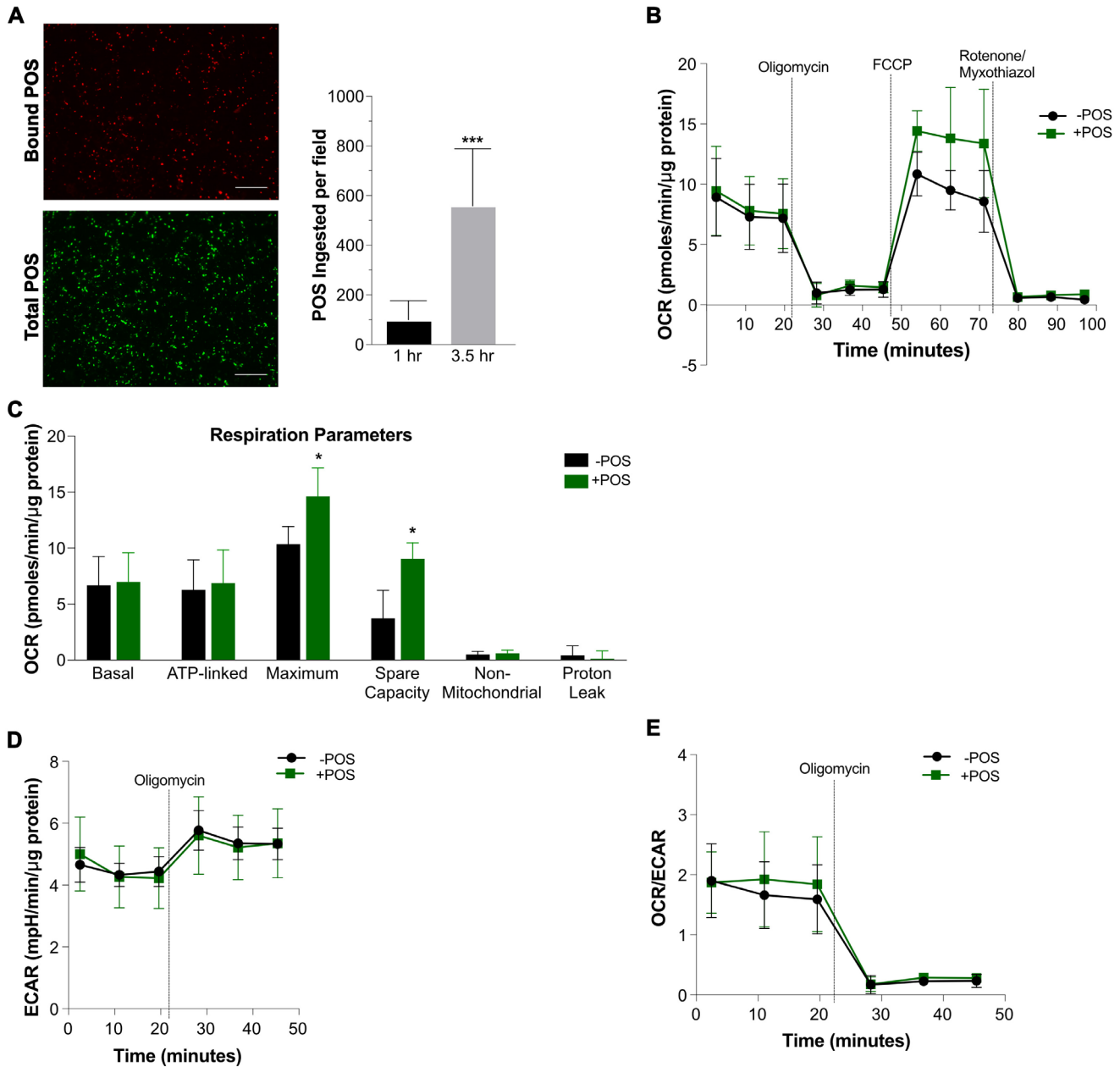


Figure 4. Increased maximum respiration and spare capacity of hfRPE cells exposed to bovine POS. **A**: Representative 20X field images of bound (red) and total (green) POS at 3.5 h, and quantification of ingested photoreceptor outer segments (POS) from six images per time point at 1.0 and 3.5 h compared with an unpaired *t* test. **B**: Mitochondrial stress tests were performed on paired samples from four different human fetal RPE (hfRPE) cell lines challenged (+POS) or unchallenged (-POS) with bovine POS 3.5 h before testing (green and black, respectively). **C**: Respiration parameters were calculated from the mitochondrial stress test measurements. **D** and **E**: Basal extracellular acidification rate (ECAR) values obtained during the mitochondrial stress test revealed no obvious differences between the POS-treated and untreated samples or in the oxygen consumption rate (OCR)/ECAR ratios. Technical triplicates were conducted for each condition for each line. **p*<0.05, ****p*<0.001. Results are expressed as mean \pm standard deviation.

sclera explants in the smaller XFP format, without the need for filter or mesh supports. Together, these examples illustrate the many potential applications of this technique.

APPENDIX 1. SEAHORSE STAMP.

To access the data, click or select the words “Appendix 1.”

ACKNOWLEDGMENTS

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