Abstract

Confluent Monolayers of Cultured Human Fetal Retinal Pigment Epithelium (hfRPE) Exhibit Morphology and Physiology of Native Tissue

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PURPOSE
To develop a reproducible method for culturing hfRPE cells that produce confluent monolayers that exhibit morphology, physiology, polarity and protein expression paterns similar to native hfRPE.
METHODS
The research followed the tenets of the Declaration of Helsinki and the institutional review board. Fetal eyes were obtained by an independent procurer (ABR; Alameda,CA) and delivered using overnight priority service. Eyes were dissected upon delivery and RPE cell sheet mechanically separated from choroid and cultured in a specifically designed medium comprised entirely of commercially available components. All physiology experiments were carried out using previously described techniques (Maminishkis A., *et al.*, IOVS, v.43: 3555, 2002). Standard techniques were used for immunohistochemisry, electron microscopy, and ELISA.
RESULTS
Confluent monolayers of RPE cell cultures exhibit epithelial morphology and heavy pigmentation and electron microscopy shows extensive apical membrane microvilli and tight junction (TJ) complexes. TJ complexes were identified using immunofluorescence labeling of ZO-1 and occludin. The mean transepithelial potential (TEP) was 2.6 ± 0.8 mV, apical positive and the mean transepithelial resistance (R_t) was 501 ± 138 $\Omega \cdot cm^2$ (mean ± SD; n = 35). Addition of 100 µM ATP to the apical bath increased net fluid absorption from 13.6 ± 2.6 to 18.8 ± 4.6 $\mu l \cdot cm^{-2 \cdot} hr^{-1}$ (mean ± SD; n = 4). We also found that VEGF secretion into the apical bath is half of the secretion to the basal bath (n = 10). In contrast, PEDF secretion is two-fold greater for the apical compared to the basal bath (n = 10).
CONCLUSIONS
A new cell culture procedure has been developed that produces confluent primary hfRPE cultures with morphological and physiological characteristics of the native tissue. Pharmacological perturbations to the apical and basolateral membranes of these monolayers were studied using microelectrode, fluorescence, and fluid transport techniques. Epithelial polarity and function of these easily reproduced primary cultures closely resembled previously studied native hfRPE.

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Methods

Human fetal tissue

The research followed the tenets of the Declaration of Helsinki and the NIH institutional review board (IRB). Fetal eyes were obtained by an independent procurer (Advanced bioscience Resources, Alameda CA), and delivered using overnight priority FedEx service. For delivery, eyes were placed in tubes containing tissue delivery media and packed on ice. All tissues were used less than 26 hrs after enucleation.

Human fetal eye delivery solution

The delivery media was prepared using 5mM KCl, 0.8mM $MgCl_2$, 113.4mM NaCl, 26.3mM NaHCO3, 1mM NaH_2PO4, 3mM Na_2HPO4, 5mM Taurine, 5.6mM Glucose, 1.8mM $CaCl_2$ after adjusting pH to 7.4 using CO2. Additional components included: 20 ml/L - MEM Amino Acid 50X (Sigma), 10ml/L - MEM Vitamin Solution 100X (Sigma), 0.11g/L - Pyruvic Acid sodium (Cell culture tested, Sigma), 10ml/L - Penicillin-Streptomycin 100X (Invitrogen), 10ml/L – GlutaMAX-I 200mM-100X (Invitrogen). Final delivery media was filtered, aliquoted into 15ml centrifuge tubes, and stored in $4^{o}C$ up to 2 months.

Cell culture media

MEM-alpha modification medium (Sigma) was used as the base media to prepare 5% and 15 % serum containing media for culturing RPE cells (RPE media). Fetal bovine serum was used in media preparation (Atlanta Biologicals), screened for toxicity and heat inactivated. Cell culture media also contained: N1 supplement (Sigma) 1:100 ml/ml, Glutamine-Penicilin-Streptomycin (Sigma) 1:100 ml/ml, and non essential amino acids solution (Sigma) 1:100 ml/ml. In addition hydrocortisone (20μg/l), taurine (250 mg/l), triiodo-thyronin (0.013μg/l) were dissolved in PBS to a final concentration 1:500 ml/ml and aliquots stored at -80 ^{o}C until added to the above media.

Ringer solutions

Ringer solutions for the physiology experiments contained the following (in mM): 120 NaCl, 5 KCl, 23 NaHCO3, 1 MgCl2, 1.8 CaCl2, 2.0 taurine, 1 glutathione and 10 glucose. This Ringer solution was bubbled continuously with 5% CO_2 / 10% O_2 / 85% N_2, to a stable pH of ~7.4 and an osmolarity of 295 ± 5 mOsm.

Cell culture preparation

Upon receipt, intact globes were rinsed in antibiotic-antimycotic solution diluted to 10X (Life Technology) plus Gentamicin (1 mg/ml) for 3-5min. Antibiotics were rinsed off twice using RPE 5% serum containing media or PBS. After removing the anterior portion of the eye, the posterior poles were incubated in dispase-I solution (Roche, 2 units/ml) in 5% serum containing media for 30 min in 37 ^{o}C, 5% CO_2. After dispase treatment, the posterior poles were transferred to a 5% serum containing RPE media, dissected into quadrants, and then retina gently removed using forceps. Single cell RPE layers were peeled off in small sheets and collected in 5% serum containing RPE media. The cells were then washed, separated from their syncitium by gently pipeting them, with or without trypsin treatment, and then put into flasks (Primaria) with a 15% serum containing RPE medium. This medium was changed after one day with 5% serum containing RPE media, and subsequent changes were made every 3-4 days. After 3 – 4 weeks the cells became confluent and uniformly pigmented. They were then trypsinized, re-suspended in 15% serum containing RPE cell culture media and seeded onto clear transwells $200x10^3$ per well (Corning Costar). Prior to seeding, the transwells were coated with human extracellular matrix (10μg per well) and cured with UV light in the hood for 2hrs. The same protocol was used to culture cells on inserts and on the flasks. Cells were used in experiments when they had a total tissue resistance of more than 200 $\Omega \cdot cm^2$ and were uniformly pigmented.

Electrophysiology

Calomel electrodes in series with Ringer solutions and agar bridges were used to measure the transepithelial potential (TEP), and an intracellular microelectrode, referenced to either the apical or basal bath, was used to measure the membrane potentials, V_A and V_B, where TEP = V_B - V_A. As previously described (Maminishkis, Jalickee et al. 2002), conventional microelectrodes were made from borosilicate glass tubing of 0.5 mm inner diameter and 1 mm outer diameter with a filament (Sutter Instrument Co., Novato, CA) and were back-filled with 150 mM KCl, and had resistances of 80-200 MΩ.

The total transepithelial resistance, R_T, and the ratio of the apical to basolateral membrane resistance (R_A/R_B) were obtained by passing 2-4 μA current pulses across the tissue and measuring the resultant changes in TEP, V_A, and V_B. Current pulses (i) were bipolar, with duration of 3 sec, and period 30 sec. R_T is the resulting change in TEP divided by current amplitude, and R_A/R_B is the absolute value of the change in V_A divided by the change in V_B ($R_A/R_B = i\Delta V_A/i\Delta V_B$). The current-induced voltage deflections were digitally subtracted from the records for clarity. In the electrophysiology experiments, the black bar indicates a solution change at the manifold outside of the recording chamber. In some cases the response onset was variably delayed because of “dead space” in the fluid delivery system and because of thickness variations in the unstirred layer at the cell membrane.

Fluid transport

Transepithelial water flow measurements were made using a refined capacitance probe technique in a modified Üssing chamber apparatus (Maminishkis, Jalickee et al. 2002; Tripathi S., Banzon T. et al. ARVO 2004 #1092). A capacitance probe (MTI Instruments Inc., Albany, New York) tracked changes in aqueous level as water moved across the RPE by measuring changes in the dielectric between the aqueous phase and the probe. Viability of the tissue was ascertained by recording of transepithelial potential (TEP), and also from transepithelial resistance (R_T) measured by injecting known bipolar currents via Ag/AgCl pellet electrodes. The current method for measuring transepithelial water flow (J_v) of epithelia utilized here combines and improves upon several features of earlier studies (Tripathi S., Banzon T. et al. ARVO 2004 #1092; Edelman and Miller, 1991; Jiang et al., 1993).

Electron Microscopy

Tissues were fixed in 4% glutaraldehide buffered solution at room temperature for 2 hrs and rinsed three times for 5 min in PBS. These tissues then were treated with 1% ice cold osmium tetroxide in PBS solution for 1 hr. After osmication, tissues were rinsed in PBS and processed thru a battery of ethanol dehydration steps (3 washes in 50 %, 70 %, 85 %, 100 % ethanol, respectively) for plastic embedding. A routine transmission electron microscopy protocol was used to obtain the RPE micrograph.

Immunohistochemistry

Coverslips were fixed in 4% formaldehyde for 1 hour. After three washes in PBS, sections were permeabilized with 0.3% Triton X-100 for 10 minutes. Coverslips were incubated in blocker solution (1% BSA, 1% FBS, and 0.3% Triton X-100 in PBS) for 30 minutes, followed by 1 hour in primary antibody (R&D Systems, Sigma, Zymed) in blocking solution. For control slides were was no primary antibody added. After washing with the blocking solution, coverslips were incubated with secondary antibodies (Alexa Fluor 488 and/or 546; Molecular Probes). Coverslips were washed in PBS and covered with antifade mounting medium (ProLong; Molecular Probes, Eugene, OR) Fluorescent images were obtained with a microscope (Axiophot, Carl Zeiss), equipped with CCD camera and Apotome module attached to Axiophot.

mRNA Quantification

Quantitative real-time polymerase chain reaction (Q-RT-PCR) was used to quantify the amount of mRNA of gene in samples. Total mRNA was extracted from the cells in each well, followed by reverse transcription using an oligo-dT primer. Q-RT-PCR was done using TaqMan® Assays-on-Demand™ for six genes of interest (PEDF, VIL2, RPE65, VEGF, CLDN3, and CLDN1); six house keeping genes: (GAPDH, UBC, SDHA, HPRT1, TBP, and YWHAZ) from Applied Biosystems with the ABI PRISM® 7900 Sequence Detection System. PCR for each gene were run in trplicates. A GAPDH standard curve using serially diluted cDNA (1, 1:10, 1:100, and 1:1000) were produced for each run. The mRNA concentration of each gene were normalized against the level of GAPDH (set as one). Ct: cycle at threshold. The relative expression level of genes in RPE grown on transwell and flask were compared as a ratio of transwell to flask (transwell/flask).

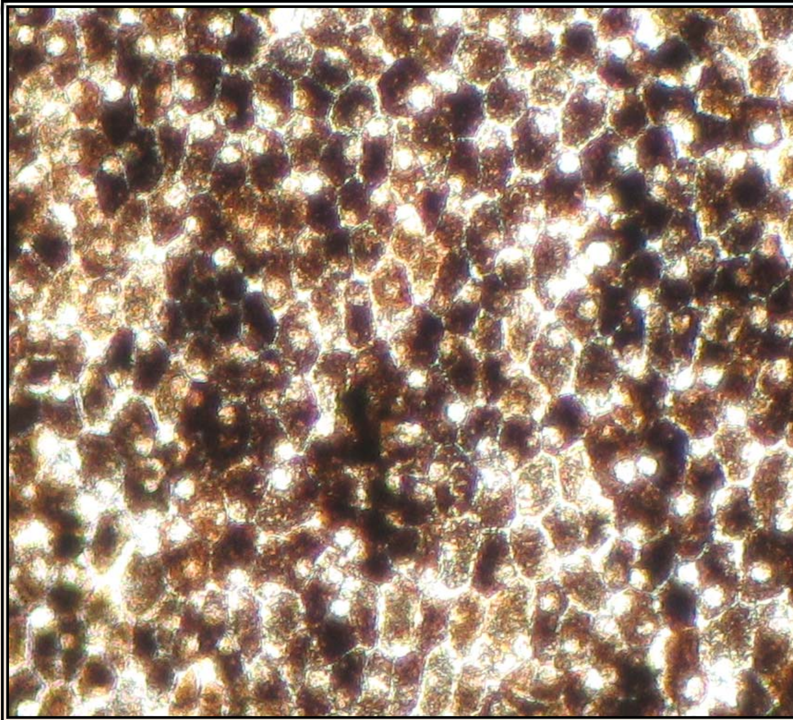
ELISA for human VEGF, PEDF

All steps were carried out at room temperature and each step was followed by three washes with 400 μl/well of washing buffer (0.05% Tween-20 in PBS, pH 7.4). A 96-well EIA plate (Costar, Cambridge, MA) was coated with 100 μl/well of 0.4 μg/ml polyclonal goat anti-human VEGF antibody (R&D System, Minneapolis, MN) overnight in PBS, pH7.4. The plate was blocked by adding 300 μl/well of PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for one hour. A set of seven point VEGF standards (10-1000 pg/well, R&D System, Minneapolis, MN) and samples (cell culture medium) in 100 μl diluent (20mM Tris-HCl, 150mM NaCl, 0.1% BSA, and 0.05% Tween20, pH 7.3) were added in triplicate and incubated for 2 hours. The plate was then incubated with 100 μl/well of 0.2 μg/ml biotinylated goat anti-human VEGF antibody (R&D System, Minneapolis, MN) in diluent for 2 hours. Streptavidin-Alkaline phosphatase (Life Technologies, Gaithersburg, MD, 100μl/well, 1:2000 in diluent) was added to the plate for 30 minutes, followed by its substrate p-nitrophenyl phosphate (pNPP, 1mg/ml in 0.2 M Tris buffer, pH9.8). Optical densities were obtained within 1- 4 hours using a microplate reader at 405 nm with wavelength correction at 570 nm and data were analyzed in Microsoft Excel (Redmond, WA).

PEDF ELISA was done according to kit instructions (Chemicon, CA). Samples were treated with 8M urea for one hour on ice, loaded to pre-coated plate after diluting 1:100 in diluent, and incubated for one hour. Biotinylated mouse anti-human PEDF was added for one hour. Detection with Streptavidin-horsereadish perioxidase and substrates was similar to VEGF ELISA described above.

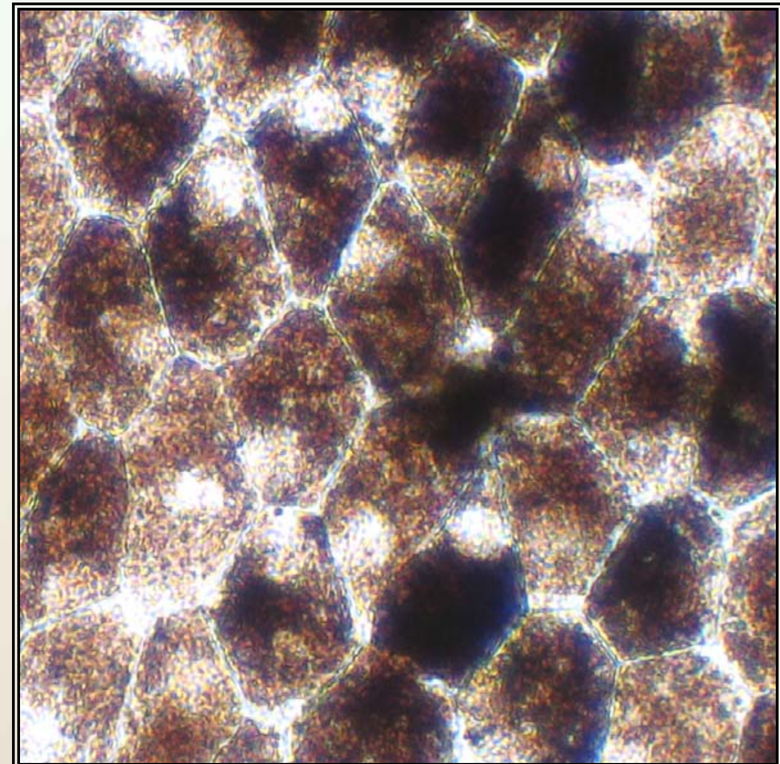
Results

10X Objective



Transmitted light microphotographs of hfRPE cell culture

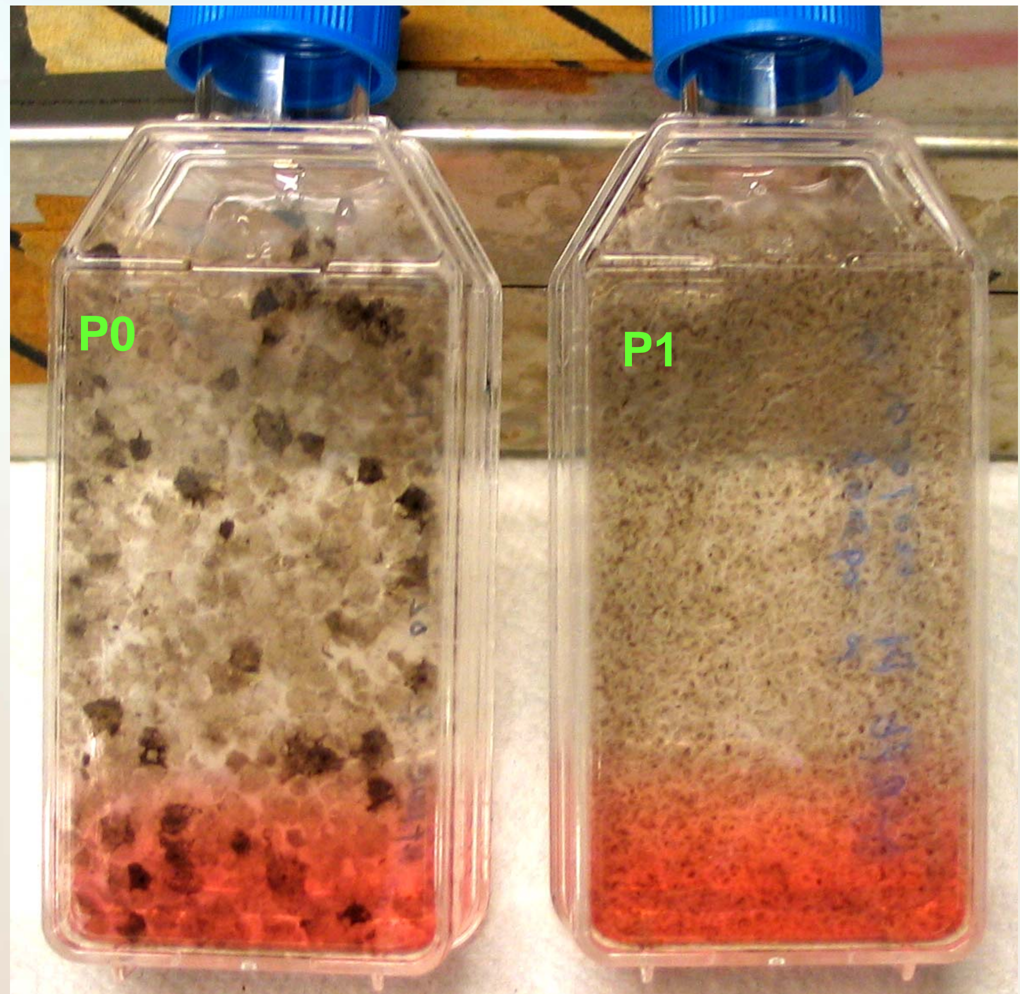
40X Objective



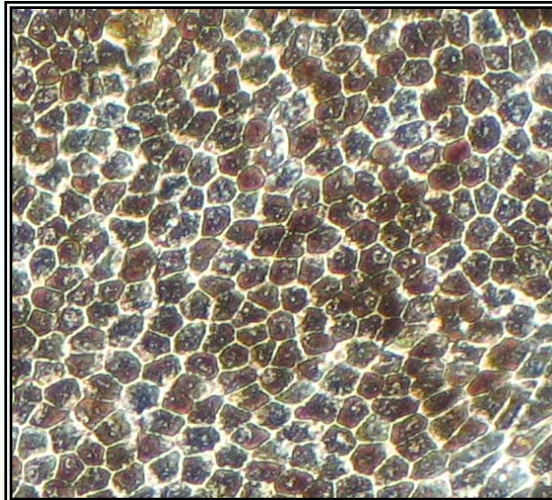
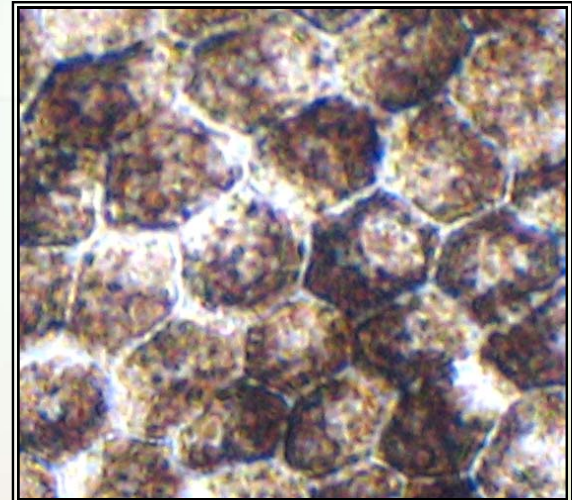
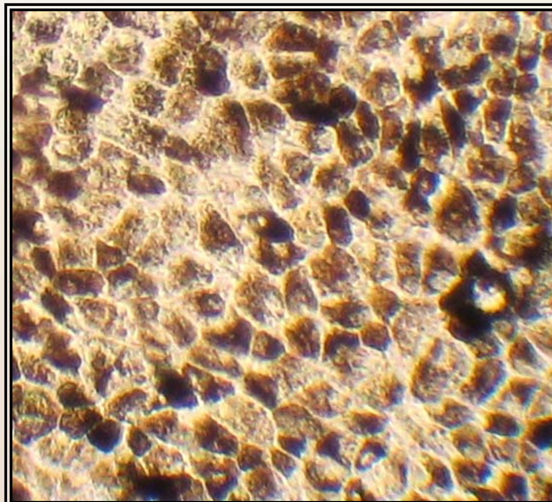
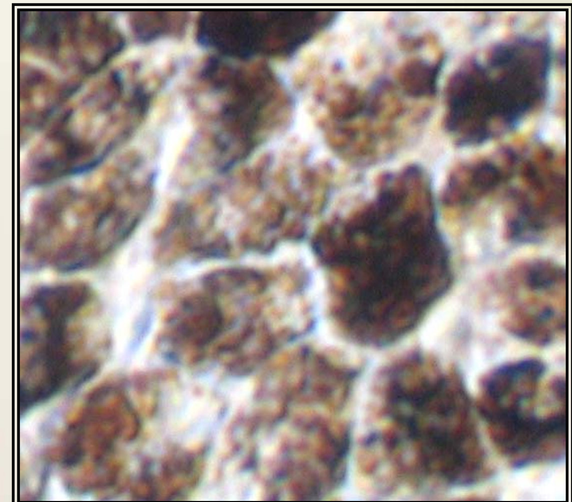
15 month old RPE cell culture growing in the flask

Six week old hfRPE cell culture growing in Primaria® uncoated flasks. For this picture, flasks are vertical (media settled to bottom). In both flasks hfRPE cells exhibit dark pigmentation. Each pigmented "island" of cells is formed from native or initially seeded cells (P0).

Trypsinization of P0, breaks hfRPE monolayer into smaller fragments that after seeding (P1) forms a more uniformly pigmented monolayer of RPE cells. The presence of contaminant cells (endothelial, fibroblasts, etc…) is noticed as large non-pigmented areas.

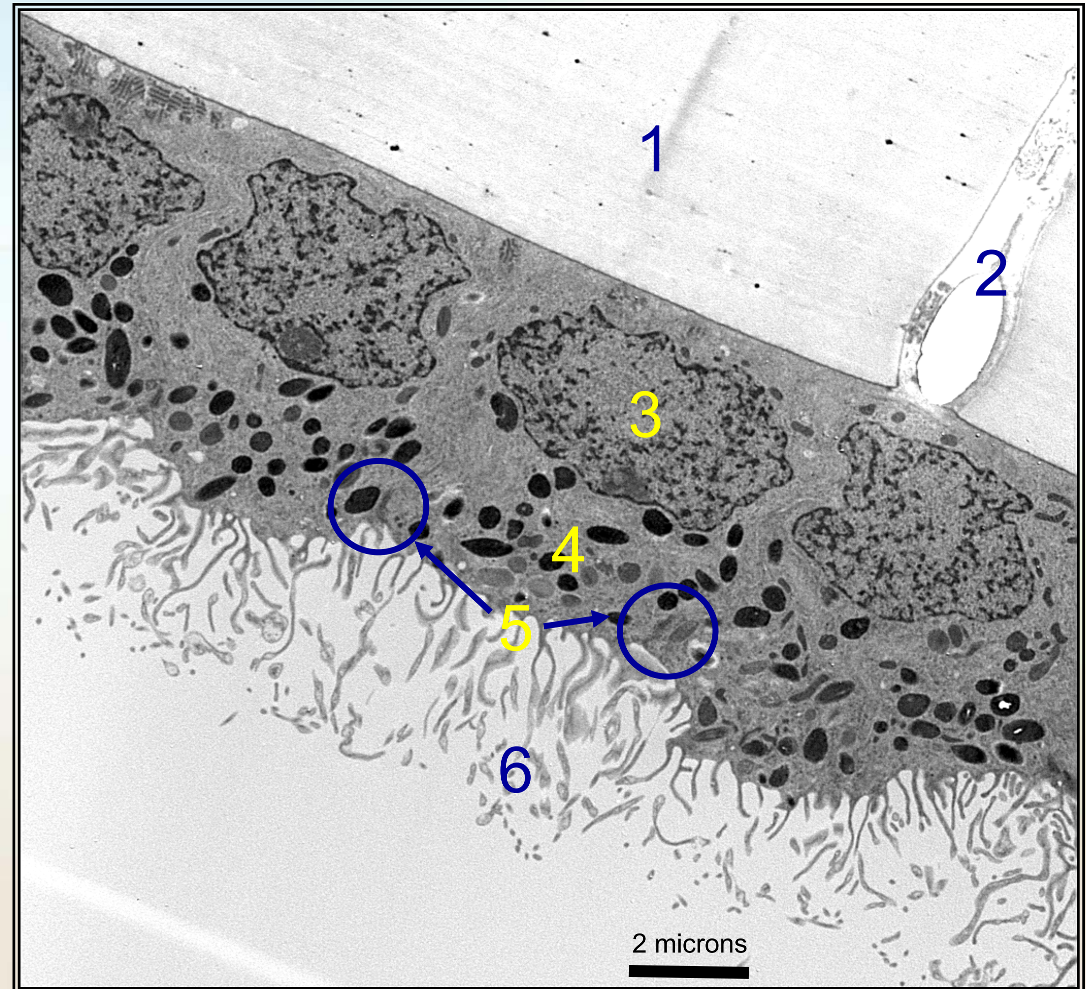


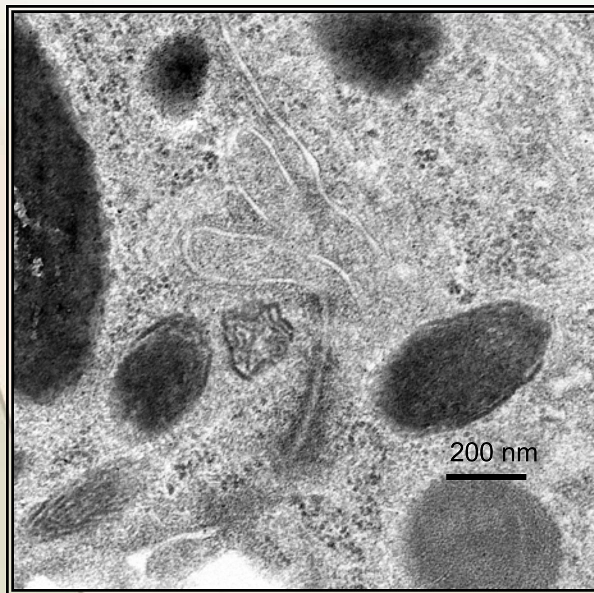
Comparison of six week old cell cultures growing in Primaria® flasks (A, B) and hfRPE cultures, also 6 weeks old, growing on cell culture inserts coated with hECM (C, D). Microphotographs of cell culture taken with 2 different microscope objectives – 10X (A, C) and 40X (B, D). Image D looks out of focus because of difficulties imaging cells on semitransparent cell culture insert with inverted microscope.

	10X	40X
Flask		
Insert		

Results

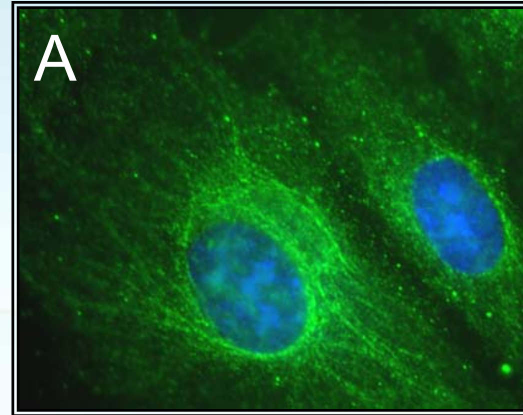
Right panel: EM (x5000) microphotograph of RPE cell culture growing on human ECM coated inserts 1 – porous plastic support; 2 – pore in plastic membrane; 3 – cell nucleus; 4 – melanin pigment; 5 – tight junctions; 6 – microvilli.

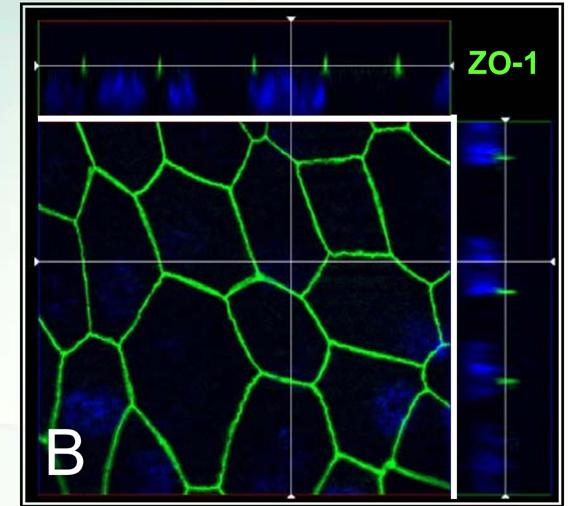




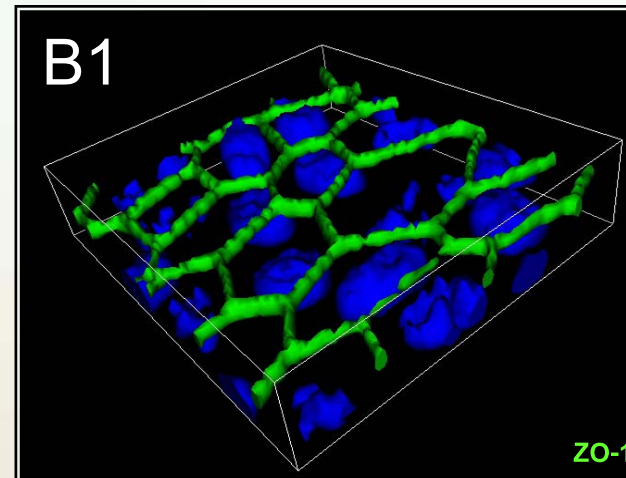
Left panel: EM (x25000) microphotograph of tight junction between cells obtained from hfRPE cell culture grown on insert.

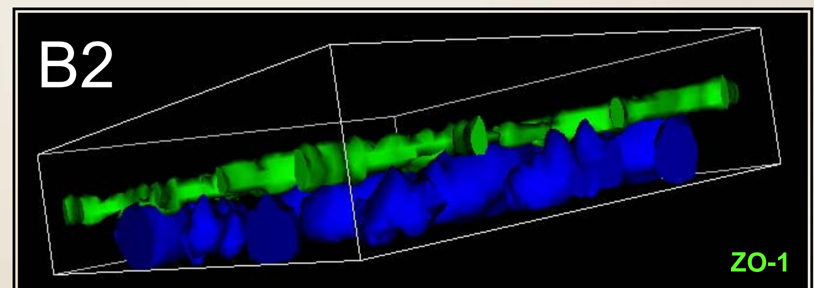
Panels A, B, and C show immunohistochemistry for RPE cell markers. Panel A shows cytokeratin labeling in non-confluent RPE cells. In panel B (B1, B2), green fluorescent antibodies label the tight junction protein ZO-1. Blue – DAPI nuclear stain. Panels B1 and B2 show 3D rendering of ZO-1 and nuclear labeling in hfRPE cell cultures. Panel C shows occludin labeled in green in tight junctions.

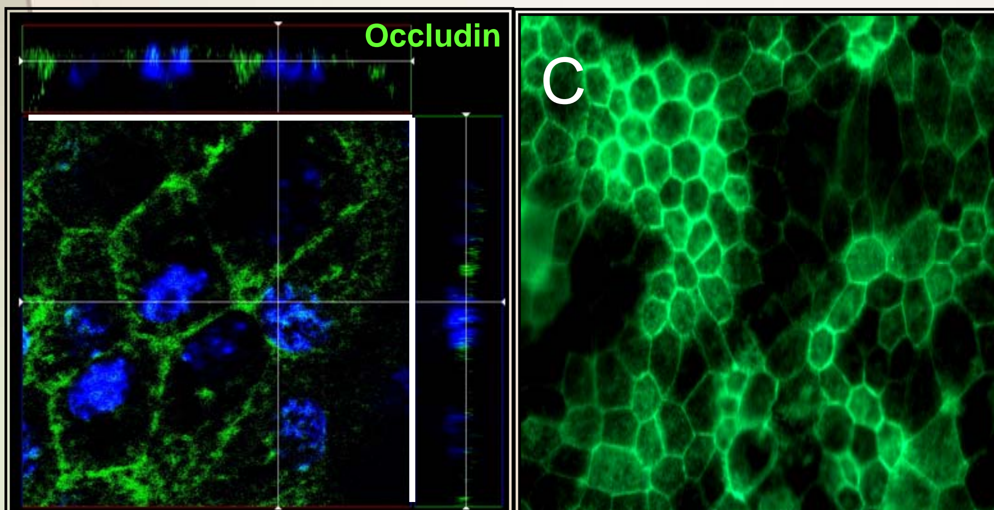




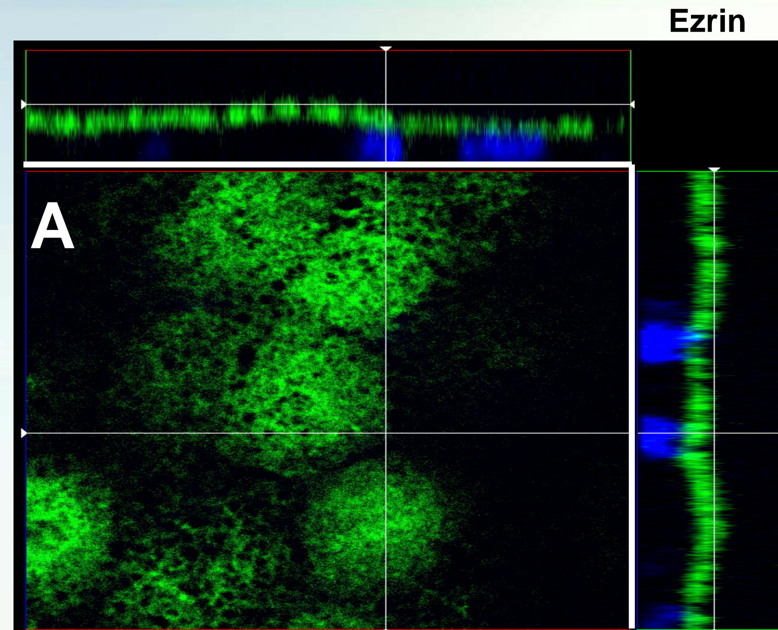
Virtual cross section through Z-stack

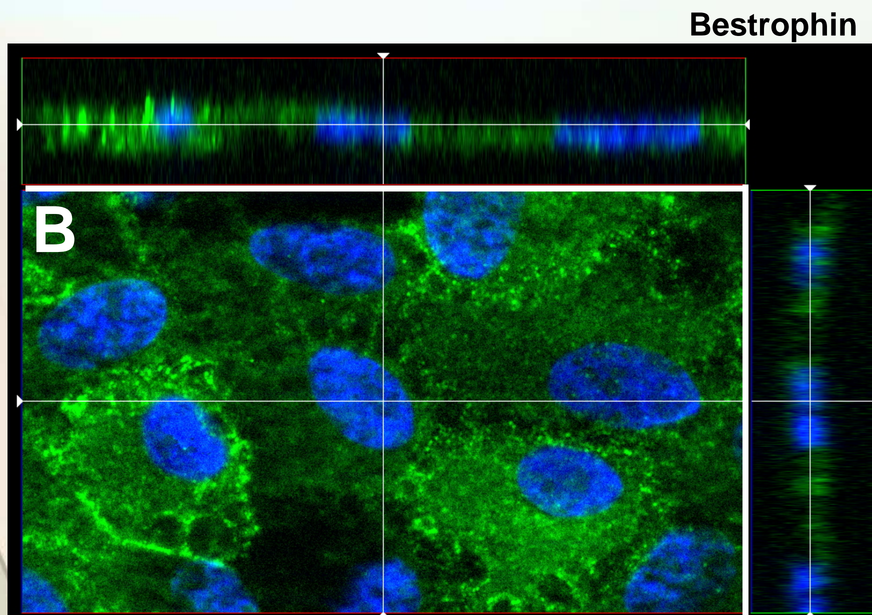


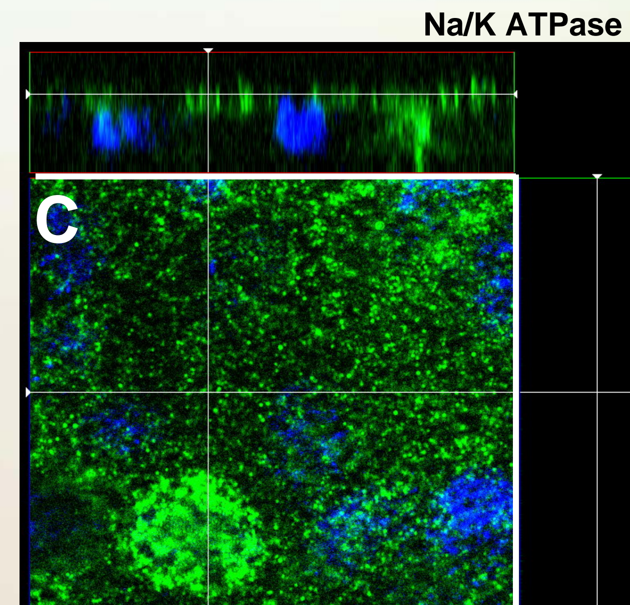


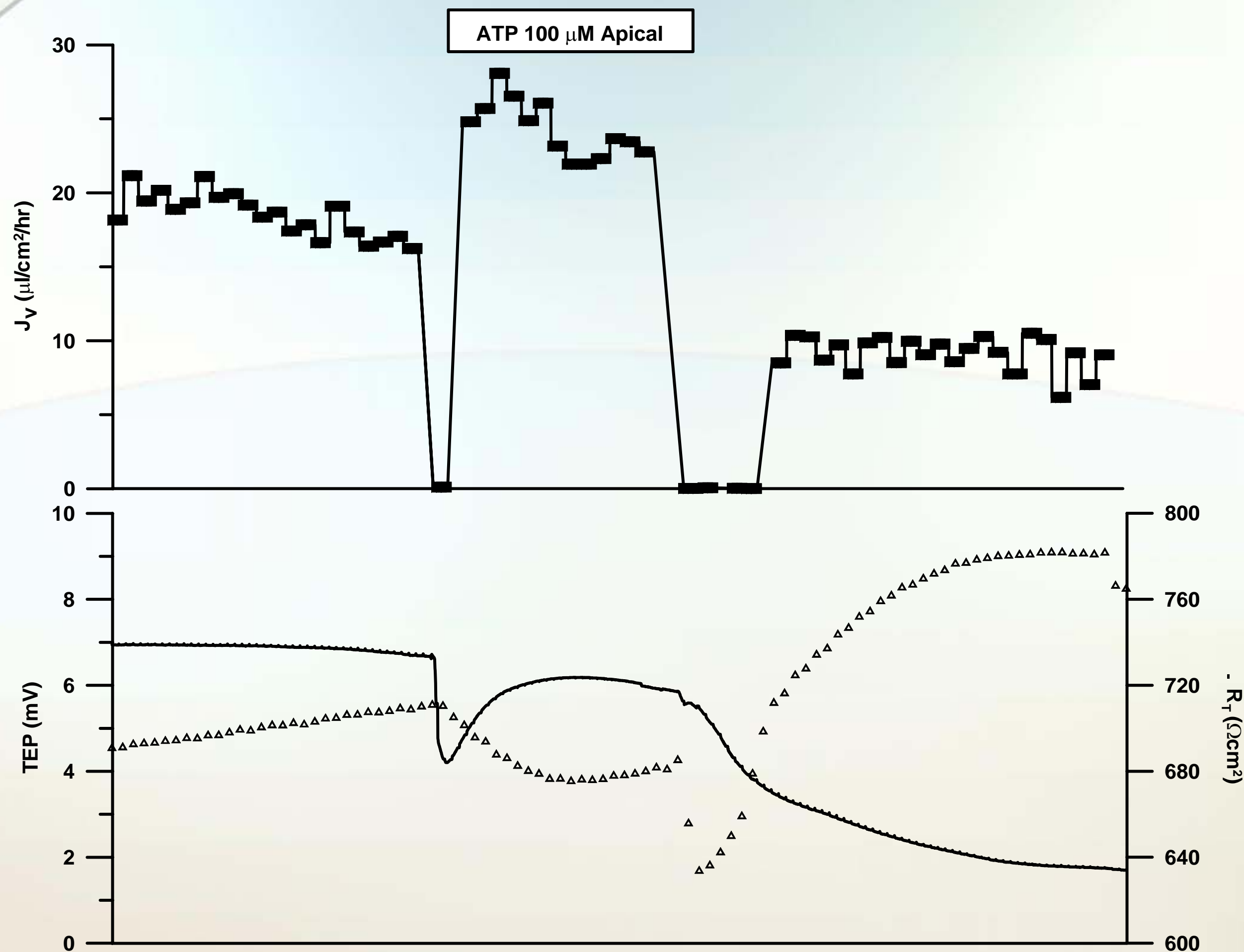


Immunohistochemistry staining of hfRPE cell cultures for A – ezrin, B – bestrophin, C – Na/K ATPase. Ezrin has been localized to the apical side of cells (above the nuclei), bestrophin to basolateral side, and Na/K ATPase primarily to apical side of hfRPE cell culture.



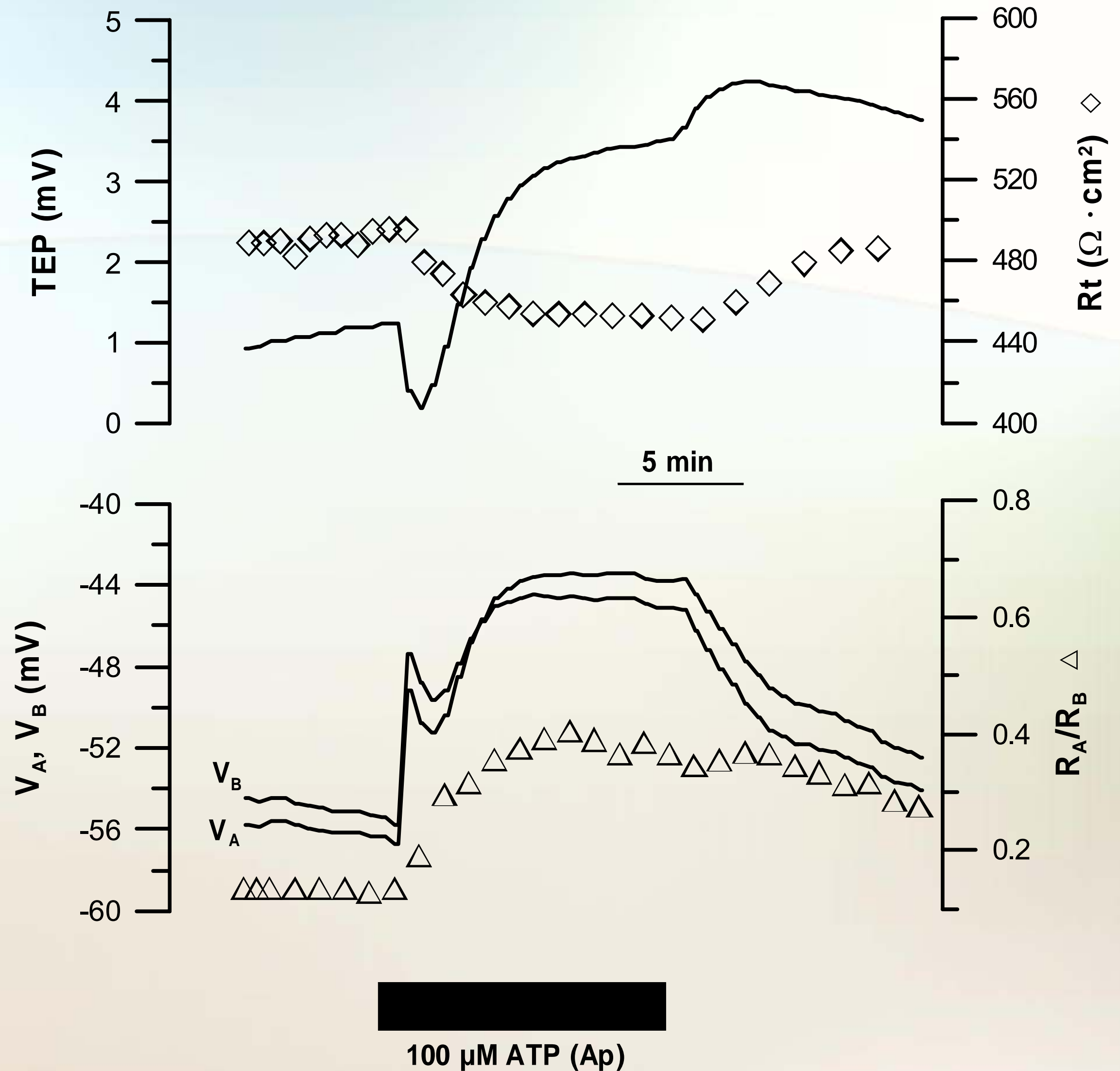




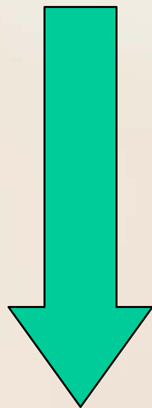


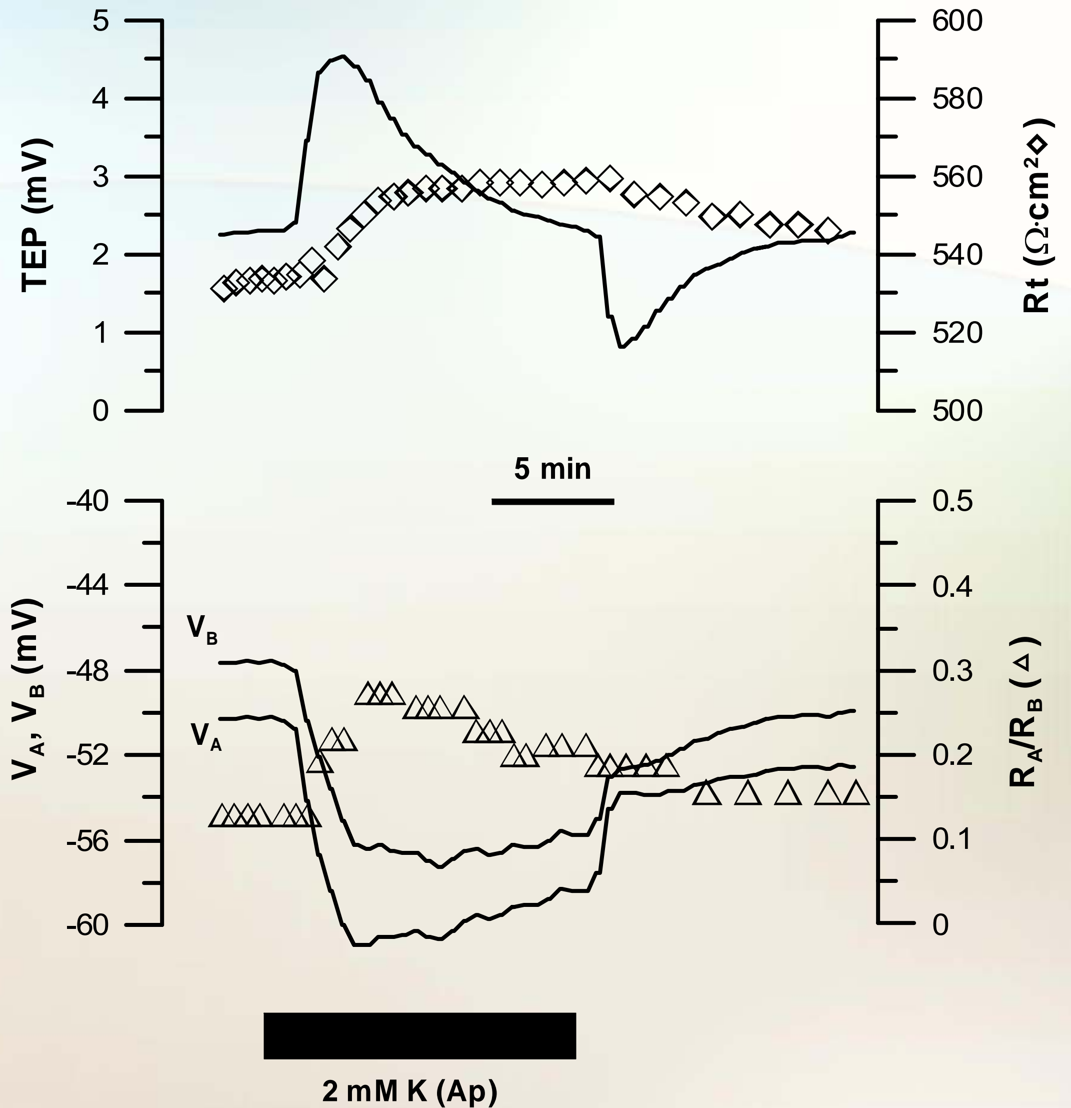
Addition of ATP to the apical side of RPE increases fluid absorption ~ 10 µl/cm²/hr. This increase in the intact adult eye (5 cm2) would lead to removal of 1.2ml of fluid per day, a clinically significant amount. Electrical responses are consistent with measurements using intracellular microelectrode techniques and indicates basolateral membrane Cl channel opening, as previously shown on native tissues.

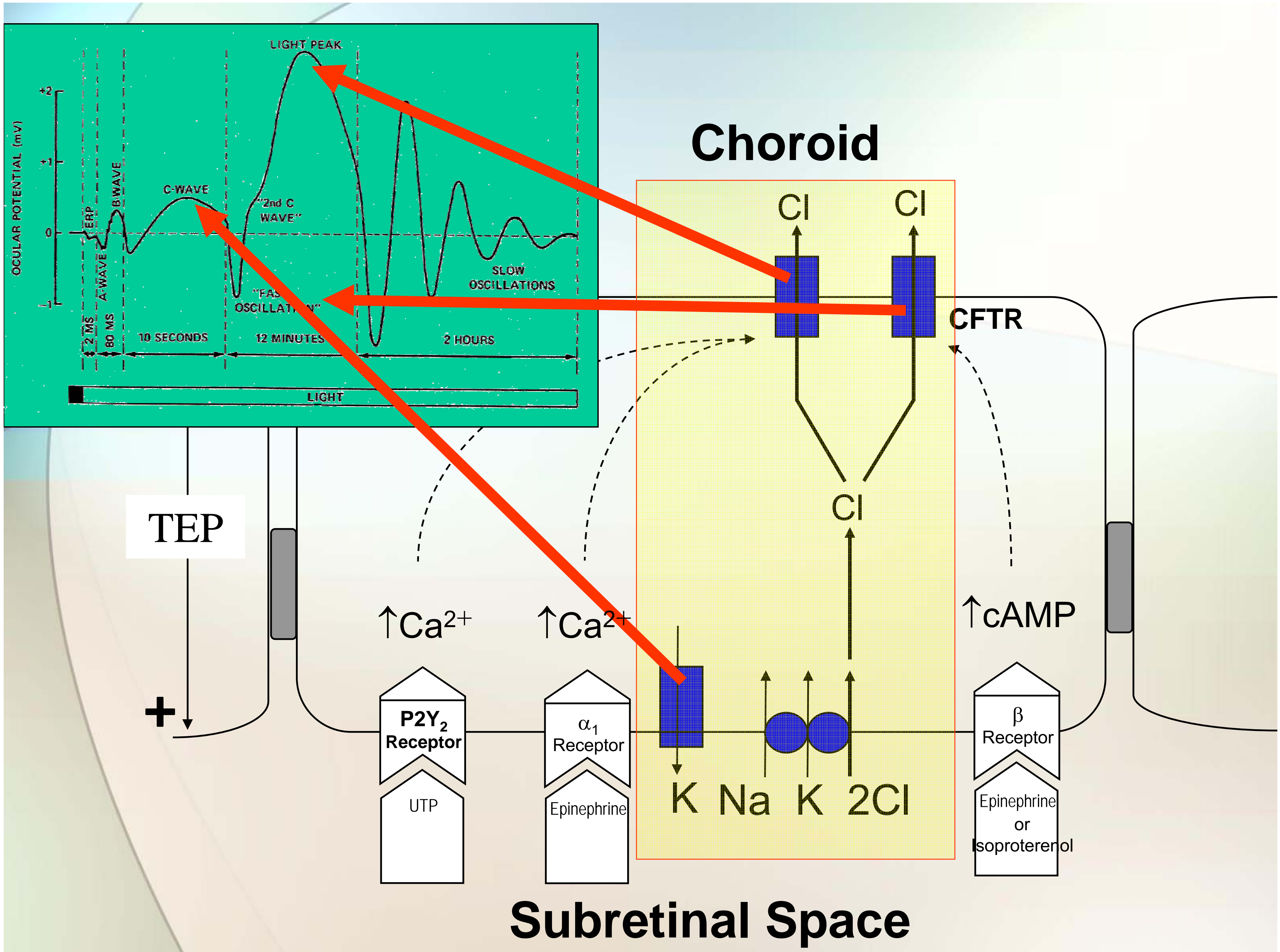
Following addition of ATP, the TEP and basolateral membrane voltages underwent changes consistent with basolateral membrane Cl channel opening. During these changes R_T decreased and R_A/R_B increased, consistent with an decrease in RB. The voltage and resistance changes show that there was an increase in basolateral membrane Cl conductance. In three experiments, similar to this figure, the mean ATP – induced depolarization of V_B was 14.8 ± 2.8 mV. Concomitantly, the decrease in R_T was 35.6 ± 14.1 $\Omega \cdot cm^2$ while R_A/R_B increased by 0.24 ± 0.06.



The apical membrane voltage response to a step decrease in apical K from 5 to 2 mM. This increase can be completely blocked by addition of Ba^{2+} to the apical bath (see bellow).

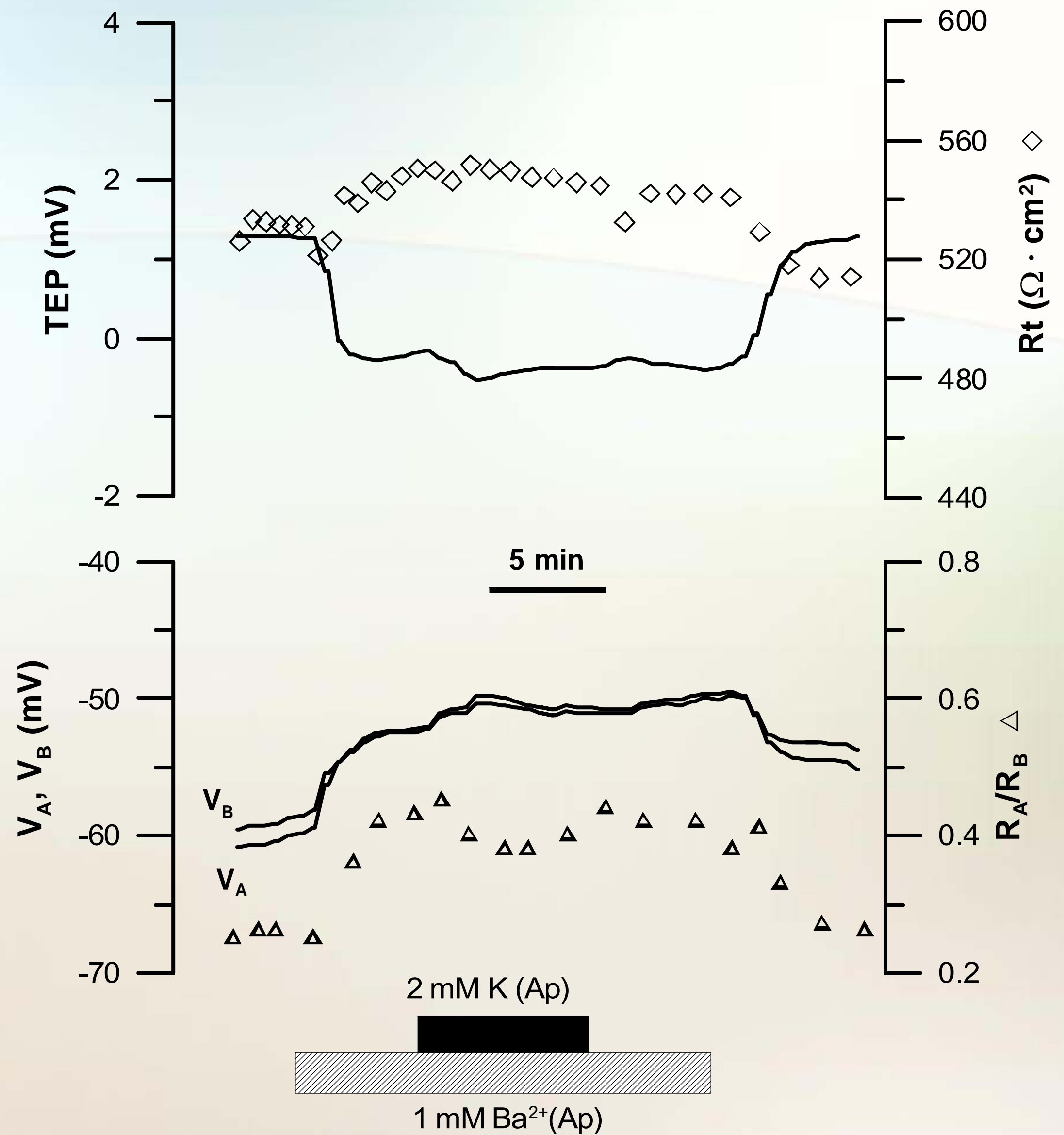


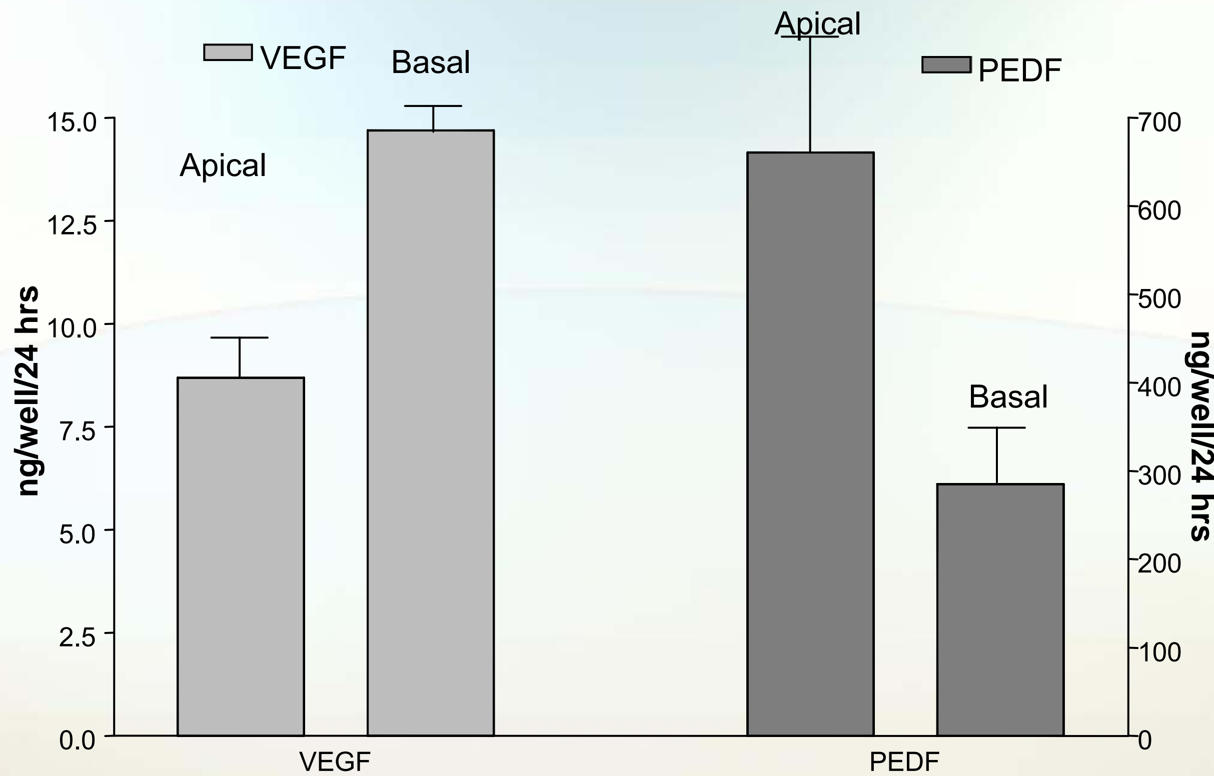




Choroid
Subretinal Space
LIGHT PEAK
OCULAR POTENTIAL (mV)
+2
+1
0
-1
ERP
A-WAVE
B-WAVE
C-WAVE
"2nd C WAVE"
"FAST OSCILLATION"
SLOW OSCILLATIONS
2 MS
80 MS
10 SECONDS
12 MINUTES
2 HOURS
LIGHT
Cl
Cl
CFTR
Cl
TEP
+
↑Ca2+
↑Ca2+
↑cAMP
P2Y2 Receptor
UTP
α1 Receptor
Epinephrine
K
Na
K
2Cl
β Receptor
Epinephrine or Isoproterenol

Apical membrane voltage response to a step decrease in apical K from 5 to 2 mM is blocked by apical Ba^{2+}.





Cultured hfRPE cells exhibit vectorial protein secretion similar to native tissue. Panel above shows secretion of VEGF and PEDF proteins in hfRPE cell culture monolayer grown on inserts.

Relative quantitation of gene expression in human fetal RPE grown on transwell and flask by Q-RT-PCR

	Transwell				Flask				Transwell / flask
Gene	Qty Mean ±SD	CV	Ct Mean±SD	CV	Qty Mean ±SD	CV	Ct Mean±SD	CV	Qty Mean
PEDF	2.5858±0.0336	1.3%	15.67±0.02	0.13%	4.1613±0.1011	2.4%	15.05±0.04	0.24%	0.62
VIL2	0.1528±0.0036	2.4%	19.96±0.04	0.18%	0.2329±0.0036	1.5%	19.43±0.02	0.12%	0.66
RPE65	0.0247±0.0016	6.3%	22.72±0.09	0.42%	0.0531±0.0060	11.3%	21.68±0.18	0.82%	0.47
VEGF	0.0242±0.0011	4.4%	22.75±0.07	0.29%	0.0370±0.0003	0.9%	22.22±0.01	0.06%	0.66
CLDN3	0.0036±0.0002	4.3%	25.63±0.06	0.25%	0.0046±0.0002	5.4%	25.39±0.08	0.32%	0.79
CLDN1	0.0022±0.0001	2.4%	26.37±0.04	0.14%	0.0021±0.0001	4.5%	26.55±0.07	0.25%	1.04
GAPDH	1.0000		17.11		1.0000		17.21		1.0000
UBC	0.1354±0.0029	2.2%	20.14±0.03	0.16%	0.1720±0.0030	1.8%	19.89±0.03	0.13%	0.79
SDHA	0.0105±0.0001	1.1%	24.02±0.02	0.07%	0.0136±0.0002	1.6%	23.74±0.02	0.10%	0.77
HPRT1	0.0048±0.0001	1.0%	25.21±0.02	0.06%	0.0050±0.0003	5.9%	25.25±0.09	0.35%	0.95
TBP	0.0019±0.0001	2.8%	26.61±0.04	0.16%	0.0024±0.0002	7.2%	26.37±0.11	0.41%	0.79
YWHAZ	0.0010±0.0000	2.8%	27.62±0.04	0.16%	0.0020±0.0000	1.5%	26.62±0.02	0.09%	0.48

Conclusions

- A newly developed cell culture method can be used to produce primary RPE cell cultures with highly reproducible morphology and physiology sustainable over 15 months.

- These cells are very similar in morphology to native hfRPE, have a regular honeycomb cell shape, express tight junction proteins (eg, ocludin, ZO-1), cytoskeletal proteins (eg, cytokeratin, ezrin) and standard transport proteins (eg, bestrophin, Na/K ATPase).

- The physiological responses of these cells, their protein expression and secretion are similar to that found in native tissue.

Acknowledgements

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