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### **Paper:**

Churm, R., Dunseath, G., Prior, S., Thomas, R., Banerjee, S. & Owens, D. (2019). Development and characterization of an in vitro system of the human retina using cultured cell lines. *Clinical & Experimental Ophthalmology*  
<http://dx.doi.org/10.1111/ceo.13578>

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1 Development and characterization of an  
2 *in vitro* system of the human retina  
3 using cultured cell lines  
4

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9 **Running title**

10 Novel triple-culture of the human retina

11

12

13 **Conflicts of interest-** None.

14 **Funding Source-** St David's Medical Foundation

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19

20 **Abstract**

21 **Background:** Previously developed *in vitro* cultures of the human retina have been solo  
22 or dual cell cultures. We developed a triple-cell culture *in vitro* model utilizing a  
23 membrane system to produce a better representation of a functional and morphological  
24 human retina.

25 **Methods:** Retinal microvascular endothelial cells (HRMVEC/ACBRI181, Cell systems),  
26 retinal pigment epithelium cells (RPE/ARPE-19, ATCC) and Müller glial cells (MIO-M1,  
27 UCL) were grown in a triple-culture. Our optimized triple-culture media contained a mix  
28 of specific endothelial medium and high glucose Dulbecco's Modified Eagle's medium  
29 (DMEM), where all three layers were viable for up to 5 days. Co-culture effect on  
30 morphological changes (cell staining) and gene expression of functional genes (pigment  
31 epithelial derived factor (*PEDF*) and vascular endothelial growth factor (*VEGF*)) were  
32 measured from RNA via real time PCR. Expression of tight junction protein 1 (*TJP1*) was  
33 measured in RNA isolated from ARPE-19s, to assess barrier stability.

34 **Results:** The triple-culture promotes certain cell functionality through up-regulation of  
35 *TJP1*, increasing *PEDF* and decreasing *VEGF* expression highlighting its importance for  
36 the assessment of disease mechanisms distinct from a solo culture which would not  
37 allow the true effect of the native microenvironment to be elucidated.

38 **Conclusion:** This model's novelty and reliability allows for the assessment of singular  
39 cellular function within the retinal microenvironment and overall assessment of retinal  
40 health, whilst eliminating the requirement of animal-based models.

41 **Key words;** Human retina, Cell culture, Triple-culture, *In vitro* model

42

43        **1. Introduction**

44        The human eye is a complex organ that is comprised of three main areas; the cornea,  
45        lens, and the retina. The retina is a highly specialised organ of photoreception, involved  
46        in translating light energy into action potentials which are relayed to the brain, where  
47        the information is processed into vision. The integrity of the retina is dependent upon  
48        its immediate microenvironment, which is reliant on cell-cell interactions of the  
49        different human retinal cellular components. Disruption of which results in a variety of  
50        retinal diseases including diabetic retinopathy (DR)<sup>1</sup>. DR is associated with the  
51        breakdown of both the inner blood-retinal-barrier (BRB)<sup>2,3</sup> and more recently linked to  
52        the outer BRB<sup>4</sup>. The inner BRB is composed of tight junctions between retinal capillary  
53        endothelial cells and the outer of tight junctions between the retinal pigment epithelial  
54        cells (RPE). The inter-relationship between vascular and endothelial cells that form the  
55        BRB is vital maintaining a specialized environment of the neural retina.

56        Current findings show that BRB breakdown is multifactorial, including; impaired  
57        endothelial cells, pericyte demise, thickening of capillary basement membrane and the  
58        alteration of tight junctions between RPE and endothelial cells<sup>5</sup>. Additionally RPE,  
59        endothelial and other cell types within the retina can be responsible for the release of  
60        neurotrophic factors that can alter the integrity of the BRB, such as vascular endothelial  
61        growth factor (VEGF) and pigment endothelial derived factor (PEDF)<sup>6-8</sup>. These factors  
62        can also be released from Müller cells<sup>8-10</sup>, which are a subset of the retinal macroglia  
63        that are strongly linked to RPEs, establishing structural connections with the subretinal  
64        space and choroidal vasculature<sup>8, 11</sup>. It is evident that research into DR requires a

65 complex system that can encompass the interaction of all cells present within both the  
66 inner and outer BRB.

67 Currently research into retinal disease utilizes two main methodologies; *in vitro*  
68 analysis of solo or dual cell cultures, and animal models. The current literature and  
69 project designs relating to RPE cultures are often limited to them encompassing a dual-  
70 culture system with endothelial cells. The culture formations published comprise of cells  
71 either in direct contact<sup>12</sup>, sandwiched with extracellular matrix proteins<sup>13, 14</sup>, or cells  
72 cultured on either side of a membrane<sup>15-17</sup>. Alternatively, there are published models  
73 that encompass a dual system where these cells do not come into contact<sup>18</sup>. There are  
74 very few multi-culture systems within literature, with those published comprising of  
75 mixed species cell sources<sup>19</sup>. We aim to expand on current *in vitro* models to develop a  
76 triple-culture which utilizes both human cell lines and primary cells, to demonstrate a  
77 better representation of a functional and morphological human retina.

78

## 79 **2. Methods**

### 80 **Cell line and isolated primary cell culture**

#### 81 **Retinal pigment epithelial cells**

82 RPE were authenticated and sourced as a commercially available cell line; ARPE-19  
83 (ATCC® CRL-2302, USA). Upon receipt were grown in Dulbecco's Modified Eagle's  
84 medium (DMEM) containing glutamine supplemented with 10% fetal bovine serum  
85 (FBS) and 1% penicillin-streptomycin (Invitrogen-Gibco, Rockville, MD). Cells were

86 incubated at 37°C at 5% CO<sub>2</sub>, with media changed every 2-3 days and sub-cultured (1:3  
87 split) at 80% confluency with 0.05% trypsin-EDTA (Invitrogen-Gibco, Rockville, MD).

### 88 **Müller glial cells**

89 Human Moorfield Institute of Ophthalmology-Müller 1 (MIO-M1) cell line were isolated,  
90 authenticated and purchased from Professor Limb (Institute of Ophthalmology and  
91 Moorfields Eye Hospital, UK)<sup>20</sup>. Upon arrival, cells were cultured in the same media as  
92 ARPE-19, incubated at 37°C at 5% CO<sub>2</sub> and media changed every 2-3 days.

### 93 **Retinal microvascular endothelial cells**

94 Primary human retinal microvascular endothelial cells (HRMVEC) are commercially  
95 available, sourced from Applied Cell Biology Research Institute (ACBRI 181, Cell Systems,  
96 Kirkland, USA). All experimental procedures completed using primary cells were in  
97 compliance with the Human Tissue Act. Cells were grown in Cell Systems Culture (CS-C)  
98 medium, incubated at 37°C at 5% CO<sub>2</sub>, with media changed every 2-3 days. Cells were  
99 passaged when confluent using Cell Systems Passage Reagent (all reagents sourced from  
100 Cell Systems, Kirkland, USA) and used in experiments at passage 8.

101

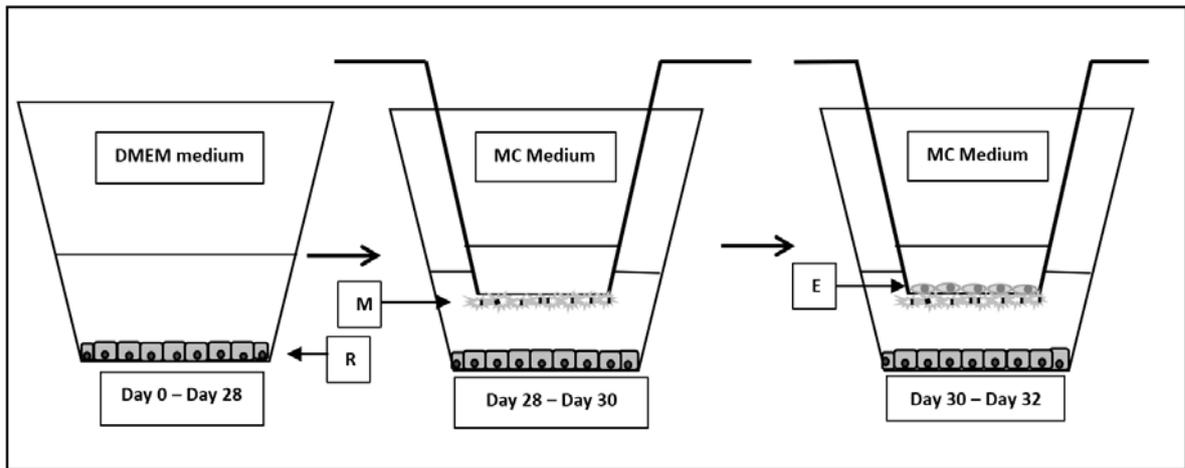
### 102 **Co-culture formation**

103 To assess cell viability in a microenvironment, cells were grown in solo, dual (MIO-M1 &  
104 ARPE-19, ARPE-19\_& HRMVEC and MIO-M1 & HRMVEC)\_and triple formations (ARPE-19,  
105 MIO-M1 & HRMVEC), using the following protocol.

106

### 107 **System set-up**

108 During solo-, dual- and triple-culture formations all cell types were grown in the same  
109 location as seen within triple-culture schematic (Figure 1). Multi-culture system utilizes  
110 a 0.4  $\mu\text{m}$  pore size polyester (PET) Transwell® membrane with a 10  $\mu\text{m}$  membrane  
111 thickness (Corning, Thermo Scientific, UK). Cells were plated according to Figure 1, using  
112 an in-house optimized multi-culture media (MC media) consisting of 2:1 DMEM with  
113 glutamine: CS-C medium.



114

115 **Figure 1.** Triple-culture schematic for ARPE-19 (R), MIO-M1 (M) and HRMVEC (E). Annotated medium is  
116 the culturing medium present in both well insert and culture dish well as described in methodology, multi-  
117 culture. (MC).

118

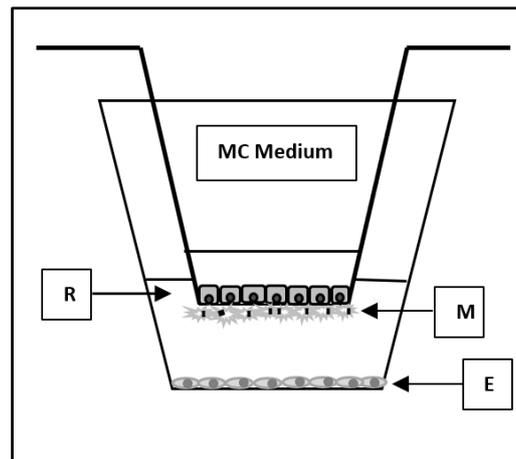
### 119 **Cell seeding and maturation**

120 ARPE-19 was grown on the base of a 6-well culture dish, as shown (Figure 1). Cells were  
121 prepared for all experiments at passage 6, split ratio 1:3, requiring maturation and tight-  
122 junction formation for 28 days with growth media changed every 2-3 days (Supp. Figure  
123 1A). At day 28, MIO-M1 cells were seeded at 100,000 cells per  $\text{cm}^2$  in 150  $\mu\text{L}$  (passage  
124 34) onto the basal side of a Transwell® membrane by inverting the membrane structure  
125 and incubated for 3 hours at 37°C at 5%  $\text{CO}_2$ . Once adhered, excess medium was

126 removed, the membrane reversed and placed in the 6-well culture plate containing the  
127 ARPE-19 cells. At day 32, HRMVEC were seeded at 100,000 cells per cm<sup>2</sup> to the apical  
128 surface of the membrane insert washed with attachment factor, and grown in 1.5 mL  
129 MC medium, cells reached optimal confluency (70%) for experimentation after 48 hours  
130 (day 34). MC media of well and insert was replenished every 2-3 days, cell viability valid  
131 for up to 5 days of construction of complete model (i.e. day 37) due to cellular  
132 characteristics and phenotypes of specific cell lines being lost after prolonged culture (>  
133 14 days)<sup>20</sup>.

134 For permeability assay, an alternative layout was assembled to assess barrier  
135 properties as original triple-culture layout does not allow for this. Cells were cultured as  
136 described previously, but with cell seeding and maturation occurring in alternative  
137 positions i.e. ARPE-19 grown on apical surface of the membrane, MIO-M1 on basal  
138 surface and HRMVEC on bottom of the culture well (Figure 2).

139



140

141 **Figure 2.** A- Alternative formation of multi-culture to assess Transwell<sup>®</sup> permeability in a multi-culture. R-  
142 ARPE-19, M-MIO-M1 and E-HRMVEC.

143

#### 144 **Morphological changes**

145 Immunocytochemical analysis was conducted to ensure the presence of correct cell  
146 types within culture (See Supp. Work 1 and Supp. Figure 1). Basic cell morphologies and  
147 relative diameter were determined using Hematoxylin (Sigma) staining and digital  
148 imaging via light microscopy. Cell quantification, density and diameter were assessed  
149 using ImageJ software (NIH, Version 2). Limitations of a basic cell imaging only allows  
150 data to be used as a relative check for cell morphology instead of a quantification of  
151 absolute size. Each experimental well produced images in 5 fields, each field was pre-  
152 defined and set prior to cell seeding to remove experimental bias. A total of 20 cells per  
153 type at x50 magnification were analysed to determine cell diameter and morphologies  
154 (n=100 cells for data analysis, per biological repeat) (representation of images provided  
155 in Supp. Figure 2). Each cell type was analysed within 3 biological repeats.

156

#### 157 **Gene expression analysis**

158 All expression data was collected from each culture layout from three biological repeats  
159 and three experimental repeats per plate (n=9 per culture layout). Individual cells types  
160 were harvested at day 32 of culture, homogenized in QIAzol lysis reagent and total RNA  
161 isolated using a RNeasy Mini Kit (Qiagen, UK). 1000ng/ $\mu$ l of total RNA was converted to  
162 cDNA using a reverse transcription kit (Invitrogen, Thermo Fisher, UK). 1:10 dilutions of  
163 cDNA transcripts were run on a CFX connect (BioRad, UK) using SYBR Green I (BioRad,  
164 UK) for each primer set (**VEGF**: Forward-ACT TCT GGG CTG TTC TC, Reverse-TCC TCT TCC  
165 TTC TCT TCT TCC; **PEDF**: Forward-TGC AGG CCC AGA TGA AAG GG, Reverse-TGA ACT CAG

166 AGG TGA GGC TC. **TJP1**: sourced and optimized from BioRad, UK) at optimal cycling  
167 conditions of; 95°C for 3min, followed by 35 cycles of 95°C for 30s, 59.5°C for 30s and  
168 72°C for 30s, followed by melt peak conditions. The average Ct value was  
169 taken from triplicate assays and normalised against the invariant expression of  $\beta$ -  
170 *actin* housekeeper gene. Result were analysed using the  $2^{-\Delta\Delta Ct}$  method to produce  
171 relative fold change values in comparison between groups, standard error of the mean  
172 (SEM) was calculated from the average Ct value for each sample produced within the  
173 experiment cohort. Fold change range of -1.5 to 1.5 is indicative of no overall change in  
174 gene expression levels.

175

#### 176 **Permeability assay**

177 Permeability assays were completed to assess barrier properties in a mixed cell  
178 environment, based on a horseradish peroxidase (HRP) (Sigma™) diffusion ELISA (n=9,  
179 per culture layout). For permeability assays, DMEM without FCS was used at all stages.  
180 An alternative layout of both dual- and triple-culture was used to assess Transwell®  
181 permeability due to the functional properties of each cell type. To assess the implication  
182 of culture formation on both ARPE-19 and HRMVEC, they were grown on the apical and  
183 basal side of the membrane, respectively. Medium within the well and insert  
184 compartments were replaced for assay media and the insert compartment dosed with  
185 HRP [1250 mU], incubated for 20 minutes and medium from the lower well  
186 compartment collected for analysis. Culture media was transferred to a 96-well clear  
187 microplate plate for colorimetric analysis via 3,3',5,5'-Tetramethylbenzidine (TMB) and  
188 stop solution (Invitron, UK) and absorbance recorded at a wavelength of 450 nm and

189 corrected at 620 nm. Membrane permeability was analysed in comparison to a control  
190 membrane with no cellular growth. Relative permeability was produced as a  
191 percentage;

$$192 \quad \text{Relative Permeability (\%)} = \frac{\text{Culture Experiment Value}}{\text{Control Value}} \times 100$$

193

### 194 **Statistical analysis**

195 Raw data was analysed using SPSS™ Version 23. Continuous data is summarized by mean  
196 and standard deviation (SD) when normally distributed, and by median and interquartile  
197 range (IQR) if not normally distributed. Normality of the data was verified by the  
198 Kolmogorov-Smirnov test and visualised on q-q plots. A Student t-test was used to  
199 compare the mean of two groups and analysis of variance (ANOVA) to compare the  
200 mean of more than two groups for normally distributed data. Alternatively, not normally  
201 distributed data was analysed using Mann Whitney U and Kruskal Wallis tests. For gene  
202 expression data, statistical analysis was run on threshold cycle (Ct) data normalised  
203 against *β-actin* housekeeper, assessed using Student t-test. P-values less than 0.05 were  
204 deemed statistically significant.

205

## 206 **3. Results**

### 207 Cell morphology

208 Across all three cell types, no significant alterations in cell morphologies were noted in  
209 any culture formation (Table 1). Analysis of HRMVEC morphologies using cellular  
210 staining and microscopy was unable to produce sufficient images within the triple-  
211 culture to assess cell diameter. RNA spectroscopy did confirm cell growth on the apical

212 surface and we believe a lack of imaging reflect limitations within methodology. Gene  
 213 expression of *TJP1* was measured in RNA isolated from ARPE-19 to assess barrier stability  
 214 in a multi-culture system. Data indicates a significant increase in fold change (FC) for  
 215 *TJP1* when RPE are grown in combination with either MIO-M1 (+11.4 FC, p<0.001),  
 216 HRMVEC (+12.9 FC, p<0.001) or both in a triple-culture (+7.8 FC, p<0.001) (Figure 3).  
 217 Barrier permeability was not assessed within the primary formation of the dual- and  
 218 triple-culture as described in methodology.

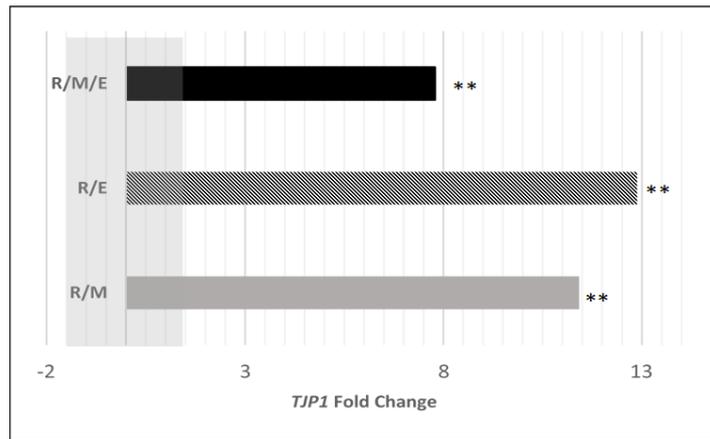
219

220 **Table 1.** Mean and standard deviation of cell diameter ( $\mu\text{M}$ ) for the three cell types grown in all culture  
 221 formations. P-value determined using one-way ANOVA.

222

Culture Formation	Cell Type		
	<u>ARPE-19</u> (n=300)	MIO-M1 (n=300)	HRMVEC (n=300)
Solo	17.5 (6.1)	62.7 (13.3)	98.9 (24.4)
Dual		61.9 (10.6)	97.8 (28.0)
Dual	18.1 (4.3)		96.0 (22.9)
Dual	17.9 (4.5)	63.8 (13.9)	
Triple	18.2 (5.3)	62.5 (14.3)	-
P-value	0.48	0.54	0.66

232



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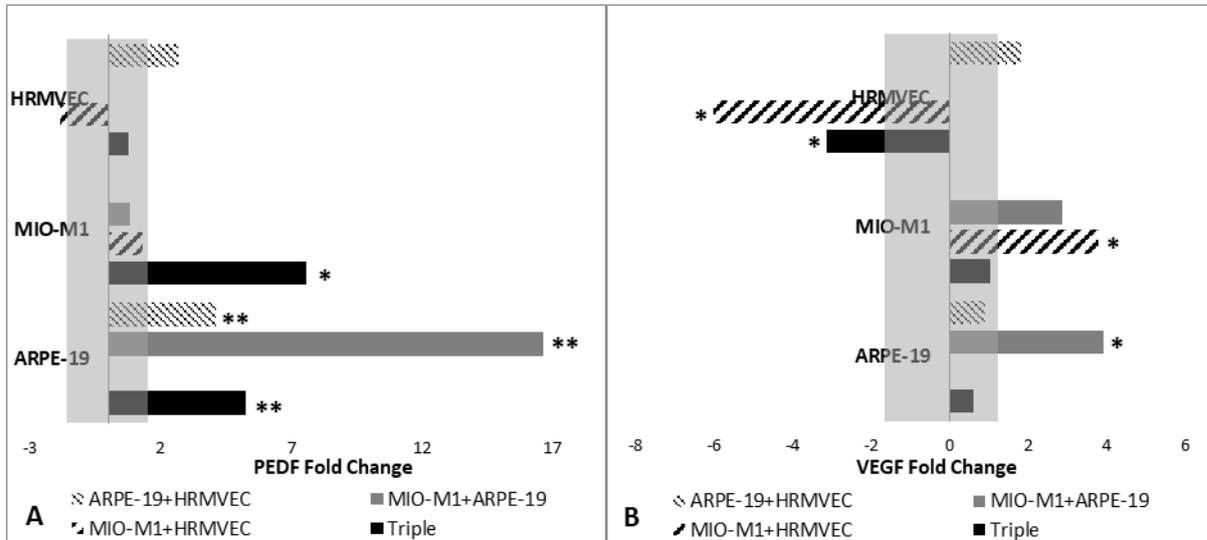
234 **Figure 3.** Relative fold change values for *TJP1* gene expression data in multi-culture formations in  
 235 comparison to solo-culture. Fold change between -1.5 and 1.5 is classed as no relative gene expression  
 236 change (grey shaded region). Statistical significance represented by asterisks (\*\* $p < 0.01$ ), determined  
 237 using an independent t-test. R-ARPE-19, M-MIO-M1 and E-HRMVEC.

238

239 *Implications of multi-culture on gene expression levels*

240 *PEDF* expression (Figure 4A) demonstrated an increase in FC in both ARPE-19 (+5.2 FC,  
 241  $p < 0.01$ ) and MIO-M1 (+7.5 FC,  $p < 0.05$ ) in triple compared to solo-culture. Triple  
 242 compared to solo-culture demonstrated no significant FC in *VEGF* expression in either  
 243 MIO-M1 (+1.0 FC,  $p = 0.49$ ) or ARPE-19 (+0.6 FC,  $p = 0.34$ ) (Figure 4B). However, expression  
 244 was significantly decreased within HRMVEC (-3.2 FC,  $p < 0.05$ ). Sub-analysis of dual-  
 245 cultures implicates MIO-M1 for the observed change in *VEGF* expression (dual-culture  
 246 of; MIO-M1+HRMVEC, -6.0 FC,  $p < 0.05$  and dual-culture; ARPE-19+HRMVEC, +1.80 FC,  
 247  $p = 0.11$ ).

248



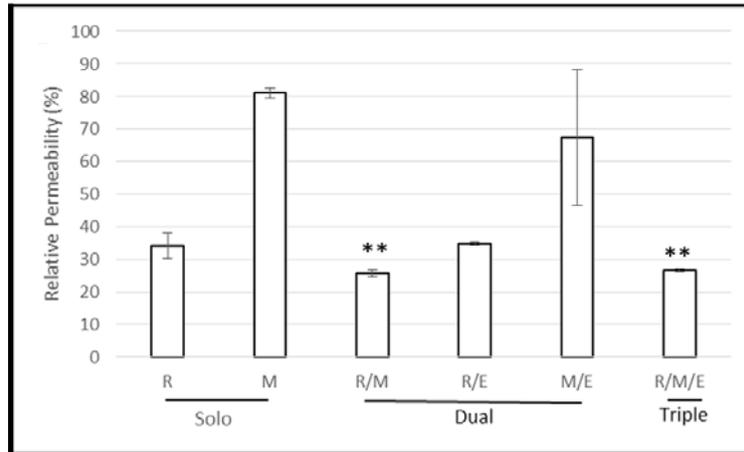
249

250 **Figure 4A-B.** Relative fold change values for gene expression data in multi-culture formations in  
 251 comparison to solo-culture. Fold change between -1.5 and 1.5 is classed as no relative gene expression  
 252 change (grey shaded region). (A) *PEDF*-fold change & (B) *VEGF*-fold change. Statistical significance  
 253 represented by asterisks (\* $p<0.05$ , \*\* $p<0.01$ ), determined using an independent t-test of the difference  
 254 of  $\Delta Ct$  in multi-culture formation of cells versus solo-cultured cells.

255

256 *Assessment of multi-culture on membrane properties*

257 HRP diffusion across the Transwell® membrane altered significantly dependent on the  
 258 cells grown on the surface. ARPE-19 monolayer formation resulted in the diminishing of  
 259 HRP passing through the membrane, this barrier was significantly strengthened in the  
 260 presence of MIO-M1 grown on the basal side of the membrane in both a dual- and triple-  
 261 culture (ARPE-19 vs ARPE-19/MIO-M1; 34.1 (3.9)% v 25.7 (1.2)%:  $p<0.01$ ; ARPE-19 vs  
 262 ARPE-19/MIO-M1/HRMVEC; 34.1 (3.9)% vs 26.5 (0.5)%:  $p<0.01$ ) (Figure 5). Diffusion of  
 263 molecules through the Transwell® membrane is significantly reduced by cell growth on  
 264 the surface, data demonstrated minimal relative permeability is 25.7% ( $\pm 1.2$ ) indicating  
 265 cross membrane molecule diffusion is still occurring.



266

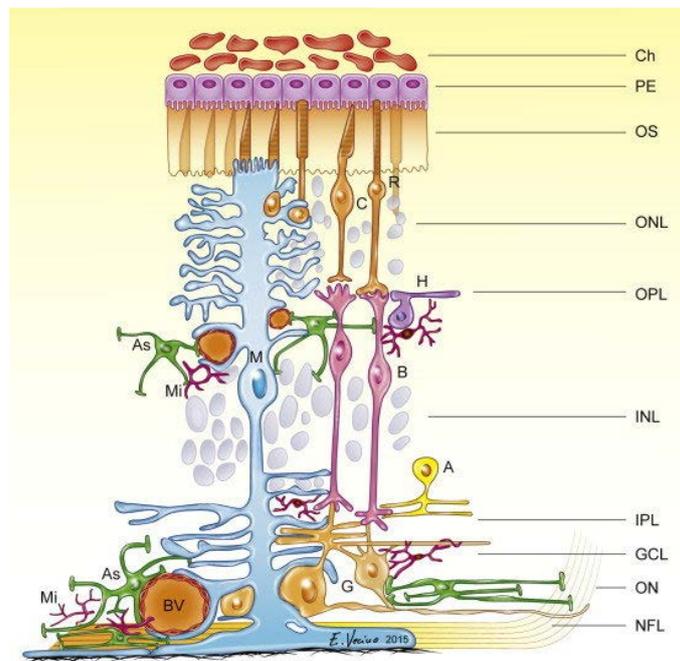
267 **Figure 5.** HRP diffusion assay, relative permeability (%) for all cell formations that require growth on the  
 268 Transwell® membrane, error bars indicate standard deviation. Statistical significance represented by  
 269 asterisks (\*\* $p < 0.01$ ), determined using an independent t-test. R-ARPE-19, M-MIO-M1 and E-HRMVEC.

270

#### 271 **4. Discussion**

272 This study presents the evidence for a novel human *in vitro* model of the retina. This  
 273 model is reproducible and provides flexibility in formation and structure to maximise  
 274 the yield of experimental research that can be conducted, including mechanistic  
 275 research into retinal disease, retina biology and pharmacology intervention. The culture  
 276 model allows for the arrangement of cellular components that mimic the retinal  
 277 microvasculature, neuronal retina and RPE *in vivo*, allowing us to explore the tissues  
 278 cellular microenvironment. The triple-culture model represents the close contacting  
 279 relationship between the Müller glial cells (MIO-M1) and the blood vessels (HRMVEC)  
 280 within the retina and the distal location of the non-contacting RPE (ARPE-19) retinal  
 281 layer (Figure 6)<sup>21</sup>. These cell types in particular play a pivotal role in the function of the  
 282 BRB and investigation of their cross-communication could lead to great enhancements  
 283 for understanding retinal disease that results in, or is the result of, BRB malfunction.

284 Our findings highlight the importance of cell to cell interaction in retinal biology,  
 285 with multi-culture systems resulting in changes in both structural and genetic alterations  
 286 when compared to solo-cultures. On the other hand, the assessment of cell morphology  
 287 resulted in no significant changes when the cells were grown in solo or in formations  
 288 with each other. However, the methodology used to assess morphology is a basic  
 289 technique and is limited in quantification and should only be utilised as an indication of  
 290 cell type presence. We propose that within future expansion of this model, investigation  
 291 into an optimized confocal analysis of cell markers across the membrane will confirm  
 292 and quantify our original findings.



293  
 294 **Figure 6.** Schematic drawing of the cellular components of the retina taken from Vecino *et al*, 2016<sup>21</sup>. The  
 295 different cell types are situated in a standard large mammalian retina, depicting the interactions between  
 296 the Müller cells in blue (M) and blood vessels (BV) (represented in triple-culture model with HRMVEC).  
 297 Different layers of the retina; optic nerve (ON), nerve fibre layer (NFL), ganglion cell layer (GCL), inner  
 298 plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), outer  
 299 segment layer (OS), retinal pigment epithelium (RPE) & choroid (Ch).

300 Structurally, these findings indicate a significant 11- and 7-fold increase in gene  
301 expression for *TJP1* or often referred to as zonula occludens (ZO1), when RPE are grown  
302 in either a solo-culture, or in combination with MIO-M1 as a dual-culture or in a triple-  
303 culture. This is also reflected within the barrier properties of the cultures, indicating a  
304 significantly strengthened barrier when MIO-M1 cells were grown on the basal side of  
305 the membrane. Previously published findings have associated Müller glial cell with  
306 tightened tight junctions and upregulated and polarized localization of specific blood-  
307 brain-barrier transporters<sup>19, 22</sup>. However, expansion of these findings would require  
308 protein analysis to determine the downstream impact.

309 Within DR and retinal disease, often researched is the alteration of key genes.  
310 Gerhardinger and colleagues report that in mice, 6 months post streptozotocin  
311 induction of diabetes, resulted in the alteration of 78 genes within Müller glial cells<sup>23</sup>.  
312 Over a third of these genes were reported to be associated with inflammation and  
313 inflammatory response including cytokines such as VEGF<sup>5, 23</sup>. These inflammatory  
314 cytokines can initiate a cascade of events within the retinal microenvironment and result  
315 in dysfunction of various cell types. The findings of this model indicate that within a  
316 triple-culture, expression of *VEGF* was significantly altered within HRMVEC; we  
317 hypothesise this is due to the presence of MIO-M1 cells. *PEDF* expression is often  
318 demonstrated in literature to promote the health of the BRB<sup>24, 25</sup>; within this model we  
319 show a marked increase in *PEDF* gene expression. We hypothesise that the triple-culture  
320 system promotes the functionality of the BRB, not only structurally through the  
321 promotion of *TJP1/ZO1*, but also by an increase in the expression of beneficial growth  
322 factors such as *PEDF* and decreasing the expression of detrimental growth factors such

323 as *VEGF*. This data highlights the significance of using a multi-culture system within the  
324 assessment of disease mechanism, as a solo-culture would not elucidate the effect of  
325 the native environment due to the implications of additional cell types. Let alone this  
326 model does not represent all cell types present within the retina, it does provide species  
327 specificity and three cell type interactions, therefore improving on the current retinal  
328 models available and published.

329 Often disputed in literature, is the use of cell lines in model design due to the  
330 presence of developmental abnormalities<sup>17, 26</sup>. The prolonged culture of both ARPE-19  
331 and MIO-M1 can result in abnormalities in characteristic features of the cell such as the  
332 loss of pigmentation in ARPE-19 and MIO-M1 exhibiting progenitor characteristics<sup>27</sup>.  
333 However, within our study design we have allowed for maturation and low passage  
334 numbers to ensure manipulation of culture conditions will result in minimal difference  
335 from parent tissue<sup>26</sup>. The superior use of an alternative cell source i.e. primary cell  
336 sources will yield a truer representation of the native tissue. However, it can also  
337 produce poor efficiency of the model in practice allowing for restricted use of repeats  
338 and minimise use in pharmacological testing. Additionally, models do not allow for the  
339 manipulation of culture environments to exacerbate a disease effect, for example;  
340 chronic exposure to high glucose and varying glycaemic levels during cell seeding and  
341 growth, to induce phenotypes seen in DR. Due to difficulties in sourcing human primary  
342 cells for the modelling of the retina, to date only a rodent *in vitro* model<sup>19</sup> and no human  
343 multi-culture system have been published. We propose the compromise of two highly  
344 characterized and routinely used cell lines ARPE-19<sup>28-30</sup> and MIO-M1<sup>20, 27</sup>; and one  
345 primary sourced cell origin ACBRI-181 within this model, allowed for an *in vitro* multi-

346 culture model to be utilized in place of either an *in vitro* mixed species model or an *in*  
347 *vivo/in vitro* animal model.

348 In conclusion, our methodology provides evidence for a novel multi-cell *in vitro*  
349 culture model of the human retina, for the assessment of retinal biology and disease  
350 mechanisms. The versatility of the model allows the assessment of both singular cellular  
351 function within the retinal microenvironment in addition to a triple-cell assessment of  
352 retinal structure and function in health and disease.

353

354

### 355 **Acknowledgement**

356 We would like to express a great gratitude to the St David's Medical Foundation for  
357 funding this project.

358

## 359 References

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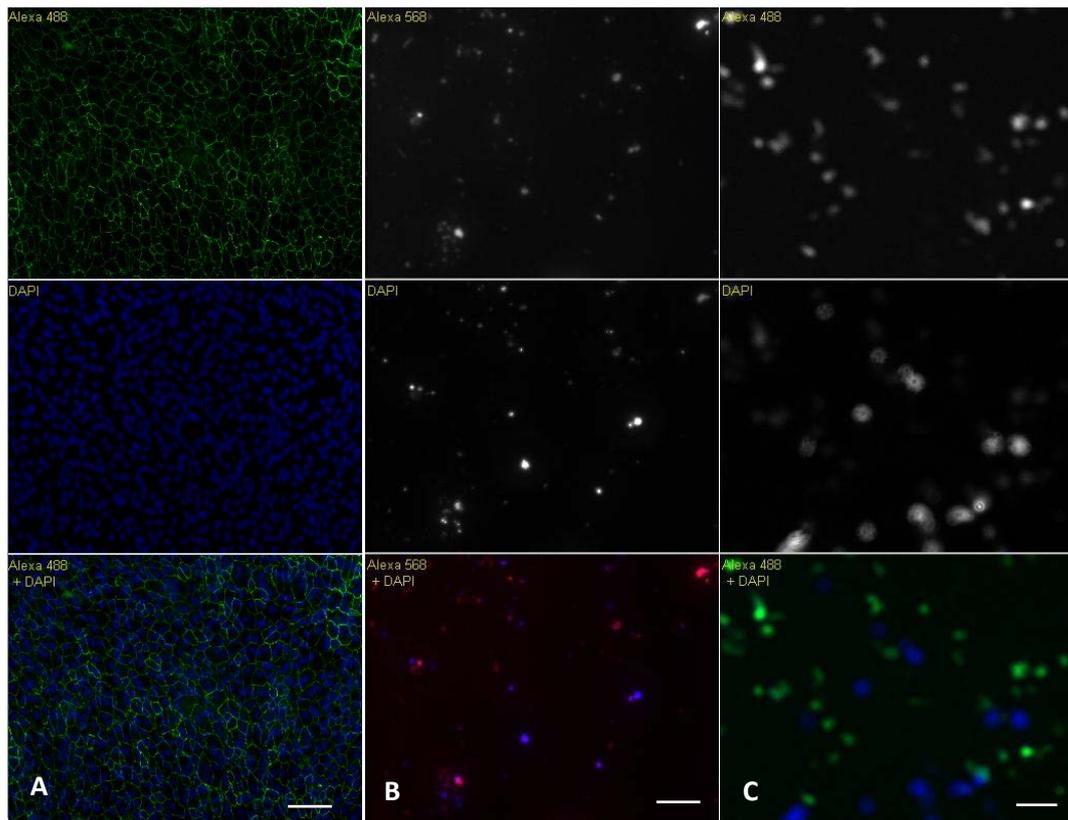
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448 **Supplementary Work**

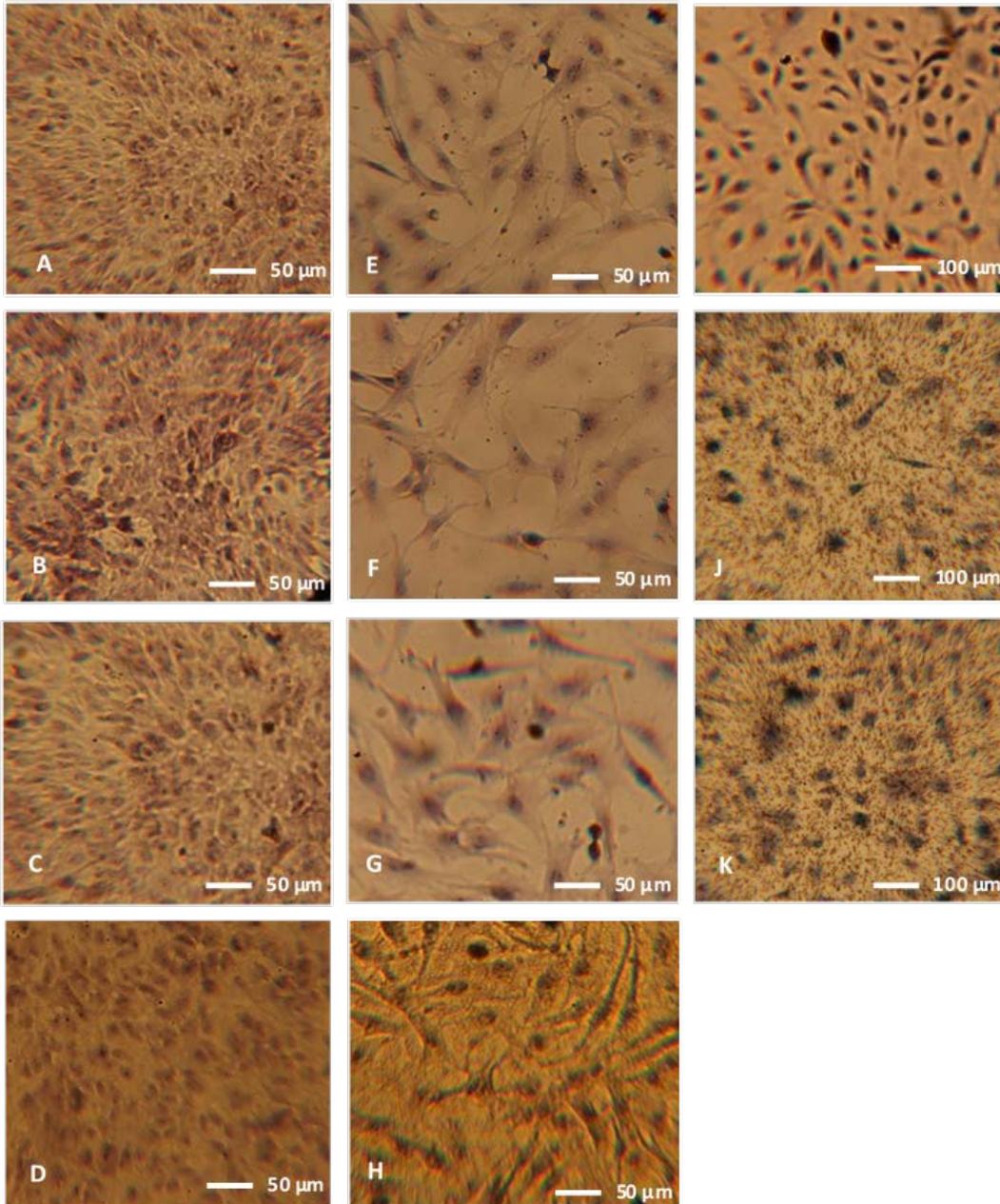
449 **Fluorescent immunocytochemical staining and imaging of cultures**

450 RPE were grown on 0.1% gelatin coated microscope cover slips and left to mature for 28  
451 days. Both MIO-M1 and HRMVEC were grown as described on Transwell® membrane.  
452 All culture formations (solo, dual and triple) followed the system set up as stated, they  
453 were then rinsed in PBS and fixed in ice-cold PBS containing 4% formaldehyde for 30  
454 minutes. Cultures were washed with wash buffer (1% BSA in PBS (Sigma Aldrich, UK)),  
455 then blocking and permeabilization were achieved by incubation with 5% BSA in 1xPBS  
456 with 0.3% Triton X-100 for 40 minutes prior to the addition of primary antibody  
457 (prepared in blocking buffer) which was incubated overnight at 4°C. The following  
458 conjugated primary antibodies were used: ZO-1 and AlexaFlour 488 (1:100, Thermo  
459 Fisher Scientific, UK), Von Willebrand factor and AlexaFlour 594 (1:1500, Novus Bio, UK)  
460 and Glutamine synthetase and AlexaFlour 488 (1:500, Novus Bio, UK). Post overnight  
461 incubation, cells were washed in wash buffer. This was followed by two washes in PBS  
462 and cultures were incubated with 1µg/ml 4', 6'-diamino-2-phenylindole (DAPI; D9542,  
463 Sigma Aldrich, UK) for 5 minutes. All cultures were washed an additional two times in  
464 PBS and once in deionized H<sub>2</sub>O and mounted using PBS and 0.3% Tween-100 as  
465 mounting medium (Sigma Aldrich, UK). RPE were mounted straight to slides by inverting  
466 coverslips, MIO-M1 and HRMVEC grown on inserts were mounted between two glass  
467 coverslips with prior to imaging with a Zeiss microscope with AxioCam MR3 and image  
468 analysis with AxioVision 4.6.



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470 **Sup.Figure 1A-C. Immunocytochemical analysis of cells grown in triple-culture, A- ZO-1 expression in**  
 471 **RPE after 28 day maturation marked with Alexa 488 (green), B- Von Willebrand factor expression in**  
 472 **HRMVEC grown on the basal side of the membrane marked with Alexa 568 (red), C- Glutamine**  
 473 **synthetase expression in MIO-M1 grown on the apical side of the membrane marked with Alexa 488**  
 474 **(green). A-C all cells counterstained with nuclear stain DAPI (blue). Scale 50  $\mu$ M.**



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476 **Sup.Figure 2. Representation of Hematoxylin stained cells used in the assessment of cell morphology**

477 **including analysis of diameter ( $\mu\text{M}$ ) by ImageJ software. A- RPE solo-culture, B- RPE dual-culture (+MIO-**

478 **M1), C- RPE dual-culture (+HRMVEC), D- RPE triple-culture, E- MIO-M1 solo-culture, F- MIO-M1 dual-**

479 **culture (+RPE), G- MIO-M1 dual-culture (+HRMVEC), H- MIO-M1 triple-culture, I- HRMVEC solo-culture,**

480 **J- HRMVEC dual-culture (+RPE) & K- HRMVEC dual-culture (+MIO-M1).**

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