1 **Human corneal stromal stem cells exhibit survival capacity following**
2 **isolation from stored organ-culture corneas.**
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26 **Abstract**

27 Purpose: To assess the suitability of human donor corneas maintained in long-
28 term organ culture for the isolation and expansion of viable and functional
29 corneal stromal stem cells (CSSCs). These cells display properties similar to
30 mesenchymal stem cells and demonstrate the ability to reproduce an organized
31 matrix in vitro. Therefore, CSSCs have great potential for the development of cell-
32 based therapies for corneal blindness or stromal tissue bioengineering.

33 Methods: Human donor corneas that had been stored either in organ culture
34 medium (OC) up to 4 weeks (n=3) or in Optisol medium (OS) up to 6 days (n=3)
35 were used for isolation of CSSCs and maintained in culture until passage four.
36 Cell phenotype of isolated CSSCs was assessed with light microscopy and
37 immunocytochemistry (PAX6, CD73 and CD90). PAX6 protein expression was
38 further confirmed with immunoblot analysis.

39 Results: A comparison of CSSCs isolated from corneas stored under OC and OS
40 conditions revealed no obvious differences in their morphology.
41 Immunocytochemistry revealed CSSCs from both OC and OS corneas maintained
42 positive staining for PAX6 and mesenchymal stem cell markers CD73 and CD90.
43 Immunoblotting confirmed protein expression of PAX6 in cells from both tissue
44 types.

45 Conclusions: Human CSSCs exhibit survival capacity by retaining their phenotype
46 following isolation from long storage, OC corneas. This advantageous property
47 enables the retrieval of CSSCs from OC corneas that are more abundantly
48 available for research than OS-stored corneas. OC corneas are also often
49 discarded for retrieval of other cell types such as corneal epithelial and
50 endothelial cells, which require high tissue quality for their preservation.

51 **Introduction**

52

53 The cornea is the outermost transparent surface of the eye and acts as our
54 window to the world, refracting light onto the retina enabling normal vision[1].
55 The stroma is the main component of the cornea and consists of a network of
56 lamellae made up of tightly packed aligned collagen fibrils[2]. This highly ordered
57 organization of collagen fibrils is thought to be essential to corneal
58 transparency[3]. Situated between these lamellae are the resident cells of the
59 corneal stroma – the keratocytes.

60

61 Keratocytes are mesenchymal-derived cells that remain quiescent for the most
62 part of adult life. Upon activation following injury or infection, keratocytes can
63 adopt a fibroblast or myofibroblast phenotype, producing a disorganized
64 extracellular matrix causing vision disrupting corneal scarring. During normal
65 wound healing these cells apoptose or dedifferentiate back into keratocytes
66 which remodel the temporary disorganized matrix into a healthy corneal
67 stroma[4]. In severe cases of corneal opacity such as limbal stem cell deficiency
68 (LSCD), the myofibroblasts remain activated causing a permanently altered
69 stromal matrix[5-6].

70

71 Currently, the most common approach to restore vision in scarred corneas is to
72 surgically replace the corneal stroma with allogeneic donor corneal tissue
73 (penetrating keratoplasty). However, alternative approaches are becoming
74 increasingly necessary as donor shortage is a major problem in most countries.
75 The supply of donor corneas in particular is expected to decrease further due to

76 current advances in corrective eye surgery, which renders potential donor
77 corneas unsuitable for transplantation. Techniques that utilize tissue
78 engineering and regenerative medicine technologies are being developed as
79 alternative approaches to using donor tissue[7-8]. There are numerous studies
80 focusing on the development of a tissue engineered human cornea using a
81 variety of substrates populated with cells of the cornea[9-11]. However, some
82 stromal cells, particularly keratocytes are difficult to culture *in vitro* as they
83 quickly differentiate into fibroblasts in response to expansion[12]. More recently, a
84 small population of human corneal stromal stem cells (CSSCs) has been
85 identified in the corneal limbal stroma, which display properties similar to
86 mesenchymal stem cells. These were the first human cells to be identified with
87 keratocyte progenitor potential[13]. Unlike keratocytes, these cells replicate *in*
88 *vitro* and maintain a corneal phenotype over a very high number of population
89 doublings. Previous studies by Funderburgh and colleagues[14] have demonstrated
90 that these cells have the ability to restore collagen fibril organization and
91 transparency in lumican-deficient mice (a mouse scar model), without immune
92 rejection. These properties make CSSCs an ideal candidate for a direct cell-based
93 therapy of corneal scarring or in the development of a bioengineered corneal
94 stromal equivalent.
95
96 Previously, it has been shown that CSSCs can be harvested from fresh donor
97 tissue that is less than 6 days old and stored in Optisol (OS), a medium typically
98 used for donor corneal tissue in America[13]. This type of hypothermic storage
99 conditions maintains the donor cornea at 4°C in a medium supplemented with
100 antibiotics and dehydrating agents such as chondroitin sulfate and dextran

101 which prevent the corneas from swelling. OS-stored corneas have been shown to
102 have minimal damage to the epithelium when stored up to 6 days with extensive
103 epithelial loss observed beyond 10 days of storage[15]. In Europe however, the
104 corneoscleral donor rims remaining after penetrating keratoplasty are
105 commonly stored in organ culture (OC) medium at temperatures of 31-37°C for
106 up to 4 weeks[16]. Tissue preserved in this way, although useful for
107 transplantation, is often disregarded for epithelial and endothelial cell isolation
108 for *in vitro* cultivation. This is because generally these cell types do not survive
109 longer storage culture conditions. Currently, OS-stored corneal rims are typically
110 used to harvest CSSCs and human limbal epithelial stem cells (HLESCs) for
111 engineering cultured corneal epithelium[17].
112
113 Given the prevalence of OC storage in the EU, the purpose of this study was to
114 assess the suitability of donor corneas maintained in long-term organ culture for
115 the isolation and expansion of viable and functional CSSCs. These tissues are
116 abundantly available but are potentially overlooked due to extended storage
117 times compared to OS-stored corneas. Cell phenotype was assessed by
118 comparing morphology of cells in culture, expression of PAX6 protein, (a
119 homeobox transcription factor specific for eye development), and expression of
120 the mesenchymal stem cell markers CD73 and CD90 (specific surface antigen
121 markers used to define mesenchymal stem cells)[18]. Differentiation of isolated
122 CSSCs into keratocytes was also investigated to confirm their progenitor
123 phenotype by assessing cells in culture for keratocyte marker expression.
124
125

126 **Methods**

127

128 **Ethical conduct of research**

129 The authors state that they have obtained appropriate institutional review board
130 approval or have followed the principles outlined in the Declaration of Helsinki
131 for all human or animal experimental investigations. For investigations involving
132 human subjects, informed consent has been obtained from the participants
133 involved.

134

135 **Human CSSCs culture and characterisation**

136 *Isolation of human corneal stromal stem cells*

137 Human corneal stromal stem cells were isolated from 6 donor corneas using a
138 method similar to that described by Du and colleagues[13]. The ages of corneal
139 donors ranged from 50-74 years old from both males and females (table 1). At
140 the time of isolation, 3 donor corneas had been stored in OC medium up to 4
141 weeks at ambient temperature and the remaining 3 donor corneas had been
142 stored in Optisol at 4°C for up to 6 days. Briefly, the superficial corneal limbal
143 region was dissected into small fragments and digested in Dulbecco's modified
144 Eagle's medium (DMEM) (Sigma-Aldrich, Dorset, UK) supplemented with
145 50ug/ml gentamicin (Gibco, Life Technologies, Paisley, UK), Penicillin-
146 Streptomycin solution (1x) (Corning Cellgro, USA), containing collagenase type L
147 (0.5mg/ml) (Sigma-Aldrich, Dorset, UK) and incubated at 37°C overnight.
148 Primary stromal cells were plated into flasks coated with fibronectin-collagen
149 (FNC) (Athena Enzyme System) and cultured in corneal stromal stem cell (CSSC)
150 medium (modified from the method described by Jiang and colleagues[19])

151 consisting of a mixture of DMEM low glucose (Gibco, Life Technologies, Paisley,
152 UK) and MCDB-201 (Sigma-Aldrich, Dorset, UK) medium, supplemented with 2%
153 fetal bovine serum (Invitrogen, Life Technologies, Paisley, UK), 10ng/ml
154 epidermal growth factor (Sigma-Aldrich, Dorset, UK), 10ng/ml platelet-derived
155 growth factor (PDGF-BB) (R&D Systems, UK), Insulin-Transferrin-Selenium (ITS)
156 solution (1x) (Gibco, Life Technologies, Paisley, UK), 0.1mM ascorbic acid-2-
157 phosphate (Sigma-Aldrich, Dorset, UK), 10^{-8} M dexamethasone (Sigma-Aldrich,
158 Dorset, UK), penicillin-streptomycin solution (1x) (Corning Cellgro, USA),
159 50ug/ml gentamicin (Gibco, Life Technologies, Paisley, UK), 100ng/ml cholera
160 toxin (Sigma-Aldrich, Dorset, UK). Cells were trypsinised and subcultured when
161 colonies of small polygonal cells were visible. Cultures were not allowed to reach
162 confluence.
163
164 *Assessing morphology of human CSSCs*
165 Cultures of human CSSCs were assessed using light microscopy. Photographs of
166 CSSCs from OC and OS-stored corneas were taken at every passage to compare
167 the morphology of cells.
168
169 *Immunocytochemistry of human CSSCs*
170 Cells were plated onto permanox slides at a density of 40,000 cells per slide,
171 cultured for a further 3-4 days in CSSC media, then fixed with 4%
172 paraformaldehyde (at passage 2-4) for 15 minutes. Cells were subsequently
173 washed with phosphate buffer saline (PBS) (Invitrogen, Life Technologies,
174 Paisley, UK) and blocked for 1 hour with 5% goat serum in PBS with 0.25%
175 Triton-X-100 (Sigma-Aldrich, Dorset, UK) at room temperature. Following a

176 further wash with PBS, samples were incubated overnight at 4°C with the
177 primary antibody diluted in 2% goat serum with PBS (PAX6 (Covance) at a
178 concentration of 1:70, CD90 and CD73 at a concentration of 10μg/ml). Samples
179 were washed three times with PBS prior to incubation with the secondary goat
180 anti-rabbit 594 Alexa Fluor antibody (1:500 dilution; Invitrogen Ltd, Paisley, UK)
181 and FITC-labelled phalloidin (1:1000 concentration; Sigma-Aldrich, Dorset, UK),
182 which binds to the actin cytoskeleton. These were incubated for 1 hour at room
183 temperature in the dark. To visualize nuclei, samples were mounted underneath
184 coverslips in Vectashield mounting medium containing DAPI (Vector
185 Laboratories Inc., Burlingame, CA). Samples were viewed and analysed on a
186 confocal Zeiss LSM 710 microscope.
187
188 *Immunoblotting of CSSCs*
189 Protein was extracted from CSSCs samples from both cultures using a lysis buffer
190 and a protease inhibitor. Samples were then separated on a 4-12% Bis-Tris gel
191 (NuPAGE gel, Invitrogen, Paisley, UK). Appropriate molecular weight markers
192 were placed into the gel together with a positive control for PAX6. Gels were
193 prepared for western blotting followed by electro-transferral to polyvinylidine
194 difluoride (PVDF) membranes. PAX6 was identified with incubation of PAX6
195 antibody (Covance, New Jersey, USA) at 1:70 concentrations. Visualisation of
196 immunoreactivity was achieved using an immunodetection kit (ECL Western
197 Blotting immunodetection reagents; Amersham Biosciences, UK).
198
199 **Keratocyte culture and characterisation**
200 *Differentiation of CSSCs into keratocytes*

201 CSSCs at passage 2-4 were cultured for 3 weeks in keratocyte differentiation
202 medium (KDM) consisting of Advanced DMEM (Sigma-Aldrich, Dorset, UK),
203 10ng/ml fibroblast growth factor (Sigma-Aldrich, Dorset, UK), 0.1mM L-ascorbic
204 acid-2-phosphate (Sigma-Aldrich, Dorset, UK), 50ug/ml gentamicin (Invitrogen),
205 Penicillin-Streptomycin solution (1x) (Corning Cellgro, USA), GlutaMAX (1x)
206 (Invitrogen, Life Technologies, Paisley, UK). Media was replaced every 2-3 days.
207

208 *Assessing morphology of keratocytes*

209 To observe changes in the morphology of CSSCs as they differentiate into
210 keratocytes, cultures were assessed with light microscopy. Photographs of
211 cultures were taken over three weeks.
212

213 *Immunocytochemistry of keratocytes*

214 Keratocyte markers keratocan, lumican and ALDH1A1 were used to confirm
215 keratocyte phenotype. Cells were seeded onto permanox slides at a seeding
216 density of 40,000 cells per slide, cultured and subsequently fixed with 4%
217 paraformaldehyde for 15 minutes. Cells were permeabilised with 0.5% Triton in
218 PBS for 15 minutes at room temperature. Samples were treated with 0.4U/ml
219 chondroitinase ABC (Sigma-Aldrich, Dorset, UK) for 1 hour at 37°C followed by
220 treatment with 0.4U/ml endo-β-galactosidase (Sigma-Aldrich, Dorset, UK) for 1
221 hour at 37°C. Cells were washed with PBS and incubated with blocking agent
222 (2% BSA in PBS) for 30 minutes at room temperature. Primary antibodies Anti-
223 Keratocan, Anti-Lumican (1:20 concentration; Sigma-Aldrich Dorset, UK), and
224 Anti-ALDH1A1 (1:50 concentration; Abcam) were prepared in blocking agent
225 and slides incubated overnight at 4°C. These were rinsed 3 times with PBS prior

226 to incubating with secondary goat anti-rabbit 594 Alexa Fluor antibody (1:500
227 dilution; Invitrogen Ltd, Paisley, UK) and FITC-labelled phalloidin (1:1000
228 concentration; Sigma-Aldrich, Dorset, UK) for 1 hour at room temperature in the
229 dark. Slides were mounted underneath coverslips in Vectashield mounting
230 medium containing DAPI (Vector Laboratories Inc., Burlingame, CA). Samples
231 were viewed and analysed on a confocal Zeiss LSM 710 microscope.
232

233 **Results**

234 **Characterisation of CSSCs**

235 *Morphology of CSSCs*

236 There was no observable difference in the morphology of CSSCs isolated and
237 cultured from organ culture and Optisol-stored corneas. Table 1 contains all
238 donor information including age, gender, time of storage and type of storage for
239 each donor used. Cells from both cultures appeared small and polygonal and
240 grew in sparsely arranged colonies (figure 1). Other stromal cells including more
241 dendritic-shaped cells indicative of the keratocyte phenotype and longer spindle-
242 shaped fibroblasts were visible in both primary cultures but disappeared at later
243 passages.
244

245 *Immunocytochemistry of hCSSCs*

246 Immunocytochemistry revealed CSSCs (up to passage 4) expressed positive
247 nuclear staining for PAX6 (positive PAX6 staining in CSSCs was expected since
248 PAX6 is a homeobox transcription factor expressed in embryonic ocular
249 precursor cells and epithelial cells). Figure 2 contains confocal micrographs
250 illustrating positive PAX6 expression in cultured CSSCs from both OC and OS-

251 stored corneas. Expression of CD73 and CD90 (mesenchymal stem cell markers)
252 were observed in CSSCs isolated from corneas in both storage conditions (figure
253 2). Expression of PAX6, CD73 and CD90 was observed in cells with a small
254 polygonal morphology, indicative of the mesenchymal stem cell phenotype.

255

256 *Immunoblotting of hCSSCs*

257 PAX6 production was demonstrated by immunoblotting of proteins collected
258 from CSSCs isolated from both OC and OS-stored corneas (figure 3). A band
259 showing the positive control of PAX6 can be seen at the predicted weight of
260 47kDa. Bands from CSSCs were slightly lower than 47kDa as observed in other
261 studies by D'elia and colleagues[20] that showed a similar pattern of PAX6
262 expression in immunoblots and could be due to post-translational modifications.

263

264 *Differentiation potential of hCSSCs into keratocytes*

265 To confirm differentiation potential, CSSCs were cultured under serum-free
266 conditions to induce their differentiation into keratocytes. Figure 4 includes light
267 microscopy images illustrating the morphology of CSSCs cultured in KDM for 1
268 and 3 weeks. The small, polygonal morphology characteristic of CSSCs changed
269 to a more dendritic morphology typical of keratocytes. Immunocytochemistry
270 analysis (figure 5) revealed CSSCs gain keratocyte characteristics through
271 exposure to KDM. Cells cultured for 3 weeks in KDM exhibited positive
272 expression of keratocyte markers ALDH1A1, keratocan and lumican. Similar to
273 recent studies by Stagos and colleagues[21], ALDH1A1 was expressed in the
274 nucleus of keratocytes.

275

276 **Discussion**

277 In this study, we have demonstrated that CSSCs can be isolated reliably from
278 stored organ-culture donor corneas. This capacity to survive such long-term
279 storage conditions has not been previously reported for CSSCs. Since human
280 CSSCs have the ability to reorganize a disorganized matrix typical of corneal
281 scarring[14], they hold great potential for bioengineering applications but have
282 only been isolated from fresh OS-stored corneas. This finding is of importance as
283 cells can now be isolated from OC-stored donor corneas, which are more readily
284 available than OS-stored corneas. OC-stored corneas are often disregarded for *ex*
285 *vivo* expansion of other cell types such as corneal epithelial and endothelial cells,
286 which require fresher tissue quality for their preservation. There is also the
287 potential to culture a sufficient number of cells from one OC-stored donor cornea
288 to treat multiple patients with corneal scarring.

289

290 Our results revealed no obvious differences between CSSCs isolated from OS and
291 OC-stored corneas when comparing cell morphology, expression of PAX6, CD73
292 or CD90. Studies by Du and colleagues[13] in which human CSSCs were isolated
293 from fresh OS-stored donor corneas, demonstrated that CSSCs exhibited a
294 morphology and positive PAX6 expression comparable to that observed in our
295 study. Since PAX6 is a transcription factor essential for ocular development and
296 present in most embryonic ocular tissues but not adult keratocytes[22], positive
297 expression in isolated cells confirms the CSSC phenotype from both OC and OS-
298 stored corneas. Positive expression of mesenchymal stem cell markers CD73 and
299 CD90, further confirms the CSSC phenotype. In addition, CSSCs isolated from OC
300 and OS-stored corneas were successfully shown to differentiate into keratocytes

301 by culturing in serum-free media. These differentiated cells exhibited positive
302 expression of keratocyte markers keratocan, lumican and ALDH1A1, similar to
303 that observed by Park and colleagues[23], confirming the keratocyte phenotype
304 and demonstrating their progenitor potential.
305
306 We hypothesise that this survival capacity demonstrated by CSSCs isolated from
307 OC-stored corneas may be attributed to their location, residing inside the matrix
308 where they are better protected from post-mortem changes than the epithelial
309 and endothelial corneal surface layers. Keratocytes are considered to remain
310 quiescent throughout adult life[24] and thus have very low metabolic activity.
311 Therefore, we could postulate that CSSCs, as the progenitor cells of keratocytes,
312 may have similar metabolic levels and require less energy for survival. This
313 property may give CSSCs the advantage over other cell types, enabling them to
314 survive extended storage conditions.
315
316 Importantly, the organ culture corneas in our study were maintained at ambient
317 temperature rather than 31-37°C for the majority of the storage duration. Donor
318 corneas are typically placed into OC medium following retrieval and stored at
319 31°C under GMP conditions, prior to being used for transplantation. Following
320 penetrating keratoplasty, the remaining corneoscleral rim is placed back into OC
321 medium in the operating theatre but stored at ambient temperature to avoid
322 compromising the sterile GMP conditions. From here on, the donor tissue is
323 maintained at ambient temperature until used for research purposes. The
324 survival of CSSCs under these storage conditions can be compared to the survival
325 of human limbal epithelial stem cells (HLESCs) in studies by Raeder and

326 colleagues[25] which showed that OC storage of cultured HLESCs at ambient
327 temperature was superior to OC storage at 31°C and Optisol-GS storage at 5°C.
328
329 **Conclusion**
330 This novel finding that CSSCs can be isolated from OC corneas and survive long-
331 term storage enables greater use of donor corneal limbal rims that would
332 otherwise be discarded. It also highlights a greater advantage CSSCs have over
333 other cell types including corneal epithelial and endothelial cells, which require
334 higher tissue quality for their preservation for *ex vivo* expansion.
335
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446 cultured corneal epithelium*. Invest Ophthalmol Vis Sci*. 2007;48(12):5484-93.
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448

449 **Figure legends**

450

451 Figure 1: Light microscope images of hCSSCs (passage 2) isolated from (A)
452 optisol or (B) organ culture-stored corneas. CSSCs (C) appear small and square
453 amongst some keratocyte (K) and fibroblast (F) cells. Scale bar represents
454 200μm.

455

456 Figure 2: Confocal micrographs of hCSSCs isolated from Optisol (Opt) or organ
457 culture (OC)-stored corneas. Cells display positive nuclear expression of PAX6
458 (gene expressed in early eye development), and CD73 and CD90 (mesenchymal
459 stem cell markers). Scale bar represents 40μm.

460

461 Figure 3: Immunoblot of PAX6 protein in CSSCs isolated from organ culture (OC)
462 and optisol (OS) donor corneas. The first lane shows a MagicMark standard (M).
463 The last 3 lanes show expression from cells known to be positive for PAX6
464 protein; human limbal fibroblasts (HLF), limbal epithelial cells (LEC) and a
465 positive (+VE) control of PAX6, consisting of rat brain lysate, respectively.

466

467 Figure 4: Light microscopy images illustrating change in morphology of CSSCs to
468 keratocytes. A) CSSCs cultured in CSSC media (passage 2), B) CSSCs cultured in
469 KDM for 3 weeks. Scale bar 100μm.

470

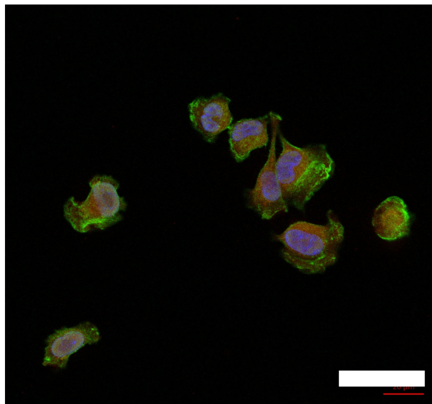
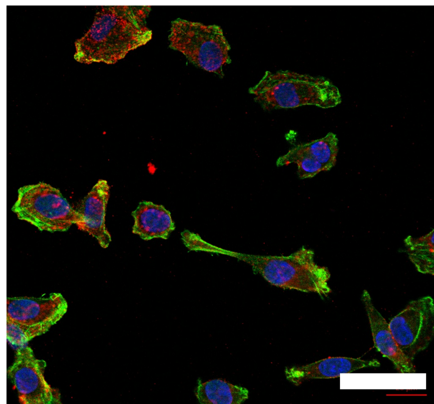
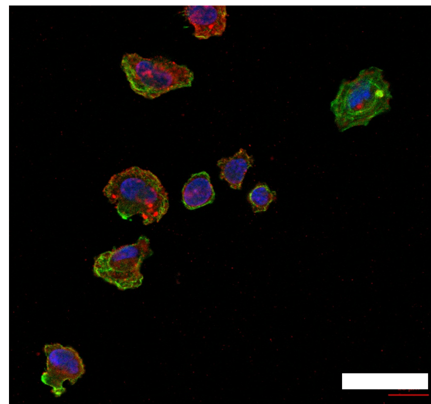
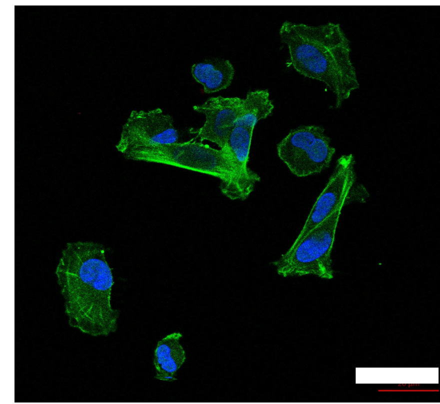
471 Figure 5: Confocal micrographs of CSSCs that have differentiated into
472 keratocytes. CSSCs were cultured in KDM for 3 weeks prior to seeding and fixing

473 on permanox slides. Positive marker expression for ALDH1A1, Keratocan and
474 Lumican are shown in red. (FITC Phalloidin staining cytoplasm in green, DAPI
475 staining nuclei in blue). Scale bar represents 20μm.

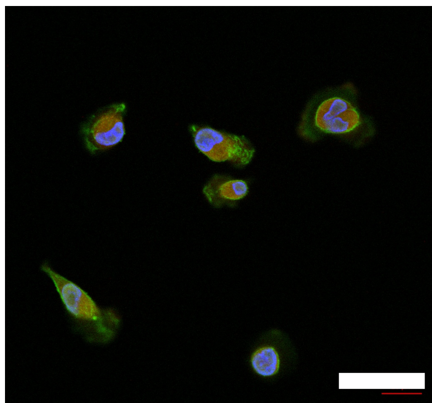
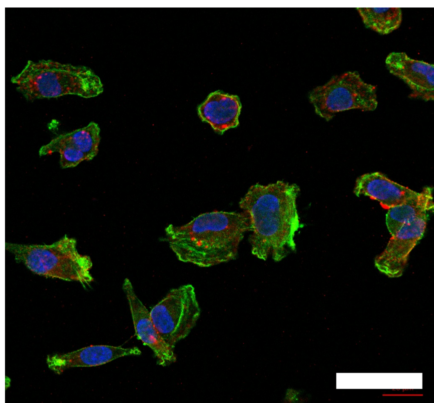
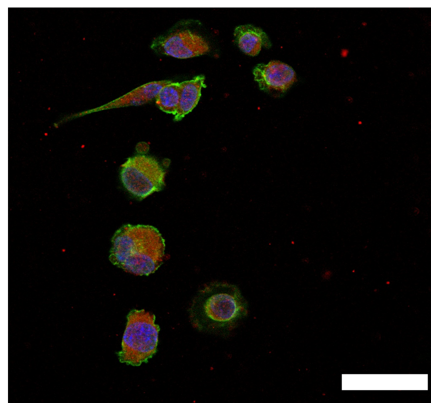
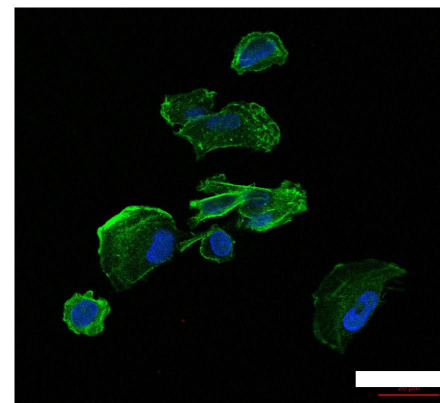
A
F
C
K
B
C
F
K

PAX6 CD90 CD73 Negative control

OC

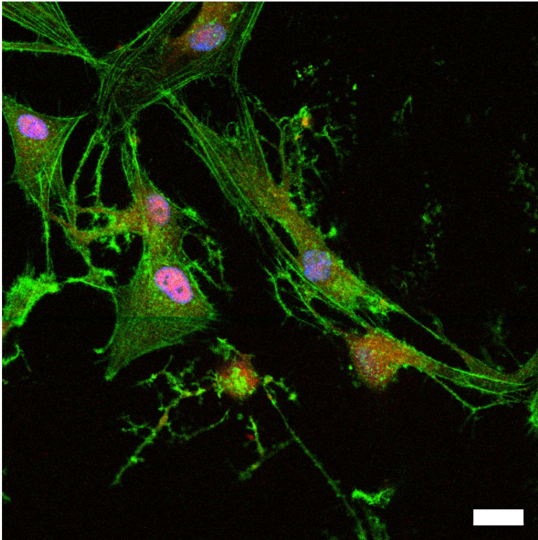
Opt

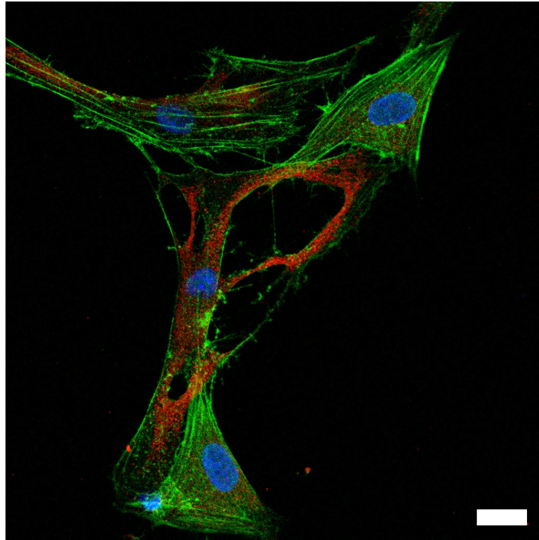





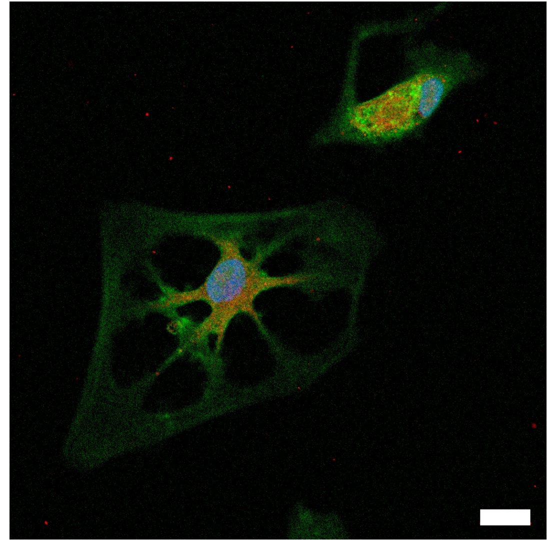
M
OS
OC
OC
OC
OS
OS
HLF
LEC
+VE
PAX6
35 kDa

A

B







ALDH1A1

KERATOCAN

LUMICAN

Tables

Donor	1	2	3	4	5	6
Storage medium	Optisol	Optisol	Optisol	OC	OC	OC
Age	58	74	50	62	51	75
Gender	F	M	M	M	F	M

Table 1: Table showing donor information of Optisol and organ-culture (OC) corneas. The age of donors ranged from 50 -74 years old and tissue was from both male and female donors.