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## An Overview of Current Glaucomatous Trabecular Meshwork Models

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23

## 24 **An overview of current glaucomatous trabecular meshwork models**

25 Abstract: The trabecular meshwork (TM) is a complex tissue that regulates aqueous humor  
26 outflow from the eye. Dysfunction of the TM is a major contributor to the pathogenesis of  
27 open-angle glaucoma, a leading cause of irreversible blindness worldwide. The TM is a porous  
28 structure composed of trabecular meshwork cells (TMC) within a multi-layered extracellular  
29 matrix (ECM). Although dysregulation of the outflow throughout the TM represents the first  
30 step in the disease process, the underlying mechanisms of TM degeneration associate cell loss  
31 and accumulation of ECM, but remain incompletely understood, and drugs targeting the TM  
32 are limited. Therefore, experimental models of glaucomatous trabeculopathy are necessary for  
33 preclinical screening, to advance research on this disease's pathophysiology, and to develop  
34 new therapeutic strategies targeting the TM. Traditional animal models have been used  
35 extensively, albeit with inherent limitations, including ethical concerns and limited  
36 translatability to humans. Consequently, there has been an increasing focus on developing  
37 alternative in vitro models to study the TM. Recent advancements in three-dimensional cell  
38 culture and tissue engineering are still in their early stages and do not yet fully reflect the  
39 complexity of the outflow pathway. However, they have shown promise in reducing reliance  
40 on animal experimentation in certain aspects of glaucoma research. *This review provides an*  
41 *overview of the existing alternative models for studying TM and their potential for advancing*  
42 *research on the pathophysiology of open-angle glaucoma and developing new therapeutic*  
43 *strategies.*

44 Keywords: glaucoma model, trabecular meshwork, 3D culture, in vitro model, tissue  
45 engineering, outflow

46

## 47 **Introduction**

48 Glaucoma is a blinding optic neuropathy affecting over 70 million people worldwide <sup>1</sup>. Its  
49 most important risk factor is elevated intraocular pressure (IOP). The trabecular meshwork  
50 (TM) plays a key role in the pathophysiology of glaucoma. This filter is located within the  
51 iridocorneal angle and constitutes the main outflow pathway for the aqueous humor. It is a  
52 fenestrated triangle-form structure in which trabecular meshwork cells (TMC) populate a  
53 multi-layered extracellular matrix. The TM tissue is not a rigid structure, but a highly  
54 dynamic, avascular filtration system that has a multitude of roles, including filtering aqueous  
55 humor of waste material, sensing and regulating IOP through the mechanical stretch, and  
56 altering ECM composition and deposition <sup>2-4</sup>. The TM contributes to the regulation of IOP by  
57 regulating the outflow of aqueous humor from the eye's anterior chamber, primarily through  
58 the juxtacanalicular tissue and the endothelium of Schlemm's canal (SC) <sup>2,5</sup>. The  
59 juxtacanalicular tissue, located adjacent to the inner wall of SC, plays a crucial role in the  
60 regulation of outflow resistance. It consists of specialized cells and extracellular matrix (ECM)  
61 components that modulate the flow of aqueous humor <sup>6</sup>. Dysfunction of the TM and the inner  
62 wall of SC is the cause of IOP elevation and represents the *primum movens* of primary open-  
63 angle glaucoma (POAG) but the precise mechanisms at the origin are still unclear. It is  
64 known that abnormal TMC function and excess of ECM deposit contribute to TM stiffening  
65 in POAG<sup>7</sup>. Alterations in the structure and composition of the TM ECM generate aqueous  
66 humor outflow resistance. Cell-ECM interactions within the TM and SC play a role in  
67 regulating outflow resistance. Cellular responses to ECM components and their remodeling  
68 can influence the contractility and overall functionality of the tissue<sup>6,8,9</sup>. The endothelial cells  
69 lining the inner wall of SC exhibit contractile properties. Contraction and relaxation of these  
70 cells regulate the diameter of the canal and thereby influence outflow resistance<sup>10,11</sup>. Tight  
71 junctions between endothelial cells of SC also contribute to the formation of a barrier that  
72 controls the paracellular movement of aqueous humor<sup>12</sup>. A second 'unconventional' outflow

73 pathways exists in the human eye, but it only accounts for less than 10% of the total outflow,  
74 and thus does not significantly contribute to the regulation of IOP in the normal eye<sup>13</sup>.

75         The development of robust models of physiologic and pathologic TM is necessary to  
76 study the different mechanisms at the origin of this deregulation but also to develop and test  
77 treatments targeting TM, which are currently rare. Several experimental models have been  
78 described with advantages and disadvantages, from animal models or perfusion-cultured  
79 anterior segments to cell culture models.

80         To study the pathophysiology of the TM or its changes under the effects of treatments,  
81 all models that artificially increase the IOP by blocking or sclerosing the outflow pathways,  
82 for example using laser photocoagulation, episcleral vein cauterization, injection of  
83 microbeads or hyaluronic acid into the anterior chamber or hypertonic saline injection into the  
84 episcleral veins are not appropriate as they do not mimic the natural course of glaucomatous  
85 dysfunction to the aqueous outflow pathway<sup>14</sup>. Indeed, a distinction must be made between  
86 animal models of ocular hypertension made to study RGC loss and those specifically  
87 developed for the study of TM.

88

89         Models of glaucoma exist in mice, rats, dogs, cats, and primates<sup>15</sup>. Each has its challenges,  
90 including the availability of genetic resources and difficulty of genetic manipulation, ethical  
91 considerations, cost, and maintenance. Translational research in glaucoma is faced with  
92 numerous challenges. One significant factor is the divergence in eye anatomy between  
93 animals and humans. Furthermore, the etiology of the disease differs between glaucoma  
94 animal models and human patients. Additionally, there are notable disparities in study design  
95 and statistical analysis methods employed in preclinical and clinical investigations. Moreover,  
96 significant differences exist in terms of dosage, scale, timing of intervention, methodologies,

97 endpoints, age groups studied, and the presence or absence of IOP-lowering treatment, which  
98 further complicates the comparison between preclinical and clinical studies in glaucoma<sup>16</sup>.  
99 The animal whose TM anatomy is the closest to humans is the primate, but their use is limited  
100 because of their expensive price, the inaccessibility of transgenic strains and the rise of ethical  
101 concern. Most preclinical studies in the field of glaucoma used mice as their outflow tract  
102 anatomy is comparable to humans. It is described as follows: TM, juxtacanalicular tissue,  
103 Schlemm canal, collector channels, and episcleral veins<sup>15</sup>. However, the main anatomical  
104 difference is that the human TM structure consists of 9 to 18 trabecular beams whereas in the  
105 mouse TM, there are only 2 to 5 layers of lamellae. This difference in the structure of the  
106 animal TM as well as the regulation of aqueous humor excretion constitutes a limitation to use  
107 of animal models. The results obtained with animal models are therefore not always  
108 transposable to humans although the majority of IOP lowering glaucoma medications in  
109 human are also effective in mouse eyes<sup>16</sup>. Moreover, the lack of cooperation of animals and  
110 the use of anesthesia to perform experimentation affects the results. The IOP values vary by  
111 animal breed, method of sedation, and measure. In general, excessive restraint, inadequate  
112 positioning, or lack of experience in the use of equipment can increase IOP. Last but not least,  
113 they are questioned from an ethical point of view. Recently, a consensus recommendation for  
114 mouse models to study the aqueous humor outflow was published in 2022 to set standard  
115 practice in this field<sup>17</sup>. The most useful models are genetic models that allow OHT by  
116 transduction of the TM with glaucoma-related genes<sup>18</sup> (e.g., *MYOC*<sup>19,20</sup>, *TGFβ*<sup>21-23</sup>, *GREM1*<sup>24</sup>,  
117 *CTGF*<sup>25</sup>, *DKK1*<sup>26</sup>, *SFRP1*<sup>27</sup>, *CD44*<sup>28</sup>). To meet the expectations of this consensus  
118 recommendation animal models used to study outflow physiology must<sup>17</sup>:

- 119 - have an open iridocorneal angle
- 120 - present decreased outflow facility
- 121 - present elevated IOP

122 - include morphologic descriptions of the conventional outflow tract (TM, Schlemm's  
123 canal, collector channels, and intrascleral and episcleral regions) preferably by  
124 electronic microscopy to be able to analyze the organization of the ECM  
125 - and assessment of TMC numbers.

126 The Myoc<sup>Y437H</sup> mouse, responsible for a juvenile glaucoma, is the best-known and most  
127 used<sup>20</sup>. The genetically modified mice express high levels of mutant myocilin in the TM and  
128 sclera resulting in a decline of AH outflow facility and a secondary increase of IOP.  
129 Morphological changes can be observed in the TM: decreased intertrabecular spaces and a  
130 progressive loss of TMC. However, the level of IOP can vary based on the genetic  
131 background of the mouse strain selected for inducing the MYOCY437H mutation<sup>29</sup>.

132 In recent years, progress in three-dimensional cell cultures and tissue engineering  
133 fabrication has offered promising approaches to reduce the use of animal models, partly  
134 encouraged by the three Rs rule (Reduce the number of animals used; Replace the living  
135 animal with alternative experimental techniques; and Refine the techniques to minimize  
136 animal suffering)<sup>30,31</sup>. While conventional 2D cell culture models represent an attractive  
137 alternative to animal models for analyzing the TM and allow for more accurate assessment at  
138 the cellular scale, they are limited by the absence of differentiation, polarization, or  
139 relationship with an extracellular matrix, making them insufficient in reflecting the actual  
140 porous architecture of the TM<sup>32,33</sup>. However, this deficient architecture is precisely the cause  
141 of its dysfunction<sup>34</sup>. *Ex vivo* models such as organ perfusion chambers, whole tissue explants,  
142 and decellularized tissues are commonly used natural sources; however, their limited  
143 availability restricts their use in perfusion studies and drug testing<sup>35,36</sup>. This is why a three-  
144 dimensional (3D) cellular model of the TM would be an interesting tool to advance research  
145 on this pathology by considering the biomechanics, which is a key element in the  
146 pathophysiology of glaucoma<sup>4</sup>. 3D cell culture would enable the recreation of the

147 microenvironment encountered *in vivo* and provide cells with an environment allowing them  
148 to interact with each other<sup>33</sup>. This, in turn, would lead to a better understanding of the  
149 physiological functioning of the TM, its behavior under conditions of stress or toxicity, and  
150 the effect of treatments<sup>37</sup>. Additionally, *in vitro* models provide a more precise analysis of cell  
151 behavior and molecular mechanisms involved in the pathology than do animal experiments<sup>38</sup>.  
152 This approach allows to investigate specific biological phenomena in isolated cells,  
153 eliminating potential confounding factors present in whole organisms. However, it is crucial  
154 to acknowledge that while *in vitro* systems provide controlled environments, they cannot fully  
155 replicate the complex conditions and interactions that occur within a living organism.  
156 Technological advances in TM *in vitro* models can help fill the gap in considering the  
157 mechanistic modifications involved in glaucoma, such as changes in porosity resulting from  
158 alterations in morphology and the mechanical properties of the interaction between TMC and  
159 ECM.

160 In this literature review, we will provide an overview of existing alternative TM  
161 models to animal experimentation. We will detail the different cell types used, culture modes,  
162 and means to obtain a pathological model. Finally, we will focus on the potential applications  
163 of these different models.

164

### 165 ***Available cell types***

166

#### 167 ***Primary cell cultures:***

168 TM is composed of three regions, from the anterior chamber to the Schlemm canal: the uveal  
169 meshwork, the corneoscleral meshwork, and the juxtacanalicular tissue, the location of  
170 greatest resistance to AH outflow. TMC have a different organization in these three regions,



171 but it is complex to isolate cells from only one of these three regions. Thus, TMC cultures are  
172 generally a mixture of cells from all three regions<sup>32</sup>.

173 Cultures of primary human TMC (HTMC) are sampled from donor eye tissue  
174 commonly from a corneal rim discarded from corneal transplant<sup>39</sup>. Whole globe or anterior  
175 segment from normal subjects, developing fetuses, or patients with glaucoma can also be  
176 purchased.

177 Cultures of TMC from patients with glaucoma are more difficult due to the accelerated  
178 loss of these cells. Nevertheless, they retain their glaucomatous characteristics after culture<sup>40</sup>.  
179 TMC change their morphology after 6 to 8 passages, thus it is recommended to use TMC  
180 from human eyes before the 7th passages. Methods used to validate that cells are TMC is  
181 required for publications including at least responsiveness of myocilin expression by cells to  
182 dexamethasone<sup>32</sup>. TMC cultures can also come from animal eyes with the limitations that this  
183 brings.

184

#### 185 *Immortalized human TM cells*

186 Immortalized TMC lines can be generated with TMC transfected with an original defective  
187 mutant of the SV40 virus<sup>41,42</sup>. However, during the immortalization process, some properties  
188 of the TMC can be lost, for example the myocilin expression. It is recommended that cell line  
189 findings be replicated with non-immortalized TMC<sup>32</sup>.

190

#### 191 *Induced pluripotent stem cell-derived trabecular meshwork (iPSC-TM)*

192 The reproducibility of primary cell cultures is a challenge and immortalized cell lines are  
193 considered poorly relevant to TMC physiology and disease patterns. The experimental  
194 transplantation of iPSC-derived TM (iPSC-TM) highlighted the huge therapeutic potential of  
195 these new human cell models, offering perspectives for toxicological or therapeutic  
196 evaluations<sup>43,44</sup>. Moreover, the pathogenesis of the glaucomatous disease is explained by a

197 TMC loss greater than the physiological age-related cell loss. This loss has been suggested to  
198 affect the ability of the human TM to regulate aqueous humor outflow and to lead to IOP  
199 elevation. In addition to providing a source of reproducible and valuable cells for the  
200 constitution of an *in vitro* model iPSC-TM cells are promising autologous cell sources that  
201 can be used to regenerate the declining TMC population and function of IOP regulation<sup>45,46</sup>.

202

### 203 *TM progenitor cells*

204 A population of progenitor cells has been identified in Schwalbe's Ring, which is located at  
205 the junction between the corneal endothelium and the anterior portion of the TM<sup>47</sup>. These  
206 progenitors can be isolated and expanded, and studies have shown that they have the ability to  
207 differentiate into both keratocytes and TMC. Zhang et al. developed an optimized method to  
208 expand multipotent progenitors from human TMC in a two-dimensional (2D) culture followed  
209 by three-dimensional (3D) culture in Matrigel using a modified embryonic stem cell  
210 medium<sup>48</sup>. The expanded cells expressed TM markers, embryonic stem cell (ESC) markers,  
211 and neural crest (NC) markers. Although some markers were lost after passage, the cells  
212 regained the markers when seeded on 3D Matrigel via activation of the canonical BMP  
213 signaling<sup>48</sup>. These cells can be used in an *in vitro* model system to help better understand how  
214 TM is affected in glaucoma and whether TM progenitor cells may have potential therapeutic  
215 applications for glaucoma.

216

### 217 ***Generation of Pathological Models:***

218

219 As discussed earlier, obtaining cells from the glaucomatous TM is a challenging task for  
220 researchers, which is why different molecules are used to induce a diseased phenotype.

221 Applying mechanical stress to the TM can induce changes similar to those observed in  
222 glaucoma. This can be done by stretching or compressing the tissue using a variety of devices.  
223 In a previous study, Schlunck et al. demonstrated that the stiffness of the ECM could alter the  
224 structure of the cytoskeleton of trabecular cells as well as the profiles of certain protein  
225 expressions<sup>8</sup>.

226 Various chemicals can be used to induce glaucomatous changes in the TM, such as  
227 transforming growth factor  $\beta$ 2 (TGF- $\beta$ 2), and have been shown to contribute to the changes in  
228 the ECM of the TM.

229 TGF- $\beta$ 2 is a profibrotic cytokine known to be elevated in the aqueous humor of  
230 patients with glaucoma<sup>49,50</sup>. It has been used in many studies as a model of pathological TM  
231 <sup>41,42,51-53</sup>. Studies have revealed that TGF- $\beta$ 2 can increase intraocular pressure (IOP) by  
232 promoting the synthesis of certain ECM components by trabecular cells (collagens,  
233 fibronectin) through epithelial-mesenchymal transition in TMC<sup>54,55</sup>. Furthermore, TGF- $\beta$ 2  
234 can increase cell rigidity by triggering the formation of Cross-linked Actin Networks (CLANs)  
235 via the Rho-GTPase pathway<sup>53,56</sup>.

236 Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), another molecule used to induce a glaucomatous phenotype, is a  
237 chemical compound with powerful oxidizing properties and has been shown to promote  
238 cellular senescence, rearrange cytoskeletal structure, and increase proinflammatory mediators  
239 such as IL-6, IL-8, and endothelial-leukocyte adhesion molecule 1 (ELAM-1) in the TM<sup>57</sup>.

240 Endothelin-1 (ET-1) is another biomarker found in the aqueous humor of patients with  
241 POAG<sup>58,59</sup>. It has been shown that ET-1 can induce TMC contraction in culture and that it can  
242 affect the outflow facility<sup>60,61</sup>. Wang et al. showed that in a cultured HTMC model, treatment  
243 with ET-1 increased the expressions of fibronectin and collagen IV, and that in an ex-vivo  
244 model, IOP increased after ET-1 administration<sup>62</sup>. Zhou et al. also used ET-1 in a whole eye

245 perfusion system and found a decreased outflow after ET-1 exposition and successfully tested  
246 several pretreatments to reverse this effect<sup>63</sup>.  
247 Benzalkonium chloride (BAK) *in vitro* induced apoptosis, oxidative stress, and also an IOP  
248 increase, with reduction of aqueous outflow *in vivo*. BAK enhances all characteristics of TM  
249 degeneration typical of glaucoma and causes degeneration in acute experimental conditions,  
250 potentially mimicking TM degeneration<sup>64</sup>. In an *in vitro* 3D TM model, Bouchemi et al.  
251 showed that BAK disorganized the TMC and decreased their number resulting in an  
252 enlargement of spaces between cells<sup>65</sup>.

253

#### 254 ***Available 3D models:***

255

##### 256 *3D scaffolds culture*

257 The first successful scaffold was a micro-fabricated SU-8 porous structure, where TMC were  
258 cultured to study steroid-induced glaucoma. Scaffolds with pores of approximately 20  
259 micrometers in thickness, which were seeded with primary HTM cells, were able to imitate  
260 some of the normal tissue functions *in vivo*. This included being able to induce or reverse  
261 glaucomatous conditions using medication<sup>66,52,67</sup>. A follow-up study showed that applying a  
262 hyaluronic acid-containing hydrogel coating to the SU-8 scaffold improved cell proliferation.  
263 Over time, various technologies and materials have been explored, including traditional  
264 polymeric filters, SU-8 membranes, electrospun nanofibers, and other methods. These  
265 methods offer precise control over morphological characteristics such as porosity and beam  
266 thickness in both 2D and 3D environments. However, the stiffness of the scaffold cannot be  
267 directly controlled using most of these methods. Despite these limitations, 3D cultures have  
268 the potential to create an *in vivo*-like microenvironment for HTM cell growth. Tissue  
269 engineering aims to produce functional biomimetic replicas of tissues of interest, but only a

270 limited number of studies have been reported on bioengineered 3D HTM *in vitro* models.  
271 These models partially mimic normal tissue function and provide a platform for drug testing  
272 and evaluating the effectiveness of different treatment options. Wlodarczyk-Biegun et al.  
273 studied the biofabrication technique of melt electrowriting (MEW), a marriage between  
274 electrospinning and 3D printing, as a means of producing fibrillar and porous scaffolds with  
275 thermoplastic polymers that replicate the multilayer and gradient structure of the natural HTM  
276 <sup>68</sup>. HTMC cultured on these scaffolds maintained the phenotype of native HTMC and  
277 infiltrated the scaffolds. However, some may argue that these models are more comparable to  
278 a 2D cell culture system rather than a true 3D model, as they cannot fully replicate the 3D  
279 cell-ECM interface apart from the ECM secreted by the HTM cells grown on top of the  
280 synthetic polymer scaffold.

281

#### 282 *Hydrogel- based TM scaffolds*

283 Recent research has focused on using hydrogels as scaffolds to study the behavior of HTMC  
284 in response to environmental changes and disease conditions<sup>69,70</sup>. Hydrogels are networks of  
285 crosslinked, hydrophilic polymers used to recapitulate the 3D architecture of organ systems in  
286 tissue engineered models. These materials are so useful in cell culture because they provide a  
287 biocompatible, degradable, hydrated microenvironment that mimics the cell-ECM  
288 interactions of natural tissues. Hydrogel scaffolds offer higher control over the morphology,  
289 stiffness, and 3D environment compared to photolithography and electrospinning, while also  
290 maintaining structural integrity.

291 Ideally, 3D cell culture matrices can reproduce the features of the ECM to closely  
292 resemble the *in vivo* environment. The interaction between cells and ECM is essential for a  
293 range of biophysical and biochemical functions, such as the transportation of signaling  
294 molecules, nutrients, and waste metabolites, as well as mechanical integrity. Thus, these

295 matrices need to reflect the specific ECM characteristics of each tissue for a given application.  
296 Moreover, the mechanical properties of 3D matrices are also significant, as they can directly  
297 influence cell adhesion, thereby affecting both the shape and response of cells<sup>71</sup>. The  
298 utilization of degradable scaffolds presents an opportunity for more molecular research, as  
299 opposed to permanent ones. Hydrogels can be created using various synthetic or natural  
300 components. In tissue engineering, natural polymers are the most commonly used approach to  
301 developing hydrogels<sup>72</sup>. Collagen, fibrin, and elastin, which are components of the ECM of  
302 the TM, have been used as attachment factors for HTMC to study specific functions and  
303 interactions<sup>65</sup>. 3D Corning® Matrigel® Matrix (Corning Life Sciences, Tewksbury, MA,  
304 United States) contains several proteins found in extracellular matrix (ECM) such as laminin,  
305 collagen IV, heparin sulfate proteoglycan, and entactin/nidogen<sup>73,74</sup>. Several studies  
306 demonstrated that unlike cells cultured on traditional 2D planar surfaces, cells in 3D scaffolds  
307 are more physiologically relevant concerning *in vivo* characteristics exhibited by *in-vivo*  
308 surrogates<sup>75</sup> (figure 1). Vernazza et al. conducted a study to compare the response of HTMC  
309 in 2D and 3D *in vitro* models following chronic stress exposure. Their results revealed that  
310 3D TMC cultured on Matrigel exhibited a higher sensitivity to the production of intracellular  
311 reactive oxygen species induced by hydrogen peroxide treatment compared to 2D cultures.  
312 Furthermore, the 3D models demonstrated a more precise regulation of apoptosis triggers and  
313 cell adaptation mechanisms than the 2D models<sup>33</sup>. Another scaffold-based approach by  
314 Osmond and colleagues utilized HTMC cultured on various collagen scaffolds containing  
315 different glycosaminoglycans (GAGs) and different pore architectures to better understand  
316 how HTMC respond to changes in their extracellular environment. The cellular response was  
317 assessed by quantifying cellular proliferation and the expression of fibronectin, an important  
318 extracellular matrix (ECM) protein. Fibronectin plays a crucial role in organizing ECM  
319 proteins both among themselves and with trabecular cells, thereby contributing to the

320 resistance of outflow<sup>76-78</sup>. The pore architecture of the scaffolds was altered using the freeze-  
321 casting technique to make both large and small pores that are aligned or with a non-aligned  
322 random structure. The composition of the scaffolds was altered with the addition of  
323 chondroitin sulfate and/or hyaluronic acid. It was found that HTMC grown on large pore  
324 scaffolds proliferate more than those grown on small pores. There was an increase in the  
325 fibronectin expression with the incorporation of GAGs, and its morphology was changed by  
326 the underlying pore architecture. These works offer a better understanding of how human  
327 TMC behave in response to alterations in their microenvironment and highlight the  
328 importance of the mimicry of the 3D structure<sup>79-81</sup>. However, the study did not explore how  
329 the constructs would react under conditions that induce glaucoma. Furthermore, if the  
330 accumulation of extracellular matrix (ECM) proteins is a characteristic feature of the  
331 pathogenic process in glaucoma, it is important to highlight that cell proliferation is not<sup>77,82</sup>.  
332 Therefore, it is crucial to determine whether cells can survive under normal conditions on  
333 these new substrates. However, it should be noted that the ability to proliferate does not  
334 necessarily indicate an appropriate glaucoma model.

335 3D bioprinting can produce a variety of architectural patterns on a wide array of biomaterials.  
336 Li and colleagues developed a hydrogel using a tissue-engineering approach for HTM. The  
337 hydrogel consisted of ECM biopolymers and normal HTMC obtained from a donor. By  
338 mixing HTMC with collagen type I, hyaluronic acid (HA), and elastin-like polypeptide (ELP)  
339 - each containing photoactive functional groups - researchers were able to create HTM  
340 hydrogels in various sizes and shapes. Short UV cross-linking, mediated by photo-initiators,  
341 was used to solidify the hydrogels. To induce glaucomatous changes, dexamethasone (DEX)  
342 was administered, and the therapeutic effects of the ROCK inhibitor Y27632 were evaluated<sup>83</sup>.  
343 To create an *in vitro* 3D TM scaffold for potential use as a tissue scaffold in glaucoma  
344 patients after trabeculectomy, Waduthanthri et al. developed a hydrogel peptide called

345 MAX8B which partly mimics the motif of cellular integrins and enables interactions with  
346 ECM components<sup>84</sup>. The scaffold material demonstrated the ability to undergo shear-thinning  
347 and exhibited biocompatibility, facilitating appropriate growth and proliferation of TMC in  
348 tightly packed cell monolayers resembling typical TMC morphology. Moreover, the MAX8B  
349 scaffold was utilized in an *in vitro* perfusion system to investigate the impact of  
350 Dexamethasone on the outflow facility of the trabecular meshwork proving the effectiveness  
351 of this three-dimensional (3D) model as a platform for drug testing<sup>84</sup>.

352

### 353 *Spheroids*

354 Although 3D culture techniques have gained popularity for their ability to better mimic *in*  
355 *vivo* environments, there are some limitations when it comes to replicating physiological and  
356 pathological conditions of human TM. This is because the use of scaffolds in 3D cultures is  
357 not reflective of the absence of such structures in human TM. However, 3D spheroid cell  
358 cultures have recently emerged as a promising alternative to conventional 2D cell cultures,  
359 particularly as *in vivo* models for various diseases. These spheroids allow for more  
360 intercellular interactions in a 3D space, potentially resulting in protein networks that resemble  
361 those found in real tissues. This makes it possible for 3D spheroids to replicate biological  
362 features associated with real tissues.

363 The spheroid model of TM refers to a 3D culture system that mimics the structural and  
364 functional properties of the TM in the eye. HTMC have been cultured as spheroids *in vitro* to  
365 study their role in glaucoma. These spheroids have been shown to exhibit features of the TM  
366 *in vivo*, such as the presence of ECM components and cytoskeletal proteins. These spheroids  
367 have been shown to respond to mechanical stress and exhibit physiological responses similar  
368 to those observed *in vivo*. These spheroids have been shown to be structurally and  
369 functionally similar to the TM *in vivo* and have been used to study the effects of various drugs



370 on TMC behavior. 3D HTM spheroids became significantly and differently smaller and stiffer  
371 in response to TGF- $\beta$ 2 or dexamethasone stimulation<sup>41,85</sup>. Watanabe et al. successfully  
372 obtained 3D HTM spheroids and found that TGF $\beta$ 2 significantly induced the down-sizing and  
373 stiffness of 3D spheroids from human orbital fibroblasts, and those effects were substantially  
374 inhibited by a ROCK-inhibitor.<sup>42,86</sup>

375

### 376 *Outflow studies*

377 Perfusion studies of outflow in HTMC began in the late 1980s and have since evolved to  
378 include a range of techniques and models. One of the earliest studies involved the use of  
379 filters to culture HTMC and measure hydraulic conductivity using a pressure/flow circuit<sup>87</sup>.  
380 This study led to further investigations into the biomechanics of HTMC. The perfusion  
381 system developed by Yubing Xie's group enabled continuous pressure monitoring at different  
382 flow rates to investigate the effects of drugs such as Lat-B, ROCK inhibitors, or TGF $\beta$ 2<sup>52,66,67</sup>.  
383 As previously mentioned, 3D culture models of TMC are superior to 2D models due to the  
384 ability to enable cell-cell and cell-ECM interactions. However, these 3D models fail to  
385 reproduce the dynamic continuous supply of nutrients, oxygen, and removal of metabolic  
386 waste products. Recent advances propose models that combine the benefits of 3D culture with  
387 milli-fluidic techniques to improve the physiological relevance of the culture and address the  
388 issues related to cell responses under static culture conditions. Microfluidic systems allow for  
389 the creation of a 3D microenvironment that mimics the *in vivo* conditions of the TM,  
390 including the presence of shear stress and fluid flow. Recently, the MAX8 peptide-hydrogel  
391 scaffold and a 3D Matrigel® model have been tested in perfusion chambers to evaluate their  
392 use as artificial TM scaffolds<sup>39,84</sup>.

393 In their closed-circuit *in vitro* model developed by Tirendi et al., 3D-HTMCs cultured  
394 in Matrigel were provided with a continuous medium supply. This was achieved by

395 connecting single-flow bioreactor culture chambers to a peristaltic pump. The milli-fluidic  
396 technology as well as the 3D culture model mimicked cell responses found *in vivo* as a result  
397 of the increase in outflow resistance<sup>57</sup>. This type of model can be used to investigate the  
398 effects of various factors on TM function, such as mechanical stress and changes in ECM  
399 composition.

400

#### 401 *Ex vivo models*

402 Ex vivo models, specifically perfusion studies, utilizing animal eyes such as pigs,  
403 cows, and primates, have been instrumental in advancing our understanding of glaucoma<sup>88-91</sup>.  
404 These models offer valuable insights into the dynamics of aqueous humor outflow and  
405 provide a platform to investigate the effects of various experimental interventions on the  
406 disease. By perfusing the enucleated eyes with a controlled flow of fluid, researchers can  
407 mimic physiological conditions and measure parameters such as intraocular pressure and  
408 outflow facility. These models have helped elucidate the mechanisms underlying glaucoma  
409 and evaluate potential treatments<sup>63,89</sup>. For example, Zhou et al. developed a platform to  
410 simultaneously evaluate outflow facility and its time- and dose-dependent responses to  
411 treatments of 20 eyes. They used whole porcine and bovine eyes to develop a perfusion  
412 system and studied the regulation of outflow facility by endothelin-1, nitric oxide donor, and  
413 sphingosine1-phosphate<sup>63</sup>.  
414 However, it is important to acknowledge the limitations of ex vivo models. They do not fully  
415 replicate the complex in vivo environment of the eye, including interactions with surrounding  
416 tissues and systemic factors. Additionally, the use of animal eyes may introduce species-  
417 specific differences that may not fully reflect human physiology. Given these limitations and  
418 the fact that they do not represent an alternative to the reliance on animal experimentation, we  
419 will primarily focus on human models.

420 The human anterior segment perfusion culture model is a valuable tool for studying the TM  
421 and aqueous humor outflow in glaucoma<sup>89,92,93</sup>. *Ex vivo* models possess several significant  
422 benefits compared to other models, including their ability to maintain the structure of  
423 pathways and their capacity to facilitate analysis in nearly ideal physiological conditions<sup>94</sup>.  
424 Outflow facility measurements can be performed *ex vivo* or *in vivo*, with *ex vivo*  
425 measurements offering a simpler approach by avoiding confounding factors that are difficult  
426 to control. However, *in vivo* measurements are more representative of real-life conditions.

427 Bahler et al. used perfusion organ culture of human anterior segments to study the  
428 effect of prostaglandin on the trabecular outflow. Since this human anterior segment culture  
429 model lacks a choroid or functional ciliary body, the uveoscleral pathway is absent. This  
430 simplification facilitates the analysis by directly assessing the sclera's impact on outflow  
431 facility<sup>93</sup>.

432 Peng et al. have created an *ex vivo* model of human corneal rim for perfusion culture  
433 experiments as an alternative to the human anterior segment perfusion culture model. This  
434 model can be used to study the TM and aqueous humor outflow in glaucoma while improving  
435 cost and availability. The corneal rims were affixed to 3D-printed perfusion culture plates and  
436 perfused in constant flow mode. Pressure changes were recorded using a computerized system.  
437 TM stiffness of corneal rims treated with dexamethasone was significantly higher than in the  
438 control group<sup>95</sup>. Additionally, the model allows histology or immunohistochemistry of the  
439 TM to investigate biomechanical changes or treatments.

440 Baudouin et al. examined TM specimens using immunohistology and reverse transcriptase–  
441 polymerase chain reaction. Trabecular specimens of glaucomatous patients showed extremely  
442 low densities of trabecular cells and the presence of cells expressing fractalkine and  
443 fractalkine receptor and their respective mRNAs<sup>64</sup>. These explants methods have the  
444 advantages to retain tissue architecture and cellular interactions closer to *in vivo* conditions as

445 opposed to traditional cell culture methods. They are suitable for studying tissue responses  
446 and drug effects at the cellular level<sup>96</sup>. The low cell count of TMC in TM explants from  
447 glaucomatous patients can be circumvented by using TM from healthy donors and exposing  
448 them to TGF- $\beta$ 2. The addition of TGF- $\beta$ 2 to healthy TM permits reproduction of the changes  
449 in TM cell cytoskeletal organization and ECM compaction, while providing sufficient  
450 material for a transcriptomic study<sup>2,82,97</sup>.

451

## 452 **Discussion**

453 This article discusses the importance of developing models of TM, a structure within the eye  
454 that plays a crucial role in regulating IOP, to study the pathophysiology of glaucoma. The TM  
455 is a dynamic filtration system that helps regulate IOP by controlling the outflow of aqueous  
456 humor.

457         Developing new 3D *in vitro* models is crucial to studying TM pathophysiology in  
458 glaucoma. They mimic the physiological microenvironment of the TM, providing a more  
459 physiologically relevant model than traditional 2D cell culture methods<sup>98</sup>.

460 One of the key advantages of these 3D models is that they reduce the need for animal studies,  
461 which can be costly, time-consuming, and ethically challenging<sup>15,30</sup>. *In vitro* models can be  
462 used as a complementary tool to animal studies, as they can provide useful data on  
463 mechanisms and drug efficacy before moving to animal models or clinical trials.

464         While *in vitro* models offer several advantages, they also have limitations that need to  
465 be considered. One of the main challenges is that *in vitro* models may not fully recapitulate  
466 the complexity of the TM *in vivo*, such as interactions with other tissues and the influence of  
467 systemic factors. To address this limitation, researchers often use a multiplicity of models to  
468 collect data for a particular question. For example, to study the modification of ECM, a  
469 natural hydrogel medium that closely resembles the components of TM ECM is more

470 interesting than a synthetic one. As it provides a more physiologically relevant environment  
471 that can better mimic the ECM interactions in the TM. Similarly, a microfluidic bioreactor  
472 can be used to study the effect of sheer stress or biomechanical impact on TMC<sup>66,99</sup>. This type  
473 of model allows researchers to control the flow of fluids and apply mechanical forces to the  
474 cells, providing more accurate simulations of the TM microenvironment. A comparison of  
475 innovative 3D TM models and measured outcomes is presented in supplementary table 1.

476 Furthermore, biomimetic 3D *in vitro* models, in addition to enhancing our  
477 understanding of TM tissue biology and outflow pathology, have the potential to be used  
478 therapeutically for restoring compromised TM function<sup>100</sup>. Promising research has  
479 demonstrated the effectiveness of stem cell therapy in repairing TM tissue and preserving  
480 vision in glaucoma patients<sup>46</sup>. Moreover, the presence of TM progenitor cells capable of  
481 differentiating into functional TM cells further supports the potential for tissue repair<sup>101,102</sup>.  
482 Advanced biofabrication techniques allow for the creation of scaffolds that closely mimic the  
483 native ECM and provide cues for stem or progenitor cell differentiation, replicating cellular  
484 responses observed *in vivo*<sup>103</sup>. By incorporating biomaterials alongside TM progenitor cells,  
485 the development of a delivery system for effective stem cell therapy can be facilitated.

486 In conclusion, the use of multiple models that can better replicate the different aspects  
487 of the TM *in vivo* can provide more robust and accurate data. By using a combination of *in*  
488 *vitro*, *ex vivo*, and *in vivo* models, researchers can gain a more comprehensive understanding  
489 of glaucoma pathology and develop better treatments for this disease.

490 However, it is important to consider the limitations of non-animal. The progress made  
491 in the alternative models presented in this study does not imply that we can completely  
492 eliminate the need for animal experimentation at present. *In vivo* experiments enable a  
493 substantial prediction of the effect of hypotonic treatment on IOP, even if the organization of  
494 their outflow system is not totally similar to that of humans<sup>16</sup>. These alternative models are

495 still in their early stages and may not fully replicate the complexity of the TM or its  
496 interactions within the eye. They may not provide the same comprehensive data as animal  
497 models, particularly in terms of assessing IOP, estimating natural flow rate, accessing the  
498 outflow facility, evaluating cellularity, tissue integrity, and capturing natural expression  
499 profiles as it would be in a living *in vivo* system. Additionally, organ culture has a significant  
500 limitation whereby the regulation of IOP relies solely on external manipulative regulations,  
501 lacking intrinsic regulation in enucleated eyes. Nonetheless, despite these current limitations,  
502 the progress made in developing these alternative models is encouraging. While they may not  
503 completely replace the need for animal models, they do hold the potential to significantly  
504 reduce their utilization, provided of course that the trabecular cells used are not derived from  
505 animals.

506 Overall, this progress in *in vitro* and *ex vivo* models offers a promising tool for studying the  
507 TM in glaucoma and reducing the need for animal studies. While it has limitations, it provides  
508 a more physiologically relevant model than traditional 2D cell culture methods, and its  
509 potential applications in drug discovery and testing make it a valuable addition to glaucoma  
510 research.

511

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840 **Figure 1: Confocal microscopy image of the 3D cultured pHTMCs in Matrigel.** The pHTMC is  
841 organized in a mesh conformation with interconnections and the formation of intercellular spaces.  
842 Actin fibers are stained in red by phalloidin, membranes with DiO (green), and nuclei with DAPI  
843 (blue). Magnification 200X.

844