1 Title: Transepithelial riboflavin absorption in an ex-vivo rabbit corneal model.

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25 **ABSTRACT**

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27 **Purpose:** To measure depth-specific riboflavin concentrations in corneal
28 stroma using two-photon fluorescence microscopy and compare commercially
29 available transepithelial corneal collagen cross-linking (CXL) protocols.

30

31 **Methods:** Transepithelial CXL riboflavin preparations – MedioCross TE,
32 Ribocross TE, Paracel plus VibeX Xtra and Iontophoresis with Ricrolin Plus –
33 were applied to the corneal surface of fresh postmortem rabbit eyes in
34 accordance with manufacturers' recommendations for clinical use. Riboflavin
35 0.1% (Vibex Rapid) was applied after corneal epithelial debridement as a
36 positive control.

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38 After riboflavin application, eyes were snap frozen in liquid nitrogen. 35µm
39 thick corneal cross-sections were cut on a cryostat, mounted on a slide and
40 imaged by two-photon fluorescence microscopy. Mean (SD) concentrations
41 were calculated from 5 globes tested for each protocol.

42

43 **Results:** Peak riboflavin concentration of 0.09 % ($\pm$ 0.01) was observed within
44 the most superficial stroma (stromal depth 0 - 10 µm) in positive controls
45 (epithelium-off). At the same depth, peak stromal riboflavin concentrations for
46 Mediocross TE, Ricrolin +, Paracel/Xtra and Ribocross TE were: 0.054 % ($\pm$
47 0.01), 0.031 % (0.003), 0.021 % ($\pm$ 0.001) and 0.015 % ($\pm$ 0.004) respectively.

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49 At a depth of 300 µm (within the demarcation zone commonly seen after
50 corneal cross-linking), the stromal concentration in epithelium-off positive

51 controls was 0.075 % ($\pm$ 0.006), while at the same depth MedioCross TE and
52 Ricrolin + achieved 0.018 % ($\pm$ 0.006) and 0.016 % (0.002) respectively. None
53 of the remaining transepithelial protocols achieved concentrations above
54 0.005% at this same 300 μ m depth. Overall, MedioCross TE was the best
55 performing transepithelial formulation.

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57 **Conclusions:** Corneal epithelium is a significant barrier to riboflavin
58 absorption into the stroma. Existing commercial transepithelial CXL protocols
59 achieve relatively low riboflavin concentrations in the anterior corneal stroma
60 when compared to gold-standard epithelium-off absorption. Reduced stromal
61 riboflavin concentration may compromise the efficacy of riboflavin/ultraviolet
62 corneal CXL.

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73 **INTRODUCTION**

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75 Corneal collagen cross-linking (CXL), using the original 'Dresden' epithelium-
76 off protocol¹ is effective in arresting disease progression in keratoconus in
77 over 90% of eyes.²⁻⁴ The corneal epithelium is removed in this protocol to
78 facilitate riboflavin absorption and hence treatment efficacy. However,
79 complications including infection, scarring, delayed healing, sterile
80 inflammatory infiltration, tissue melting and resultant vision loss have all been
81 described in association with corneal epithelial removal in CXL.⁵ Due to these
82 complications, CXL is normally reserved for patients with demonstrable
83 disease progression.

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85 Transepithelial CXL, in which riboflavin is applied through an intact epithelium,
86 aims to deliver the benefits of standard epithelium-off treatments without the
87 painful rehabilitation and complications of epithelial removal. However, the
88 only published randomised controlled trial of this newer technique⁶ recently
89 concluded it was not effective compared with epithelium-off CXL. Most
90 commercially-available riboflavin formulations for transepithelial CXL are
91 designed to enhance epithelial permeability by the addition of epithelial-toxic
92 agents (table 1). Another method involves iontophoresis, in which negatively-
93 charged riboflavin is driven through the epithelium down an electrical
94 gradient.⁷⁻⁹

95

96 Previous studies to quantify riboflavin penetration through an intact epithelium
97 have used high-performance liquid chromatography (HPLC)¹⁰⁻¹² and
98 fluorescence microscopy.¹³⁻¹⁷ HPLC, in which the sample is dissolved in a
99 solvent for analysis, can accurately quantify the concentration of riboflavin in a

100 whole block of tissue, but is unable to provide information about the
101 concentration at different depths within the corneal stroma (unless lamella
102 sections are prepared and separately dissolved).¹⁸ Fluorescence microscopy,
103 both single-photon excitation (i.e. confocal) and multi-photon excitation^{13–17}
104 has the added advantage of quantifying concentration at depth within the
105 cornea; both excitation techniques, however, suffer from signal attenuation
106 with increasing scan depth, principally attributable to scattering and
107 aberration.^{14–16} We have previously reported¹⁴ a novel time-lapse
108 measurement approach to correct for two-photon fluorescence (TPF) signal
109 attenuation; although applicable to epithelium-off absorption, this method has
110 not proven suitable when imaging through an intact epithelium.¹⁹

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112 Here we describe a new method for quantifying riboflavin concentration within
113 the cornea using an ex vivo animal rabbit eye model and compare the efficacy
114 of current commercially available protocols for transepithelial CXL in
115 promoting riboflavin absorption.

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121 **MATERIALS AND METHODS**

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123 Ethical approval for corneal two-photon fluorescent microscopy studies in an
124 ex vivo model was granted by University College London Institute of
125 Ophthalmology (ref. 10/H0106/57-2012ETR27).

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127 **Sample preparation**

128 Adult pigmented rabbit heads transported on ice in a phosphate buffered
129 saline (PBS) bath were received within 5 hours post-mortem (First Link Ltd.,
130 Wolverhampton, UK). Intact globes were enucleated and examined under a
131 low-magnification light microscope to rule out obvious epithelial trauma or
132 scars. The globes were warmed to room temperature in balanced salt solution
133 before different commercially-available riboflavin solutions (table 1) were
134 applied to the corneas according to the manufacturers' protocols. Corneas
135 soaked for 30 minutes with 0.1 % riboflavin in HPMC and saline (VibeX Rapid,
136 Avedro, Inc) after epithelial debridement served as positive controls and
137 unsoaked corneas served as negative controls.

138

139 Commercial transepithelial CXL protocols we compared were (n=5 for each
140 protocol):

141 *1. Medio-Cross TE (Peschke Meditrade GmbH, Germany)*

142 A 9 mm vacuum well secured on the cornea was filled with approximately 0.5
143 ml of Medio-Cross TE solution for 30 minutes. Riboflavin was not rinsed from
144 the cornea.

145 *2. Ribocross TE (IROS Srl, Napoli, Italy)*

146 A 9 mm vacuum well secured on the cornea was filled with approximately 0.5
147 ml of Ribocross TE solution for 30 minutes. Riboflavin was not rinsed from the

148 cornea.

149 *3. ParaCel and VibeX Xtra (Avedro, Inc., Waltham, MA, USA)*

150 ParaCel drops were applied at a rate of 1 drop every 90 seconds for 4
151 minutes. The cornea was then rinsed with VibeX Xtra completely coating the
152 cornea. Additional VibeX Xtra was applied at a rate of 1 drop every 90
153 seconds for 6 minutes (total riboflavin soak time 10 minutes). Riboflavin was
154 not rinsed from the cornea.

155 *4. Ricrolin+ (Sooft Italia S.p.A. Montegiorgio, Italy)*

156 The iontophoresis system comprised 2 electrodes: a negatively-charged metal
157 grid housed within the corneal applicator and a positively-charged 20G needle
158 inserted through the sclera into the vitreous. The corneal applicator was
159 vacuum-attached to the cornea. The reservoir was filled with Ricrolin + above
160 the level of the metal grid before beginning a 5 minute treatment with a
161 continuous direct current of 1 mA (figure 1A). After the iontophoretic
162 procedure, the applicator was removed and riboflavin rinsed from the cornea.

163

164 **Section preparation**

165 At the end of the riboflavin soak, the globes were immediately immersed in
166 liquid nitrogen for 5 minutes and stored overnight at -25 degrees Celsius. A
167 broad incision across the posterior globe was made just before immersion to
168 prevent the cornea from splitting open on freezing. For each globe, three
169 35 µm corneal cross-sections were cut on a cryostat 1 mm apart
170 (Supplementary Fig. S1). With the frozen section on the blade platform
171 (maintained at -21°C), a knife was used to cut the cornea free and the
172 remaining tissue was brushed away. The cornea itself was meticulously

brushed to ensure no ice remained attached that might allow riboflavin to leak out once thawed. The corneal section was then mounted on a slide and covered with fluorescence-free immersion oil (Immersol 518 F, Carl Zeiss Ltd.) to prevent any leakage of riboflavin out of the tissue. A coverslip was placed on top prior to imaging under the microscope objective (figure 1B). The time taken from the tissue thawing onto the slide to image acquisition on the microscope was approximately 1 minute, and we refer to this earliest imaging time point as $t = 0$ minutes. To investigate riboflavin migration within the thawed slide-mounted tissue, serial images were captured every minute for selected samples. For all other measurements, data was acquired as soon as possible following tissue thawing, i.e. after approximately 1 minute.

Two-photon microscope set-up and imaging protocol

We have previously described the use of two-photon fluorescence (TPF) microscopy for imaging corneal cross-sections.¹⁴ In brief, a Ti:Sapphire laser, operating at a wavelength of 890 nm with a 140 femtosecond pulse duration and 80 MHz pulse repetition rate, was used as the excitation laser source. The excitation laser beam was guided to a Leica DM6000CS upright microscope where it passes through two galvoscaners, allowing scanning in the x-y plane, before being focused into the sample by a Leica 10 X /0.3 NA water immersion objective. Theoretical (full-width half-maximum) axial and lateral resolutions were calculated at 16 μm and 1.1 μm respectively. TPF excitation light of 890 nm wavelength was chosen to correspond with the highest riboflavin absorption peak (445 nm) as determined by spectrophotometry. Emitted riboflavin fluorescence was collected between

198 525 nm and 650 nm to avoid overlap with the absorption spectrum of
199 riboflavin. 553 μm x 553 μm (512 x 512 pixel) images were captured at a scan
200 rate of 600 Hz (line average 16) with the pinhole wide open.

201

202 **Image analysis**

203 Grey scale images (figure 1C) were exported and analysed using Java-based
204 imaging software (ImageJ, 1.48v, [http:// imagej.nih.gov.ij](http://imagej.nih.gov/ij); provided in the
205 public domain by the National Institutes of Health, Bethesda, MD, USA). For
206 each image, three separate rectangular regions of interest each 40 pixels
207 wide in the x-direction (see figure 1C) were manually selected. The intensity
208 profiles along the z-direction for each region of interest were then exported to
209 a .txt file (Excel for Mac, 2011; Microsoft Corp, Redmond, WA, USA) (figure
210 1D). The epithelial/stromal junction was then identified in each trace by the
211 abrupt change in signal and confirmed with reference to the corresponding
212 image region of interest. This information was then used to align all three plots
213 along the z-axis and a mean intensity plot was then generated representing
214 the average TPF signal for that image as a function of depth (z). TPF signals
215 were converted to riboflavin concentration by normalizing to the TPF signal
216 achieved in a well-slide reservoir of 0.1 % riboflavin solution during the same
217 experimental session. Mean (SD) concentrations were calculated from 5
218 globes tested for each protocol.

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220

221 **RESULTS**

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223 **Riboflavin migration within thawed tissue**

224 Serial images were captured at time = 0, 1 and 2 minutes (t = 0 being the first
225 scan approximately 1 minute after the tissue thawed on the slide). Figure 2
226 confirms fluorophore migration within the sample, redistributing posteriorly
227 with time including from the epithelium into the underlying stroma. Measured
228 at the beginning of the stroma, this drop in signal is approximately 7% within
229 the first minute. Therefore, all subsequent data was acquired as soon as
230 possible following thawing, i.e. after approximately 1 minute.

231

232 **Depth-specific corneal riboflavin absorption**

233 No fluorescence was detected by TPF microscopy in the negative (untreated)
234 controls. The maximum riboflavin concentration in positive controls
235 (epithelium-off) was 0.09 % (± 0.01). Maximum stromal riboflavin
236 concentrations for Mediocross TE, Ricrolin +, Paracel/Xtra and Ribocross TE
237 were: 0.054 % (± 0.01), 0.031 % (0.003), 0.021 % (± 0.001) and 0.015 % ($\pm$
238 0.004) respectively. Depth-specific riboflavin concentrations are displayed in
239 figures 3-5. Stromal absorption of riboflavin by iontophoresis was not
240 homogenous, with areas of relative hypo- and hyper fluorescence within each
241 section. In all samples, the epithelial concentration was higher than that in the
242 underlying stroma. A film of higher signal riboflavin adherent to the epithelium
243 was detected in samples treated with Ribocross TE.

244

245 At a depth of 300 μm (within the demarcation zone commonly seen after
246 corneal cross-linking), the stromal concentration in epithelium-off positive

247 controls was 0.075 % ($\pm$ 0.006), while at the same depth MedioCross TE
248 and Ricrolin + achieved 0.018 % ($\pm$ 0.006) and 0.016 % (0.002) respectively.
249 None of the remaining transepithelial protocols achieved concentrations
250 above 0.005% at this same 300 μ m depth.

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252

253 **DISCUSSION**

254

255 These data suggest that absorption of riboflavin into the corneal stroma is
256 significantly reduced in current commercial transepithelial CXL protocols in
257 comparison with epithelium-off CXL.

258

259 Since most tissue cross-linking is thought to take place in the anterior
260 cornea,²⁰ determining the availability of key photochemical ingredients
261 (riboflavin, UV light, oxygen) at specific depths may add useful additional
262 information. The method we describe above provides these depth-specific
263 measurements of riboflavin, with the microscope objective and image size
264 determining the resolution available. An additional advantage of our technique
265 is the ability to quantify riboflavin concentration within the epithelium which, as
266 our results demonstrate, exceeds that in the underlying stroma. In clinical
267 practice, the consequences of loading the corneal epithelium with riboflavin in
268 transepithelial CXL could include epithelial toxicity and UV light shielding. The
269 normal epithelium filters out an average 20% of UVA light radiation passing
270 through the cornea.²⁰ This UV light attenuation is increased by riboflavin
271 absorption within the epithelium in transepithelial CXL, and stromal

272 concentrations of riboflavin are reduced. Enhanced UV absorption with free
273 radical generation within the epithelium could result in 'arc eye' type epithelial
274 toxicity. Epithelial defects are commonly observed on day 1 after
275 transepithelial CXL using currently available protocols,²¹ and punctate
276 epithelialopathy is evident in all cases (personal communication, David
277 O'Brart, February 2015).

278

279 The inability to measure concentration at specific depths in the epithelium and
280 stroma is a significant limitation of HPLC. Furthermore, unless this riboflavin-
281 rich epithelium is specifically removed prior to dissolving the sample, the
282 concentration result will be inappropriately increased. This may have
283 contributed to the observations described by Ostacolo et al.¹¹ in which
284 Ribocross TE was tested using modified Franz-type diffusion cells with freshly
285 excised porcine corneas (both with and without epithelium). After 40 minutes,
286 riboflavin accumulation through an intact epithelium (0.394 ± 0.02 nmol/mg)
287 matched that achieved epithelium-off (0.396 ± 0.03 nmol/mg). This contrasts
288 with our results in which Ribocross TE performed poorly compared to other
289 protocols tested. As demonstrated in figures 4 & 5, we observed TFP signals
290 within the epithelium to be higher than in the underlying stroma in all tested
291 protocols. Furthermore, the pre-corneal film of riboflavin solution exhibited
292 even higher fluorescence signal. Dissolving a full-thickness specimen,
293 including a riboflavin-loaded epithelium and pre-corneal film, will lead to
294 overestimation of corneal stromal riboflavin concentrations by HPLC. This
295 may explain why Ostacolo et al.¹¹ recorded only a 4-fold reduction in stromal
296 concentration when their control riboflavin solution (i.e. no vitamin E) was

297 applied to an intact epithelium, compared with epithelium-off application
298 (0.098 ± 0.04 nmol/mg epithelium-on; 0.396 ± 0.03 nmol/mg epithelium-off).
299 Cassagne et al.¹² also used HPLC in an in vivo rabbit eye study of
300 iontophoresis, testing both the dissolved cornea and aqueous sample. Again,
301 the investigators did not remove the epithelium prior to analysis. This may, in
302 part, explain the discrepancy in their results in which they observed 45% less
303 riboflavin in corneas treated using iontophoresis (compared to epithelium-off
304 controls), but 95% less riboflavin in the aqueous of iontophoresis treated
305 eyes.

306

307 There are a number of limitations to this study. Firstly, results in ex-vivo rabbit
308 corneas, although a better anatomical match to human corneal epithelial
309 thickness than porcine eyes, may be affected by post-mortem changes in
310 epithelial layer integrity. Measures taken to minimize this compromise
311 included a short postmortem interval, immersion in chilled PBS immediately
312 postmortem, and warming to physiological temperature prior to preparation of
313 corneas for experimentation. We observed no obvious stromal swelling
314 (indicative of endothelial pump failure) in negative controls, and mean corneal
315 thicknesses to those published for rabbit corneas in vivo ($400 \mu\text{m}$).²² Despite
316 this, some epithelial degradation and enhanced permeability is likely, and our
317 results may overestimate stromal riboflavin absorption in clinical
318 transepithelial CXL. Secondly, migration of riboflavin within the snap frozen
319 tissue will have started as soon as the section thawed on the slide. Even with
320 the cryostat next to the microscope, there was still a delay of up to a minute
321 before image acquisition. This would tend to result in an underestimation of

322 stromal riboflavin concentrations. Finally, imaging through two media of
323 different refractive indices (oil and water either side of the coverslip) may have
324 increased optical aberrations as the laser light passes through. We chose oil
325 to ensure no (water soluble) riboflavin leaked out of the tissue. When we tried
326 to embed excised corneas in optimal cutting temperature (OCT) compound
327 we observed very prompt dye leakage once thawed on the slide (data not
328 shown). This may have impacted the study of Cassagne et al.¹². For lack of
329 an appropriate oil-immersion objective, we placed a coverslip on top and used
330 a water-immersion objective. Any induced aberrations may have resulted in a
331 small absolute loss of signal; but since this same method was employed for all
332 imaged samples, no relative drop between samples should be present.

333

334 Notwithstanding the above laboratory studies, results from preliminary clinical
335 case series using non-iontophoretic transepithelial riboflavin preparations
336 have been equivocal, with some showing similar efficacy to epithelium-off
337 CXL,^{23,24} whilst most have demonstrated less pronounced effects.^{25-27,6,28} 12-
338 month results from one recently published randomised controlled trial⁶
339 concluded that transepithelial CXL using Ricrolin TE (Sooft Italia S.p.A.
340 Montegiorgio, Italy) was not effective at stabilising corneal shape compared
341 with epithelium-off treatment. For iontophoresis, up to 15 month follow-up data
342 have been published, with reported cessation of disease progression and
343 improvements in keratometric and visual parameters.⁷⁻⁹ The longer-term
344 relative efficacy of all these transepithelial techniques compared to epithelium-
345 off CXL remains unknown.

346

347 In conclusion, the methodology described above provides a quantitative
348 means of measuring riboflavin across the whole cornea in an ex vivo model.
349 We present evidence that the corneal epithelium represents a significant
350 barrier to riboflavin absorption into the stroma. Existing commercial
351 transepithelial CXL protocols load the corneal epithelium with riboflavin and
352 achieve relatively low riboflavin concentrations in the anterior corneal stroma
353 when compared to epithelium-off CXL. Although a key rate-limiting step, the
354 absolute stromal concentration of riboflavin required for effective CXL is not
355 known. Without further evidence, the concentration achieved 'epithelium-off'
356 remains the gold standard. The differences in transepithelial absorption
357 demonstrated above may yield different degrees of tissue cross-linking and
358 are, therefore, likely to be clinically significant. The method described above
359 may prove useful in testing candidate protocols prior to future clinical trials.
360

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362
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Table 1 Commercially available riboflavin preparations

Riboflavin formulation	Composition
Vibex Rapid	0.1% wt/vol riboflavin 5'-monophosphate, saline, HPMC
Ribocross TE	0.125% wt/vol riboflavin 5'-monophosphate, D-alpha-tocopheryl poly(ethylene glycol) 1000 succinate
Medio-Cross TE	0.25% wt/vol riboflavin 5'-monophosphate, HPMC, benzalkonium chloride 0.01%
Ricrolin +	0.1% wt/vol riboflavin 5'-monophosphate, sodium edetate, trometamol, sodium dihydrogen phosphate dihydrate, sodium phosphate dibasic dehydrate
ParaCel	0.25% wt/vol riboflavin 5'-monophosphate, HPMC, sodium edetate, trometamol, benzalkonium chloride, saline
VibeX Xtra	0.25% wt/vol riboflavin 5'-monophosphate, saline
HPMC, hydroxypropyl methylcellulose	

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508 **FIGURE LEGENDS**

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510 **Figure 1**

511 (A) Iontophoresis schematic showing a riboflavin-filled corneal well housing a
512 negative electrode with the return electrode connected to a needle passed into the
513 vitreous cavity. The riboflavin solution acts as an electrical contact between the
514 electrode and the eye. The generator self-checks the continuity and a warning signal
515 sounded in case of current disruption. (B) Slide mounted 35µm tissue sections were
516 covered in immersion oil with a coverslip placed on top and coupled to the
517 microscope objective with distilled water. (C) Grey scale two-photon fluorescence
518 image analysed in ImageJ with 3 box-plots (40 pixels wide) from which to average
519 the signal (D) in each section. The edges of the images were not analysed to avoid
520 areas of vignetting (grey scale image formatted to increase brightness and clarity).

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523 **Figure 2**

524 Fluorophore migration within the corneal section investigated with serial
525 images of the same tissue location at time = 1, 2 and 3 minutes (**A-C**). Dark
526 circles are air bubbles within the oil overlying the tissue. All images have been
527 formatted to increase brightness by the same amount to improve view. (**D**)
528 Corresponding TPF signal across samples. Note the loss of the epithelial
529 peak as riboflavin moves into the stroma.

530

531 **Figure 3**

532 Color photographic globe and section examples from each group tested
533 allowing naked eye comparative view of riboflavin absorption through the
534 cornea into the anterior chamber. Upper row: Sagittal sections of treated
535 globes mounted on a cryostat during section preparation. Lower row: 35 µm
536 corneal sections prepared and laid on white paper for photographic contrast
537 (visible shadows indicate section lifting off paper). (**A**) negative control; (**B**)
538 positive control (epithelium-off) 0.1% riboflavin 30 minutes; (**C**) Ribocross TE
539 0.125% 30 minutes; (**D**) MedioCross TE 0.25% 30 minutes; (**E**) ParaCel
540 0.25% 4 minutes, VibeX Xtra 0.25% 6 minutes; (**F**) Ricrolin + 0.1% 1 mA 5
541 minutes iontophoresis.

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545 **Figure 4**

546 Two-photon fluorescence images of tissue sections (grey scale). (**A**) negative
547 control (B) positive control (epithelium-off) 0.1% riboflavin 30 minutes; (**C**)
548 Ribocross TE 0.125% 30 minutes; (**D**) MedioCross TE 0.25% 30 minutes; (**E**)

549 ParaCel 0.25% 4 minutes, VibeX Xtra 0.25% 6 minutes; (F) Ricrolin + 0.1% 1
550 mA 5 minutes iontophoresis. e, epithelium; DM Descemet's membrane; †,
551 Ribocross TE 'riboflavin film' on top of epithelium. All mages have been
552 formatted to increase brightness by the same amount to improve view. DM
553 scroll artifact visible in some preparations.

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556 **Figure 5**

557 Mean concentrations (standard deviation error bars) of riboflavin achieved
558 using different transepithelial protocols, compared with epithelium-off
559 absorption (n = 5 for each protocol). Shaded box denotes epithelium.

560

561 **Supplementary figure S1**

562 Section preparation. (A) Globe adhered onto specimen stage (with optimal
563 cutting temperature (OCT) compound) and secured within cryostat chuck.
564 Yellow cornea visible with white crystalline lens marked with '96.' Note
565 disrupted posterior segment from relieving incision made prior to immersion in
566 liquid nitrogen to prevent anterior globe splitting (white arrow). (B) 35 µm
567 whole-globe section laid on blade platform. (C) Lens and aqueous brushed
568 away leaving sclero-corneal shell (arrow-head) and iris (arrows). (D) Blade
569 used to cut cornea out before meticulously brushing away attached ice on the
570 back. Note slight corneal crimp from suction well (*). The surface riboflavin
571 film - measured by Wollensak et al.²⁹ at up to 300 µm in cellulose-containing
572 formulations - was also brushed away (Ribocross TE film was particularly

573 adherent to the epithelium and was still present on imaging despite attempts
574 to remove it).

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Fig. 1 **A**

B

C

D

Fig. 2

Fig. 3

Fig. 4

Fig. 5