Title: A comparison of different corneal iontophoresis protocols for promoting transepithelial riboflavin penetration.

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ABSTACT

Purpose: To measure corneal riboflavin penetration using different transepithelial iontophoresis protocols.

Methods: Freshly enucleated rabbit eyes were divided into 9 treatment groups of 4 eyes. One group, in which 0.1% w/v riboflavin was applied for 30 minutes without iontophoresis after corneal epithelial debridement, acted as a control. The remaining groups were treated with an intact epithelium using different riboflavin formulations and varying iontophoresis current, soak and rinse times. After riboflavin application, eyes were snap frozen in liquid nitrogen. Thirty-five μm thick corneal cross-sections were then imaged immediately by two-photon fluorescence microscopy, using image processing software to quantify stromal riboflavin concentration at different corneal depths.

Results: In the epithelium-on iontophoresis treatment groups, greater stromal riboflavin penetration was achieved with higher concentration riboflavin solutions, greater iontophoresis dosage and longer solution contact times. A protocol utilizing 0.25 % w/v riboflavin with benzalkonium chloride (BAC) 0.01 % and two cycles of applied current and subsequent soaking (1 mA 5 minutes, soak 5 minutes, 0.5 mA 5 minutes, soak 5 minutes) achieved similar stromal riboflavin penetration to epithelium-off controls. The best performing non-BAC containing protocol produced stromal riboflavin penetration approximately 60% that of epithelium-off controls. Riboflavin solutions containing saline resulted in minimal stromal penetration. Riboflavin loading

within the epithelium was equivalent to, or higher than that in the subjacent stroma, despite rinsing the ocular surface with balanced salt solution.

Conclusion: Modified iontophoresis protocols can significantly improve transepithelial riboflavin penetration in experimental corneal collagen cross-linking.

INTRODUCTION

Riboflavin/Ultraviolet A corneal collagen cross-linking (CXL), is the first intervention shown to halt disease progression in keratoconus.[1–4] Riboflavin acts as a photo-sensitizer for the production of free radicals that drive the cross-linking process.[5] Whilst riboflavin is hydrophilic with a molecular weight of 340 Dalton (Da), the corneal epithelium is lipophilic and has a reduced permeability to molecules larger than 180 Da.[6,7] Thus standard CXL requires debridement of the central of epithelium to facilitate stromal riboflavin penetration and achieve adequate efficacy.[7] Epithelial debridement, however, is associated with post-operative pain, delayed visual recovery and increases the risks of infection, corneal melt and scarring.[8] As a result, CXL is normally reserved for patients with documented disease progression.

Transepithelial (epithelium-on) CXL aims to avoid the pain, delayed recovery and complications of epithelial removal inherent in epithelium-off CXL. The original dextran-containing solutions have been shown in both laboratory and clinical studies to be ineffective for transepithelial cross-linking.[9–12] A number of formulations of riboflavin designed to facilitate penetration across an intact corneal epithelium are currently marketed.[13] Most contain toxic agents to increase epithelial permeability, including benzalkonium chloride (BAC), high concentration sodium chloride, sodium ethylenediaminetetraacetic acid (EDTA) or trometamol. Despite these chemical enhancers, results in clinical studies with transepithelial riboflavin preparations have been equivocal: some

studies report similar efficacy to epithelium-off CXL,[14,15] but most report inferior results.[10–12,16–18]

Iontophoresis, in which an electrical gradient is used to drive negatively-charged riboflavin molecules across the intact epithelium, may further enhance riboflavin penetration in transepithelial CXL. Laboratory studies of iontophoresis have been encouraging, demonstrating enhanced transepithelial riboflavin penetration and improvement of corneal biomechanics.[19–23] Initial clinical studies report improvements in keratometric and visual parameters.[24–26]

We have previously investigated transepithelial stromal riboflavin penetration of commercially-available protocols in an ex vivo rabbit eye model using two-photon fluorescence (TPF) microscopy, and have found that none are able to match the stromal riboflavin concentrations achieved epithelium-off.[13] Here, using the same method, we investigate whether modified iontophoresis protocols designed by one of the authors (DO'B) can enhance riboflavin penetration across an intact epithelium.

METHODS

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

Sample preparation

Adult pigmented rabbit heads transported on ice in a phosphate buffered saline (PBS) bath were received within 5 hours post-mortem (First Link Ltd., Wolverhampton, UK) to minimise epithelial cell layer degeneration following death. Intact globes were enucleated and examined under a low-magnification light microscope to rule out obvious epithelial trauma or scars. The globes were warmed to room temperature in PBS before the iontophoresis system was set up.

The iontophoresis system comprised 2 electrodes: a negatively-charged metal grid housed within the corneal applicator and a positively-charged 20G needle inserted through the sclera into the vitreous (figure 1A). The corneal applicator was vacuum-attached to the cornea. Once set-up, intra-ocular pressure was assessed manually by touch to ensure globe tension remained grossly normally. The reservoir was filled with differing riboflavin formulations (table 1) to above the level of the metal grid before beginning the iontophoresis treatment. Protocols varied either by iontophoresis dosage, soak time or riboflavin formulation and full parameters are given in table 2. Four globes

were studied for each protocol except for protocol F where n = 5. After the iontophoretic procedure, the applicator was removed and the corneas were rinsed with PBS to both remove any riboflavin on the corneal surface and to try to reduce riboflavin concentration within the epithelium. Corneas soaked for 30 minutes with 0.1 % w/v riboflavin in HPMC and saline (VibeX Rapid, Avedro, Inc) after epithelial debridement served as controls.

Section preparation and imaging protocol

We have previously described the use of TPF microscopy for imaging transepithelial riboflavin penetration.[13,27] Briefly, following iontophoresis the globes were immediately immersed in liquid nitrogen. 35 µm corneal cross-sections were cut on a cryostat 1 mm apart (including the central meridian) and 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. For lack of an appropriate oil-immersion objective, we placed a coverslip on top prior to imaging under a Leica 10x/0.3 NA water immersion objective. The time taken from the tissue thawing on the slide to image acquisition on the microscope was approximately 1 minute. This interval was kept as short as possible to minimize migration of fluorescein within the thawed tissue. A model-locked Ti:Sapphire laser, operating with a 140 femtosecond pulse duration and 80 MHz pulse repetition rate, was used as the excitation laser source. TPF excitation light of 890 nm wavelength was chosen to correspond with the highest riboflavin absorption peak (445 nm) as determined by spectrophotometry.[28] Emitted riboflavin fluorescence was collected in the backward (epi) configuration onto a de-scanned Leica HyD

detector with the pinhole opened to its maximum setting. To avoid overlap with the absorption spectrum of riboflavin, fluorescence was detected in the range between 525 nm and 650 nm. (We previously investigated[13] the presence of endogenous fluorescence within this spectral band by imaging negative controls without riboflavin and detected negligible TPF signal).

Image analysis and concentration calibration

Grey scale images were exported and analysed using Java-based imaging software (ImageJ, 1.48v, http:// imagej.nih.gov.ij; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). For each image, three separate 40-pixels wide rectangular regions of interest were manually selected (Figure 1B). The intensity profiles with depth for each region of interest were then exported to a .txt file. The epithelial/stromal junction was then identified in each trace by the abrupt change in signal and confirmed with reference to the corresponding image of the region of interest. This information was used to align all three plots and a mean intensity plot was generated representing the average TPF signal for that image as a function of depth.

In order to convert measured TFP signal into riboflavin concentration, we performed a calibration measurement. Each point on the calibration curve was produced by placing a known concentration of riboflavin solution on a well slide. The riboflavin solution was then covered with a layer of immersion oil and a coverslip in order to exactly replicate the measurement conditions used for imaging tissue. Image z-stacks were then acquired throughout the full

depth of the well slide using the same Leica 10x/0.3 NA water immersion objective as used for the tissue imaging. The resulting TPF signals are plotted as a function of depth in Figure 1C for selected riboflavin concentrations. As expected, the TPF signal is constant with depth. The average raw TPF signal measured at 60 μm below the riboflavin surface was then plotted against riboflavin concentration (Figure 1D). A quadratic equation was then fitted to the experimental data and the resulting curve was used to convert the TPF signal measured in corneal sections into riboflavin concentration. The TPF signal from a well slide reservoir of 0.1 % w/v riboflavin solution was acquired for every measurement session and the resulting data used to correct for drifts caused by variations in laser output power.

Mean (SD) concentrations were calculated from 4 globes tested for each protocol. Unpaired Student *t*-tests were used to compare riboflavin concentrations achieved at a depth of 300 μm. A *p* value less than 0.05 was considered significant. Analyses were performed in Excel for Mac, 2011; Microsoft Corp, Redmond, WA, USA).

RESULTS

Peak riboflavin concentration of 0.094 (± 0.002) % w/v was recorded in epithelium-off controls. At a depth of 300 μm corresponding to the demarcation line commonly observed after corneal cross-linking, the riboflavin

concentration was 0.082 (± 0.005) % w/v. Stromal riboflavin concentrations achieved through an intact epithelium with different iontophoresis protocols, along with corresponding color photograph and fluorescence microscopy images, are shown in figures 2-4 and summarised in table 3. Higher concentrations were achieved with longer iontophoretic dosage and longer solution contact (diffusion) time with the cornea. The best protocol (A), utilizing 0.25 % w/v riboflavin with BAC and two separate iontophoretic treatments followed by soak periods of 5 minutes to allow diffusion of riboflavin from the sub-epithelial tissues deeper into the stroma (the 'St. Thomas'/Cardiff Iontophoresis protocol', total time 20 minutes), was not significantly different with respect to stromal riboflavin concentrations compared with epithelium-off controls (0.082 [± 0.005] % w/v vs. 0.075 [± 0.007] % w/v at a depth of 300 μm, p = 0.63, table 3). The best performing protocol (C) not containing BAC but with 0.25 % w/v riboflavin and the same iontophoresis treatments and soak times as protocol A, achieved a mean stromal concentration of 0.049 (± 0.008) % w/v at 300 μm (table 3).

Near-uniform stromal riboflavin penetration with depth was observed in longer-duration protocols (A-C), as compared with shorter treatments in which the concentration fell by up to 40 % within the anterior 300 μm (protocols D-F). Riboflavin solutions containing saline (protocols G, H), resulted in minimal stromal penetration (maximum 0.01 %). Of note, corneal thickness was observed to increase by approximately 50 μm following these saline-based iontophoretic applications.

The concentration of riboflavin within the epithelium was equivalent to or higher than that in the immediate underlying stroma. Extended rinsing of the surface effectively washed away any pre-corneal riboflavin film but did not selectively reduce the fluorophore load within the epithelium itself. A 5 minute corneal rinse after protocols A and B (MedioCross TE) resulted in a wash-out effect of both the epithelium and anterior stroma up to an approximate depth of 200 μm.

DISCUSSION

In this study we have demonstrated that corneal stromal riboflavin penetration in iontophoretic transepithelial CXL can be improved by increasing soak time, solution concentration and iontophoretic dosage.

The corneal epithelium has been previously shown to be a significant barrier to riboflavin penetration into the stroma.[9,29,30] This has been confirmed by a series of experiments using spectrophotometry as an indirect measure of stromal riboflavin concentration. Using our TPF methodology, which can directly quantify riboflavin concentration through the entire depth of the corneal,[13,28,31] we previously confirmed these findings, demonstrating very limited riboflavin diffusion through an intact epithelium with any of the several current commercially available transepithelial riboflavin formulations and protocols.

Riboflavin is water soluble and negatively charged at physiological pH, and laboratory studies suggest that iontophoresis can be applied effectively to enhance riboflavin penetration in transepithelial CXL. Several groups have reported similar increases in corneal biomechanics[19,22,23] in animal models as compared with epithelium-off CXL. Preliminary clinical studies have also been encouraging, with reported cessation of disease progression with up to 15 month follow-up and improvements in keratometric and visual parameters.[24–26] However, the relative efficacy of this technique compared to epithelium-off CXL remains to be determined especially over longer term follow-up, and

current randomized prospective studies comparing the two techniques (clinicaltrials.gov.uk NCT02117999, NCT01868620, ISRCT 04451470) have yet to be reported.

Existing recommendations for iontophoresis in transepithelial CXL utilize 1 mA for 5 minutes with a 0.1 % w/v riboflavin solution. Improved riboflavin penetration can be obtained by modifying these parameters. Stromal riboflavin concentrations using protocol A here were similar to epithelium-off controls ($p = 0.63$) (figure 4, table 3). This protocol utilized a solution of 0.25 % w/v riboflavin with BAC and the 'St. Thomas'/Cardiff Iontophoresis protocol'. This protocol was derived in collaborative pilot experimentation at St Thomas' Hospital and Cardiff University using spectrophotometry, and comprises two cycles of iontophoresis each followed by a 5 minute soak period to allow time for riboflavin to diffuse more posteriorly. The use of the cationic surfactant BAC has been shown with percutaneous iontophoresis to have a synergistic effect on the transport of anions.[22] We similarly observed this synergistic effect with higher stromal concentrations achieved with protocol A (0.25 % w/v riboflavin, BAC) compared to protocol C (0.25 % w/v riboflavin, no BAC) (figure 4, table 3). A further variable in iontophoresis relates to the zeta potential and electrophoretic mobility of different riboflavin solutions that may, in part, explain our results. Although beyond the scope of this study, this warrants further investigation in the future.

A key advantage of our TPF imaging method, as compared to whole-tissue analysis (high-performance liquid chromatography, HPLC;[19,32–34]

spectrophotometry[9,29,30]) is the ability to quantify riboflavin concentration within the epithelium. We have previously demonstrated in transepithelial protocols (without rinsing the cornea) that epithelial riboflavin concentration often exceeds that in the underlying stroma.[13] Analysing a full-thickness specimen, including a riboflavin-loaded epithelium and pre-corneal film, will lead to significant overestimation of corneal stromal riboflavin concentrations.[13] Clinically, riboflavin loading in the epithelium may reduce CXL efficacy by attenuating UV light transmission to the stroma. It may also result in an 'arc-eye' type reaction, particularly where riboflavin preparations including BAC are used, accounting for the epithelial defects seen in up to two-thirds of patients after transepithelial CXL using Medio-Cross TE.[35] The ideal transepithelial protocol would load the stroma with riboflavin, while leaving the epithelium relatively clear. To this end, in this study we rinsed the surface of the globe after iontophoresis in a bid to wash out some of the riboflavin from within the epithelium. Figures 3 and 4 and table 3 show that this approach did not work since there was no significant difference in fluorescence between the epithelium and the subjacent stroma. Extended rinsing (5 minutes) of the ocular surface in protocols A and B (MedioCross TE) also reduced corneal stromal concentrations of riboflavin up to an approximate depth of 200 μm. Stromal riboflavin elution was less evident with non-BAC containing solutions (protocols C and D), where the epithelial tight junctions were still probably largely intact. Although iontophoresis is not commercially promoted to be used with BAC-containing solutions, this stromal wash-out effect with prolonged rinsing of the ocular surface may compromise tissue cross-linking within the anterior cornea.

It is important to note that iontophoresis was ineffective with riboflavin formulations containing saline (Protocols G and H) that produced minimal riboflavin penetration and some stromal swelling.

There are a number of limitations to this study. Firstly, results in ex-vivo rabbit corneas, although a better anatomical match to human corneal epithelial thickness than porcine eyes, may be affected by post-mortem changes in epithelial layer integrity. Despite steps to minimize this as described in the Methods section, some epithelial degradation and enhanced permeability is likely, and our results may overestimate stromal riboflavin absorption in clinical transepithelial CXL. Secondly, migration of riboflavin within the snap frozen tissue will have started as soon as the section thawed on the slide, resulting in an underestimation of stromal riboflavin concentrations. Finally, imaging through two media of different refractive indices (oil and water either side of the coverslip) may have increased optical aberrations as the laser light passes through. Any induced aberrations may have resulted in a small absolute loss of signal; but since this same method was employed for all imaged samples, no relative change in signal between samples should be present. Given these limitations, we are unable to guarantee an absolute concentration from this TPF data alone.

In conclusion, this study confirms that transepithelial riboflavin penetration can be improved by increasing soak time, riboflavin concentration in the soak preparation and iontophoretic dosage. While the optimum tissue concentration

for effective CXL is unknown, Protocol C (0.25 % w/v riboflavin [BAC-free], St. Thomas'/Cardiff Iontophoresis protocol) achieves more than 50% greater stromal penetration compared to the standard iontophoresis protocol, as well as far higher concentrations than we have seen with non-iontophoresis transepithelial protocols.[24] Although less than that achieved epithelium-off, its considerable stromal riboflavin penetration, without relying on epithelial-toxic additives, may represent the best transepithelial technique to date. Clinical trials will determine whether this level of stromal riboflavin penetration produces effective CXL.

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Table 1 Riboflavin formulation

Brand name (manufacturer)	**Composition**
Vibex Rapid (*Avedro, Inc., Waltham, MA, USA*)	0.1 % w/v riboflavin 5'-monophosphate, saline, HPMC
Medio-Cross TE (*Peschke Meditrade GmbH, Germany)*	0.25 % w/v riboflavin 5'-monophosphate, HPMC, 0.01% benzalkonium chloride, disodium hydrogenphosphate, sodium dihydrogenphosphate, water
*Ricrolin + 0.25% (*Sooft Italia S.p.A. Montegiorgio, Italy)*	0.25 % w/v riboflavin 5'-monophosphate, sodium edetate, trometamol, sodium dihydrogenphosphate dihydrate, sodium phosphate dibasic dehydrate
Ricrolin + 0.1% (*Sooft Italia S.p.A. Montegiorgio, Italy)*	0.1 % w/v riboflavin 5'-monophosphate, sodium edetate, trometamol, sodium dihydrogenphosphate dihydrate, sodium phosphate dibasic dehydrate

*Non-commercial formulation

Table 2 Treatment protocols

Protocol	Solution	Current dose I	Soak I	Current dose II	Soak II	Rinse
Control	Vibex Rapid	-	30 min	-	-	-
A	MedioCross TE	1mA 5 min	5 min	0.5 mA 5 min	5 min	5 min*
B	MedioCross TE	1mA 5 min	15 min	-	-	1 min
C	Ricrolin+ 0.25%	1mA 5 min	5 min	0.5 mA 5 min	5 min	5 min*
D	Ricrolin+ 0.25%	1mA 10 min	-	-	-	5 min
E	MedioCross TE	1mA 5 min	-	-	-	1 min
F	Ricrolin + 0.1%	1mA 5 min	-	-	-	1 min†
G	Vibex Rapid	1mA 5 min	-	-	-	1 min
H	Vibex Rapid	1mA 5 min	5 min	0.5 mA 5 min	5 min	5 min

Min, minute; n = 4 for all protocols except F (n = 5), †previously published[13] * St. Thomas'/Cardiff Iontophoresis protocol

Table 3 Corneal riboflavin concentrations

Protocol	Riboflavin concentration ± SD % w/v			P value[1]
	Epithelium	Peak stroma	300 µm*	
Control	-	0.094 ± 0.002	0.082 ± 0.005	-
A	0.054 ± 0.004	0.078 ± 0.008	0.075 ± 0.007	0.63
B	0.042 ± 0.006	0.054 ± 0.005	0.053 ± 0.005	0.016
C	0.050 ± 0.008	0.057 ± 0.008	0.049 ± 0.008	0.001
D	0.035 ± 0.004	0.039 ± 0.007	0.026 ± 0.003	< 0.001
E	0.029 ± 0.003	0.029 ± 0.002	0.016 ± 0.003	< 0.001
F	0.021 ± 0.002	0.018 ± 0.003	0.010 ± 0.001	< 0.001
G	0.005	0.008	0.007	< 0.001
H	0.005	0.006	0.005	< 0.001

[1]Unpaired Student t-test comparing riboflavin concentration vs. control at a depth of 300 µm (depth chosen to correlate with demarcation zone commonly seen after corneal cross-linking).

FIGURE LEGENDS

Figure 1

(**A**) Iontophoresis riboflavin delivery system modified for use in ex-vivo eyes. The system comprised two electrodes: a negatively-charged metal grid housed within the corneal applicator and a positively-charged 20G needle inserted through the sclera into the vitreous. (**B**) Grey scale two-photon fluorescence image analysed in ImageJ with 3 box-plots (40 pixels wide) from which to average the signal in each section. The edges of the images were not analysed to avoid areas of vignetting. **(C)** TPF signals achieved with depth through diluted riboflavin solutions in a well slide. **(D)** Calibration curve used to calculate corneal riboflavin concentration from TPF signal.

Figure 2

Color photographic globe and section examples from each group tested allowing macroscopic comparative view of riboflavin penetration through the cornea into the anterior chamber. Upper row: Sagittal sections of treated globes mounted on a cryostat during section preparation. Lower row: 35 µm corneal sections prepared and laid on white paper for photographic contrast (visible shadows indicate section lifting off paper). **A-H** protocols as per table 2.

Figure 3

Two-photon fluorescence images of tissue sections (grey scale). **A-H**

protocols as per table 2. e, epithelium; DM, Descemet's membrane. All images have been formatted to increase brightness by the same amount to improve view. DM scroll artifact visible in some preparations. Apparent epithelial damage with BAC-containing protocol A should not be inferred as a treatment effect - our TPF methodology was not designed to qualify epithelial structural changes and a processing-induced artefact cannot ruled out.

Figure 4

Mean concentrations (standard deviation error bars) of riboflavin achieved using different transepithelial protocols, compared with epithelium-off penetration. Shaded area denotes epithelium. **A-H** protocols as per table 2. Note the increased concentrations achieved at 300um with protocols A and C compared to B and D due to the second iontophoresis/soak cycle.

Fig. 1

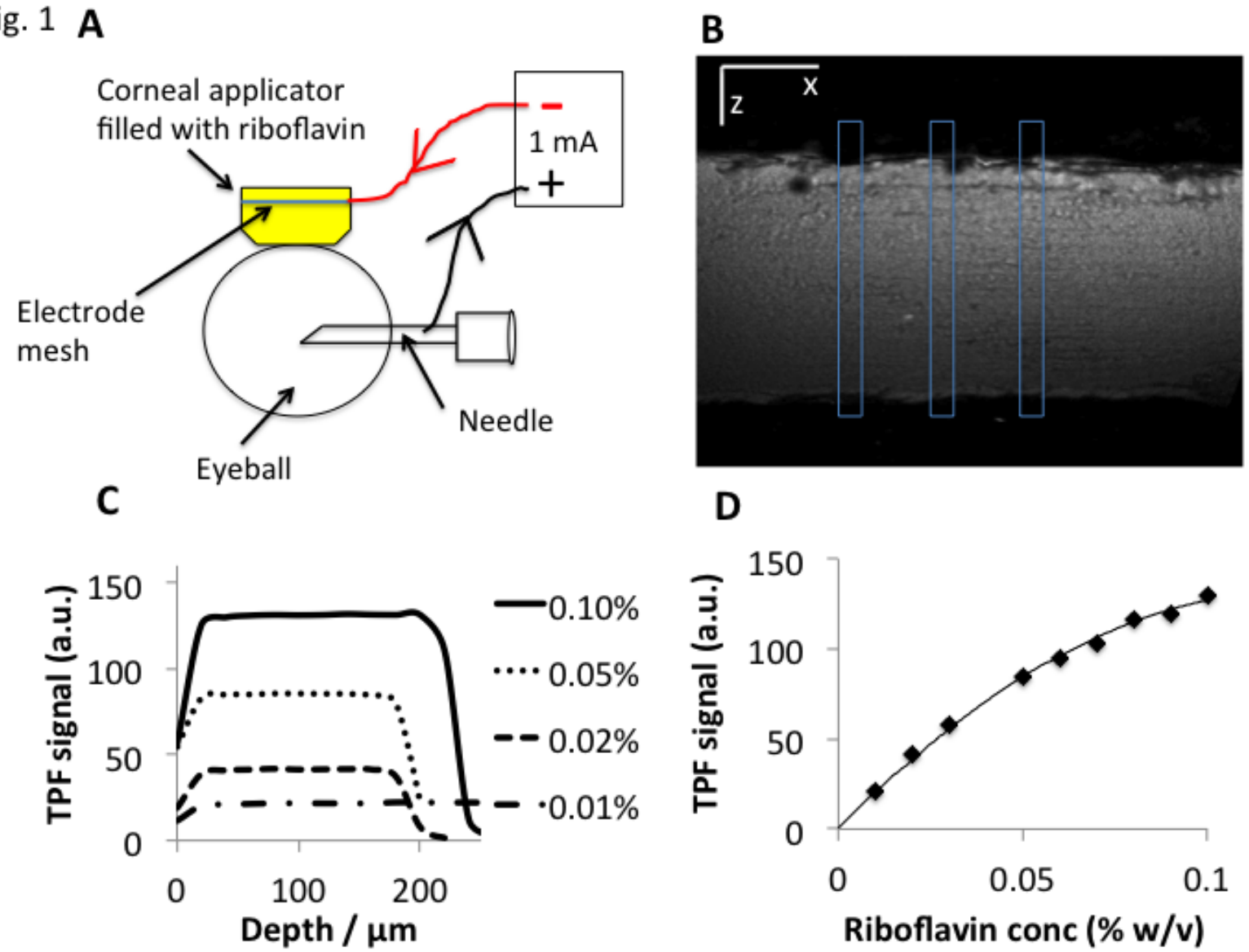

A
Corneal applicator
filled with riboflavin
1 mA
Electrode
mesh
Needle
Eyeball
B
z
x
C
TPF signal (a.u.)
150
100
50
0
0
100
200
Depth / µm
0.10%
0.05%
0.02%
0.01%
D
TPF signal (a.u.)
150
100
50
0
0
0.05
0.1
Riboflavin conc (% w/v)

Fig 2

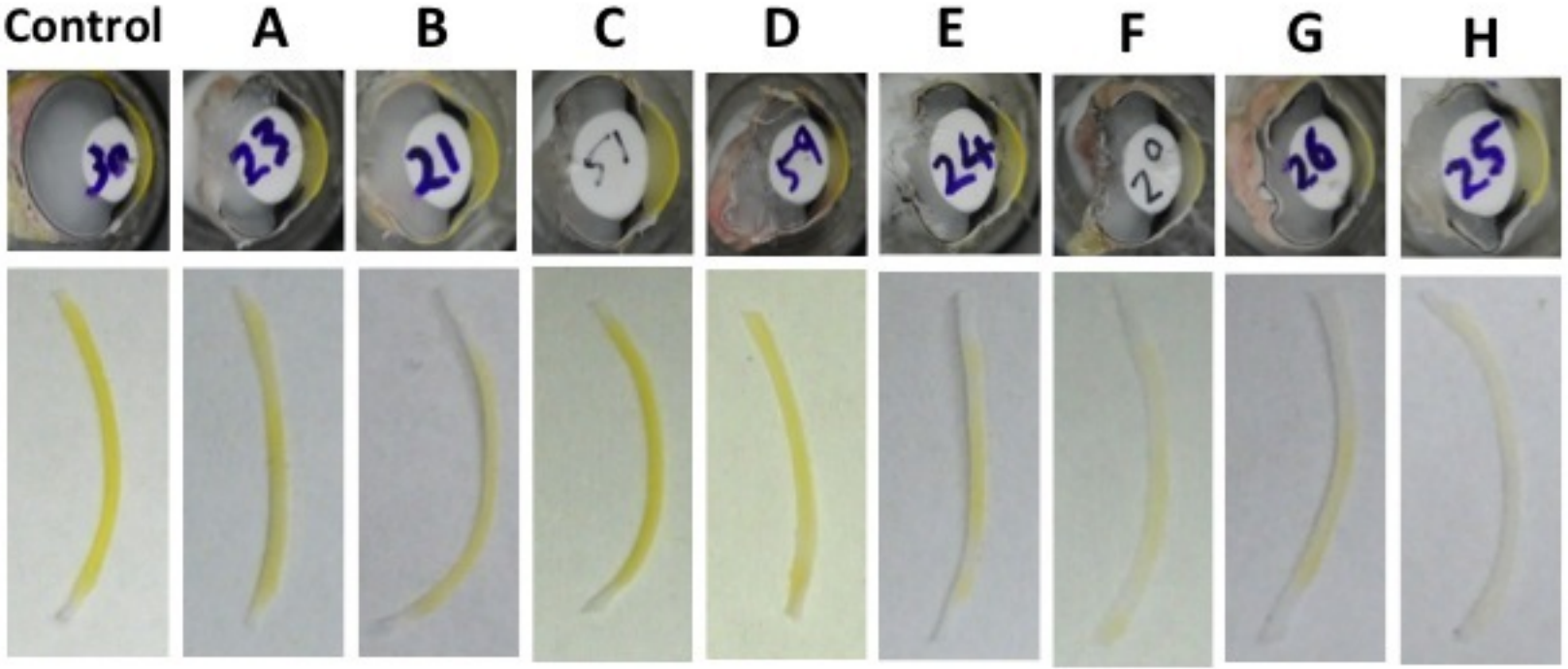


Fig 3

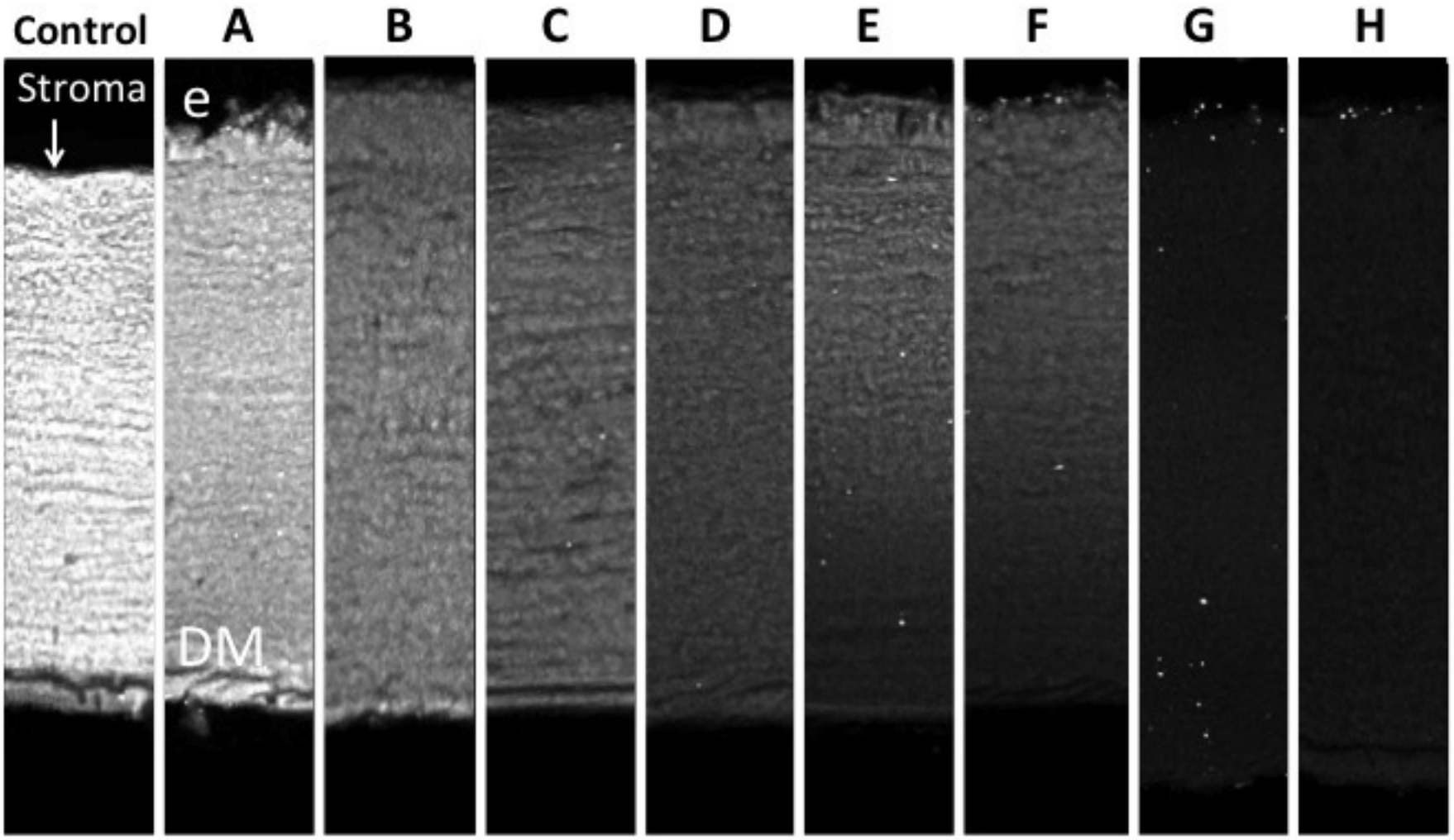
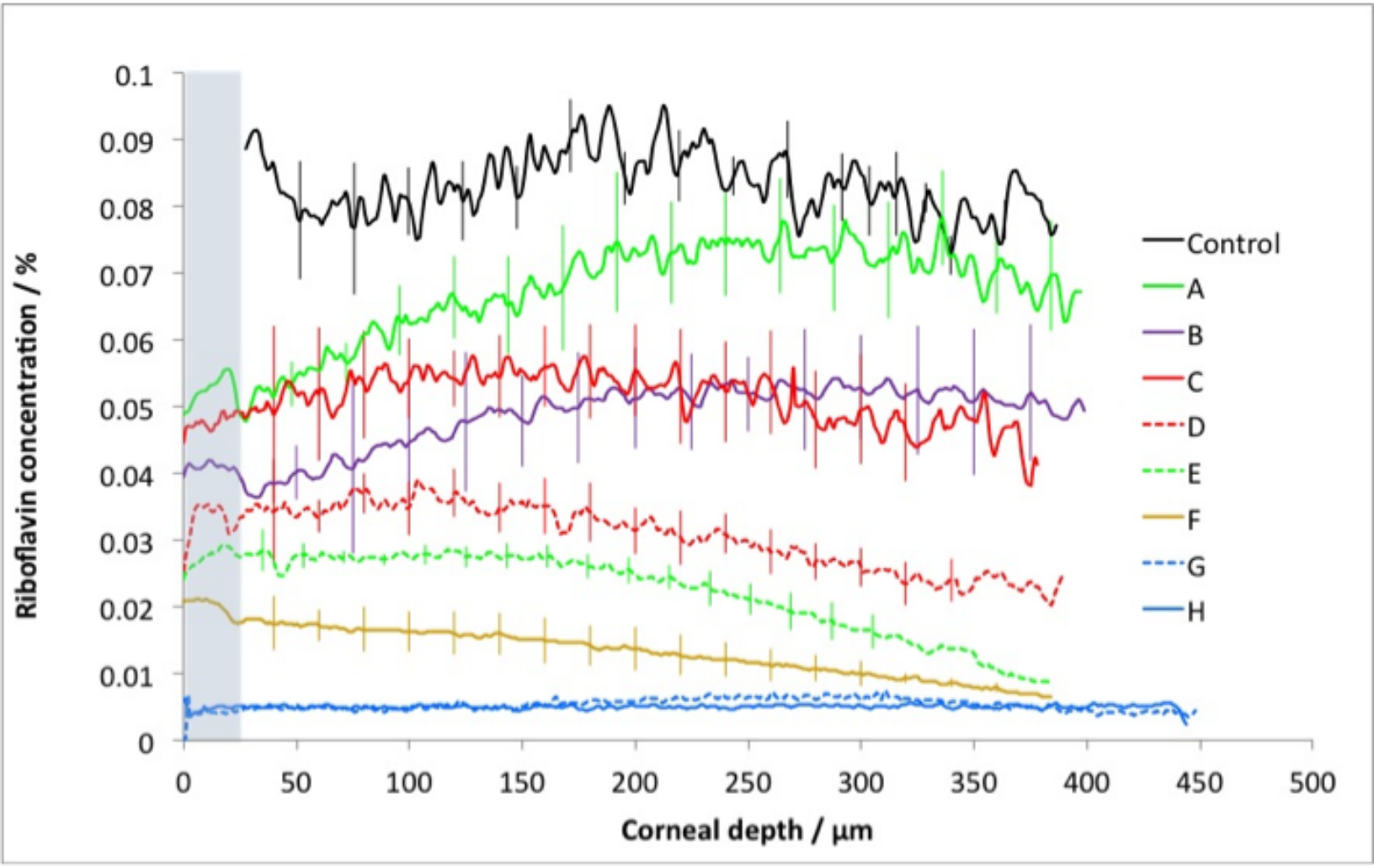


Fig 4


Riboflavin concentration / %
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
50
100
150
200
250
300
350
400
450
500
Corneal depth / µm
Control
A
B
C
D
E
F
G
H