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Article

Fluorophotometric Determination of Riboflavin Concentrations in a Human Artificial Anterior Chamber Model

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Purpose: Corneal cross-linking (CXL) requires an adequate corneal riboflavin impregnation, which is clinically assessed by verification of a riboflavin "flare" in the anterior chamber. We set out to replace this subjective assessment with an objective measurement method and evaluated fluorophotometry as an apparatus-based technique for riboflavin detection in the anterior chamber.

Methods: In an artificial anterior chamber model using human corneas and a modified Fluorotron fluorophotometer, we determined the detection limits of riboflavin concentrations across native corneas by comparison measurements of the same concentrations in glass cuvettes. Subsequently, standard CXL procedures with corneal application of riboflavin were simulated and the proportions of riboflavin entering the anterior chamber were measured fluorophotometrically.

Results: The measurement results of the riboflavin dilution series in the artificial anterior chamber showed a very high concordance with the results obtained in a glass cuvette (Pitman test $P = 0.329$). In the CXL simulation, the mean riboflavin concentration measured in the anterior chamber increased within 15 minutes from 5 ($\pm$1) to 903 ($\pm$204) ng/mL and stood at 1089 ($\pm$56) ng/mL after 30 minutes.

Conclusions: Fluorophotometry is able to measure riboflavin in an artificial anterior chamber across human corneas over a wide range of concentrations and it reliably detects the increasing riboflavin signal in simulated CXL procedures.

Translational Relevance: The replacement of the subjective riboflavin detection by a technically straightforward, objective detection method might increase patient safety and treatment efficiency in CXL.

Introduction

Corneal cross-linking (CXL) represents a de facto standard to halt the progression of ectatic corneal disorders.[1] A documented corneal riboflavin impregnation is a prerequisite for the procedure, as riboflavin not only serves as a photosensitizer, but also as an ultra-violet (UV) blocker that protects the intraocular lens and retina from radiation damage.[2] However, up to the present day, no technique for reliable quantification of stromal riboflavin impregnation has been established in clinical routine. Approaches such as two-photon fluorescence microscopy[3,4] and high-performance liquid chromatography[5,6] have been shown to measure riboflavin concentrations in the corneal stroma but are limited to ex vivo approaches or are too elaborate to be used in everyday clinical practice. Instead, slit-lamp biomicroscopy with a positive riboflavin "flare" detection in the anterior chamber serves as a surrogate for corneal impregnation. Detecting the weak signal of a positive "flare" behind a yellow-soaked corneal surface can be difficult and is highly dependent on the examiner's experience.

Fluorophotometry could be an alternative technique to objectively detect riboflavin molecules in the

anterior chamber, and thus prove corneal impregnation with feasible effort. The development of fluorophotometers about three decades ago allowed the study of the fluorescence of the cornea, crystalline lens and retina, the investigation of the blood–aqueous and the blood–retina barrier, as well as the investigation of corneal epithelial and endothelial cell functions in a clinical environment.[7] While this work was mainly carried out with fluorescein as tracer,[8] the literature about fluorophotometric corneal penetration studies using riboflavin is limited to conference abstracts (Viennet A, et al. *IOVS*. 2011;52:ARVO E-Abstract 5201),[9] and may be impaired by instrument settings that have not been adjusted to register anterior chamber fluorescence signals behind a strongly fluorescent cornea. Therefore, with the long-term goal of an objective riboflavin detection in the anterior chamber as part of cross-linking treatments, we set out to evaluate a modified Fluorotron Master FM-2 fluorophotometer to quantify riboflavin fluorescence in an artificial anterior chamber model using human corneas.

Materials and Methods

We evaluated the detection limits of riboflavin solutions in artificial anterior chambers across native corneas and simulated the influence of a precorneal riboflavin film on the measurement by application of a riboflavin-saturated contact lens. Subsequently, a standard CXL procedure with corneal application of riboflavin was simulated and the proportion of riboflavin entering the anterior chamber was measured fluorophotometrically. The study was approved by the local Ethics Committee (#2018-00439).

Modification of Fluorophotometer

Fluorescence measurements were performed with a modified Fluorotron Master FM-2 fluorophotometer (OcuMetrics, Mountain View, CA). A detailed description of the device and the measuring principle are published elsewhere.[10–12] In brief, an optical illuminating path (excitation wavelength 420–490 nm) intersects with an optical pick-up path (emission wavelength 530–630 nm). The rhomboid intersection ("focal diamond"[13]) represents the instrument's depth of focus and sampling volume, which is sequentially placed at 148 discrete locations along the axis of the eye (4 per millimeter), starting from anterior to the cornea to posterior to the crystalline lens. A photodetector circuit counts the photons emitted at each location and a computer creates a fluorescence concentration profile along the scanning axis by converting photon counts to concentration of fluorescence in nanograms per milliliter. The 28° default angle between excitation and emission path was increased to 44° to narrow the cross-section of the focal diamond: as the leading parts of the diamond move through the autofluorescent cornea into the anterior chamber, the following parts are still registering the corneal fluorescence and influence the overall signal considerably. The angle modification kept the influence of this "tailing" effect as small as possible (Fig. 1). In addition to the standard cuvette holder we equipped the device with a multijoint adapter to accommodate corneas mounted on artificial anterior chambers (Moria, Antony, France) in the optical path of the instrument (Fig. 2).

Riboflavin Dilution Series

By diluting commercially available riboflavin (Peschke TE 0.25 %; Peschke, Huenenberg, Switzerland) in balanced salt solution (BSS; Serag-Wiessner, Naila, Germany), serial dilutions of 1, 5, 10, 50, 100, 1000, 10,000, 50,000, and 100,000 ng/mL were produced using calibrated research pipettes with disposable tips (Eppendorf, Hamburg, Germany). The solutions were protected from light and used within 2 hours of manufacture.

Corneal Tissue

Thirteen human corneal grafts not suitable for transplantation and with consent for use in research were provided by the Eye Bank of the Lucerne Cantonal Hospital. Mean donor age was 59 years (range, 40–70 years). Mean storage time was 15 days (range, 9–30 days). In microscopic examination, the stroma was clear and compact, mean endothelial cell count was 2344 cells/mm^2 (range, 1575–2927 cells/mm^2) on the date of preparation.

Detection Limits of Riboflavin Dilutions Across Native Corneas

Nine corneas were mounted onto an artificial anterior chamber that was filled with BSS to obtain an applanatorial pressure of 15 mm Hg. Because the condition of the epithelium varied between corneas it was removed for uniformity reasons. The corneas were swiveled into the optical path and centered. After a fluorophotometric baseline measurement, the artificial chamber was stepwise flushed with riboflavin solutions of increasing concentration (Figs. 3a–c). For

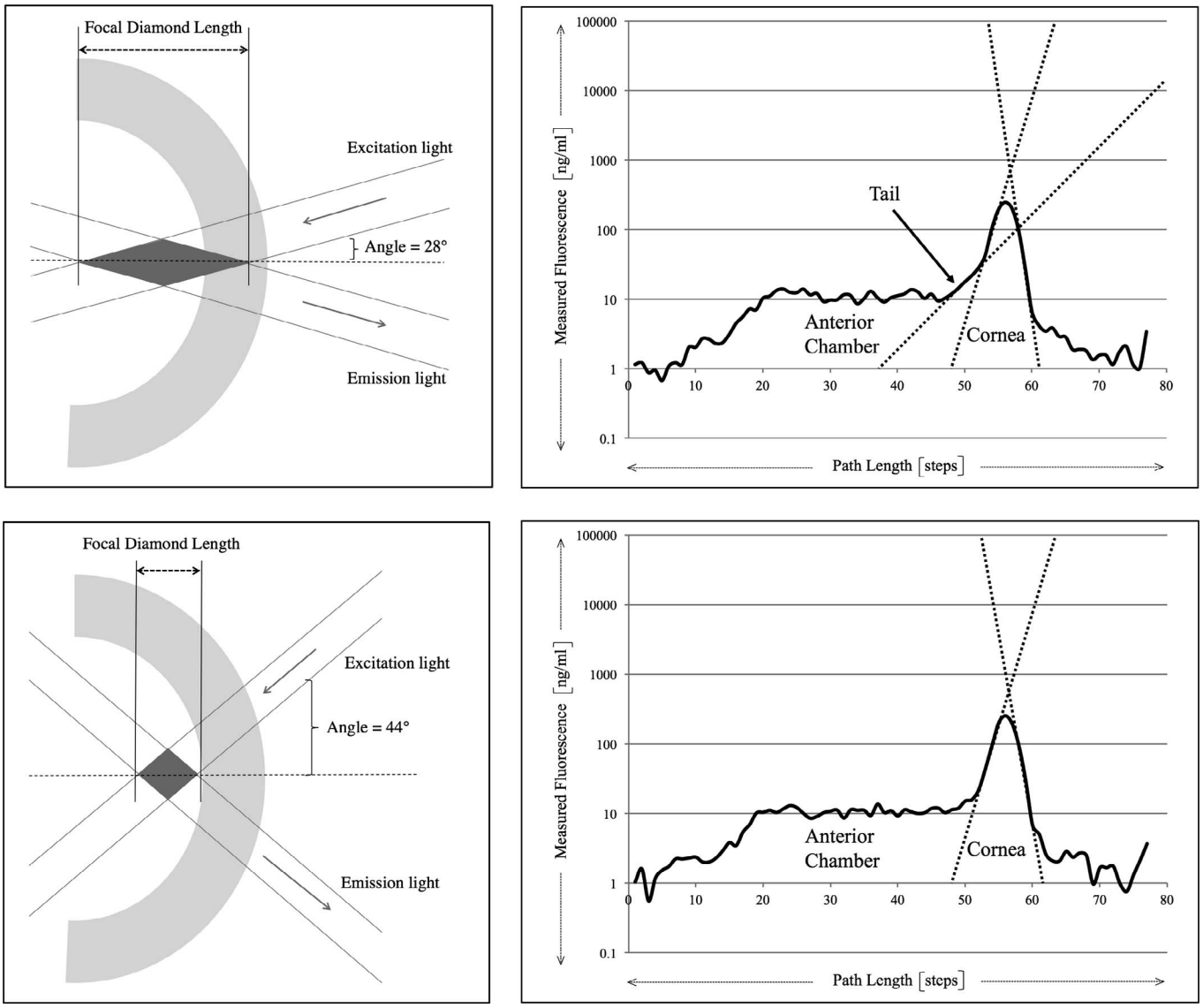


Figure 1. Schematic of diamond modification (*left*) and corresponding fluorophotometry scans (*right*), illustrating the difference between the standard (*above*) and the modified (*below*) Fluorotron Master FM-2 fluorescence concentration profiles. As a standard diamond's main intersection plane travels through the cornea (from the *right* to the *left*) and reaches the anterior chamber, the diamond's trailing part still picks up a corneal fluorescence signal that causes a "tail" after the corneal peak. In contrast to the standard diamond size, the modified diamond has a steeper angle and a shorter focal length. As a consequence, most of the diamond is situated in the area of interest, so its main signal is less influenced by the noise of the adjacent tissue.

each individual concentration level, a set of three fluorophotometric scans was acquired. At the end of the measurement series, a riboflavin soaked contact lens (Pure Vision 2; Bausch & Lomb, Rochester, NY) was placed on the cornea to investigate to which extend a simulated, maximum riboflavin-saturated epithelium with a precorneal riboflavin film interferes with the quantification of riboflavin concentrations in the anterior chamber (Fig. 3d).

Corneal thickness was measured by ultrasound pachymetry (Avia Tonopen; Haag-Streit, Wedel, Germany) immediately after epithelial removal and after the final measurement. This experiment was repeated nine times, using a fresh donor cornea for each cycle.

Simulation of Standard Epi-Off CXL Procedure

In clinical practice, riboflavin impregnation of the cornea is achieved by the application of riboflavin eye

Figure 2. Optical head of the Fluorotron Master FM-2 fluorophotometer fitted with multijoint adapter accommodating an artificial anterior chamber with two irrigation ports attached to the base. The cornea (mounted on the artificial anterior chamber's tissue pedestal and therefore not visible from this perspective) is aligned with the lens and the centering is checked via the alignment window in the side housing.

drops. Based on the Dresden protocol, which includes epithelial ablation ("epi-off") and topical riboflavin application before and during irradiation,[14] we removed the epithelium of four corneas, took fluorophotometric baseline measurements (Fig. 4a) and applied 0.25% riboflavin drops every 2 minutes for 30 minutes (application phase, Fig. 4b). At half-time and at the end of the application phase (i.e., after 15 and 30 minutes) the precorneal riboflavin film was washed off with BSS (Fig. 4c) to enable fluorescence measurements (Fig. 4d). Further measurements were taken 15, 30, 45, and 60 minutes after the end of application phase (Fig. 4e).

Corneal thickness was measured after epithelial removal and after the final measurement. The experiment was repeated four times, using a fresh donor cornea for each cycle.

Analysis

The measurement result of an individual scan was determined by identifying on the graphic output the linear 2-mm segment, which followed the anterior cuvette wall (for cuvette measurements) or followed the corneal tail in the transition zone between cornea and anterior chamber (for anterior chamber measurements; Figs. 5a, 5b). The numeric results of the digital output within the 2 mm were then averaged. At very high-riboflavin concentrations, a graphic fusion of corneal peak and anterior chamber segment occurred on the one hand, and an exponential decrease in light transmission on the other hand, according to Lambert Beer's law. For these cases, the effective riboflavin concentration was semiautomatically extrapolated by the analysis software (Fig. 5c).[15] All measurements were taken in triplicates and the results averaged.

Statistical Analysis

For the detection limits of riboflavin dilutions across native corneas the concordance of the logarithmically transformed concentration values (glass cuvette versus anterior chamber) were graphically assessed using Bland-Altman plots and statistically tested using the Pitman test. The level of statistical significance was set at $P < 0.05$. The range of highest concordance was identified visually by plotting the logarithmically transformed dilution series with the corresponding fluorescence measurements.

Results

Detection Limits of Riboflavin Dilutions Across Native Corneas

Representative examples of fluorescence concentration profiles recorded with increasing riboflavin concentrations are illustrated in Figure 3. The mean riboflavin concentrations measured fluorophotometrically in the glass cuvette and in the artificial anterior chamber are shown in Table 1. Bland-Altman plots analyzing the concordance between logarithmically transformed cuvette and anterior chamber riboflavin concentrations are shown in Figure 6.

The baseline measurement with BSS showed formally higher results for the artificial anterior chamber than for the glass cuvette, which was considered noise. The lowest concentrations of 1 and 5 ng/mL could be measured in the glass cuvette, but could hardly be distinguished from noise in the anterior chamber. Consequently, the noise influenced anterior chamber readings for baseline, 1, and 5 ng/mL were higher than cuvette readings, but from a concentration of 10 ng/mL on upward, the magnitude of the differences was essentially constant with narrow limits of agreement. Across the whole spectrum of concentrations (Fig. 6a), the corresponding correlation coefficient between difference and mean was $r =$

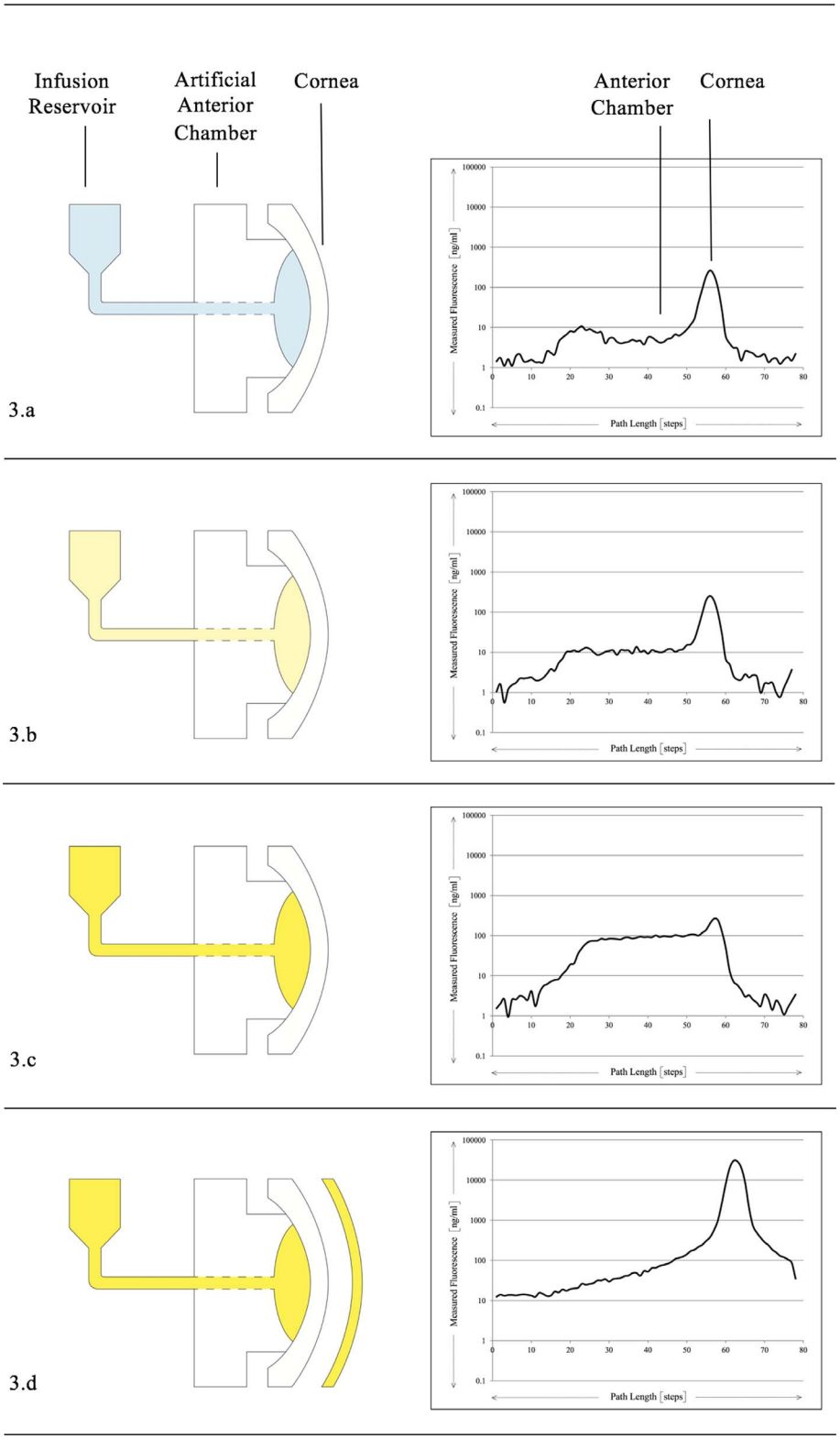


Figure 3. Schematic of artificial anterior chamber model filled with increasingly higher riboflavin concentrations and corresponding fluorophotometry scans. (a) Baseline measurement with balanced salt solution; (b) riboflavin 10 ng/mL; (c) riboflavin 100 ng/mL; (d) additional application of riboflavin-soaked contact lens.

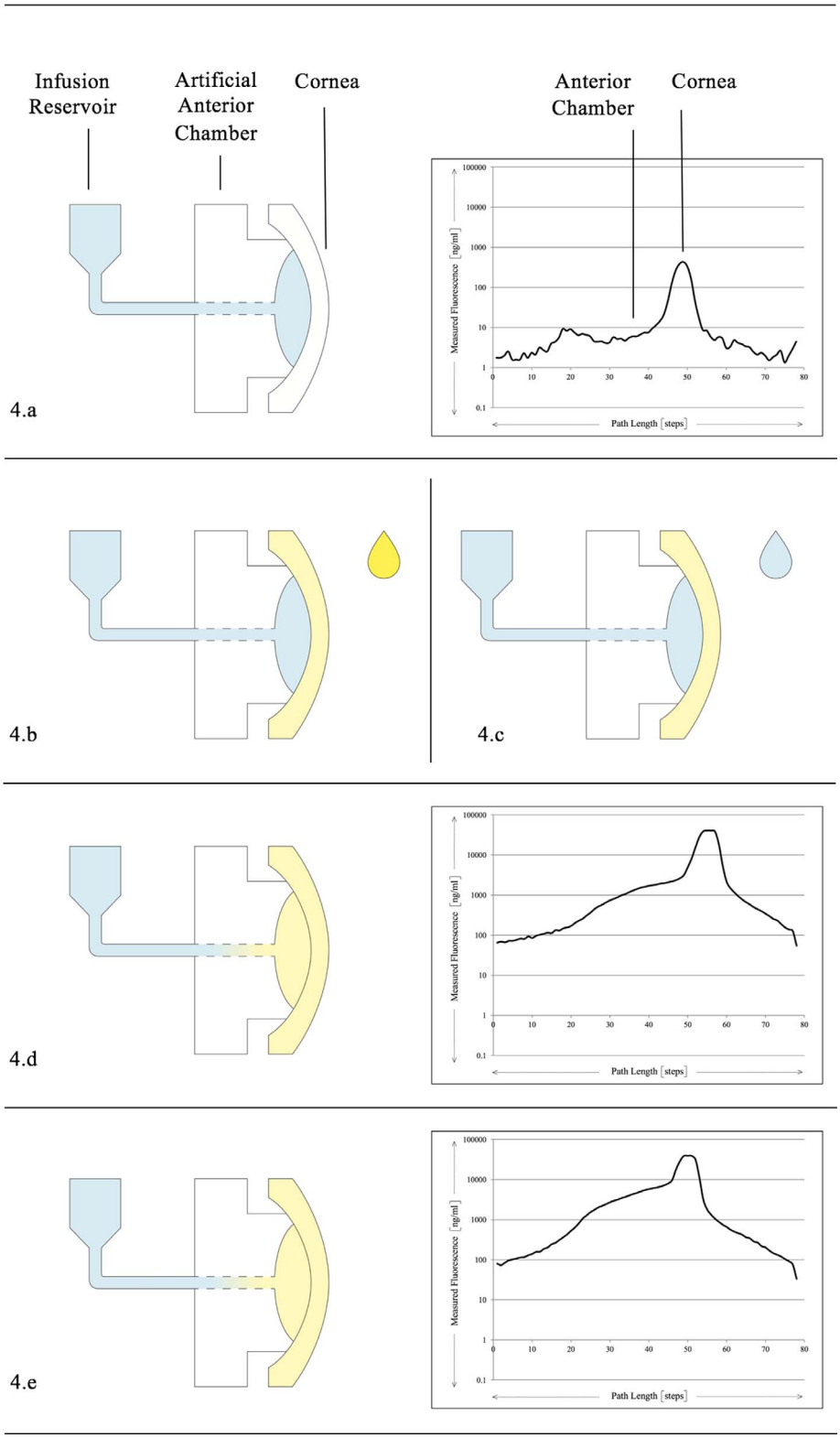


Figure 4. Experimental setup and corresponding fluorophotometry scans for simulation of cross-linking treatment. (a) Baseline measurement with BSS; (b) application of riboflavin drops; (c) rinsing riboflavin film with BSS for fluorophotometric measurements at half-time (15 minutes) and at the end of riboflavin application phase (30 minutes); (d, e) increasing riboflavin concentration in anterior chamber due to diffusion from impregnated cornea.

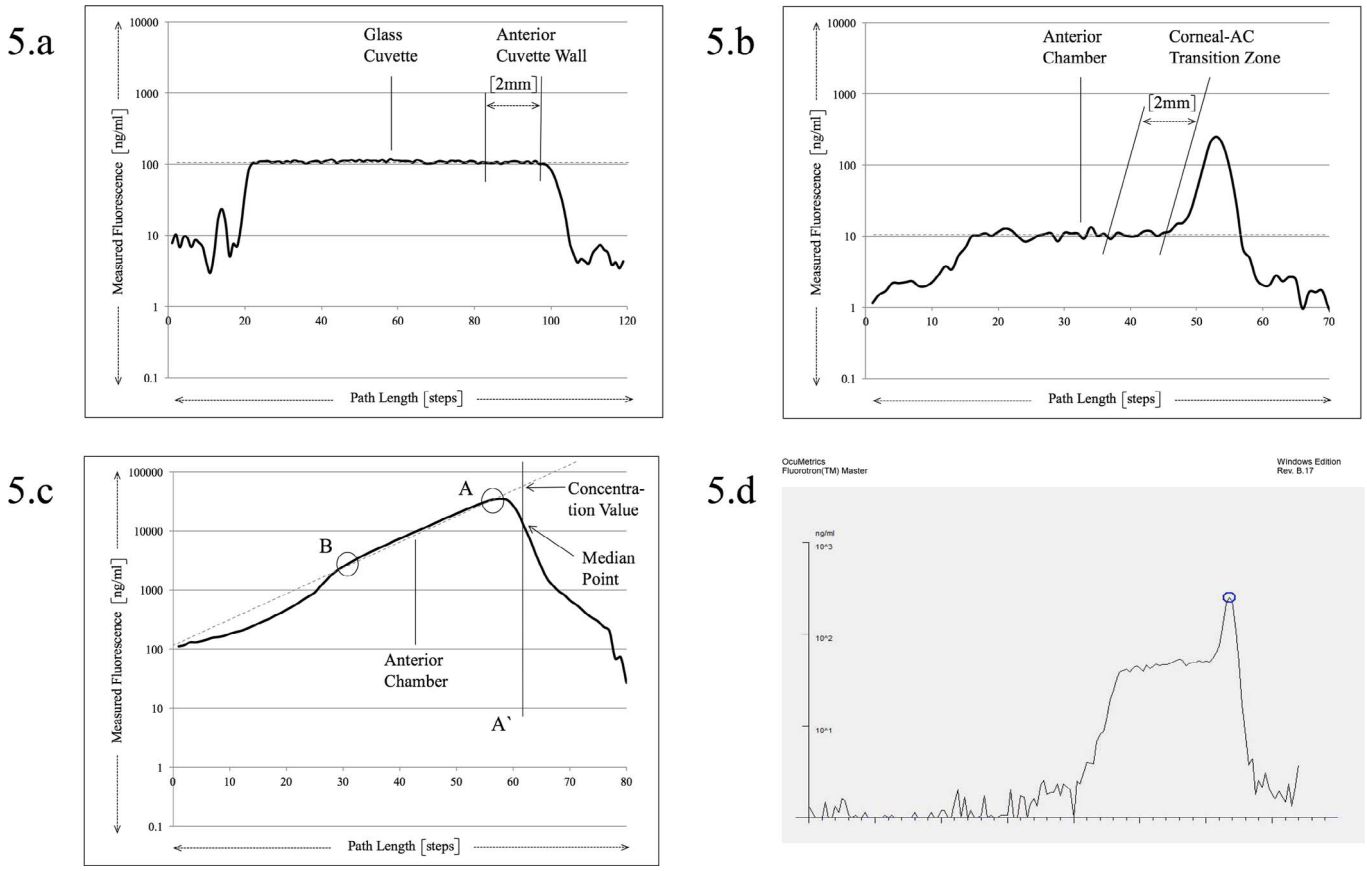


Figure 5. Derivation of riboflavin concentrations from fluorescence concentration profiles of scans across cuvette (a) and corneas mounted on artificial anterior chamber (b–d) with y axis indicating the amount of fluorescence in nanograms per milliliter (logarithmic scale) and x axis indicating individual measurement positions along the scan axis. The measurement result was determined by averaging the eight data points over the first 2 mm behind the anterior cuvette wall or behind the transition zone between cornea and anterior chamber, respectively. For very highly concentrated riboflavin solutions with indistinguishable corneal peaks and obvious signal extinction (c), the riboflavin concentration was semiautomatically extrapolated by connecting the anterior (A) and posterior (B) peak fluorescence by a line A to B and by drawing a vertical anterior fluorescence line A′ through the median point of the anterior slope (due to the logarithmic scale the median point appears high on the slope). The numeric result of the concentration value corresponds to the intersection of the fluorescence line A′ with the line A to B. (d) Graphic output of scan data in the analysis screen of the Fluorotron Master software.

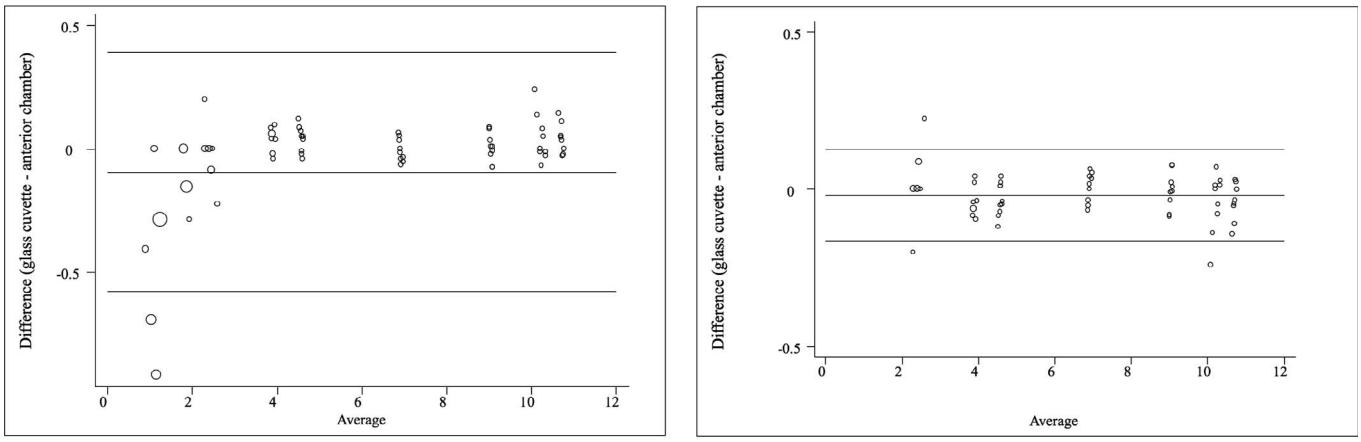
−0.205 (Pitman test $P < 0.001$), indicating discordance. When excluding the low dilutions representing the noise and focusing on the data range between 10 and 100,000 ng/mL (Fig. 6b), the two measurements were highly concordant as indicated by a nonsignificant Pitman test P value. The mean difference was 0.020 (95%CI: 0.001–0.038, $P = 0.329$).

At a reference concentration of 10,000 ng/mL, the fluorophotometer underestimated the value by 15%, and at the high end of the concentration spectrum (50,000 and 100,000 ng/mL), a strong relative loss of the fluorescence signal or false-deep measured values were observed in relation to the manufactured reference concentrations, along with a pronounced increase of the standard deviations. The application of a riboflavin-soaked contact lens finally led in all cases to a fluorescence profile with a high initial peak and a subsequent steep drop from which no anterior chamber riboflavin concentration could be derived (Fig. 3d).

Mean pachymetry values were 536 µm (±92 µm) after epithelial ablation and 512 µm (±92 µm) after the last fluorophotometric measurement ($P = 0.005$).

Simulation of Standard Epi-Off CXL Procedure

Concentration profiles recorded during corneal riboflavin application are shown in Figure 4. Changes in corneal stroma and anterior chamber fluid concentrations are summarized in Table 2. Corneal riboflavin concentrations increased significantly during the



6.a 6.b

Figure 6. Bland-Altman plots showing the difference against the average of logarithmically transformed, paired riboflavin concentrations measured in a glass cuvette and in the fluid of an artificial anterior chamber bank. (a) Riboflavin dilution series (0, 1, 5, 10, 50, 100, 1000, 10,000, 50,000, 100,000 ng/mL) in nine corneas with three paired measurements per concentration. (b) Plot with the same data set, but limited to concentrations from 10 to 100,000 ng/mL (i.e., without low concentrations influenced by background fluorescence). *Middle line*: mean of difference; *outer lines*: 95% limits of agreement, size of data points corresponds to number of observations with identical values.

application phase, and thereafter decreased very slowly. In contrast, anterior chamber fluid concentrations kept increasing during the entire observation period of up to 3 hours after the application phase. Mean pachymetry values at the beginning of the measurement series were 652 µm (±86 µm) and 755 µm (±31 µm) after completion ($P = 0.163$).

Discussion

We have found that fluorophotometry was able to measure riboflavin in an artificial anterior chamber across human corneas over a wide range of concentrations and we have shown that corneal application of riboflavin drops leads to a measurable increase of riboflavin in the anterior chamber.

In the first experimental set-up, the artificial anterior chamber was filled with riboflavin solutions of known concentration and the anterior chamber concentration measured through a clear cornea was compared with the measurement result of the same solution in a glass cuvette. A good concordance was observed with the exception of extremely low concentrations: the signal strength of the 1- and 5-ng/mL concentrations differed only marginally from

Table 1. Riboflavin Concentrations: Planned (Manufactured) Concentrations of the Dilution Series Compared With Fluorophotometrically Measured Concentrations in the Glass Cuvette and in the Artificial Anterior Chamber

Planed Concentration, ng/mL	Concentration Measured in Cuvette, ng/mL		Concentration Measured in Artificial Anterior Chamber, ng/mL	
	Mean	SD	Mean	SD
1	2.81	0.06	3.63	0.16
5	6.22	0.18	6.93	0.72
10	10.78	0.56	11.26	1.58
50	50.38	2.39	48.35	2.36
100	99.18	3.04	95.51	5.16
1000	1004.68	25.16	1011.95	61.85
10,000	8534.02	141.37	8505.35	501.87
50,000	28,295.59	1673.24	27,241.29	3219.61
100,000	45,814.56	944.37	44,064.59	2825.03

Table 2. Fluorophotometrically Measured Riboflavin Concentrations in Corneal Stroma and Anterior Chamber Fluid During a Thirty-Minute Topical Application Phase and Subsequent Observation Phase ($n = 4$, except where indicated otherwise)

	Cornea, ng/mL		Anterior Chamber, ng/mL	
Time, min	Mean	SD	Mean	SD
With riboflavin application				
0	496	97	5	1
15	40,520	2938	904	201
30	41,430	1183	1089	56
Without riboflavin application				
45	41,627	1048	1890	92
60	41,501	452	2716	439
75	41,449	2467	4444	822
90	41,236	1826	5483	491
150	40,542 ($n = 3$)	1233	10,966 ($n = 3$)	2253
210	31,277 ($n = 1$)	-	13,606 ($n = 1$)	-

the background fluorescence of the baseline measurements and was formally higher in the artificial anterior chamber than in the cuvette. Background fluorescence originates from the autofluorescence of the samples and instrument parameters, such as the light of the excitation source, camera noise, and remaining ambient light. The order of magnitude measured here corresponds to values found earlier in human anterior chambers[16] and cuvettes with distilled water.[7] In view of the fact that anterior chamber fluorescence in corneal riboflavin application approaches a value of 1000 ng/mL after 15 minutes already and then increases further (Table 2), this measurement uncertainty in the low-concentration range can be neglected for clinical applications.

At the other end of the spectrum, at the very high concentrations of 50,000 and 100,000 ng/mL, the fluorometrically measured concentrations no longer increased proportionally to the concentration of the dilution series prepared until finally the application of a riboflavin-soaked contact lens made it completely impossible to derive a concentration determination from the anterior chamber. We speculate that the riboflavin molecules inside the contact lens material were even closer packed than in the high-concentration solutions and blocked the beams by reflection, scattering,[17] and self-absorption.[18,19] The clinical implication of this finding is that any precorneal riboflavin film should to be washed off before taking a fluorescence measurement to avoid the observed shielding effect. In contrast, the fact that the concentrations of 50,000 and 100,000 ng/mL are outside the reliable measuring range of the fluorophotometer has no immediate clinical consequences, as these riboflavin concentrations are 50 to 100 times higher than those measured in the anterior chamber after 30 minutes of corneal application (Table 2).

Applying this knowledge to the simulation of an epi-off CXL procedure, a significant increase of the fluorescence signal in the anterior chamber could already be measured after 15 minutes of corneal riboflavin application. The cornea also showed a very rapid increase of the fluorescence signal within a quarter of an hour, which remained approximately constant in the further course and only began to weaken two hours after the end of the riboflavin application. In contrast, the fluorescence signal from the anterior chamber increased steadily during the entire observation period, which can be explained by a continuous drainage of riboflavin from the corneal stroma into the anterior chamber.

To the best of our knowledge, literature on fluorophotometric riboflavin measurements is limited to two conference abstracts by Chiambaretta and colleagues: in two in vivo CXL studies using the same fluorophotometer, the corneal riboflavin impregnation and its outflow into the anterior chamber was documented in 25 patients (Viennet A, et al. *IOVS*. 2011;52:ARVO E-Abstract 5201) and a comparison between iontophoresis-assisted CXL and conventional CXL showed higher corneal riboflavin levels for the latter.[9] Because no data have been published, a comparison with the present study is not possible. However, we would expect the anterior chamber concentrations measured by us to be somewhat lower as we reduced the noise of the corneal tailing effect by

narrowing the focal diamond (Fig. 1). In earlier fluorescence studies on aqueous humor dynamics using intravenously applied fluorescein as a tracer, tailing was less of a problem because the anterior chamber fluorescence was higher than the corneal autofluorescence. In CXL, however, the fluorescence of the riboflavin-impregnated cornea is significantly higher than the anterior chamber fluorescence and the corneal tailing effect becomes relevant, especially at low anterior chamber concentrations.

This study has potential limitations. First, the concentrations of the produced riboflavin dilution series were not verified by an external reference method. Due to restrictions with regard to the availability of donor corneas and the shelf life of the fluorescent solutions, several dilution series had to be manufactured at different points in time. Despite the use of a commercially available stock solution and a uniform production process, some deviations of the effectively produced from the planned concentration can therefore not be excluded. However, the paired measurements for the comparison of cuvette with anterior chamber fluid readings were carried out with identical batches in each case, so that the assessment of concordance was still valid. Similarly, the anterior chamber fluid concentrations measured after corneal application of riboflavin drops were not checked with a reference method. In contrast to measurements taken across clear corneas, the riboflavin concentration profile behind riboflavin-impregnated corneas did not show a strictly horizontal segment, but a subtle descending slope behind the corneal peak. It remains to be shown how accurately the averaged results of the digital output within these 2 mm correspond to the effective anterior chamber riboflavin concentration. However, the fluorescence concentration profiles obtained were qualitatively similar to those that were measured through clear corneas and the uniform increase in anterior chamber fluorescence over time corresponded to the naturally occurring riboflavin outflow from the corneal stroma into the aqueous. We therefore consider the study purpose of an objective riboflavin detection in the anterior chamber to be achieved. To develop a full picture and to optimize the accuracy of the concentration measurements, future investigations could compare fluorophotometry readings with those obtained by analytical chemistry methods.

The findings of this first in vitro study about fluorophotometric riboflavin measurements might make some contributions to future CXL practice: the proof of sufficient corneal riboflavin impregnation, and thus sufficient UV protection of the intraocular structures may no longer be dependent on the clinical expertise of the examiner, but could be provided by the objective and largely automatically calculated output of a fluorophotometer. This would increase not only safety but also efficiency, as the operation of the fluorophotometer is comparable with that of an aberrometry device, it would in principle be delegable to nonmedical staff. However, the implementation of fluorophotometry in routine CXL might be hampered by the fact that the precorneal riboflavin film makes fluorophotometric measurements in the anterior chamber fluid impossible and must therefore be rinsed off.

Finally, as CXL continues to generate an impressive amount of research interest, fluorophotometry could play a helpful role in corneal penetration studies of different riboflavin formulations and administration modalities.

In conclusion, we present evidence that fluorophotometry provides a quantitative means of measuring riboflavin in an artificial anterior chamber across human corneas in an ex vivo model. The methodology described may prove useful in riboflavin penetration studies.

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