

Two-Photon Fluorescence Microscopy for Determination of the Riboflavin Concentration in the Anterior Corneal Stroma When Using the Dresden Protocol

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PURPOSE. To determine the riboflavin concentration gradient in the anterior corneal stroma when using the Dresden protocol with different dextran solutions.

METHODS. Three different groups of porcine corneas, five each, were compared regarding the riboflavin concentration in the anterior stroma. Before all experiments, stable hydration conditions were established for the corresponding solution. All groups were treated with 0.1% riboflavin in different dextran solutions (15%, 16%, 20%). After imbibition, two-photon microscopy was used to determine fluorescence intensity. For signal attenuation and concentration determination corneas were saturated and measured a second time by two-photon microscopy. Additionally, the distribution was calculated mathematically and compared to the empiric results.

RESULTS. Riboflavin concentration is decreasing with depth for all dextran solutions. A nearly constant concentration could be determined over the first 75 μm . Analysis of the fit functions leads to diffusion coefficients of $D = 2.97 \times 10^{-7} \text{ cm}^2/\text{s}$ for the 15% dextran solution, $D = 2.34 \times 10^{-7} \text{ cm}^2/\text{s}$ for the 16% dextran solution, and $D = 1.28 \times 10^{-7} \text{ cm}^2/\text{s}$ for the 20% dextran solution.

The riboflavin gradients of the 20% dextran group were statistically significantly different from 15% dextran starting at a depth of 220 μm and deeper ($P = 0.047$). The 16% dextran group differed statistically at a depth of 250 μm and deeper ($P = 0.047$). These results show a significant difference to those published previously.

CONCLUSIONS. With correct settings two-photon microscopy is a precise way to determine the concentration of riboflavin in cornea. The measured gradient is excellently fit by a Gaussian distribution, which comes out as a solution of Fick's second law.

Keywords: riboflavin distribution, cross-linking, cornea, two-photon microscopy, concentration

Corneal cross-linking appears to be a safe and effective procedure to halt the progression of keratectasias, such as keratoconus or post-LASIK ectasia.¹⁻³ In clinical routine, cross-linking is achieved by using the Dresden protocol: After removing the epithelium, riboflavin is applied to the surface of the cornea.^{1,4} Diffusion leads to a spreading of riboflavin in the stroma of the cornea and anterior chamber. Since riboflavin acts as a photosensitizer, it can be activated by ultraviolet A (UV[A]) light and forms new chemical bonds within the extracellular matrix.^{1,5} These newly formed bonds (cross-links) increase the mechanical stiffness of the cornea. The riboflavin acts also as UV(A) light protection for the endothelium, lens, and retina.⁶⁻⁸ Therefore, the success of the cross-linking is highly dependent on an adequate riboflavin concentration in the cornea. In several publications, the distribution of riboflavin through the corneal stroma has been investigated with different approaches, such as

(two-photon) fluorescence microscopy⁹⁻¹¹ and high-performance liquid chromatography.^{12,13} In our described work, two-photon fluorescence microscopy was used for this purpose as well, but the methodology was changed in comparison with previous publications.

In two-photon microscopy, pulsed infrared laser light is focused into the cornea. Due to the high intensity inside the focus, nonlinear processes as two-photon absorption or second harmonic generation (frequency doubling) can take place.^{14,15} Two-photon absorption transfers a molecule (here, riboflavin) into an excited state, that returns to the ground state by emission of fluorescence. Since two-photon absorption is restricted to the focal volume, the laser can be scanned in a defined plane and a consecutive pointwise signal detection results in a two-dimensional image. Variation of the focal position over depth (z-position) allows imaging in

different planes and facilitates a three-dimensional reconstruction of a defined volume.¹⁶⁻¹⁸ Generated fluorescence light is absorbed by the extracellular matrix and by the riboflavin itself on its way back to the surface. In addition, scattering occurs at keratocytes and collagen inhomogeneities.¹⁹ These losses must be compensated by an appropriate correction model.

Some groups already reported two-photon microscopy as a suitable technique for the determination of riboflavin content inside the cornea.⁹⁻¹¹ However, due to methodical errors all three groups underestimated or did not address all different sources of loss of the fluorescence and, therefore, come to erroneous conclusions.

In this report we demonstrated the riboflavin distribution in the porcine cornea after imbibition with 0.1% riboflavin drops with variable dextran content.

MATERIALS AND METHODS

Cornea Preparation

A total of 15 corneas from fresh enucleated porcine bulbi delivered by a local slaughterhouse were divided into 3 groups, each with 5 eyes. Central ultrasound pachymetry (SP-100; Tomey, Nagoya, Japan) was performed before and after every step of the experiment. After abrasion, a corneoscleral disk was excised and mounted into an artificial anterior chamber (Barron Precision Instruments, Grand Blanc, MI, USA) as shown in Figures 1a and 1b. To guarantee that hydration of every cornea in its group was the same, the corneas were deswollen by means of different dextran T500 solutions of 15% and 16%, respectively, 20% wt/wt (Sigma-Aldrich Corp., St. Louis, MO, USA). To achieve this, the corresponding dextran solution was injected into the anterior chamber as well as into a reservoir on top of the cornea. Steady state was assumed if corneal pachymetry did not change more than 3% in 3 consecutive measurements each 5 minutes apart. This was achieved in all corneas within the first 90 minutes. Afterwards, the reservoir mounted on top of the cornea was filled with 0.1% riboflavin-5-monophosphat (Streuli Pharma AG, Uznach, Switzerland) solution with the corresponding dextran solution for 30 minutes, simulating the repeated riboflavin application in compliance with the Dresden protocol. After 30 minutes, the riboflavin reservoir was removed and the corneal surface was single wiped clean without liquid followed by the first two-photon measurement through the center of the cornea. For individual calibration, the corneas were saturated with riboflavin by applying the corresponding dextran/riboflavin solution into the reservoir and anterior chamber. After 120 minutes, another stack was measured to determine the signal of the saturated cornea. After each step of the experiment, the contents of the reservoirs were replaced by freshly prepared solutions. During the whole procedure, physiological pressure was applied to the anterior chamber.

Imaging Setup and Methods

Two-photon fluorescence imaging of riboflavin treated corneas was performed with the commercially available multiphoton microscope TriM Scope II (LaVision Biotec GmbH, Bielefeld, Germany). This microscope system was equipped with the laser system Chameleon Ultra II (Coherent, Inc., Santa Clara, CA, USA) providing fs-laser pulses in the near infrared. For imaging experiments, the artificial anterior chamber was fixed in a custom made holder beneath the objective lens (W Plan-Apochromat $\times 20/1.0$; Zeiss GmbH, Oberkochen, Germany). This objective lens was used for

focusing the laser into the corneas and is chromatically corrected for coverslips with a thickness of 0.17 mm. The coverslip separated the stromal surface from the immersion medium (water), which we used with the objective lens to reach its full numerical aperture (NA) and to prevent alteration of the diffused riboflavin in the cornea through undesired dilution by the immersion medium. The coverslip was mounted on a custom made holder (Figs. 1b, 1c). One of the riboflavin absorption maxima is approximately 445 nm. Since scattering inside the cornea becomes smaller with increasing wavelength, a central laser wavelength of 900 nm was used for riboflavin excitation in all experiments.^{10,20} For signal detection, the microscope system was equipped with two photomultiplier tubes arranged in backward direction (behind the objective lens). To restrict the detectable wavelength range, bandpass filters were used in front of every photomultiplier tube. For detecting the riboflavin fluorescence, a bandpass filter with a transmission window of 525 ± 25 nm was used. In the second detection pathway, second harmonic signals generated at the stromal collagen could be detected simultaneously when using a bandpass filter with a transmission window of 450 ± 35 nm in front of the second photomultiplier tube. A scheme of the total setup is shown in Figure 1c. For each cornea, images were recorded in z-stacks with a step size of 10 μm over a depth of 350 μm . One single image inside the stack covered an area of $400 \times 400 \mu\text{m}^2$ with a pixel resolution of 1042×1042 . In addition, one dark image (same settings, but without laser irradiation) was taken after every z-stack. After saturation, each cornea was imaged again in a z-stack with the same parameters as described before. The saturation process was necessary to correct our data for absorption and scattering effects.

Image Analysis

For image evaluation, each z-stack of the riboflavin-treated corneas and the corresponding dark image was loaded into the image processing software ImageJ (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Subtracting the corresponding dark image from every image inside one stack reduced the electronic noise. Afterwards a region of interest (ROI) was defined in the center of each image throughout the stack and the mean gray value representing the signal intensity in arbitrary units was determined. Likewise, signal intensity analysis in the corresponding saturated cornea allowed correction of the data for absorption and scattering effects. Thus, the resulting data were only affected by the riboflavin distribution over the depth of the corneas.

Diffusion in Corneal Tissue

Before we present our experimental results, we will briefly introduce and summarize the theory of diffusion of riboflavin in cornea. This theoretical contemplation helps to interpret the results and gives characteristic values to describe the diffusion behavior of riboflavin in corneal tissue.

The diffusion of the applied riboflavin solution into the stroma can be described by Fick's second law of diffusion:

$$\frac{\partial c(z, t)}{\partial t} = D \frac{\partial^2 c(z, t)}{\partial z^2}.$$

Here, $c(z, t)$ stands for the riboflavin concentration, D for the diffusion coefficient, z for the depth in the cornea, and t for time. To solve this differential equation, useful boundary conditions must be assumed. Taking the experiments into account, it can be assumed that $c(z, 0) = 0$ for all z that are not

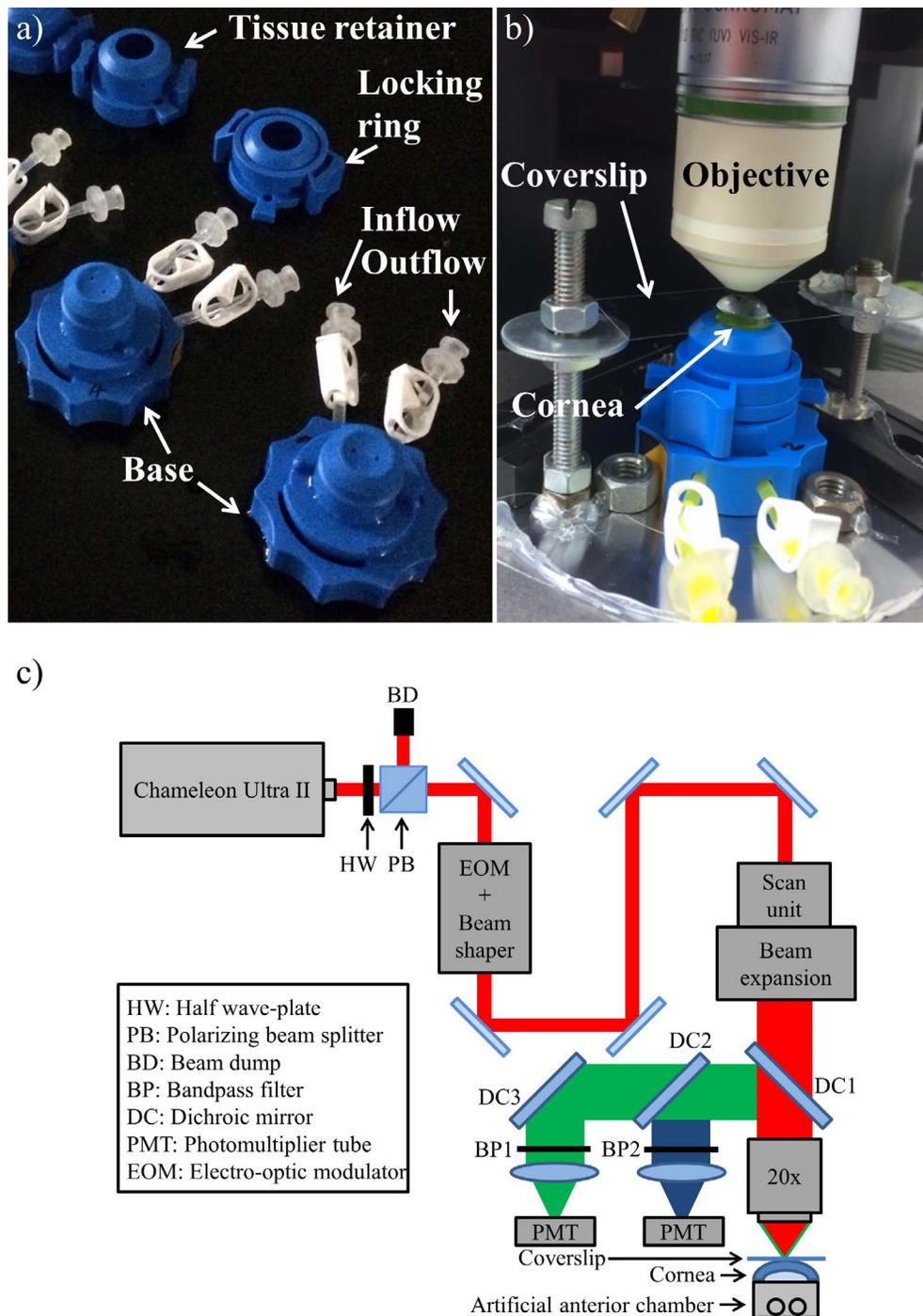


FIGURE 1. Experimental setup. (a) Anterior chamber. (b) Measuring interface. (c) Schematic illustration of the experimental setup. Simultaneous detection of two-photon fluorescence and second harmonic signals was performed in backward direction.

equal to 0. The concentration c tends to infinity for $z = 0$. This means that all molecules whose number can be described by N are located in an infinitesimal volume V at the surface of the stroma. Since $c = N/V$ and $V \rightarrow 0$, the concentration is tending to infinity. With these boundary conditions, Fick's second law can be solved by

$$c(z, t) = \frac{N}{2\sqrt{\pi Dt}} \exp\left(\frac{-z^2}{4Dt}\right).$$

Since the eyes were treated with riboflavin for 30 minutes (Dresden protocol), t is a constant. To determine the diffusion coefficient from our measurements, the obtained data can be fitted with an equation like

$$c(z, 30 \text{ min}) = \frac{B}{\sqrt{(30 \text{ min})\pi D}} \exp\left(\frac{-z^2}{4D(30 \text{ min})}\right).$$

In this equation, B stands for $N/2$ and reduces the number of factors.

TABLE. Pachymetry With Standard Error

US Pachymetry, μm	Intact Bulbi	After Mounting and Applying IOP	Before Riboflavin Application	Before First Measurement	After Saturation
15% dextran	854 \pm 20	770 \pm 13	625 \pm 5	607 \pm 12	565 \pm 22
16% dextran	836 \pm 27	778 \pm 12	585 \pm 11	556 \pm 11	518 \pm 10
20% dextran	865 \pm 21	780 \pm 18	516 \pm 12	499 \pm 13	474 \pm 10

Statistical Analysis

To determine statistical significance between concentrations in corresponding depths, Mann-Whitney U tests were performed (Winstat; R Finch, Bad Krozingen, Germany). Significance was accepted if $P < 0.05$.

RESULTS

Hydration Condition

Ultrasound pachymetry was performed to control stable hydration of the corneas (see Table). There is slight reduction

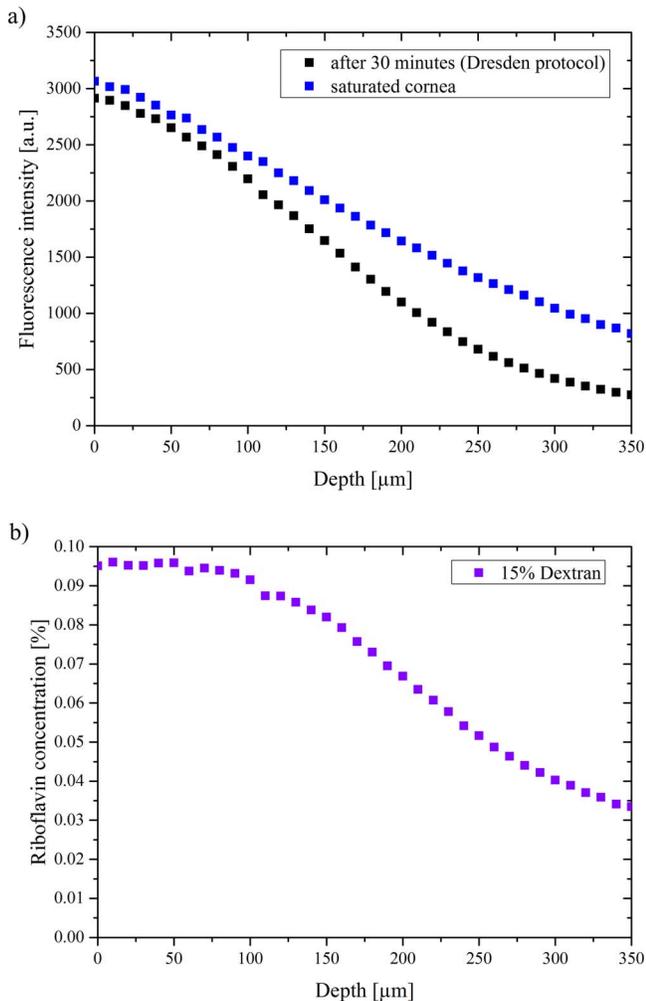


FIGURE 2. Calculation of riboflavin concentration from measured fluorescence intensities. (a) Fluorescence intensity values from a riboflavin-treated cornea (Dresden protocol) and values from the corresponding riboflavin-saturated cornea were obtained. Pairwise calculation of the ratio between these intensity values led to the riboflavin concentration over corneal depth as shown in (b).

in thickness after applying the 0.1% riboflavin solution into the reservoir (3%-5%) as well as a reduction during saturation (5%-7%).

Fluorescence Signal

Profiles along the z -axis measured from riboflavin-treated corneas were corrected by data obtained from the same corneas in a saturated state. Figure 2 shows exemplarily the fluorescence intensities obtained from a cornea treated with 15% dextran after 30 minutes of riboflavin application and the corresponding data obtained from the same cornea in a saturated state. As expected, measured intensities from the saturated cornea are higher compared to intensities after applying riboflavin for 30 minutes. To determine the riboflavin concentration as a function of corneal depth, the resulting intensity values in the various depths were divided pairwise (fluorescence intensity after 30 minutes of treatment/fluorescence intensity of saturated cornea) and multiplied with the concentration of the riboflavin solution [0.1%].

The overall results we got for different dextran/riboflavin solutions are presented in Figure 3 and are based on $n = 5$ corneas treated with 15% dextran, $n = 5$ corneas treated with 16% dextran, and $n = 5$ corneas treated with 20% dextran. In all cases, the experimentally obtained data (means \pm SE) and the corresponding fit functions (solution of Fick's second law) are displayed.

Riboflavin concentration is decreasing with depth for all dextran solutions. Nevertheless, a nearly constant concentration could be determined over the first 75 μm in all samples. Analysis of the fit functions leads to diffusion coefficients of $D = 2.97 \times 10^{-7} \text{ cm}^2/\text{s}$ for the 15% dextran solution, $D = 2.34 \times 10^{-7} \text{ cm}^2/\text{s}$ for the 16% dextran solution, and $D = 1.28 \times 10^{-7} \text{ cm}^2/\text{s}$ for the 20% dextran solution. To compare the results of the different groups with each other, Figure 4 shows line diagrams of the mean values from Figure 3.

Comparison of Riboflavin Gradients

The riboflavin gradients of the experiments performed with 15% and 16% dextran were not statistically significant different ($P = 0.917$). The riboflavin gradients of the 20% dextran group were statistically significantly different from 15% dextran starting at a depth of 220 μm and deeper ($P = 0.047$). The 16% dextran group differed statistically from the 20% dextran group at a depth of 250 μm and deeper ($P = 0.047$).

DISCUSSION

The main findings of the study are: (1) a relative constant riboflavin concentration of 0.07% to 0.09% as deep as 100 μm after 30 minutes of imbibition, (2) the following decay is the steeper the higher the concentration of dextran, (3) measurement data fit well to the theory proposed enabling valid prediction of riboflavin gradients under various conditions, and (4) significant differences to previously published data.

The relatively flat gradient during the anterior 75 μm of the stroma is explained by the Gaussian function that we found in

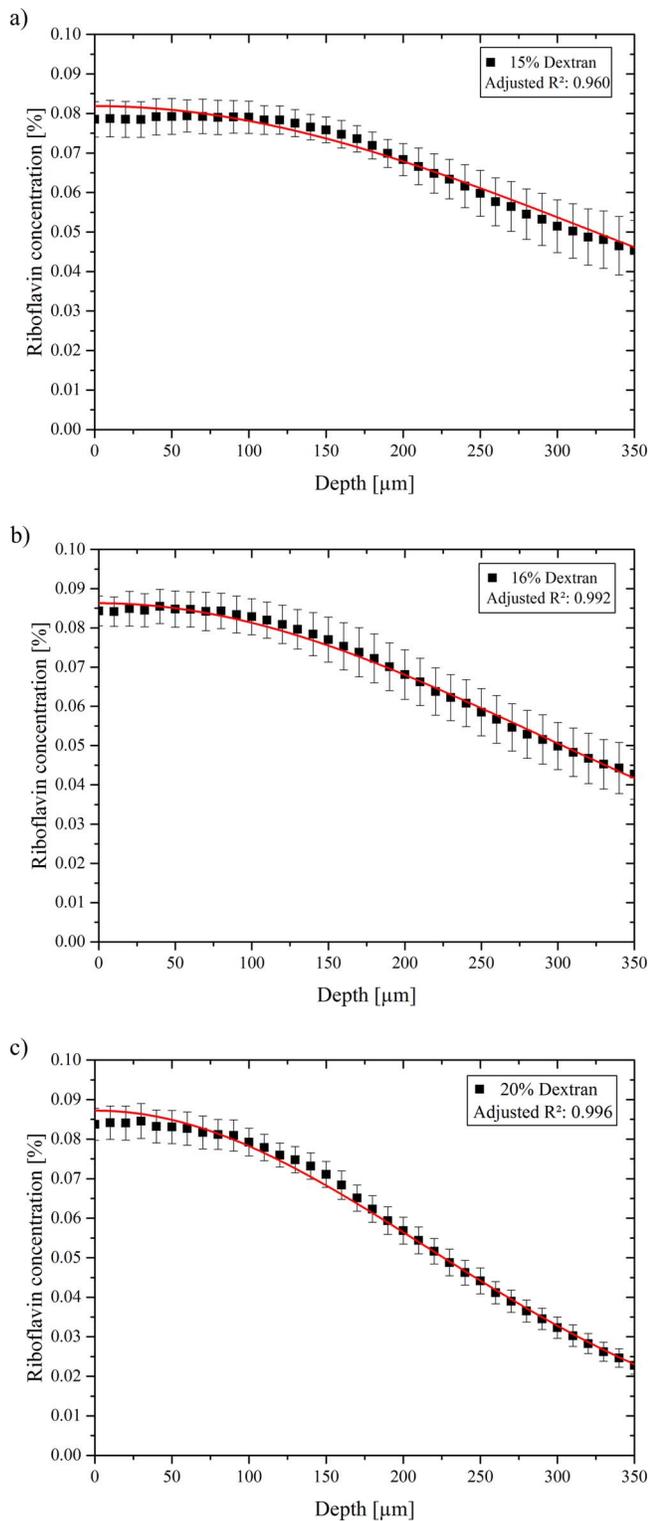


FIGURE 3. Riboflavin concentration after 30 minutes over corneal depth when applying the Dresden protocol. *Black squares* represent the mean values of the measured data after correction for scattering and absorption effects ($n = 5$). The *red line* corresponds to the fit function resulting from the solution of Fick's second law. (a) Results for 15% dextran solution. (b) Results for 16% dextran solution. (c) Results for 20% dextran solution. The riboflavin concentration is nearly constant over the first 75 μm . For bigger depths, the concentration is decreasing.

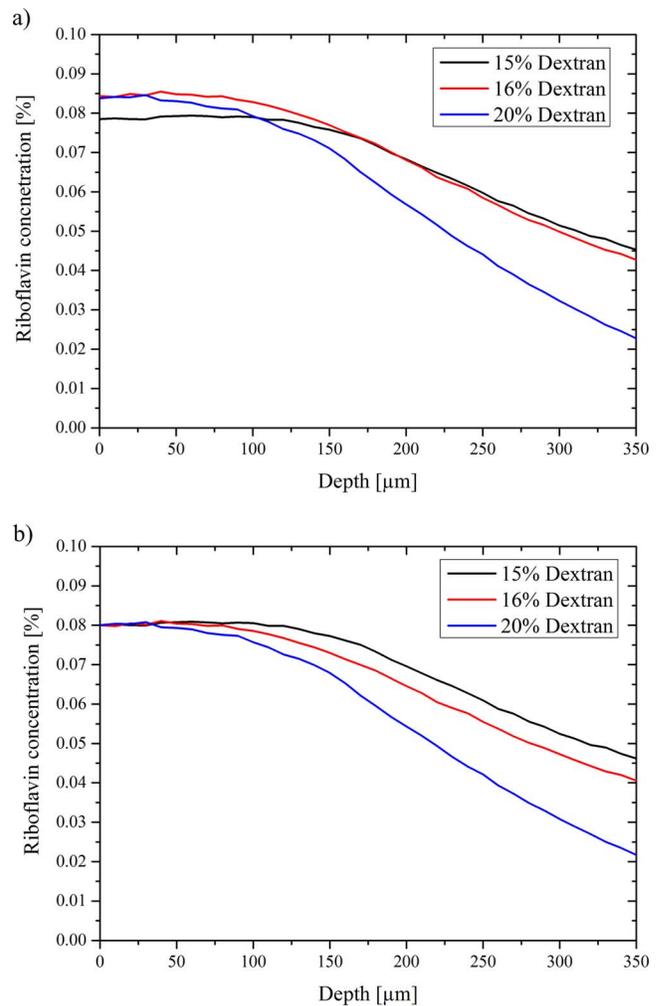


FIGURE 4. (a) Summary of the data points shown in Figure 3. (b) Standardized graphs for the starting concentration of 0.08% for easier comparability.

the theoretical evaluation. This is quite in contrast to gradients presented before, such as the study of Kampik et al.¹¹ that proposed an exponential gradient with steep slope at the surface, or that of Gore et al.,¹⁰ who found a relatively constant concentration throughout the whole corneal thickness after 30 minutes.

Surprisingly, higher dextran concentrations lead to a steeper decay of the riboflavin gradient (Fig. 4). According to basics in physical chemistry, the partial pressure of solved riboflavin is the driving force for diffusion, independent of the osmotic agent. The steeper gradient could be explained by a diffusion reduction due to a denser cornea. In detail, a higher collagen content per volume. This also is reflected by a smaller diffusion constant of $D = 1.28 \times 10^{-7} \text{ cm}^2/\text{s}$ for the 20% dextran solution compared to $D = 2.97 \times 10^{-7} \text{ cm}^2/\text{s}$ for the 15% dextran solution. From x-ray scattering experiments²¹ in corneal stroma, we know that primarily the collagen molecule is superficially cross-linked. However, Hayes et al.²¹ also found cross-links within and between the proteoglycans. In a deswollen cornea, proteoglycans and collagen are more densely packed and, therefore, intermolecular cross-links are facilitated. Therefore, for clinical efficacy of corneal cross-linking (CXL) a higher concentration of dextran is preferable. On the other hand, a steep gradient avoids sufficient riboflavin

concentration in the deeper layer, and, therefore, causing less cross-linking in the posterior cornea.

In our experiments, we assumed constant hydration within the cornea and throughout all measurements. This was necessary to ensure a reproducible environment; however, it does not depict clinical reality. In clinical cross-linking, after the abrasion, the dextran content of the drops is supposed to prevent swelling of the cornea during the imbibition of riboflavin. In porcine cornea a 15% dextran solution in the anterior chamber as well as precorneal resulted in a steady central corneal thickness of approximately 600 μm without epithelium, which is comparable with in vivo measurements.²² We assumed that the slight reduction in thickness results from a constant dextran concentration throughout the experiments, because we refilled the riboflavin/dextran reservoir with fresh solution after each measurement step.

As expected, the riboflavin concentration is decreasing with corneal depth. The obtained data points obviously form the shape of a Gaussian curve, which correlates well with the solution of Fick's second law (Fig. 3). Fitting the experimentally obtained data points led to diffusion coefficients with a magnitude of 10^{-7} cm^2/s . In the literature, diffusion coefficients of the stroma are reported with a magnitude of 10^{-7} cm^2/s as well.²³ Thus, the diffusion coefficients obtained from the fit functions are comparable to those reported by Araie et al.²³

To our knowledge, this is not the first work about the quantitative measurement of riboflavin diffusion into the cornea by means of two-photon fluorescence microscopy.

Kampik et al.¹¹ used an excitation wavelength of 880 nm in their studies and detected the riboflavin fluorescence as well as the second harmonic signal generated at collagen fibrils present in the stromal region simultaneously. To correct for scattering effects of laser light and fluorescence emission, the intensity loss of the second harmonic was determined and used as a reference for the depth-associated reduction of signal. An incident laser wavelength of 880 nm leads to a resulting second harmonic generation at 440 nm. Since riboflavin has an absorption maximum at 445 nm the generated second harmonic is absorbed by the riboflavin itself. Thus, using the second harmonic signal is not a good reference signal to compensate scattering effects in riboflavin treated corneas.

Gore et al.¹⁰ tried to correct the two-photon fluorescence signal by imaging a cornea that was treated with riboflavin for 50 minutes. In their setup, riboflavin was stored in a sole reservoir on top of the cornea during the treatment. After 50 minutes of treatment, they assumed an uniformly soaked cornea, as fluorescence signals almost did not change any more, and used the signal obtained from this for signal correction over depth. This assumption is wrong, especially when considering the relatively big volume of the anterior chamber, which acts as a sink during the diffusion process. As they used full globes, the concentration gradient after 50 minutes cannot be constant and the assumption of a uniformly soaked cornea after 50 minutes cannot be made.

Furthermore, Sondergaard et al.⁹ used confocal microscopy to estimate the riboflavin distribution inside the cornea before cross-linking. In their experiments, an excitation wavelength of 458 nm was used. This wavelength is absorbed strongly by corneal tissue.⁹ The results obtained were not corrected for absorption and scattering effects in the cornea. Thus, the data presented mainly demonstrated a loss of signal over the depth due to absorption of the incident light only (Lambert Beer's Law).

In summary, all earlier published results of two-photon microscopy were not interpreted correctly and, therefore, are misleading in the understanding of the real distribution of riboflavin after imbibition in CXL.

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