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Enhanced Transepithelial Riboflavin Delivery Using Femtosecond Laser-Machined Epithelial Microchannels

Samantha Bradford[1], Eric Mikula[1], Yilu Xie[1], Tibor Juhasz[1], Donald J. Brown[1], and James V. Jester[1]

[1] Department of Ophthalmology and Biomedical Engineering, University of California, Irvine, Irvine, CA, USA

Correspondence: Samantha Bradford, University of California, Irvine, 843 Health Sciences Road, Irvine, CA, USA. e-mail: smbradfo@uci.edu

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Purpose: This study describes a femtosecond laser (FS) approach to machine corneal epithelial microchannels for enhancing riboflavin (Rf) penetration into the cornea prior to corneal crosslinking (CXL).

Methods: Using a 1030-nm FS laser with 5- to 10-µJ pulse energy, the corneal epithelium of slaughterhouse rabbit eyes was machined to create 2-µm-diameter by 25-µm-long microchannels at a density of 100 or 400 channels/mm^2. Rf penetration through the microchannels was then determined by applying 1% Rf in phosphate-buffered saline for 30 minutes followed by removal of the cornea and extraction from the central stromal button. Stromal Rf concentrations were then compared to those obtained using standard epithelial debridement or 0.01% benzalkonium chloride (BAK) to disrupt the epithelial barrier.

Results: Microchannels formed using a 5-µJ/pulse at a density of 400 channels/mm^2 achieved a stromal Rf concentration that was 50% of that achieved by removal of the corneal epithelium and imbibing with 1% Rf. Stromal Rf levels were also equal to that of debrided corneas soaked with 0.5% Rf, threefold higher than those soaked with 0.1% Rf, and twofold higher than corneas soaked in BAK without epithelial debridement. Organ culture of treated corneas showed a normal corneal epithelium following FS machining while BAK-treated corneas showed extensive epithelial and stromal damage at 24 hours posttreatment.

Conclusions: FS corneal epithelial machining can be used to enhance penetration of Rf into the stroma for corneal CXL.

Translational Relevance: The creation of epithelial microchannels allows for stromal Rf concentrations high enough to perform true transepithelial crosslinking.

Introduction

Strengthening the corneal stroma by inducing the production of chemical crosslinks within the collagen structure has been shown to be an effective treatment for keratoconus and is currently being investigated as a treatment for low refractive errors.[1,2] The standard, US Food and Drug Administration–approved version of this technique uses ultraviolet-A (UVA) irradiation to excite the photosensitizer, riboflavin (Rf), imbibed into the patient's corneal stroma, causing the production of oxygen free radicals that in turn induce collagen crosslinking (CXL) within the corneal stroma.[3,4] While this technique is clinically successful in causing corneal stiffening and flattening,[1,5–15] it is still far from ideal for many reasons. Chief among these reasons are the low precision of the technique and the method of Rf application.

Keratocyte damage within the volume of CXL is inevitable with this procedure, and endothelial cell damage is a risk in corneas thinner than 400 µm.[16] Unfortunately, the single-photon nature of UVA CXL allows for very little precision with regard to the volume of cornea treated. Even when UVA CXL is customized by altering radiation profiles across the treatment area,

the reaction must begin at the surface, covering the entire exposed area of the cornea, and quenches with depth into the stroma.[17] A previously tested technique that improves upon the precision of corneal CXL uses focused femtosecond (FS) infrared laser pulses to produce CXL within a highly defined region, with micron-level precision in every direction. This alternative technique, termed *nonlinear optical crosslinking* (NLO CXL), has been shown to produce comparable results to traditional UVA CXL, namely, increased corneal stiffness and the generation of blue collagen autofluorescence due to the formation of collagen crosslinks.[5,6]

Another important concern about traditional UVA CXL involves the method of Rf application to the corneal stroma. Because the corneal epithelial barrier function prevents Rf molecules from penetrating into the corneal stroma, traditional UVA CXL requires epithelial debridement. This is painful for the patient, lengthens the recovery time, and exposes the cornea to possible infection.[14,18] The development of transepithelial UVA CXL is a highly active area of research, with many different methods being tested both in the laboratory and clinically. Common methods used to avoid debridement of the epithelium include the addition of chemicals such as benzalkonium chloride (BAK) to weaken epithelial tight junctions,[2,19–23] the addition of vitamin E or C,[24,25] or using iontophoresis during Rf application.[26–29] Whatever the method of Rf application, however, transepithelial UVA CXL has yet to reproduce the results of traditional UVA CXL.[14,23,30–32] Specifically, a review of the literature shows that patients with transepithelial crosslinking show improved Best spectacle corrected visual acuity (BSCVA) compared to traditional crosslinking but do not show a halting of the progression of ectasia.[31] This could be due to the reduced concentration of Rf within the corneal stroma when the epithelium is left intact. It could also be due in part to the nature of the single-photon reaction used, which begins at the surface, in this case within the epithelium itself. That is, the corneal epithelium acts as a barrier not only to Rf penetration but also to the necessary UVA irradiation.

To address both issues, we propose a new method of Rf application to be used in combination with our existing NLO CXL technology. We hypothesized that FS laser-based micromachining of channels through the corneal epithelium would increase Rf penetration into the corneal stroma, avoiding the cellular damage often associated with eye drops containing BAK.[23,33,34] This method would also have the added advantage of being highly compatible with NLO CXL after Rf application, which can produce CXL at any depth and pattern.

Methods

Tissue Preparation

A total of 64 ex vivo, intact rabbit eyes were shipped overnight (Pel-Freez, Rogers, AR) and prepared for use upon arrival as previously reported.[5,6,35] Briefly, they were rinsed in phenol-free, low-glucose, Dulbecco's modified Eagle's medium (Sigma Aldrich, St. Louis, MO), inspected for epithelial damage using Lissamine green staining (10 mg/mL in phosphate-buffered saline [PBS]; Sigma Aldrich), and placed in a 37°C, 5% CO_2 humidified incubator for 1 to 2 hours prior to treatment. Eyes showing corneal Lissamine green staining, indicating epithelial damage, were discarded or used as control corneas treated by epithelial debridement. After incubation, eyes were soaked with Rf solution using one of three techniques: the traditional Dresden protocol with epithelial debridement,[1] intact epithelium treated with a Rf solution containing 0.01% BAK, or epithelial micromachining. The full Rf solution contents and group sizes, including subcategories of each technique, are listed in the Table.

Epithelial Micromachining

Epithelial micromachining was achieved by directing a 1030-nm, 1-kHz amplified FS laser beam (One Five Origami; NKT Photonics, Birkerød, Denmark) into the same delivery system previously described for NLO CXL (Fig. 1).[6] A polarizing beam cube and half-wave plate were used to adjust the pulse energy to 5 µJ or 10 µJ. The pulse depth was aligned carefully for each experiment to avoid contact of the focal volume with the stroma by micromachining holes into a sheet of silicone. As shown in Figure 2, the pulse was aligned so only the bottom 25 µm of the focal volume reached below the contact glass, cutting only the surface epithelium. Finally, the spacing of the holes for each treatment group was adjusted to either 50 or 100 µm (100–400 channels per mm^2) using the custom Labview software (National Instruments, Austin, TX), which controls the delivery mirrors. Additionally, only the central 6-mm diameter area of the cornea received FS laser-based micromachining.

Treatment

Eyes were segregated into three main treatment groups, as outlined in the Table. The first group (DRES (the Dresden protocol, as used in standard UVA CXL); 23 eyes) was the only group treated with epithelial removal. The central corneal epithelium, 8

Table. Rf Solutions

Characteristic	Power	Epithelium	Media	BAK (%)	Rf (%)	No.
DRES	NA	OFF	20% Dextran	0	0.1	9
					0.5	9
					1.0	5
BAK	NA	ON	1% MC	0.01	0.1	5
					0.5	5
					1.0	11
Microchannels	5 µJ	50 µm	1% MC	0	1.0	5
		100 µm	1% MC	0	1.0	5
	10 µJ	50 µm	1% MC	0	1.0	5
		100 µm	1% MC	0	1.0	5

NA, Not applicable.

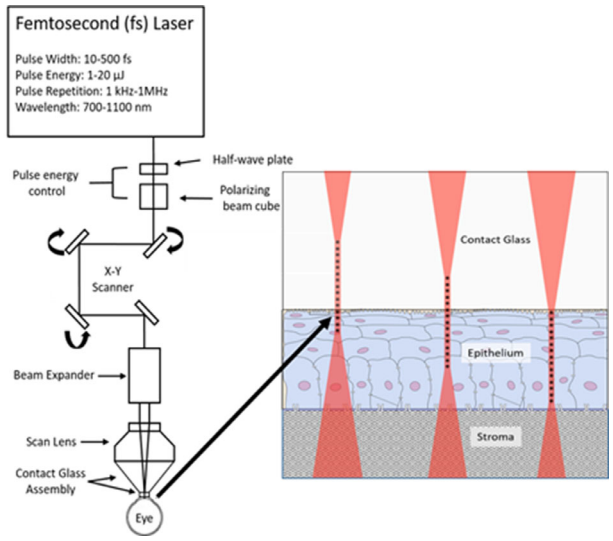

Figure 1. Schematic of laser-drilling delivery device. To drill microchannels into the corneal epithelium, a 1030-nm, 1-kHz laser is directed into our NLO CXL delivery device. The pulse energy is controlled using a half-wave plate and polarizing beam cube for 1 to 20 µJ.

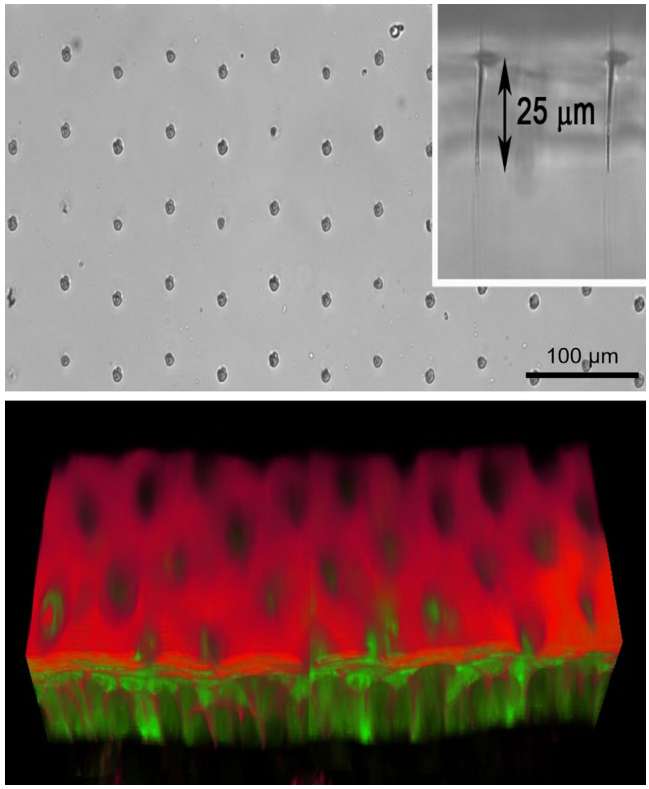

Figure 2. Channel spacing in silicone and corneal epithelium. A grid of microchannels with 50-µm spacing and 25-µm depth was cut into a silicone sheet for demonstration of the pattern (*top*). A three-dimensional reconstruction of a corneal section stained with phaloidin and propidium iodide shows the same grid placed on the surface of the corneal epithelium.

mm in diameter, was removed with a Tooke knife, and eye drops containing Rf and high-fraction dextran (450–650 kDa; Sigma-Aldrich) in PBS, according to the concentrations listed in the Table, were dropped onto the corneal surface every 2 minutes for 30 minutes. This group contained three subgroups, treated according to the standard Dresden protocol with 0.1%, 0.5%, and 1.0% Rf. These alterations were examined because increased Rf concentration of 0.5% was used for NLO CXL in all previous work, as an increased concentration has been shown to offset lowered dwell times.[5,6,36] The second group (BAK; 21 eyes) was treated using the same 2-minute interval dripping schedule as the DRES group, but the Rf drops contained 1% methylcellulose (MC) and 0.01% BAK. The eyes in this group were also treated with three different Rf concentrations as listed in the Table. The third group of eyes was treated using various patterns of epithelial microchannels followed by Rf eye drops containing 1% Rf and 1% MC

(Table) using the same dripping schedule as the DRES group.

A few additional eyes, not tested for stromal Rf concentrations, were used to access cellular damage due to the varying dripping protocols. These eyes were also subjected to NLO CXL after either BAK Rf or microchannel Rf treatment. NLO CXL was performed as described in previous works, using 760-nm amplified FS pulses directed into the same delivery device that was used for the placement of microchannels. These eyes were organ cultured for either 3 hours or overnight using previously reported methods.[37] Corneas were then fixed in 2% paraformaldehyde and then processed for phalloidin (1:100) and propidium iodide (0.01 mg/mL) staining to assess corneal cell viability posttreatment.

Measuring Stromal Rf Concentration

To measure stromal Rf concentration, the epithelium was first removed using a Tooke knife or excess Rf removed using a cotton gauze. Next, the entire cornea was removed from the globe around the limbus, and a central corneal button was cut using an 8-mm trephine. The button was then placed in 1 mL PBS solution and left to soak overnight, allowing Rf to elute from the button into the PBS. Care was taken to make sure that the corneal button was removed and placed in fluid within 4 minutes or less to avoid loss of Rf into the anterior chamber.

The concentration of Rf in the eluate was then measured using a Spectramax Gemini XPS fluorescence plate reader (Molecular Devices, Sunnyvale, CA) with 380 nm excitation and 540 nm emission. This model of plate reader has a reported photometric precision of less than ±0.003 OD (Optical density) and stray light of less than 0.05% at 340 nm, according to the manufacturer's website. Serial dilutions of the stock Rf solution were used to create a standard curve for the calculation of stromal Rf concentration, which was then used to convert the measured intensity values of each sample. Standard curve calculations were performed each time the experiment was repeated to avoid any day-to-day errors, and all data were compared to the standard curve taken on the day the data were taken. R^2 values for the linear regressions ranged from 0.906 to 0.999.

In contrast to other methods of measuring stromal riboflavin concentration such as fluorescence imaging, slit-lamp examination, or high-performance liquid chromatography (HPLC), this method was able to provide a real-time measurement without relying on fluorescence measurements taken within the corneal stromal and produced comparative measurements

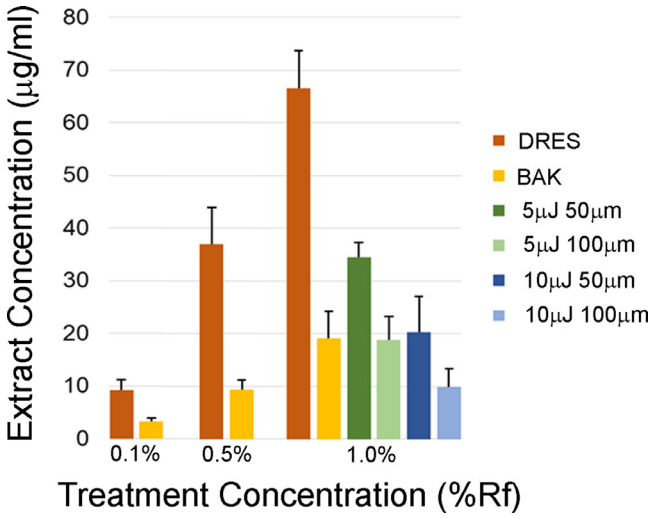

Figure 3. Stromal Rf concentration. Stromal Rf concentration within the transepithelial groups was highest using 5-μJ, 50-μm spaced channels, 34.4 ± 3.0 μg/mL. This was not significantly different from DRES using 0.5% Rf required for NLO CXL. Channels created with 10-μJ pulse energy but allowed a significantly lower concentration of Rf to diffuse into the stroma.

to other techniques.[27,36,38,39] Mastropasqua et al.[27] reported using iontophoresis and additives to achieve stromal riboflavin concentrations that were 21% and 44%, respectively, compared to the standard Dresden protocol using HPLC. Additionally, Cui et al.[36] reported a peak stromal concentration of 50% standard measured via two-photon fluorescence after transepithelial BAK treatment. Preliminary experiments had determined that riboflavin fluorescence measured within the stroma were quenched with depth, making measurements through the entire depth unreliable. Furthermore, the serial dilution of riboflavin for the production of a standard curve ensured that the measurements could be kept within a linear range of fluorescence. It also prevented the movement of riboflavin out of the tissue during measurements from becoming a complication.

Results

Riboflavin Extract Concentration

A comparison of the concentration values of the different treatment groups is shown in Figure 3. The values of Rf measured within the stroma of DRES controls using 0.1%, 0.5%, or 1.0% Rf eye drops were 9.2 ± 1.9, 37.0 ± 6.9, and 66.6 ± 7.1 μg/mL, respectively. BAK treatment using 0.1%, 0.5%, and 1.0% Rf achieved stromal concentrations of 3.2 ± 0.7, 9.3 ± 1.8, and 19.2 ± 5.0 μg/mL or approximately 25% to 35% of

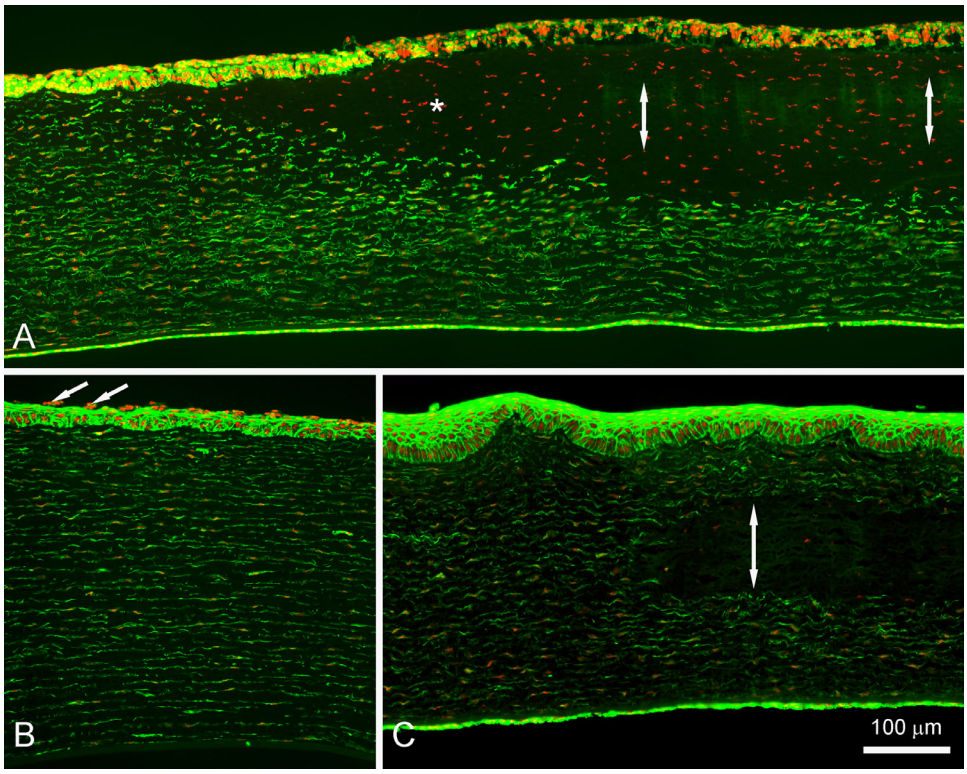

Figure 4. Cellular staining after BAK and microchannel Rf administration. (A) Cross section of a cornea treated with 0.5% Rf eye drops containing 0.01% BAK, followed by transepithelial NLO CXL. The cornea was rinsed and cultured for 24 hours and then stained with phalloidin (*green*; 1:100) and propidium iodide (*red*; 0.01 mg/mL). (B, C) Cross sections of corneas treated with 5-μJ pulse energy microchannel Rf delivery alone (B) and followed by NLO CXL (C), cultured for 3 hours (B) or 24 hours (C), and stained with phalloidin and propidium iodide. Regions of CXL and examples of visible microchannels are marked with *arrows*, and cellular death outside this region is marked with an *asterisk* (A).

the level achieved when the epithelium was removed. On average, FS laser-based micromachining using 5 μJ/pulse at 50-μm spacing (400 channel/mm^2) achieved a stromal Rf concentration of 34.4 ± 3.0 μg/mL or nearly twofold higher than that achieved with BAK and 50% of the level achieved when the epithelium was completely removed. Furthermore, this concentration was not significantly different from that achieved with epithelial removal and treatment with 0.5% Rf, the same concentration used for previously reported NLO CXL using amplified pulses. Wider channel spacing of 100 μm or 100 channels/mm^2 resulted in only a 50% drop in stromal Rf concentration, equal to that achieved with 0.01% BAK. Increasing the pulse energy to 10 μJ resulted in stromal concentrations of 20.2 ± 6.8 and 9.9 ± 3.4 μg/mL for 50-μm and 100-μm channel spacing, respectively, only 50% of the 5-μJ/pulse counterparts.

Cellular Staining

Staining of 24-hour organ-cultured samples posttransepithelial NLO CXL using either BAK with 0.5% Rf or microchannels to detect intact epithelial cells and keratocytes is shown in Figure 4. As shown in Figure 4A, the cornea treated with BAK and 0.5% Rf followed by NLO CXL showed extensive damage to the overlying epithelium and stromal keratocytes (asterisk) that were outside the area of corneal CXL (double-headed arrows). It should be noted that the acellular zone extended beyond the region of CXL in all directions in the cornea treated with BAK and 0.5% Rf. Additionally, the corneal epithelium appears highly damaged and was only loosely attached to samples after culture. The cornea shown in Figure 4B was treated with microchannels without BAK, followed by 3 hours of organ culture. In this case, there is no obvious acellular region or visible damage to the epithelium following micromachining. Arrows indicate examples of spots where channels were drilled. After only 3 hours of culture, they are easily identified by the nuclei on the surface of the epithelium. The image in Figure 4C shows a cornea 24 hours post-FS laser-based micromachining followed by transepithelial NLO CXL. In contrast to the cornea treated with BAK, the only region of cellular damage corresponded to the region of NLO CXL (double arrowheads). Both the surrounding stromal keratocytes and epithelial

cells appear undamaged by both the method of Rf application and the NLO CXL procedure.

Discussion

To date, no method of transepithelial corneal collagen crosslinking has been wholly satisfactory. Treatment results are not as effective as traditional UVA CXL, and the epithelial layer is often damaged in the process, leading to little improvement in the delay of recovery, pain, and risk of infection.[14,23,30] Because the epithelial layer acts as a natural barrier to both Rf penetration and UVA exposure, it was thought that NLO CXL would be better suited than UVA CXL for a transepithelial treatment. The two-photon paradigm utilized in NLO CXL allows for highly controlled CXL at any depth within the cornea and any location without affecting regions anterior or posterior to the focus of the beam, allowing for personalized CXL dependent on the patients topography.[5,6] As long as a sufficient concentration of Rf penetrates into the stroma, the epithelium can be left untouched by the CXL portion of the procedure.

This study establishes for the first time that micromachining of the corneal epithelium to form microchannels permits diffusion of Rf into the stroma that achieves 50% or greater of the levels obtained following epithelial removal and twofold higher than that achieved by the addition of 0.01% BAK. Our findings show that epithelial microchannels not only allowed for higher stromal Rf concentrations but also produced markedly less cellular damage to the epithelium and stroma compared to the enhanced penetration using BAK. Rf eye drops containing the additive BAK did achieve stromal concentrations equivalent to the traditional epithelium off Dresden protocol (DRES) when Rf concentration was increased beyond 0.5%, but this increase is still considered unsatisfactory for several reasons. First, the achieved concentration was only 19.2 ± 5.0 μg/mL if 1.0% Rf eye drops were used, which is not sufficient to perform NLO CXL. Second, cellular staining of samples cultured for 24 hours postprocedure revealed extensive epithelial and stromal cellular damage beyond the area of CXL, which is likely caused by the toxicity of the BAK alone. This amount of cellular damage to the epithelium would likely lead to defocusing of the laser beam as it is scanned across the corneal surface, reducing the efficiency of two-photon excitation of Rf. By contrast, the microchannels produced in this study showed no evident stromal cellular damage after culture. Furthermore, microchannels were only detectible by the presence of nuclei on the surface of the epithelium in samples cultured for 3 hours and at distances equivalent to the channel spacing. These regions were no longer visible in samples cultured for 24 hours, suggesting that channels heal quickly after microengineering. Clearly, further work is needed to establish the long-term safety of this procedure and identify the full response of the corneal epithelium and epithelial nerves.

It should also be noted that, contrary to what was expected, channels created using higher-energy, 10-μJ pulses resulted in consistently and significantly lower stromal concentrations compared to those created using lower-energy, 5-μJ pulses. It was thought that larger holes, created using larger energies, would allow for more Rf to enter the stroma. It is possible that the higher energy may have hindered the diffusion of Rf by generating greater epithelial damage. Again, additional study is needed to identify the effects of epithelial micromachining using different pulse energies and channel separation profiles on intrastromal delivery of Rf or other photoinitiators.

One limitation of this study has to do with the method of Rf extraction used during experiments. It is likely that the 8-mm corneal buttons taken from BAK and DRES samples resulted in Rf concentrations that were unfairly high compared to microchannel samples, since epithelial microengineering was only performed within the central 6 mm. During Rf imbibition, it was noticed that stromal Rf appeared predominantly within the 6-mm central region under the microengineered corneal epithelium. Since BAK and DRES treatments covered the entire 8-mm button, microchannel treatments had a 2-mm ring of untreated cornea around each sample, potentially diluting the final results. Even with this considered, microchannels were able to produce higher concentrations of Rf than any other treatment. Therefore, it is possible that a lower initial concentration of Rf solution or a higher channel spacing and lower density might be used to produce the same results.

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