Noninvasive Intratissue Refractive Index Shaping (IRIS) of the Cornea with Blue Femtosecond Laser Light

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PURPOSE. To test the feasibility of intratissue refractive index shaping (IRIS) in living corneas by using 400-nm femtosecond (fs) laser pulses (blue-IRIS). To test the hypothesis that the intrinsic two-photon absorption of the cornea allows blue-IRIS to be performed with greater efficacy than when using 800-nm femtosecond laser pulses.

METHODS. Fresh cat corneas were obtained postmortem and cut into six wedges. Blue laser pulses at 400 nm, with 100-fs pulse duration at 80 MHz were used to micromachine phase gratings into each corneal wedge at scanning speeds from 1 to 15 mm/s. Grating lines were 1 μm wide, 5 μm apart, and 150 μm below the anterior corneal surface. Refractive index (RI) changes in micromachined regions were measured immediately by recording the diffraction efficiency of inscribed gratings. Six hours later, the corneas were processed for histology, and TUNEL staining was performed to assess whether blue-IRIS causes cell death.

RESULTS. Scanning at 1 and 2 mm/s caused overt corneal damage in the form of bubbles and burns. At faster scanning speeds (5, 10, and 15 mm/s), phase gratings were created in the corneal stroma, which were shown to be pure RI changes ranging from 0.037 to 0.021 in magnitude. The magnitude of RI change was inversely related to scanning speed. TUNEL staining showed cell death only around bubbles and burns.

CONCLUSIONS. Blue-IRIS can be performed safely and effectively in living cornea. Compared with near-infrared laser pulses, blue-IRIS enhances both achievable RI change and scanning speed without the need to dope the tissue with two-photon sensitizers, increasing the clinical applicability of this technique. (*Invest Ophthalmol Vis Sci.* 2011;52:8148–8155) DOI:10.1167/iov.11-7323

The use of lasers in corneal refractive surgery benefits from the transparency of ocular media. As early as 1981, 193-nm laser pulses emitted from argon-fluoride lasers were used for photoablation of the corneal epithelium.^{1,2} Shortly thereafter, Trokel et al.³ demonstrated the feasibility of excimer laser

surgery in the cornea with precise control of incision depth. This led to the rapid development of corneal refractive surgeries, including photorefractive keratectomy (PRK)^{4,5} and laser in situ keratomileusis (LASIK).⁶ Conventional excimer laser refractive surgery works in the far-ultraviolet range, based on the fact that the cornea natively absorbs ultraviolet light. In this modality, corneal tissue is photoablated via one-photon absorption, altering the curvature and thickness of the tissue and thus its optical power.^{7–10} In PRK, excimer laser ablation can reshape the cornea; however, this process causes stromal haze and pain, as well as major epithelial disruption. In LASIK, a corneal flap is first created, followed by photoablation of the exposed corneal bed. This procedure reduces epithelial disruption and stromal wound healing, which in turn, decreases complications after surgery.^{6,10}

The emergence of near infrared (NIR) femtosecond laser technology has provided a powerful tool for less invasive and highly localized corneal surgeries and is now successfully used in corneal flap cutting and intrastromal vision correction.¹¹ Femtosecond laser pulses significantly decrease the threshold for laser-induced optical breakdown and minimize collateral damage and the creation of large bubbles. NIR laser pulses can pass through transparent corneal tissue without significant one-photon absorption. They affect only tissue at the focus of these laser pulses, creating plasma, shock waves, and small bubbles.^{11–13} NIR femtosecond lasers are now clinically used for corneal flap cutting.^{11,14–24} Although flaps created with a femtosecond laser lead to better visual outcomes than flaps created with mechanical microkeratomes,^{25,26} femtosecond flap cutting is more expensive, and there are still debates over which method is better.^{27–29}

In 2008, we developed a new approach for changing corneal refractive properties—intratissue refractive index shaping (IRIS).³⁰ Instead of ablating tissue via photodisruption, a 27-fs NIR laser at 800 nm was used to locally modify the refractive index (RI) of ocular tissues with low scattering loss. The RI changes achieved ranged between 0.005 and 0.01 in fixed, postmortem cornea and 0.015 and 0.021 in fixed lenses. Importantly, changes were retained after 1 month of storage in an aqueous solution. However, the scanning speed needed to induce these RI changes was very slow (0.7 $\mu\text{m}/\text{s}$), which limited the use of this technique in clinical applications. We recently showed that IRIS is significantly more effective in living corneal tissue in terms of both achievable RI changes and scanning speeds, if the cornea is first doped with sodium fluorescein (Na-Fl), which enhances its two-photon absorption properties.³¹ The RI changes attainable in doped, living cornea ranged from 0.004 to 0.020, with the largest RI change being 0.020 at a scanning speed of 0.5 mm/s in tissue doped with 1% Na-Fl. The RI change was inversely related to scanning speed and monotonically increased with Na-Fl doping concentration.³¹ However, although IRIS is significantly enhanced by Na-Fl doping of the cornea, the corneal epithelium acts as a barrier^{32,33} and needs to be scraped off to allow Na-Fl to penetrate into the corneal stroma. Epithelial removal creates a

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Supported by an unrestricted grant to the University of Rochester's Department of Ophthalmology from Research to Prevent Blindness Inc., Grant R01 EY015836 (KRH) and Core Grant 08P0EY01319F (Center for Visual Science) from the National Institutes of Health, and grants from Bausch & Lomb, Inc., and the University of Rochester's Center for Electronic Imaging Systems, an NYSTAR-designated Center for Advanced Technology.

Submitted for publication February 3, 2011; revised May 18 and August 11, 2011; accepted September 11, 2011.

Disclosure: L. Xu, Bausch & Lomb, Inc. (F); W.H. Knox, Bausch & Lomb, Inc. (F); M. DeMagistris, Bausch & Lomb, Inc. (F); N. Wang, Bausch & Lomb, Inc. (F); K.R. Huxlin, Bausch & Lomb, Inc. (F)

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surface wound and a wound-healing response in the cornea^{33–37} that disrupts optical quality and creates a significant complication both for live animal studies and human applications.

Presently, we describe a modification of IRIS, which achieves even better optical results in native, live corneas, without the need for epithelial removal or enhancement of two-photon absorption. We have termed this process blue intratissue refractive index shaping (blue-IRIS), as it uses blue femtosecond laser pulses at 400 nm rather than NIR laser pulses at 800 nm (NIR-IRIS). To create useful changes of optical power in the eye, it is first necessary to determine the magnitude of the localized RI changes, and second, to design and write a refractive structure that can generate such refractive changes. The present article deals with the first of these issues. We are currently writing devices of various refractive powers in various materials, and the results of these experiments will be published in a future article.

MATERIALS AND METHODS

Extraction and Preparation of Corneas

Two enucleated feline eye globes (Liberty Research Inc., Waverly, NY) were maintained in storage medium (Optisol-GS; Bausch & Lomb, Inc., Rochester, NY) at 4°C overnight after extraction, to keep the corneas alive until micromachining was done the next day.^{38,39} All animal procedures were conducted in accordance with the guidelines from the University of Rochester Committee on Animal Research, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the NIH Guide for the Care and Use of Laboratory Animals. Corneas were dissected from the enucleated globes, and each was then cut into six wedges, as previously described.³¹ Each of the wedges was a pie sector that subtended approximately 60°, with a thickness of approximately 800 μm (due to slight corneal swelling after cutting) and a radius of approximately 8 mm. The corneal wedges were then placed on a glass slide (25 $\times$ 75 $\times$ 1 mm; Fisher Scientific, Inc., Pittsburgh, PA) and coverslipped (25 $\times$ 25 $\times$ 1, Fisher Scientific, Inc.) with storage medium (Optisol-GS; Bausch & Lomb). The corneal epithelium was facing up and the endothelium was facing down, so that laser pulses irradiated the corneal tissue from the anterior surface, as they would in live animal eyes. The corneal tissue was also bathed in the storage medium in a sandwich structure, to keep the tissue alive and hydrated, since dehydration would affect corneal transparency and RI.⁴⁰

Blue-IRIS in Undoped, Living Cornea

After proper sample preparation, blue-IRIS was performed using blue femtosecond laser pulses at 400 nm. The laser source was a mode-locked Ti:Sapphire laser (Mai Tai HP; Spectra Physics, Mountain View, CA) with a 100-fs pulse width and a 80-MHz repetition rate operating at 800 nm. Second-harmonic generation (SHG) pulses at 400 nm were created with a frequency doubler kit (model 3980; Spectra Physics). The blue femtosecond laser pulses were then attenuated by a metallic variable attenuator and focused through a water-immersion objective (20 $\times$, NA 1.0; W Plan-Apochromat; Carl Zeiss MicroImaging GmbH, Dublin, CA) into the stromal layer of each cornea wedge at a depth of $\sim$ 150 μm below the corneal epithelial surface (Fig. 1A). The average output power from the doubler was approximately 500 mW. It was attenuated so that the power at the focus of the objective was 80 mW. The corneal wedges were then mounted on a scanning platform consisting of two piezo stages (M-663; Physik Instruments, Auburn, MA) set on top of a three-dimensional scanning system consisting of three linear servo stages (VP-25XA; Newport, Mountain View, CA).

The micromachining process was monitored with a CCD camera. The corneal wedges were micromachined with a fixed pulse energy ($\sim$ 1 nJ) at different scanning speeds. One of the corneal

FIGURE 1. Blue-IRIS experimental setup. (A) Blue femtosecond laser pulses were focused through a 1.0-NA water-immersion objective, into living corneal tissue sandwiched between a glass slide and a no. 1 glass coverslip, and bathed in storage medium (Optisol-GS; Bausch & Lomb). The whole slide assembly was then mounted on a 3D scanning platform. (B) Blue-IRIS gratings inside a corneal wedge. Five sets of grating lines were micromachined into most pieces. The two outermost lines of each grating line set were micromachined at 0.5 mm/s to create reference damage marks, and the central lines were drawn at the indicated speeds.

wedges from each cornea was used for testing the effects of multiple scanning speeds from 1 to 15 mm/s for the purpose of performing TUNEL-staining studies. Five grating line sets of different scanning speeds, at 1, 2, 5, 10, and 15 mm/s, were created in the stromal layer of the corneal tissue (Fig. 1B). Each grating line set consisted of 20 lines with inter-line spacing of 5 μm . The two outermost lines of each line set were micromachined at a low speed of 0.5 mm/s to intentionally cause visible corneal damage. Because micromachined lines that consist of pure RI change are not visible under traditional microscopic imaging conditions, having visible damage lines flanking each line set allowed us to easily locate the boundaries of the IRIS lines sets for subsequent phase contrast imaging and TUNEL staining.

Measuring RI Change

Eight corneal wedges were used to measure the RI change induced at each scanning speed. Each measurement was repeated three times. Finally, three wedges were also used to write larger gratings (1 $\times$ 3-mm area) with 5- μm inter-line spacing at each of the chosen scanning speeds, under the same experimental conditions. These larger gratings were used to measure the diffraction efficiencies of the micromachined pattern using a custom-built scatterometer with a low-power He-Ne laser source. A digital camera captured the diffraction images,

FIGURE 2. Phase-contrast micrographs of blue-IRIS grating lines in excised cornea. The central lines of each grating set were micromachined at scanning speeds of 1, 2, 5, 10, and 15 mm/s. The two outermost lines of each grating set (arrows) were micromachined at 0.5 mm/s to cause damage. For scanning speeds above 5 mm/s (C, D, E), clear, undamaged IRIS lines predominated within the line set, whereas lower scanning speeds (A, B) caused plasma luminescence and blebs within the line set.

and a power meter measured the intensity distribution of the diffraction patterns. The ratio of the first- to zero-order intensity was used to extract the magnitude of RI change along the grating lines, as described previously.⁴¹

TUNEL Assay for Dying Cells

To assess whether blue-IRIS was toxic to living corneal pieces, we performed TUNEL staining. TUNEL labels dying cells, including apoptotic cells, as little as 4 hours after corneal surgeries such as LASIK and PRK.^{42,43} To provide a positive control for this experiment, the entire corneoscleral rim of each live corneal piece was manually crushed with forceps immediately before IRIS was performed. After blue-IRIS and RI/scatterometry, the tissue was replaced into storage medium (Optisol-GS; Bausch & Lomb) at 4°C for 6 hours. After this time, corneal pieces were drop fixed in 1% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4) for 10 minutes and then transferred to 30% sucrose in 0.1 M PBS at 4°C for 1 day. Each corneal piece was then mounted in OCT compound (Tissue Tek, Sakura Finetek, Torrance, CA) and frozen, and serial 20-μm-thick cross-sections were cut on a cryostat (2800 Frigocut E; Leica, Bannockburn, IL), mounted on microscope slides, and stored at -20°C until ready to stain.

For TUNEL staining, corneal sections were air dried and rinsed in 0.1 M PBS. The tissue was then processed according to the directions in the cell-viability kit (S7165 ApopTag *In Situ* Apoptosis Detection Kit; Chemicon International, Millipore Inc., Temecula, CA). Briefly, mounted sections were postfixated in precooled ethanol:acetic acid 2:1 for 5 minutes at -20°C and drained. After rinsing, equilibration buffer was applied to the sections and briefly incubated at room temperature. The sections were then incubated with terminal deoxynucleotidyl transferase enzyme for 1 hour in a humidified chamber. The reaction was halted by incubating the sections in stop/wash buffer for 10 minutes followed by another wash in PBS. Warmed anti-digoxigenin conjugate (rhodamine-labeled) was applied to the sections and incubated in a dark, humidified chamber for 30 minutes. After a final wash in PBS, the stained sections were coverslipped with mounting medium containing DAPI (Vectashield; Vector Laboratories, Burlingame, CA). The stained sections were imaged by fluorescence microscopy (AX70 Microscope; Olympus, Lake Success, NY), and the photomicrographs were obtained with a high-resolution digital camera (Microfire; Olympus) interfaced with image-acquisition software (ImagePro software; MediaCybernetics, Silver Spring, MD).

RESULTS

Blue-IRIS Gratings in Undoped, Living Corneal Tissue: Effect of Scanning Speed

At a given pulse energy, scanning speed determines optical outcome. At the very low scanning speed of 0.5 mm/s, which was used to create flanking lines for each line grating set, tissue destruction and bubbles were created (Fig. 2 arrows). At a scanning speed of 1 mm/s under our experimental conditions, we observed strong plasma luminescence emission and major photodisruption in the cornea (Fig. 2A). Large blebs formed along the laser-scanned lines, covering almost the entire band of grating lines and rendering each individual line almost invisible. At a scanning speed of 2 mm/s, there was still damage to the corneal tissue, with plasma emission and strong scattering captured by the CCD camera (Fig. 2B). Smaller blebs were formed in the grating area, overlaying several lines. Individual lines became more visible but still not well localized, which is

FIGURE 3. Using diffraction patterns to measure RI change attained with blue-IRIS. (A) Diffraction image of a blue-IRIS grating micromachined at 5 mm/s in a piece of live cornea. (B) Plot of first-order diffraction efficiency versus corresponding RI change. For the grating written at 5 mm/s, first-order diffraction efficiency was measured relative to the 0th order. The corresponding RI change is plotted in the figure with horizontal error bar (light gray).

in contrast to the three-dimensional structuring capability of femtosecond laser micromachining. Increasing scanning speed further decreased the accumulated heat in the focal volume and thus the photodisruption effect. At scanning speeds from 5 to 15 mm/s, there was no damage to the corneal tissue and pure RI changes were obtained (Figs. 2C-E). The faster the scanning speed, the smaller the magnitude of RI change obtained.

Blue-IRIS Gratings in Undoped, Living Corneal Tissue: Magnitude of RI Changes Attained

Immediately after femtosecond laser micromachining, the corneal pieces with the larger 1 × 3-mm gratings written inside the stroma were placed into the diffraction setup and the first-order diffraction efficiency was measured. This was then used to calculate the corresponding RI change associated with the grating lines.⁴¹ The higher the diffraction efficiency, the larger the RI change. At the scanning speeds that yielded pure RI change, the highest diffraction efficiency found corresponded to a scanning speed of 5 mm/s (Fig. 3). The RI changes obtained were 0.037 ± 0.0005, 0.030 ± 0.001, and 0.021 ± 0.001 at scanning speeds of 5, 10, and 15 mm/s, respectively (Table 1).

TUNEL Staining of Undoped Corneas after Blue-IRIS

The crushed rim of each corneal piece was thinned, with clearly disrupted epithelium and a large incidence of TUNEL-

TABLE 1. RI Changes Induced by Blue-IRIS in Corneas

Na-Fl Conc. (%)	NIR-IRIS in Living Cornea ³⁰					Blue-IRIS in Undoped Living Cornea					NIR-IRIS in Undoped Fixed Cornea ²⁹	
	Scanning Speed (mm/s)					Scanning Speed (mm/s)					Scanning Speed (mm/s)	
	0.1	0.5	1	2	5	1	2	5	10	15		
0.0	0.005 (0.0001)	—	No detectable change	—	—	Damage	Damage	0.037 (0.0005)	0.030 (0.001)	0.021 (0.001)	0.0007	0.008 (0.002)
0.25	Damage	0.015 (0.004)	0.014 (0.001)	0.008 (0.0001)	0.004 (0.001)							
1.0	Damage	0.020 (0.004)	0.019 (0.001)	0.012 (0.0001)	0.008 (0.001)							
1.5	Damage	Damage	Damage	0.017 (0.001)	0.013 (0.001)							
2.5	Damage	Damage	Damage	Damage	0.015 (0.0001)							

Corresponding values previously obtained by NIR-IRIS in both fixed corneas²⁹ and doped living corneas³⁰ are shown for comparison. Values are the mean (SD).

positive cells in both the epithelium and stroma (red and pink cells in Fig. 4A). The IRIS line sets inscribed at the lowest speed (1 mm/s) exhibited positive TUNEL staining across the entire area of damage and bubbles (Fig. 4B, set A; see also Fig. 2A). For all other IRIS line sets (2, 5, 10, and 15 mm/s, corresponding to line sets B-E in Fig. 2), TUNEL-positive cells were seen in the flanking damage lines rather than among lines of pure RI change (Figs. 4B, 4C). The only exception was a single TUNEL-positive cell in the middle of IRIS line set C, which generated the largest RI change. Having the IRIS line sets in the same corneal sections as the crush sites was an excellent internal control for the efficacy of the TUNEL assay within the experimental time frame in which IRIS was performed and allowed us to compare cell death in the different line sets. It should be noted, however, that the flanking lines inscribed in each line set at the slower scanning speed of 0.5 mm/s, causing bubbles of tissue destruction, only resulted in the appearance of TUNEL-positive cells when lines physically crossed these cells. If, as in line set E in Figure 4C, the flanking (damage) lines failed to cross keratocytes in their path, no positive TUNEL staining was observed. Nor were TUNEL-positive cells seen above or below the plane in which individual IRIS line sets were written, $\sim 150 \mu\text{m}$ below the epithelial surface. Only at the crush sites, were epithelial TUNEL-positive cells found (Fig. 4A).

DISCUSSION

The present study describes a new approach for performing IRIS less invasively in living cornea using blue femtosecond laser pulses at 400 nm, which we termed blue-IRIS. Blue-IRIS was more effective than NIR-IRIS in doped corneal tissue³⁰⁻³¹ both in terms of scanning speed and RI change

achieved. A significant advantage of blue-IRIS over NIR-IRIS is that two-photon sensitization is not needed to attain high efficacy.

Effectiveness of Blue-IRIS Compared with NIR-IRIS

In prior studies, NIR-IRIS was performed in both paraformaldehyde-fixed cat corneal tissue³⁰ and living cat corneal tissue (both undoped and doped with Na-Fl).³¹ For NIR-IRIS in lightly fixed cornea, the attainable RI change ranged from 0.005 ± 0.001 to 0.01 ± 0.001 , averaging 0.008 ± 0.002 ³⁰ (Table 1). These RI changes were actually not small compared with those in previous studies of femtosecond laser micromachining in optical materials such as glass, where typical RI change ranged between 1×10^{-2} and 1×10^{-4} .⁴⁴⁻⁴⁶ In cornea, which accounts for the largest portion of the total refractive power of the eye, a RI change of ~ 0.01 could have a significant refractive impact if the proper refractive structure is inscribed. However, IRIS in fixed cornea had to be performed at an extremely slow scanning speed of $\sim 0.7 \mu\text{m/s}$ ³⁰ (Table 1), which was impractical for in vivo applications. Consistent with the notion that IRIS should be more effective in fresh rather than fixed tissues, NIR-IRIS induced an average RI change of 0.005 at a scanning speed of $100 \mu\text{m/s}$ in living cornea.³¹ Doping fresh, living cornea with Na-Fl, an enhancer of two-photon absorption, generated larger RI changes of up to ~ 0.02 at larger scanning speeds³¹ (Table 1). Nevertheless, the maximum RI change ever achieved in doped, live corneas was 0.02, and the maximum scanning speed at which this could be attained was 0.5 to 1 mm/s. However, the cornea's most significant absorption is in the ultraviolet range.⁴⁷ Since

FIGURE 4. TUNEL staining after the blue-IRIS procedure. (A) Micrograph of a TUNEL-stained section of a corneal piece schematically shown in Figure 1B, with the forceps crush site at the corneal rim. Red: TUNEL-positive staining; blue: nuclear DAPI counterstain. Pink cells are both TUNEL- and DAPI-positive. Note the tissue disruption induced by the crush and the large number of TUNEL-positive cells (arrows) in the epithelium and stroma. Stromal cells mostly possessed the morphology of keratocytes. (B) Adjacent region of the same corneal slice, showing strong, diffuse, TUNEL-positive staining of IRIS line set A, created at 1 mm/s. This line set caused bubbles of damage in the micromachined region (see Fig. 2A). Note, however, the lack of TUNEL-positive staining in areas of stroma and epithelium directly above and below the micromachined (damaged) line set. Cross section of line set B, created at 2 mm/s with flanking (damage) lines at 0.5 mm/s shows TUNEL-positive cells only in regions of stroma crossed by the flanking burn lines, not the intervening IRIS lines. (C) Distal region of the same corneal

slice shown in (A) and (B), illustrating TUNEL staining for line sets (C-E), inscribed at 5, 10, and 15 mm/s, with flanking (damage) lines written at 0.5 mm/s. Once again, a general pattern is observed, whereby TUNEL-positive cells are observed wherever flanking (damage) lines intersect stromal keratocytes. Only one TUNEL-positive cell is seen in the middle of a pure RI change line set (set C, 5 mm/s). All other line sets are free of such cells, suggesting that blue-IRIS, when it induces RI changes in a living cornea, generally does not cause a significant amount of cell death.

femtosecond lasers operating at 800 nm are widely used and readily available, and since frequency doubling using a nonlinear crystal is a standard nonlinear optical procedure today, it is conceivable that femtosecond laser pulses at 400 nm could be used to induce stronger two-photon absorption around 200 nm in native cornea. The present results confirmed this hypothesis, as blue-IRIS in undoped cornea generated the largest RI change ever achieved in this tissue (0.037) at a scanning speed of 5 mm/s. In contrast, the largest RI change attained at a scanning speed of 5 mm/s in corneas doped with 2.5% Na-Fl was only 0.015. However, consistent with previous results using NIR-IRIS, scanning speed for blue-IRIS was also inversely related to the RI change achieved. At scanning speeds up to 15 mm/s, the lowest RI changes attained were ~ 0.021 , about the same as the maximum RI change attained in prior studies, but with a 30 $\times$ increase in scanning speed. We conclude that the enhanced effectiveness of IRIS using blue femtosecond laser pulses is probably owing to the stronger two-photon absorption of the cornea at 400 nm.

Our previous work on femtosecond laser micromachining in hydrogel polymers showed a strong wave-guiding effect along the RI change line, which could only be true when the RI change is positive.⁴⁸ RI changes in cornea appear to occur via the same principles as in hydrogel samples. Indeed, our preliminary results with electron microscopy of the RI change in the cornea showed a localized densification of the collagen fibrils in the corneal stroma (results not shown). The term "densification," as used presently, refers to increased density of the collagen matrix, caused primarily by decreased interfibrillar spacing. Other studies have shown that densification of collagen fibrils (as in dehydration) increases the cornea's RI.^{40,49,50} However, by varying the device design and laser scanning parameters, it will ultimately be possible to create refractive corrections in the corneal stroma with a net positive or negative dioptric change.

The Minimally Invasive Nature of Blue-IRIS

The high speed with which significant RI changes can be inscribed with blue-IRIS means that the time needed for writing a large pattern in the cornea is diminished. Another advantage of blue-IRIS for in vivo applications is that the tissue does not need to be doped with an enhancer of two-photon absorption, such as Na-Fl. Such doping normally requires epithelial removal, which temporarily destroys this layer's barrier function in the living eye.^{32,33} Since the epithelium renews itself continuously, it regrows over the denuded cornea, and its barrier function is restored within 1 to 1.5 days after epithelial wound closure.⁵¹ However, epithelial removal significantly and immediately decreases the optical quality of the cornea. In addition, it makes the cornea more susceptible to infection, persistent erosion and increased stromal haze due to transformation of stromal keratocytes into fibroblasts and myofibroblasts.^{37,43,52-54} Because blue-IRIS does not require a change in the cornea's native absorption range, as NIR-IRIS did, there is no need for doping or disruption of the epithelium in any way.

Toxicity of Blue-IRIS and Long-Term Applications

The present study has shown that blue-IRIS can locally alter the RI inside the corneal stroma with greater efficacy than previously achieved using NIR-IRIS. From an optical point of view, this is a great advance that could lead to the development of customized visual correction with exquisitely detailed 2D or 3D patterns inscribed noninvasively into the cornea. However, it is important to understand the short- and long-term biological responses to IRIS. In this study, we assessed the short-term

toxicity of blue-IRIS by using the TUNEL assay, an established method of detecting cell death since its first demonstration by Gavrieli et al.⁵⁵ After corneal surgery, dying TUNEL-positive cells are seen as early as 4 hours after surgery.⁴² To verify the validity of this time frame in the context of our experimental paradigm, we intentionally injured (via forceps crush) the periphery of two corneal pieces in which blue-IRIS was also performed. Within regions of crushed cornea, there were numerous TUNEL-positive cells in the epithelium and stroma, the latter representing dying keratocytes for the most part. In contrast, there was no substantial cell death (i.e., no TUNEL-positive cells), where blue-IRIS induced pure RI changes. The only TUNEL-positive cells observed in association with the IRIS line sets were due to the flanking damage lines, created at a slow scanning speed of 0.5 mm/s, or in line sets created at 1 and 2 mm/s—basically, anywhere there was damage to the corneal tissue, with plasma luminescence and formation of blebs. Note that a similar phenomenon is also seen during LASIK and femtosecond laser flap cutting.^{6,56} Thus, our present experiments confirmed that blue-IRIS did not cause substantial cell death when inducing RI changes in the corneal stroma, whereas corneal injury with forceps crush did. The only exception was one TUNEL-positive cell in the line set inscribed at 5 mm/s. A likely explanation for this is that there is a sharp transition when scanning speed goes from below to above 5 mm/s (where the unique TUNEL-positive cell was observed). At 5 mm/s, the amount of laser power deposited hovers just below the damage threshold of the tissue. It is conceivable that there is some unevenness in the lines and that at one location the laser energy went above the damage threshold, thus killing the cell in question. By slightly increasing the scanning speed, such TUNEL-positive cells can be avoided. Furthermore, even when damage and cell death were induced in the stroma, they occurred only when IRIS lines actually traversed a keratocyte. There was no detectable collateral damage and TUNEL-positive cells above or below the IRIS lines or between line sets. The epithelial layer immediately above stromal IRIS lines appeared similarly untouched, with no TUNEL-positive cells.

Thus, to the best of our knowledge, there are two potential advantages of blue-IRIS over LASIK and PRK. One is that IRIS does not require a flap to be cut or the epithelium to be removed. Second, blue-IRIS appears capable of inducing changes in refraction without causing tissue damage and thus without inducing a wound-healing reaction in the cornea. This is a distinct advantage of blue-IRIS over all current refractive surgical procedures. Of course, the possibility remains that there could be a delayed biological reaction to blue-IRIS—either in the stroma or the epithelium. To ascertain the long-term effects, it will be necessary to perform blue-IRIS in situ in the cornea of living animals and then monitor them for several months. These studies are currently under way and will be reported in the future. Finally, another important consideration is that the creation of clinically useful refractive correction within the range of ± 2 to 3 D will require writing of more dense and complex structures than we have written in the present study. We are currently experimenting with such structures in vitro and in vivo and will report the results in future publications.

Acknowledgments

The authors thank Tracy Bubel for excellent technical assistance in performing the histology, Yuhong Yao for great help in diffraction experiments for the study, and Zeyu Zhao for the custom-built scatterometer setup.

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