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Intra-tissue Refractive Index Shaping (IRIS) of the cornea and lens using a low-pulse-energy femtosecond laser oscillator

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Abstract

Purpose—To assess the optical effect of high-repetition-rate, low energy femtosecond laser pulses on lightly-fixed corneas and lenses.

Methods—Eight corneas and eight lenses were extracted post-mortem from normal, adult cats. They were lightly fixed and stored in a solution that minimized swelling and opacification. An 800nm Ti:Sapphire femtosecond laser oscillator with a 27fs pulse duration and 93MHz repetition rate was used to inscribe gratings consisting of 20-40 lines, each 1μm wide, 100μm long and 5μm apart, 100μm below the tissue surface. Refractive index changes in the micromachined regions were calculated immediately and after one month of storage by measuring the intensity distribution of diffracted light when the gratings were irradiated with a 632.8nm He-Ne laser.

Results—Periodic gratings were created into the stromal layer of the corneas and the cortex of the lenses by adjusting the laser pulse energy until visible plasma luminescence and bubbles were no longer generated. The gratings had low scattering loss and could only be visualized using phase microscopy. Refractive index changes measured 0.005 ± 0.001 to 0.01 ± 0.001 in corneal tissue and 0.015 ± 0.001 to 0.021 ± 0.001 in the lenses. The gratings and refractive index changes were preserved after storing the micromachined corneas and lenses for one month.

Conclusions—These pilot experiments demonstrate a novel application of low-pulse-energy, MHz femtosecond lasers in modifying the refractive index of transparent ocular tissues without apparent tissue destruction. Although it remains to be verified in living tissues, the stability of this effect suggests that the observed modifications are due to long-term molecular and/or structural changes.

Keywords

laser refractive surgery; femtosecond laser; feline cornea; feline lens

Introduction

Conventional ultraviolet nanosecond excimer lasers have been successfully used for corneal refractive surgery, including photorefractive keratectomy (PRK), laser-assisted in situ keratomileusis (LASIK) and laser sub-epithelial keratomileusis (LASEK). By ablating corneal

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tissue through direct, one-photon absorption of ultraviolet light, these lasers alter the curvature, thickness and ultimately, the optical power of the cornea^{1, 2}.

The rapid development of femtosecond laser technology has provided an additional tool for corneal refractive surgery. In contrast to the photo-ablative ultraviolet lasers, femtosecond laser pulses in the near infrared can pass through transparent corneal tissue without significant one-photon absorption. Only when pulses are focused inside the cornea, is the intensity of the beam sufficient to cause nonlinear, typically multi-photon absorption, and a range of modifications to the tissue. Because the absorption is strongly nonlinear, the laser-affected region tends to be highly localized, leaving the surrounding region unaffected, or minimally affected³⁻⁵. This unique capability for three-dimensional, high-precision micromachining is the primary reason for the introduction of femtosecond lasers to refractive surgery, where their main application has been in corneal flap cutting⁶⁻¹². For this application, femtosecond laser pulses with a low repetition rate (Hz-kHz range) are used to induce photo-disruption and destructive, optical breakdown of corneal tissue. This is generally associated with high-density microplasma generation, bubble formation and shock-wave emission, often extending beyond the focal region. Compared with mechanical blade microkeratomes, femtosecond lasers are better able to define the depth of the cut, eliminating some flap-related complications and generally improving visual outcomes¹³⁻¹⁶. However, like any cut, the creation of a femtosecond laser flap causes biomechanical changes in the cornea, and since tissue is destroyed, a wound healing reaction ensues^{13, 17, 18}. This wound healing reaction includes regeneration of the protective corneal epithelium and the differentiation of the usually quiescent and supportive stromal keratocytes into reactive, inflammatory and contractile myofibroblasts^{19, 20}. Myofibroblasts appear responsible for most of the negative side-effects of laser refractive surgery, including haze or loss of corneal transparency, and unintended changes in corneal shape, which negatively impact the optical quality of the eye^{19, 21}. While increasingly popular, femtosecond laser flap cutting remains limited by its high cost, accessibility, as well as uncertainty about its long-term photochemical, mechanical and biological effects^{17, 22}. Recent reports have detailed negative side-effects of this technique, particularly in terms of tissue destruction, which, at some laser settings, appears stronger than following mechanical microkeratome cuts^{17, 22}.

The ability to alter corneal shape or optics without causing tissue destruction (and thus, a wound healing response), would significantly improve laser refractive correction by decreasing or eliminating the negative side effects that currently compromise optical outcomes and ocular health. To date, most clinical femtosecond lasers employ μJ or mJ pulses with a low-repetition-rate (Hz-kHz range) and spot diameters in the range of several μm ^{6, 7}. This contrasts with femtosecond laser parameters that have been established for other biomedical applications³. High-repetition-rate ($>1MHz$) femtosecond laser oscillators with pulse energies on the order of nJ have successfully been used to micromachine artificial media and perform cellular nanosurgery²³⁻²⁶. However, in clear ocular tissues such as the cornea, nJ femtosecond laser pulses with 170fs pulse duration and 80MHz repetition rate have only been reported to induce destructive optical breakdown²⁷. For such lasers, the diffusion time of non-linearly-absorbed laser energy is longer than the time interval between laser pulses. As a result, absorbed energy accumulates locally, with destructive consequences²⁸.

In the present study, we reduced femtosecond laser pulse energies below the optical breakdown threshold of lightly-fixed cat corneas and lenses to examine the optical consequences of the resulting tissue modifications. In both silicone and non-silicone-based hydrogels, this approach induces a significant change in refractive index without visible plasma luminescence or bubble formation²⁹. Changing the refractive index of the cornea or lens without tissue destruction, a phenomenon we termed Intra-tissue Refractive Index Shaping (IRIS), could represent a major advance in the field of laser refractive correction.

Materials and methods

Extraction and preparation of cat corneas and lenses

Eight corneas and eight lenses were extracted under surgical anesthesia from five normal, adult domestic short-hair cats (*felis cattus*). All animal procedures were conducted in accordance with the guidelines of 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. Feline corneas and lenses are similar to human corneas and lenses in terms of histological structure, molecular composition and optical properties³⁰. However, in contrast with the problems associated with obtaining post-mortem human eyes, using cat eyes allowed us to precisely control post-mortem extraction time and tissue processing parameters. To avoid decomposition and opacification prior to femtosecond laser micromachining, extracted feline tissues were immediately drop-fixed for 10 minutes (corneas) or one hour (lenses) in a solution consisting of 1% paraformaldehyde in 0.1M phosphate buffered saline (PBS), pH 7.4. Lenses were then cut into 500 μ m thick slices using a HM650V vibratome (Microm International), after which lens sections and whole corneas (also ~500 μ m thick) were immersed in a mixture of 30% ethylene glycol + 30% sucrose in 0.1M PBS, pH7.4 at 4°C. Storage in this solution minimized tissue swelling and loss of transparency. Small pieces of tissue, ~1cm² were then flattened onto a clear glass slide (1×3 inches, 1mm thick, Surgipath Medical Industries Inc., IL). In the case of corneal pieces, this was done with the epithelium facing up and the endothelium facing down. A glass coverslip (Corning No. 0211 Zinc Titania glass) was placed on the top of each piece, stabilizing it for the duration of the experiment. The ethylene glycol/sucrose storage solution was used as mounting medium to minimize dehydration of the cornea and lens tissue samples since these effects are known to alter the refractive index and transparency of both these tissues³¹⁻³³.

Femtosecond laser micromachining

Femtosecond laser micro-machining was conducted as previously described in hydrogels²⁹. The laser source was a Kerr-lens mode-locked Ti:Sapphire laser (K-M Labs). The laser oscillator generated pulses averaging 300mW, 27fs in duration, with a 93MHz repetition rate at 800nm wavelength. A continuously variable, metallic, neutral density filter was inserted into the optical path and used to adjust the incident laser power onto each cat cornea and lens piece. Pulses were focused 100 μ m below the tissue surface using a 60X, 0.70NA Olympus LUCPlanFLN microscope objective with an adjustable working distance of 1.5-2.2mm. Because the large amount of glass within the microscope objective induced significant chromatic dispersion into the femtosecond laser pulses, broadening the pulse durations, a standard extra-cavity-prism double-pass configuration was used to compensate for the dispersion and maintain the ultra-short pulse duration. By carefully adjusting this dispersion compensator, we obtained nearly transform-limited 27fs duration pulses at the focal point of the focusing objective, as measured by a collinear auto-correlator using 3rd order surface harmonic generation^{34, 35}. During IRIS, the slide containing the biological tissue samples was mounted on a 3D scanning platform consisting of a Physik Instrumente P-622.2CD XY scanning stage with 250 μ m travel range and 0.7nm close-loop resolution, and a Newport VP-25XA linear servo Z-axis scanning stage with 25mm travel range and 100nm resolution. An infrared CCD camera was used to monitor the micromachining process and the generation of visible plasma luminescence in real-time.

Experiments were conducted at room temperature (~25°C). It took about 40 minutes to create a 100 μ m×50 μ m grating and conduct the immediate post-micromachining measurements. Corneal trimming and mounting did not exceed 10 minutes in duration, and the corneal tissue was exposed to ambient air during the trimming process for at most 2 minutes. Since we saw no significant changes in cornea and lens transparency or thickness at the end of our

micromachining experiments, we concluded that our manipulations did not cause significant corneal or lenticular dehydration or swelling.

The first experimental step was to establish thresholds for the optical breakdown of lightly-fixed feline cornea and lens. The neutral density filter was first adjusted to minimize the focused incident laser power on the cornea and the lens below their breakdown thresholds^{4, 5}. Adjusting the neutral density filter then progressively increased the incident laser power. The breakdown threshold power was reached when visible plasma luminescence suddenly appeared and strong scattering light as well as laser-induced damage became visible (Figs. 1 and 2). With our 0.70NA long-working-distance objective, the measured breakdown thresholds for cat cornea and lens were ~55mW and 75mW average laser power respectively, which corresponds to pulse energies of 0.6nJ and 0.8nJ.

Once tissue breakdown thresholds were established, the focused laser power was lowered gradually by carefully adjusting the neutral density filter until lines could be micromachined without the induction of bubbles or burns. Average laser power settings at which this could be done were 30mW in the cornea and 45mW in the lens, corresponding to pulse energies of about 0.3nJ and 0.5nJ respectively. These values lay between those used for imaging and our measured breakdown thresholds. The gratings were micromachined in the horizontal plane within the stromal layer of each corneal piece and the cortex of each lens at a constant speed of 0.7μm/s for the cornea and 1μm/s for the lens. The spherical aberration at the laser focus induced by refractive index mismatch was compensated by adjusting the correction collar of the focusing microscope objective in order to achieve the smallest possible laser-affected region along the laser propagation direction²⁹.

Measurement of refractive index (RI) change

To assess whether the gratings generated in corneal and lens pieces were associated with a change in RI, the slides containing the tissue were first placed under an Olympus BX51 optical microscope where gratings were localized under differential interference contrast (DIC) imaging. A low-power 632.8 nm He-Ne laser was then used to irradiate the gratings, generating a diffraction pattern that was captured by a digital camera and used to calculate RI changes attained as described previously²⁹. In brief, a power meter measured the intensity of the 0th - 3rd order diffracted light from the gratings and the different order diffraction efficiencies were obtained by calculating the ratios between the intensity of the 1st, 2nd and 3rd to the 0th order diffraction light. Since the intensity distribution of the diffraction pattern of a phase grating is proportional to the square value of the Fourier Transform of the transmittance function of the grating³⁶, one particular value of RI change matches only one particular diffraction efficiency value²⁹. To reduce measurement error of the diffraction order intensities, we collected five measurements on each grating, calculating the average value obtained and its standard deviation. In principle, the spatial distribution of the RI change within the micromachined region was a small-scale gradient-index structure. However, for the purpose of the present investigation, we presumed the index profile to be uniform within the grating lines, which were only 3μm deep because the spherical aberration at the focal point was corrected²⁹.

The micromachined corneal and lens pieces were then stored in the ethylene glycol/sucrose solution at 4°C. After one month, each piece was re-mounted onto a new glass slide for imaging and a repeat of the diffraction light intensity measurements. This allowed us to assess whether the RI change initially observed had been maintained during storage.

Results

Patterns created by femtosecond laser pulses below the optical breakdown thresholds are visible only with phase microscopy

Exposure of lightly-fixed cat corneal and lenticular tissue to 0.3nJ or 0.5nJ femtosecond laser pulses (30mW or 45mW average laser power) respectively resulted in the reliable creation of line gratings about 100µm below the epithelial surface or 100µm below the lens surface in all test samples (Figs. 3 and 4). When imaged immediately after micromachining, individual grating lines could be clearly observed and distinguished with DIC microscopy, but they were practically invisible when viewed under bright field transmission microscopy. This could be interpreted as the grating lines having very low scattering properties, which is in contrast to the destructive tissue changes observed when laser energy was increased above the optical breakdown threshold (Figs. 1 and 2). Using the knife-edge method³⁷, we ascertained that the laser focus diameter was 2.5µm in air, which was much bigger than the micromachined line-widths. Thus, it appears that only the central part of the laser focal area had sufficient intensity to modify corneal and lens tissue.

Low-pulse-energy femtosecond laser micromachining alters the RI of cat corneal stroma and lens

Because displacement of the stromal collagen lamellae as a result of post-mortem corneal swelling could not be completely avoided the scattering effect from the 0th order diffraction light was very strong obscuring the 1st order diffraction light³³. Thus, only the 2nd and 3rd order diffraction efficiencies of each grating could be measured and used to calculate an approximate RI change in corneal pieces (Fig. 5B). Because tissue swelling and opacification were minimal in slices of lens cortex, the 0th through 3rd order diffraction light could be measured clearly (Fig. 6A) and 1st and 2nd order diffraction efficiencies were used to calculate the induced RI change (Fig. 6B). Although single diffraction efficiency is usually sufficient to calculate refractive index, here we measured 1st/2nd or 2nd/3rd combinations to confirm that the RI calculated were consistent through different diffraction orders, assuming that the RI of cat corneal stroma and lens cortex were 1.376 and 1.400 respectively³⁰. For corneal stroma, the RI changes induced by the laser in our multiple samples ranged between 0.005±0.001 and 0.01±0.001 (Fig. 5B). For cat lens cortex, RI changes (and scanning speeds) were larger, ranging between 0.015±0.001 and 0.021±0.001 (Fig. 6B).

Long-term maintenance of IRIS in fixed corneas and lenses

After micromachining, each cornea and lens piece was stored in an aqueous solution for one month to determine if IRIS could be maintained long-term. After one month, the tissue pieces were removed from storage and re-examined. Our first observation was that although the storage solution significantly slowed corneal swelling and opacification, it did not completely prevent either. In spite of this, DIC microscopy was able to reveal the grating structures initially micromachined (Figs. 7A and 8A).

For both corneas and lens slices, the diffraction light distribution of one-month old gratings (Figs. 7B and 8B) was no different than that obtained right after the gratings' creation (Figs. 5B and 6B). In the corneal pieces, the scattering light from the 0th order diffraction still obscured the 1st order diffraction. However, the 2nd, 3rd, and even 4th order diffractions were visible and measurable. In the lens pieces, the 1st, 2nd and 3rd order diffraction were visible. The RI change after one month of storage ranged between 0.005±0.001 and 0.01±0.001 for corneal pieces and between 0.015±0.001 and 0.021±0.001 for lens slices.

Discussion

The present study reports on the optical consequences of focusing a high-repetition-rate, low-pulse-energy femtosecond laser onto clear, biological tissues. Gratings were micromachined onto the stroma and cortex of excised, lightly-fixed cat corneas and lenses. The inscription of gratings into these tissues induced small, but significant and persistent RI changes with low scattering loss, a phenomenon we termed IRIS. The main difference between the present results and previous uses of femtosecond lasers in corneal flap cutting is that our application was designed to avoid tissue destruction. We believe that this may be the first report in which the RI of the corneal stroma and lens cortex has been modified non-invasively with a femtosecond laser.

Tissue Breakdown Threshold Vs. Pulse Duration and Repetition Rate

Choosing the right laser parameters was critical to performing IRIS in the cat cornea and lens. Not only did the femtosecond laser fluence at the objective focus have to be below the optical breakdown threshold of the tissue, it also had to be strong enough to induce some nonlinear changes. To achieve this, the average power of the incident laser beam was adjusted to lie between the power for nonlinear imaging (5-10 mW for 100fs pulses – which causes no tissue disruption^{3, 38}) and the breakdown threshold (80mW for corneas when using 170fs pulses and 1.30NA focusing²⁷; 55mW for corneas and 75mW for lenses when using 27fs pulses and 0.70NA focusing in the present study)

In the past two decades, extensive experimental and theoretical work has been done to characterize laser-induced optical breakdown thresholds in different materials, including corneal tissue^{3-5, 39-43} and lens⁴⁴⁻⁴⁷. With the exception of the study by Krueger et al.⁴⁷, most of this work centered on continuous wave (CW) lasers or on single pulses from low-repetition-rate lasers in which thermal diffusion time is much shorter than the time interval between adjacent pulses. Thus, each pulse is responsible for a change in the material. Indeed, it has been established that for pulses longer than 10ps, the optical breakdown threshold fluence scales as the square root of the pulse duration⁴². For pulses shorter than 10ps but longer than about 100fs, the experimental results show a departure from this dependence. However, whether threshold fluence increases or decreases as pulse durations get shorter remains a challenging question^{39, 41, 43}. Some models predict that the threshold would first increase, then decrease when pulse duration becomes shorter than 100fs, but there is no solid experimental evidence to support this⁴³. More recently, it has been claimed that for corneal stroma, the breakdown threshold is almost plateau-like when the pulse duration is between 100fs and 1ps, with a rapid decrease in threshold for pulse durations in the low end of the femtosecond range⁵. However, insufficient experimentation on cornea and lens using sub-100fs pulses makes it difficult to support this prediction and furthermore, existing data were collected using single pulses from low-repetition-rate lasers.

When high-repetition-rate femtosecond laser pulses are used, cumulative, free-electron-mediated chemical effects, photochemical bond breaking and thermal effects contribute to the laser-tissue interaction. As a result, the breakdown threshold fluence may be quite different from that predicted by current models³. Several studies on the effects of high-repetition-rate femtosecond lasers on fused silica and borosilicate glass have found that laser pulses greatly increased the temperature of the materials at the laser focus⁴⁸. Vogel calculated the temperature change in water would be $>10^{\circ}\text{K}$ with a 0.6NA focusing lens and 100fs laser pulses³, assuming that with each pulse, an energy density of $1\text{J}/\text{cm}^3$ at the center of the initial temperature distribution is deposited. In the present experiments, using very high-repetition-rate (93MHz), ultra-short laser pulses (27fs), we found the optical breakdown threshold for the 0.70NA focusing condition in lightly-fixed corneal stroma and lens cortex to be 55mW and 75mW average laser power respectively. This corresponds to 0.6nJ and 0.8nJ pulse energies

respectively, both lower than the optical breakdown power reported by König and colleagues using 1nJ pulse energy, 170fs pulse duration and 1.30NA focusing in porcine corneas²⁷. By using 30mW and 45mW average laser power (0.3nJ and 0.5nJ pulses), we were able to induce IRIS, without accompanying photo-disruption and tissue destruction.

Mechanisms of IRIS in the cornea and lens

While the present results show that we can reproducibly attain small RI changes in lightly-fixed corneas and lenses, they reveal little about the mechanism(s) likely to underlie these changes, other than that they depend critically on incident laser pulse energy, laser repetition rate and pulse duration. While our laser pulses did not cause overt tissue damage, the possibility remains that they induced changes in extracellular, subcellular and molecular structure. König and colleagues have in fact used femtosecond lasers to carry out sub-cellular manipulations (transfections, intracellular nanosurgery) on live cells^{25, 26}. However, they did not discuss whether these manipulations were associated with optical changes, such as those reported in the present study. An important next step for our work will be to test the feasibility of IRIS in living cornea and lens, where the mechanisms and magnitude of the effects may yet be different from those observed presently.

Since 93MHz femtosecond pulse trains and sub-micron/second scanning speeds were used in our experiments, each region of the gratings was exposed to millions of femtosecond laser pulses. The interval time between these successive pulses was about 11ns, which is much shorter than the thermal diffusion time out of the focal volume. Thus, a cumulative thermal effect should be considered a possible mechanism of IRIS in the biological tissues tested. However, our data suggest that the cumulative thermal effect is likely localized within the focal volume, and has negligible impact on surrounding regions. As such, and compared to the overall thickness of the tissue (~500µm), micromachining is also unlikely to affect tissue thickness.

Given the critical role played by hydration in maintaining the RI and clarity of the cornea and lens^{32, 33}, one possibility is that the femtosecond laser pulses used in the present study exerted their main optical effect by displacing water from the corneal or lenticular tissue. However, this is unlikely because RI changes attained after micromachining were maintained even after storing the tissue pieces in an aqueous solution for one month. The stored corneas and lens sections swelled clearly over that period, suggesting that they were exchanging (and especially incorporating) water from the aqueous solution in which they were immersed. Thus, micromachining likely caused sustained molecular and/or structural changes in the collagen/proteoglycan extracellular matrix and/or the cells contained within the micromachined portions of tissue. Ongoing experiments with Raman spectroscopy⁴⁹ and electron microscopy are attempting to ascertain the nature of these changes.

Conclusions – implications for clinical practice and future research

The present study demonstrates, for the first time, that it is possible to cause low-scattering-loss, RI modifications in lightly-fixed cat cornea and lens using 93MHz repetition rate, 27fs laser pulses with 0.3nJ and 0.5nJ pulse energies. These modifications are visible only using DIC microscopy and are not associated with apparent tissue damage. They represent RI changes between 0.005 ± 0.001 to 0.01 ± 0.001 for the corneal stroma and 0.015 ± 0.001 to 0.021 ± 0.001 for the lens cortex. Preservation of IRIS over a month of refrigerated storage suggests that the femtosecond laser-induced modifications are likely to involve relatively long-term molecular/structural alterations.

Although the RI changes themselves were small, their impact on optical power was significant. Based on published values for the power (39D) and native RI (1.376) of the cat cornea³⁰, IRIS

should generate a change in corneal power ranging between 0.14D and 0.28D (assuming that the RI change affects the thickness of the cornea uniformly). Similarly, for the cat lens (power=53D, RI of the homogeneous lens=1.554)³⁰, the RI changes induced by micromachining should theoretically alter lenticular power by between 0.5D and 0.7D. However, the scanning speeds used (0.7µm/s - 1µm/s) made the micromachining process too slow for most practical applications. These observations and the fact that fixation and storage of ocular tissues post-mortem likely affects the mechanisms and magnitude of RI changes attained, make it necessary to measure the impact of IRIS in *living* corneas and lenses. Our goal is not only to detail the cellular and molecular mechanisms underlying IRIS, but to further manipulate the size, placement and design of micromachined patterns, as well as the magnitude of the RI changes with which they are associated. The ability to alter the native RI of the cornea and lens without causing significant damage has important practical implications. Not only could this change our approach to laser refractive surgery, but also to vision correction in general. For instance, the preservation of tissue clarity during the treatment allows the application of IRIS for refractive corrections in a closed-loop approach, e.g. for the correction of higher-order aberrations. In conclusion, the feasibility of IRIS in clear ocular media demonstrated in the present study offers new possibilities for long-term, non-invasive alterations, marking or pattern-inscription within biological tissues.

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Figure 1. Femtosecond IRIS in lightly-fixed cat corneal stroma just around the tissue breakdown threshold

(A)(C) Differential interference contrast (DIC) images of lines created in the stroma of two different, lightly-fixed cat corneas with 0.6nJ pulses and a scanning speed of 10μm/s. Note dark spots of tissue destruction (arrowed) and the "bubbles" along the micromachined lines (clear, horizontal lines within stromal tissue). (B)(D) Bright Field (BF) images of the corneal region in (A)(C) illustrating the visibility of brown spots of tissue destruction (arrowed) and the relative invisibility of the rest of the lines that are clearly seen under DIC conditions (see A, C).

Figure 2. Femtosecond IRIS in lightly-fixed cat lens cortex just around the tissue breakdown threshold

(A) DIC image of lines created with 0.8nJ pulses and a scanning speed of 10µm/s. Note dark spots of tissue destruction (arrowed) and the bubbles created. (B) BF image of the lens region in (A) illustrating the visibility of the bubbles and dark spots of tissue destruction (arrowed), and the relative invisibility of the rest of the lines that are clearly seen under DIC conditions (see A).

Figure 3. Femtosecond IRIS in lightly-fixed cat corneal stroma below the tissue breakdown threshold

(A)(C) DIC images of periodic line gratings created using 0.3nJ pulses and a scanning speed of 0.7µm/s into the stromal layer of two different cat corneal pieces. Note the absence of tissue destruction (no dark spots or bubbles). The insert in A shows a magnified portion of the grating. (B)(D) BF images of corneal regions shown in A and C, including the rectangular area magnified in the insert in A. Note the poor visibility of the micromachined gratings under transmitted, bright light microscopy, which contrasts with the high visibility of the burn spots and bubbles created when using laser power above the tissue breakdown threshold (see Fig. 1).

Figure 4. Femtosecond IRIS in lightly-fixed cat lens cortex below the tissue breakdown threshold (A) DIC image of a periodic line grating created using 0.5nJ pulses and a scanning speed of 1μm/s. Note the absence of tissue destruction and bubbles. (B) BF image of the lens region shown in A. Note the poor visibility of the micromachined grating under transmitted, bright light microscopy, which contrasts with the high visibility of the dark spots and bubbles created when using laser power above the tissue breakdown threshold (see Fig. 2).

Figure 5. Measuring refractive index change in IRIS-treated corneas immediately after the treatment

(A) DIC image of a periodic line grating created using 0.3nJ pulses and a scanning speed of 0.7µm/s in the stromal layer of the piece of cat cornea illustrated in Fig 3. Note that some of the lines appear sharper than others - this is due to the whole grating not being on the same focal plane when imaged under the microscope. (B) Graph plotting the 2nd and 3rd order diffraction efficiencies ($\times 10^{-3}$) and the corresponding laser-induced refractive index changes of eight gratings micromachined in different corneal samples immediately after they were created. The insert is a photograph of the diffraction pattern obtained when illuminating the grating shown in A with a 632.8nm He-Ne laser.

Figure 6. Measuring refractive index change in IRIS-treated cat lenses immediately after the treatment

(A) DIC image of a periodic line grating created using 0.5nJ pulses and a scanning speed of 1 μm/s in the cortex of the piece of cat lens illustrated in Fig 4. (B) Graph plotting the 1st and 2nd order diffraction efficiencies and the corresponding laser-induced refractive index changes of eight gratings micromachined in eight different lens cortices immediately after they were created. The insert is a photograph of the diffraction pattern obtained when illuminating the grating shown in A with a 632.8nm He-Ne laser.

Figure 7. Measuring refractive index change in IRIS-treated corneas 1 month after the treatment (A) DIC image of a periodic line grating created using 0.3nJ pulses and a scanning speed of 0.7μm/s into the stromal layer of the cat corneal piece shown in Fig. 3A and B. Note that after one month, the grating is still visible, but the clarity of the grating lines is markedly decreased, a likely result of corneal swelling and opacification. (B) Graph plotting the diffraction efficiencies ($\times 10^{-3}$) and the corresponding refractive index changes of eight gratings measured one month after they were created. The insert is a photograph of the diffraction pattern obtained when illuminating the grating shown in A with a 632.8nm He-Ne laser.

Figure 8. Measuring refractive index change in IRIS-treated cat lenses one month after the treatment

(A) DIC image of a periodic line grating created in lens cortex shown in Fig. 4 after one month of storage. Note that the grating's appearance has not changed significantly from its presentation in Fig. 4. (B) Graph plotting the diffraction efficiencies and the corresponding refractive index changes of eight gratings in eight lens cortex pieces measured one month after they were created. The insert is a photograph of the diffraction pattern obtained when illuminating the grating shown in A with a 632.8nm He-Ne laser.