

# Femtosecond Laser Photodisruption of Primate Trabecular Meshwork: An Ex Vivo Study

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**PURPOSE.** The femtosecond laser has been realized as an advantageous tool for micromachining, and the feasibility of employing it for surgical procedures has been investigated. A prior study demonstrated dose-dependent femtosecond laser photodisruption of peripheral corneal tissue through a gonioscopic lens in enucleated porcine eyes and of the trabecular meshwork (TM) in human corneoscleral rim tissues, with little collateral damage. The present study was undertaken to extend these efforts to ex vivo primate eyes.

**METHODS.** Photodisruption of the TM in enucleated baboon eyes and human donor eyes was performed with a gonioscopic lens and a custom femtosecond laser ablation delivery system. Laser ablation was executed with a Titanium:Sapphire laser (800-nm wavelength), focused with a 0.15-NA lens, with the following settings: pulse duration, 45 fs; pulse energy, 60 to 480  $\mu$ J; pulse repetition rate, 1 kHz, total exposure time, 0.001 to 0.3 seconds. The laser lesions were evaluated by two-photon microscopy.

**RESULTS.** Laser-induced lesions were consistently observed in the TM of the baboon and human eyes and visualized by two-photon microscopy. These oblique, trough-shaped lesions, which did not penetrate the juxtacanalicular region, had sharp edges and showed no evidence of thermal coagulation. The dimensions of the lesion increased linearly with both pulse energy and exposure time.

**CONCLUSIONS.** The present study demonstrates that laser ablation of the TM ab interno in ex vivo primate eyes is feasible by a custom femtosecond laser ablation system with a gonioscopic lens. (*Invest Ophthalmol Vis Sci.* 2009;50:1198-1204) DOI:10.1167/iovs.07-1536

There is great need in glaucoma therapy for a simple, effective, and incisionless surgical procedure. Laser trabecular ablation, an example of such a procedure, has been attempted. This technique is intended to create a direct communication between the anterior chamber and Schlemm's canal with an ab interno approach.<sup>1</sup> Using this procedure, one could, in principle, increase the outflow facility by creating a fistula through

the trabecular meshwork (TM), which accounts for approximately 75% of the outflow resistance.<sup>2</sup> The lasers most widely used for trabecular ablation are pulsed Nd:YAG,<sup>3-9</sup> Er:YAG,<sup>10-15</sup> and excimer<sup>16</sup> lasers. Nd:YAG laser trabecular ablation was studied in primates and humans, with the laser pulse delivered through a gonioscopic lens. In monkeys<sup>5</sup> there was an immediate decrease in intraocular pressure (IOP), but the outflow facility returned to baseline levels in 8 days to 3 weeks. Short-term human studies have revealed variable lowering of IOP, with a return to baseline IOP in most patients and gradual filling of the laser holes with scar tissue.<sup>4</sup> These treatments, therefore, were ineffective or not widely accepted.<sup>17</sup> The primary reason for their failure is related to the thermal effects of the lasers used for treatment.<sup>17-21</sup> For pulse durations of several nanoseconds or longer, thermal and mechanical relaxation occur during the laser pulse and compete with material ablation, resulting in collateral damage of tissues and scar formation.<sup>22</sup>

More recently, laser trabecular ablation has been performed with the pulsed 2.94  $\mu$ m Er:YAG laser. The longer wavelength, lying near the peak of the water absorption band, requires the use of an endoprobe, necessitating surgical entry into the anterior chamber to perform the ablation, while visualizing the TM through a gonioscopic lens or endoscope.<sup>12,15</sup> Feltgen et al.<sup>15</sup> reported endoscopic Er:YAG goniopuncture in humans to be a successful adjunct to cataract surgery in lowering IOP by 30% in the treatment group for more than 12 months. The 308-nm XeCl excimer laser has also been used for laser trabecular ablation. The distinct advantage offered by excimer laser trabecular ablation is reduced levels of thermal damage and necrosis. Vogel and Lauritzen<sup>16</sup> demonstrated increased outflow facility in vitro, and they showed modest lowering of IOP (median 7 mm Hg) at 7 months in a study in humans. Huang et al.<sup>23</sup> showed minimal trabecular scarring in a rabbit model of ab interno excimer laser trabecular ablation. The drawbacks of Er:YAG laser and excimer laser trabecular ablation include the need for an incision to introduce the delivery system and difficulty in manipulating the endoprobe in the anterior chamber to visualize the meshwork. The absence of controlled clinical studies may have also contributed to the lack of extensive use of this laser.

The femtosecond Ti:Sapphire laser is a potentially useful tool for laser trabecular ablation for several fundamental reasons. First, the 800-nm wavelength of the Ti:Sapphire laser falls within the absorption window of most tissues. The multiphoton nature of the transition allows the radiation to be focused to a point within the target without damaging the outer layers.<sup>24</sup> Second, the use of subpicosecond pulses allows the radiant energy to be absorbed on a time scale that is much shorter than both the thermal diffusion and shock wave propagation times. This property leads to thermal and stress confinement, thereby reducing the region of material damage to the vicinity of the laser focus.<sup>24</sup> Third, the lower fluence threshold for femtosecond laser ablation reduces the overall thermal and mechanical load on the tissue.<sup>24</sup> Fourth, the femtosecond laser can be applied to the TM without using endoprobes.

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Previously, our laboratory showed that photodisruption by a femtosecond laser can be used to create lesions in the human TM in nonfixed corneoscleral rim tissues of donor eyes without damaging the surrounding tissues.<sup>25</sup> In addition, using enucleated porcine eyes and a gonioscopic lens, we demonstrated that femtosecond laser energy can be delivered to the peripheral inner surface of the cornea adjacent to the TM, which is bridged anteriorly by robust pectinate ligaments.<sup>26</sup> The present study was undertaken to extend our previous efforts to ex vivo primate eyes. Using enucleated baboon and human donor eyes, we demonstrate the feasibility of laser trabecular ablation through a gonioscopic lens.

## MATERIALS AND METHODS

### Laser and Delivery System

In this study, we used an optical delivery system reported by us in a previous publication (Fig. 1).<sup>26</sup> Briefly, a Ti:Sapphire oscillator (Tsunami; Spectra-Physics Lasers, Newport Corp., Tucson, AZ) pumped by a diode laser (Millennia V; Spectra-Physics Lasers) generated 82-MHz, 45-fs pulses. The oscillator output was amplified by a regenerative amplifier (Spitfire; Spectra-Physics Lasers) to produce 800 nm, 45-fs pulses with energies of 2 mJ/pulse at a 1-kHz repetition rate with a beam diameter of 9 mm. A half-wave plate (H) and polarizer (P) were used to attenuate the pulse energy to a desirable level. To obtain a high numerical aperture (NA), the beam was expanded to a diameter of 30 mm, producing an NA of 0.15 at lens L5. We used an He:Ne (632.8 nm) laser as a guiding beam because the surgical femtosecond laser beam is barely visible at 800 nm. The He:Ne laser beam was expanded to match the diameter of the surgical beam. Both the guiding He:Ne and the surgical femtosecond laser beams were combined at beam splitter BS2 to produce coaxially propagating beams. Both beams were then focused by lens L5 ( $f = 100$  mm) and directed by the beam splitter BS3 to the enucleated eye with a gonioscopic lens, as described later. The focal area of the surgical laser beam is  $154 \mu\text{m}^2$ . The illumination beam from the light bulb of a surgical microscope (OPMI-6; Carl Zeiss Meditec, Inc., Dublin, CA) was transmitted through the same beam splitter and focused onto the target by the gonioscopic lens. The

procedure was viewed by an operator through the eyepiece of the microscope or via a television monitor. The focal points of both laser beams were finely adjusted by lens L5, which was mounted on a motorized computer-controlled three-dimensional translation stage. The laser treatment time was precisely controlled by a computer, which triggered the Pockell's cell in the amplifier.

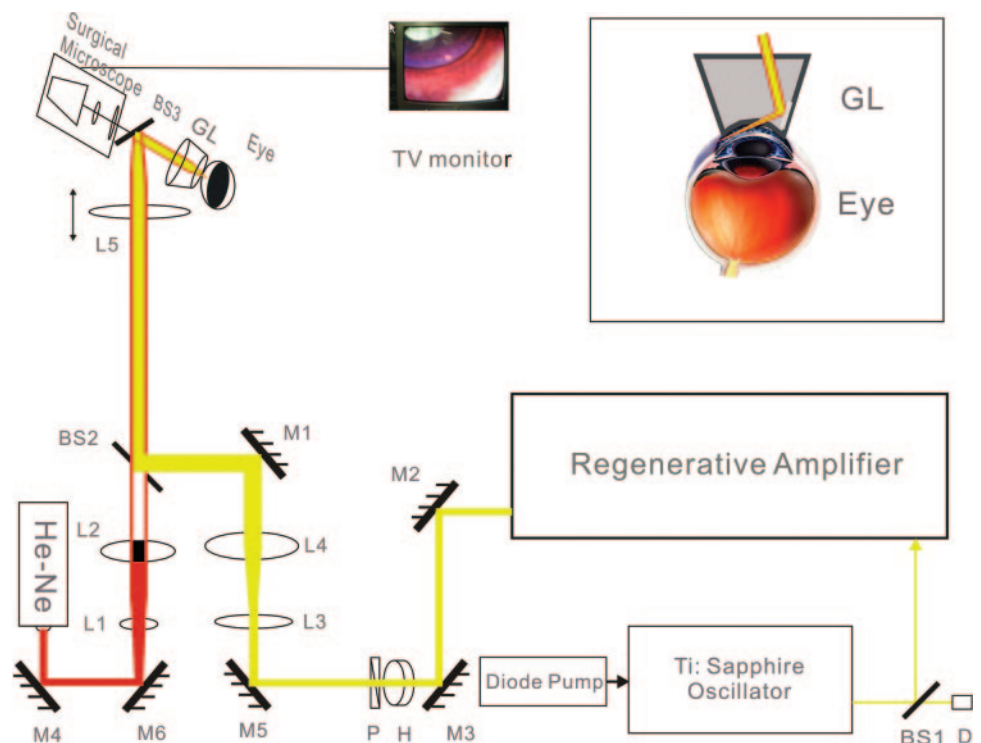
### Laser Procedure

Use of baboon eyes and a human donor eye was in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Declaration of Helsinki on research involving human subjects, respectively. Three baboon eyes were enucleated within 4 hours of death, after the animals were killed for other experiments by other investigators at the Biological Resources Laboratory (BRL) at the University of Illinois at Chicago. A human eye (56 years of age) was obtained within 24 hours of death from the Illinois Eye Bank in Chicago. The eyes were kept at  $4^\circ\text{C}$  for several hours before use. Three baboon eyes were examined (1) to assess the feasibility of producing an incisionless trabecular ablation by a femtosecond laser through a gonioscopic lens and (2) to evaluate the relationship between lesion volume in the primate TM and laser parameters. Only the first objective was possible for the human donor eye, because its cornea was insufficiently transparent to achieve perfect focusing of the ablation laser.

The eyes were placed on a gauze pad, with the cornea facing upward, in a Styrofoam orbital model, to approximate the customary positioning of the eye for laser surgery. A gonioscopic lens (Magna View Gonio Laser Lens, OMVGL; Ocular Instruments Inc., Bellevue, WA) was manually placed on the cornea with 2.5% hypromellose ophthalmic demulcent solution (Gonak; Akorn Inc., Buffalo Grove, IL). The TM was observed through the gonioscopic lens with a surgical microscope incorporated in the laser delivery system (Fig. 1).

TM photodisruption was conducted in baboon and human eyes using a gonioscopic lens and the femtosecond laser delivery system described in the Laser and Delivery System section. A two-beam focusing technique, developed in our laboratory,<sup>26</sup> was used. The He:Ne guiding beam, which is coaxial with the surgical femtosecond laser beam, was split into two beams at lens L2. When the guide beam was focused onto the target, the two visible beams coalesced. A single spot

**FIGURE 1.** The optical delivery system for ex vivo laser surgery. M, mirror; BS, beam splitter; L, lens; D, detector; H, half-wave plate; P, polarizer; GL, gonioscopic lens; He:Ne, Helium-Neon laser. *Inset:* Sketch showing the path of the laser beam in the eye. The He:Ne and Ti:sapphire laser beams are indicated by red and yellow lines, respectively. Adapted with permission from SLACK Incorporated: Liu Y, Nakamura H, Witt TE, Edward DP, Gordon RJ. Femtosecond laser photodisruption of porcine anterior chamber angle: An ex vivo study. *Ophthalmic Surg Lasers Imaging*. 2008;39(6):485-490.



of the guiding beam indicated precise focusing on the target, as these beams overlapped each other only when the target was at the focal point.

No perfusion system was used during the laser procedure. Air bubbles produced by photodisruption formed in the anterior chamber under the cornea occasionally obstructed the visibility of the TM through the gonioscopic lens. These bubbles were removed with a 1-mL syringe and a 30-gauge needle through a self-sealing paracentesis. The IOP was maintained by the injection of balanced salt solution, also through a self-sealing paracentesis. The pressure was assessed digitally and was found to be approximately 10 to 30 mm Hg. Maintenance of the IOP prevented the eye from either collapsing or being overly compressed. Variable settings of the laser energy (60–480  $\mu$ J) and exposure times (0.001–0.3 seconds) were applied to the baboon eyes to investigate the relationship between lesion extent and laser parameters. The laser treatment process was recorded by a video system (DXC-760MD; Sony, Tokyo, Japan) to monitor visible effects of femtosecond laser treatment on the TM and surrounding tissues.

### Imaging of the Lesion

Baboon and human eye bank eyes were fixed with 10% buffered formalin for 24 hours after laser surgery. The eyes were then dissected, and the iris and ciliary body were gently removed from the corneal scleral rim for observation of the TM by two-photon microscopy.

Two-photon images of the TM were acquired with a multiphoton microscope (Radiance 2100MP; Bio-Rad, Hercules, CA). Two-photon fluorescence from collagen fibers and similar molecules provides a useful tool for imaging structures within the TM. All images were acquired with the output of a Ti:Sapphire oscillator (780 nm, 80 fs, 1.2 nJ/pulse, 82 MHz), focused with a 60  $\times$  1.3-NA oil immersion objective, and scanned with a resolution of 0.1 to 0.3  $\mu$ m to produce the image. For each lesion, images were obtained from the surface of the TM to the bottom of the lesion in 3- to 5  $\mu$ m-intervals. In addition, a z-scan (a stack of images at different depths) with a 0.5- $\mu$ m interval between adjacent images, was performed to reconstruct the 3D image by Voxo, a voxel-based 3D imaging program.<sup>27</sup>

Fixed and dissected baboon and human tissues including the TM were processed and embedded in paraffin. Sequential 5- $\mu$ m sections were stained with hematoxylin and eosin to examine the lesions histologically.

### Measurement and Statistical Analysis

Each lesion in the baboon TM was evaluated using images obtained by two-photon microscopy. The area, perimeter, maximum diameter, and minimum diameter of each lesion produced in the surface tissue of the TM were measured with the Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). The depth of the lesion was defined as the distance between images of the surface and the bottom of the lesion.

The relationship between lesion extent in the baboon eyes and laser parameters (pulse energy and exposure time) was analyzed statistically in a linear regression model (SPSS, ver. 15.0; SPSS Inc., Chicago, IL). The independent variables were area, perimeter, maximum diameter, minimum diameter, depth, and nominal volume (area multiplied by depth). The pulse energy and exposure time were taken as dependent variables, with the exposure time fixed at 0.1 second for measurements with variable energy, and the laser energy fixed at 800  $\mu$ J/pulse for variable exposure time.

### RESULTS

During laser surgery, bubble formation around the focal point of the femtosecond laser was observed to increase with laser energy and exposure time.<sup>26</sup> These bubbles were promptly dissipated into the aqueous humor, as described previously. Tissue fluorescence, channel formation, and (de)pigmentation of the TM were not observed through the gonioscopic lens.

During each laser treatment, no vibration or movement of the TM and surrounding tissue was observed by the operator or detected later in digital videos recorded during the procedure.

Areas of ablation were consistently observed in two-photon microscopic images of the TM of the baboon and human eyes. These lesions were observed to have a trough-like shape (Fig. 2, Movie S1/baboon and Movie S2/human for z-scanned images and Movie S3/baboon and Movie S4/human for 3D images; movies are online at <http://www.iovs.org/cgi/content/full/50/3/1198/DC1>). The edges of the lesions were sharp, and no evidence of a coagulative effect was found in the collagenous beams of the baboon or human TM.

The results of the statistical analysis are shown in Figure 3. Significant positive correlations were obtained between all the dimensional parameters of the lesions and the pulse energy ( $P < 0.01$  for area and nominal volume and  $P < 0.05$  for perimeter, maximum diameter, minimum diameter, and depth). Significant positive correlations were also obtained between area ( $P < 0.05$ ), depth and nominal volume ( $P < 0.01$ ), and exposure time. For both pulse energy and exposure time, nominal volume showed the strongest correlation, with  $R^2 = 0.868$  and  $0.719$ , respectively.

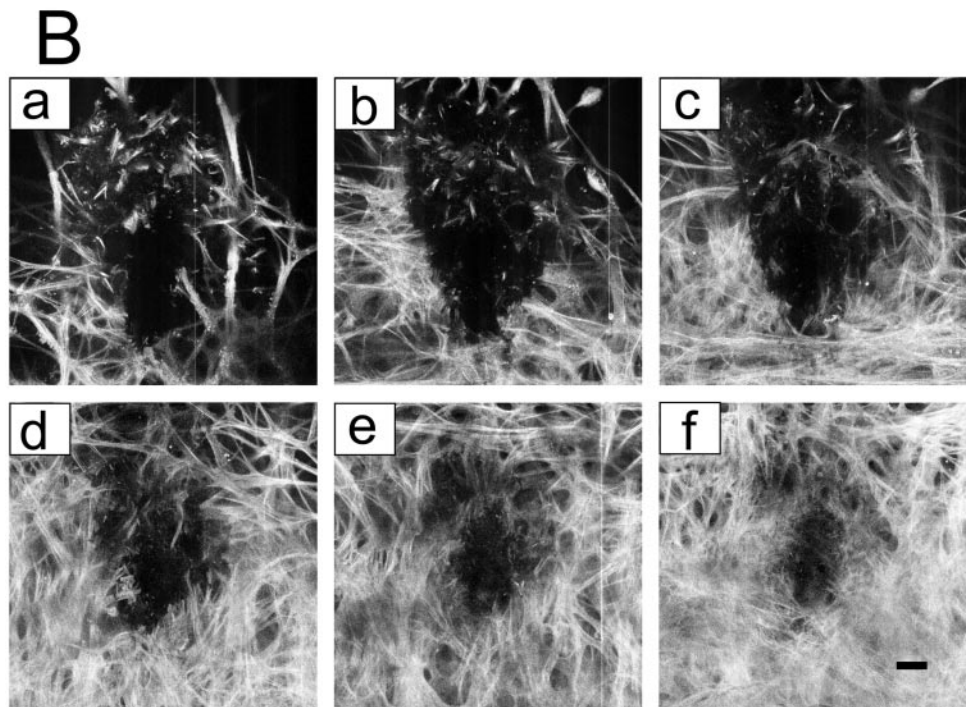
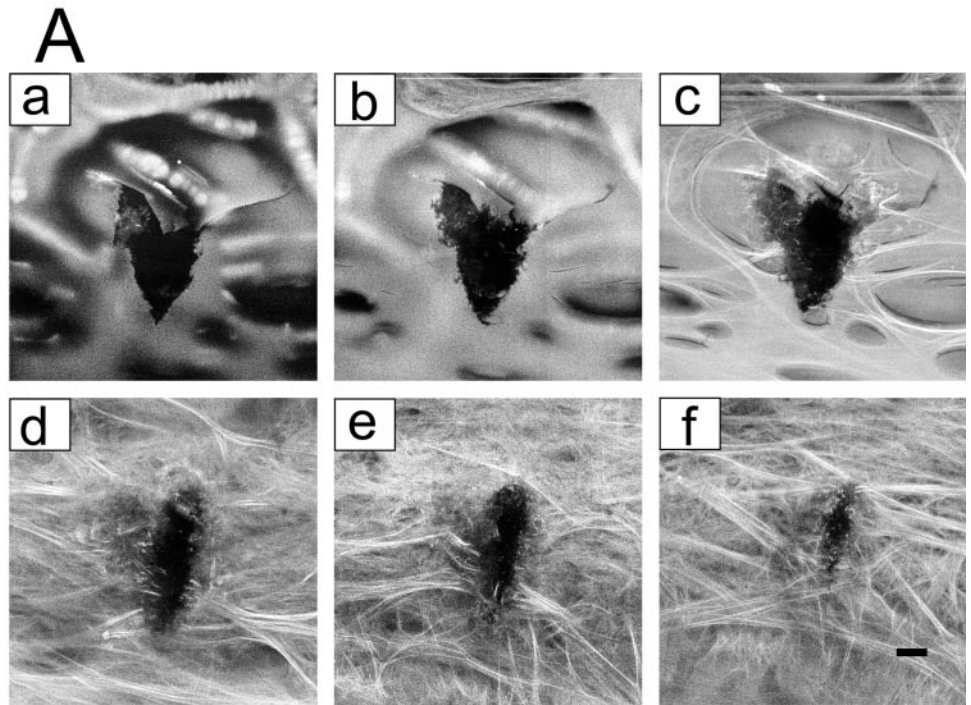
Histologic analysis of sequential sections revealed only a few lesions that were not artifacts. These lesions demonstrated trabecular beam disruption that was oblique in shape and did not reach the juxtacanalicular region (Fig. 4).

### DISCUSSION

Primate eyes were treated in this study to examine whether a femtosecond laser could be used to create lesions in the trabecular meshwork. The femtosecond laser is a useful tool for micromachining because it produces extremely precise ablation of all types of materials with little collateral damage.<sup>28</sup> Femtosecond lasers have also been used in various medical fields, such as ophthalmology to prepare corneal flaps for refractive surgery, otolaryngology to treat ossicular bones, and cardiology to treat arteriosclerosis.<sup>28</sup> In a previous study,<sup>26</sup> we demonstrated that femtosecond laser radiation could be delivered to the anterior chamber angle of ex vivo porcine eyes by means of a gonioscopic lens to produce sharp lesions in the peripheral inner surface of the cornea.

We report successful ablation of the TM by an ab interno approach in ex vivo baboon and human eye bank eyes with a femtosecond laser (45 fs, 800-nm laser pulses). This result was achieved using a gonioscopic lens to deliver the radiation. Other methods of applying femtosecond laser radiation to glaucoma surgeries, such as ab externo procedures,<sup>29</sup> have also been investigated recently (Chai D, et al. *IOVS* 2008;49:ARVO E-Abstract 1227; Pogorelov P, et al. *IOVS* 2008;49:ARVO E-Abstract 2881). The laser lesions after ablation were consistently observed in primate TM tissue by two-photon microscopy. The edges of lesions were sharp, and there was no evidence of a coagulative effect, indicating that an incisionless laser trabecular ablation is feasible with a femtosecond laser.

Autofluorescence of the primate TM tissue makes it possible to obtain high-quality 3D images by means of two-photon fluorescence microscopy.<sup>25</sup> Quantitative analysis of the lesions in baboon eyes revealed a significant positive linear correlation between the nominal volume and both pulse energy and exposure time, with higher correlation coefficients and without the saturation effects observed in our previous study.<sup>26</sup> We determined that there is a simple correlation between the photodisruption effect and total laser energy in the primate TM, at least up to 480  $\mu$ J of laser energy and 0.3 seconds of exposure time, for a total energy dose of 14.4 mJ. The linear correlation without saturation in the present study is attribut-

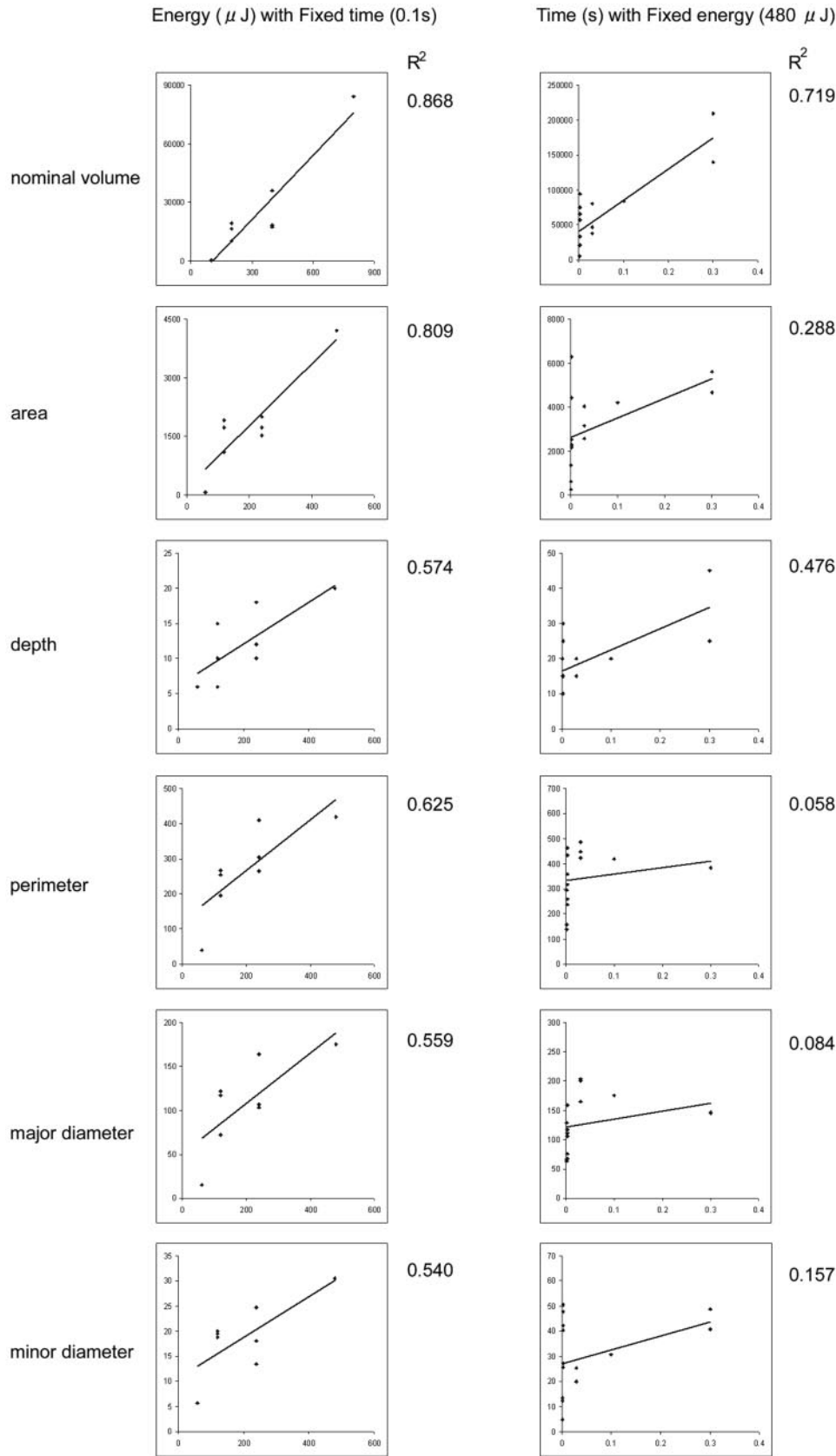


**FIGURE 2.** Two-photon microscopic images for typical lesions at different depths from the surface of the trabecular meshwork with the same magnification. (A) Baboon; (B) human: (a), 0  $\mu\text{m}$  (on the surface of the trabecular meshwork); (b) 5  $\mu\text{m}$ ; (c) 10  $\mu\text{m}$ ; (d) 15  $\mu\text{m}$ ; (e) 20  $\mu\text{m}$ ; and (f) 25  $\mu\text{m}$  depth from the surface. Both lesions demonstrated trough-shaped craters. Their edges were sharp, and there was no evidence of a coagulative effect in any of the photodisruptive lesions examined. Laser energy/exposure time was (A) 480  $\mu\text{J}/0.003$  second and (B) 480  $\mu\text{J}/0.5$  second. Areas of lesions at the surface of the TM were (A) 2514.8 and (B) 3219.2  $\mu\text{m}^2$ . Depth of both lesions was 35  $\mu\text{m}$ . Detailed z-scanned images of the lesions were also obtained by two-photon microscopy, and 3D images were reconstructed. Both z-scanned (Movie S1/baboon and Movie S2/human) and 3D images (Movie S3/baboon and Movie S4/human) are posted. Scale bar, 20  $\mu\text{m}$ .

able to three major factors that differed from our porcine study. First, the target tissue is different; the TM in the baboon eye is a much softer tissue than the cornea. Second, the laser ablation parameters were different. In our previous study in porcine eyes, the dimension of the lesion increased nonlinearly with both pulse energy and exposure time, showing saturation at high energy and long exposure times. Because we concluded that fewer pulses and/or higher energy would be more effective, shorter laser exposure time (0.1 second, compared with 0.5 second) or higher laser energy (480  $\mu\text{J}$  compared with 240  $\mu\text{J}$ ) was set in this study as the fixed laser parameter to investigate the relationship between lesion extent and variable

laser parameters. Third, a better method to evaluate lesions, namely two-photon imaging, was used in this study. This diagnostic yields a three-dimensional image of the lesion, which provides a more reliable measure of the lesion properties. Nevertheless, a more systematic study of the effects of different laser parameters on primate laser trabecular ablation is still needed.

In contrast, the laser lesions were difficult to demonstrate by conventional histologic sectioning using formalin-fixed and paraffin-embedded specimens. Based on observed alterations in sequential sections, only a few lesions were judged not to be artifacts. We believe that it was difficult to distinguish between

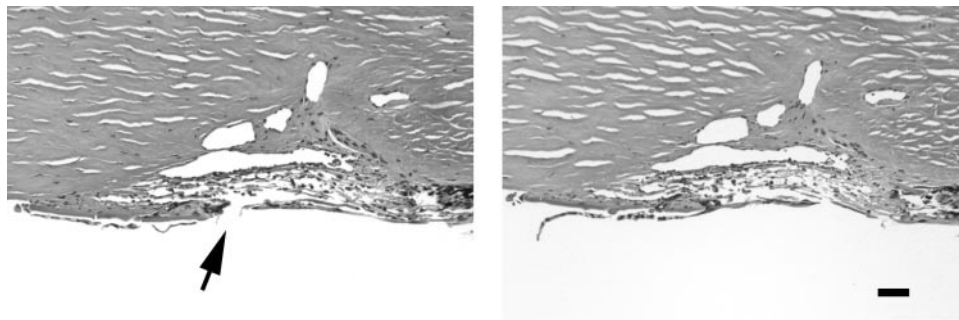


**FIGURE 3.** Line graphs for linear regression model. *Left:* relationship between laser energy and lesion parameters, nominal volume, area, depth, perimeter, major diameter, and minor diameter under fixed laser exposure time (0.1 second). *Right:* relationship between laser exposure time and lesion parameters under fixed laser energy (480 μJ). R<sup>2</sup> is shown on the top right side of each graph. For both pulse energy and exposure time, nominal volume has the highest R<sup>2</sup> values.

lesions and artifacts because of the oblique and shallow nature of the lesions in this experiment. In our previous in vitro data, the laser beam was directed perpendicular to the tissue sur-

face, and it was therefore easier to demonstrate the lesions by conventional histologic techniques.<sup>25</sup> Although seven monkey and two human specimens were examined, in which there

**FIGURE 4.** Histologic appearance of a lesion in baboon trabecular meshwork. *Left:* section at lesion; *right:* adjacent to lesion. *Arrow:* laser lesion. The lesion was oblique and did not reach the juxtacanalicular region. Its edge was sharp, and there is no evidence of a coagulative effect. Laser energy/exposure time is 480  $\mu\text{J}/0.1$  second. Scale bar, 50  $\mu\text{m}$ .



were at least five lesions created in each specimen, only a few sections were judged to show nonartificial lesions. The data could not therefore be analyzed statistically. We suggest that two-photon microscopy may have distinct advantages in evaluating laser lesions of the TM.

In our previous study, a two-beam focusing technique was used to overcome some unfavorable conditions, namely blurred observation of the TM though a hand-held gonioscopic lens and a mildly edematous cornea in the enucleated porcine eye. This focusing technique proved to be useful in our work as well. Although bubble formation was observed in the present study, consistent lesions of trough-shaped craters were nevertheless created in the primate TM, as was previously observed in porcine peripheral corneas.<sup>26</sup> Because the femtosecond laser energy is delivered on a much shorter time scale than that required for thermal or mechanical energy propagation, it is unlikely that mechanical or shock wave effects, evidenced by the formation of air bubbles, have significant effects on the ablation efficiency of the laser. Moreover, our finding that the lesion dimensions are positively and significantly correlated with the pulse energy and exposure time suggests that bubble formation has only a limited secondary (e.g., shielding) effect. Our results reaffirm that precise focusing is the most critical factor, which can compensate for bubble interference with succeeding pulses.

The lesions in both baboon and human eyes displayed trough-shaped craters, as was observed in our previous experiments in porcine eyes.<sup>26</sup> In addition, the trough-shaped lesion did not reach the juxtacanalicular tissue (JCT) region or the endothelium of the inner wall of Schlemm's canal, which are believed to account for aqueous humor outflow resistance.<sup>30,31</sup> We attribute this effect to the fact that the laser beam was focused on the target by the gonioscopic lens at a shallow angle with respect to the tissue surface and/or that the pulse energy and exposure time were insufficient to achieve full penetration. Although the lesions did not penetrate far enough from the surface of the TM to reach the JCT region, the lesion dimensions were demonstrated to have a positive correlation with the laser parameters. In future research, three strategies of laser delivery will be used to create lesions in the JCT region. First, by using a larger angle of incidence of the laser, it should be possible to create more cylindrically shaped lesions that penetrate the entire thickness of the TM. Second, the focal point will be readjusted by computer control of the focusing lens during the laser procedure. Because precise focusing is critical in the femtosecond laser application, continuous adjustment of the focal point from the surface of the TM into the JCT region will be effective to create full-thickness lesions. A third approach is to create deep lesions around the JCT region without damaging the inner surface of the TM. This may be accomplished by focusing the 800-nm laser radiation more deeply into the tissue without damaging the outer layers. In addition to further improvement in delivery techniques, optimization of the laser parameters is needed. Laser energy and/or

exposure time can be increased because our results demonstrate a significant positive linear correlation between laser dimensions and either pulse energy or exposure time without saturation effects.

In summary, the present study successfully demonstrates TM photodisruption ab interno produced by a femtosecond laser ablation system using a gonioscopic lens in baboon and human ex vivo eyes. It is anticipated that lesions produced by a femtosecond laser may decrease the resistance of aqueous humor outflow in glaucomatous eyes. In the future, we plan to study the biological response to such lesions produced in vivo in primate eyes as a step toward developing an incisionless glaucoma therapy with femtosecond lasers.

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