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Characterization of the Fluorescence Spectra Produced by Excimer Laser Irradiation of the Cornea

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The epithelium and stroma of bovine and human corneas were exposed to both ablative and subablative levels of 193-nm excimer laser radiation and the spectra of the induced fluorescence recorded. Two broad peaks in the emission spectra were observed, at 310 nm and 460 nm, with a difference in the relative height of these peaks between epithelium and stroma. The lower cut-off for fluorescence (260 nm) was similar for both tissues. Time-resolved measurements showed that fluorescence occurs on the nanosecond time scale, and an estimate gives the quantum yield for photons emitted in the 260–350-nm range as approximately 1×10^{-5}. The significance of these results in the evaluation of the safety of surgery with an argon fluoride excimer laser is discussed. Invest Ophthalmol Vis Sci 31:1512–1518, 1990

The argon fluoride (ArF) excimer laser (λ = 193 nm) has been proposed as an alternative to the surgical knife for corneal surgery because it excises tissue with a high degree of precision and minimal damage to adjacent tissue.[1-3] Although normal repair processes appear to follow laser excision of corneal tissue in the rabbit,[4] primate,[5] and human,[6] questions remain with regard to the effects of highly energetic ultraviolet irradiation on cells surviving adjacent to the site of ablation. It has been proposed that a mutagenic or carcinogenic effect could result if the DNA of these cells was damaged by either direct irradiation with sublethal doses of 193-nm photons, or by the longer wavelengths of secondary fluorescence produced at the site of ablation.

Results using several different experimental systems to address the question of ArF laser safety have been published. They show that direct exposure to a subablative dose of 193-nm laser radiation can be cytotoxic to mammalian cells in culture. Although Green et al[7] and Trentacoste et al[8] did not observe a mutagenic effect after direct irradiation of mammalian cells, Rasmussen et al[9] have demonstrated sister chromatid exchange in exposed cells and a mutagenic effect in one of two genetic markers studied. The effect of irradiation on an area peripheral to the site of photoablation, whether it be the result of photons scattered by the target, or induced fluorescence, has also been examined. The production of what is believed to be pyrimidine dimerization in yeast cells[10] and the induction in bacteria of the lambda prophage[11] has been reported adjacent to laser-ablated substrates, but excision repair of pyrimidine dimers in tissue—as demonstrated by unscheduled DNA synthesis—has been found neither in epithelial cells nor in fibroblasts at the margin of an ablated wound.[12,13]

Because fluorescence may be an important mechanism in producing DNA damage, we have characterized the spectrum emitted during the irradiation of corneal tissue with an ArF laser. Unlike previous experiments,[14] the fluence (energy/area/pulse) has been kept to values near to or below the ablation threshold, such that the principal luminescence can be attributed to the intact tissue rather than to the ablation products or to laser-induced plasma effects.

Materials and Methods

Laser Parameters

Fresh bovine and human corneas were irradiated with ArF excimer lasers emitting short-duration

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(~15 nsec full-width at half maximum) pulses at 193 nm. The experiments on bovine corneas were carried out with a home-built laser operating at 2 Hz, and those on human corneas with a Questek Series 2000 laser (Questek, Bedford, MA) at 10 Hz. In both cases the laser output was passed through a circular iris to select a region of uniform fluence from the beam, and a fused-silica lens used to produce an image of this iris at the surface of the tissue sample (ie, the tissue surface lay in the conjugate plane of the iris). This arrangement provided a sharp-edged, 4-mm-diameter irradiation zone of uniform radiant exposure. Energy measurements were made with a Gen-Tec (Ste-Foy, Canada) pyroelectric Joulemeter and the fluence calculated from the energy and irradiation spot size.

Fluorescence Measurements

Fluorescent light emitted by the tissue was collected at 45° to the side of the incident beam with a 10-cm focal length fused-silica lens and imaged at unity magnification onto the entrance slit of a visible–ultraviolet (UV) monochromator (Bentham, Reading, UK). The latter was equipped with a 1200-lines/mm holographic diffraction grating and an EMI 9813QB photomultiplier detector with a bi-alkali photocathode (EMI, Ruislip, UK). To obtain time-integrated spectra the photomultiplier current pulse was passed to an integrating amplifier (~1-sec time constant) and the output voltage displayed on a CR652 recorder (J. J. Lloyd, Southampton, UK). The monochromator was scanned with a stepper-motor drive from 180 to 600 nm at 0.15 nm/sec for the 2-Hz laser and 0.75 nm/s for the 10-Hz laser; in the scanned mode the spectral resolution was about 1 nm. No corrections have been applied to the spectra reported for the variation of sensitivity with wavelength for the combined monochromator–photomultiplier detection system. The relative spectral response r(λ) of the detection system was calculated from the product of the radiant sensitivity of the EMI 9813QB photomultiplier and the grating efficiency provided by the manufacturers. This, as shown in Figure 1, exhibited a broad peak at ~350 nm, with upper and lower wavelength detection limits set by the photocathode response and photomultiplier window transmission, respectively.

Scattered laser radiation entering the monochromator produced strong lines in first (193 nm), second (386 nm) and third (579 nm) order, and for some spectra these were removed by placing a 1-mm-thick Lucite sheet in the beam path of the fluorescence. This acted as a cut-off filter, essentially removing all radiation below 250 nm but becoming fully transmissive above 370 nm (~50% transmission at 300 nm).

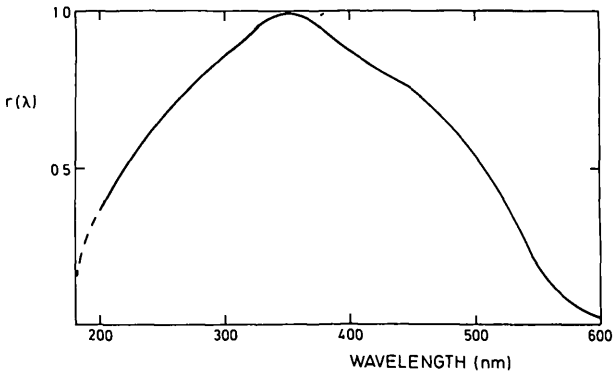

Fig. 1. Relative response (rλ) of the monochromator detector system as a function of wavelength.

Time-Resolved Measurements

Time-resolved measurements of the fluorescence signal with approximately 3-nsec resolution were made on bovine corneas with the arrangement described above, by directly amplifying the photomultiplier output with a Tektronix 7A19 plug-in and 400 MHz bandwidth Tektronix 7834 storage oscilloscope (Tektronix, Beaverton, OR). In this case the monochromator was fixed at the desired wavelength, and the resolution (~0.6 nm) was dictated by the slit width. Measurements of the laser pulse shape, for comparison with the fluorescence signal, were made with a fast-time response (~1 ns) vacuum photodiode.

Spectra were recorded first from the epithelium and then from the front and back surfaces of the stroma, either after debridement of the epithelium and endothelium or after mechanical keratectomy. In most experiments the ArF laser fluence was kept well below the ablation threshold[15,16] of ~50 mJ/cm^2 to prevent loss of the target material during spectral recording and to prevent the interfering effects of luminescence from ablation and plasma products.[14] One set of experiments was carried out on human corneas at 52 mJ/cm^2, but in this case only spectra of stromal tissue could be obtained since, at this fluence, removal of the epithelium occurred under the multishot irradiation conditions necessary to acquire spectra. Therefore, for these experiments the epithelium was debrided prior to the irradiation.

The absence of interfering signals from fluorescence in the optical components was checked by removing the tissue sample from the holder and replacing it with a black paper. On running the monochromator, strong scattered laser lines in first, second, and third order were still observed, but no significant fluorescence features were observed between these orders.

Results

Bovine corneal epithelium irradiated at 5 mJ/cm^2 produced a broad spectrum of fluorescence with a

short wavelength cut-off in the region of 260 nm, as shown by the unfiltered spectrum in Figure 2A. There were two peaks of emission, at 310–320 nm and ~460 nm, the latter being the higher. The relative spectral response of the detection system tended to underestimate the relative height of the 460 nm peak. Although the pattern of fluorescence from the corneal stroma was similar, the relative magnitude of the two peaks was reversed; this is illustrated in Figure 2B, which shows the unfiltered spectrum for the debrided front surface of the cornea. There was no difference detected in the fluorescence spectra of stroma after either debridement of the epithelium or mechanical anterior keratectomy, and the spectra recorded for the front and the back surfaces of the stroma were similar.

Results for human cornea of the filtered spectra obtained at 2.5 mJ/cm^2 are shown in Figures 3A and 3B, and for unfiltered spectra at 8 mJ/cm^2 in Figures 4A and 4B. Here, unlike in Figure 2, the fluorescence spectra have not been redrawn and are reproduced directly from the recorder traces. The small level of fluctuation and occasional sharp spikes are due to noise in the detection system. These results show that the low-fluence fluorescence response of the human cornea to 193-nm ArF laser radiation closely resembles that of the bovine samples. Both the epithelium and stromal tissue show broad peaks around 310 nm and ~460 nm, with the UV peak being the stronger of the two in stroma.

Figure 5 shows the fluorescence spectrum for human stroma at a fluence of 52 mJ/cm^2, which is close to the 193-nm laser ablation threshold. No visible sign of ablation (eg, plume of vapor or luminous products) was observed at this fluence, although a small degree of material removal did take place. This was evident visually, for example, by the loss of the characteristic fluorescence color of the epithelium as it was removed by ablation. Because of this, only stroma was studied at this higher fluence. Figure 5 shows that the fluorescence emission again extends down to ~260 nm, with broad peaks at ~310 and 460 nm; however, unlike the lower fluence results, the visible peak is the larger of the two.

The temporal evolution of the fluorescence signal was found to be similar to the ArF laser pulse. This is

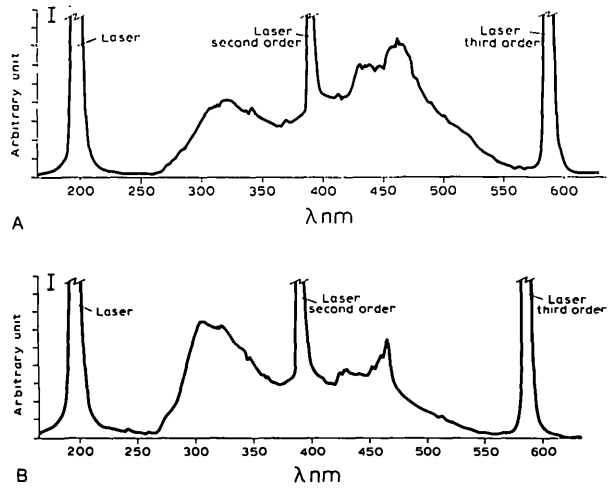

Fig. 2. Fluorescence spectra recorded for bovine samples irradiated at 5 mJ/cm^2 with an ArF excimer laser. (**A**) Epithelium. (**B**) Stroma.

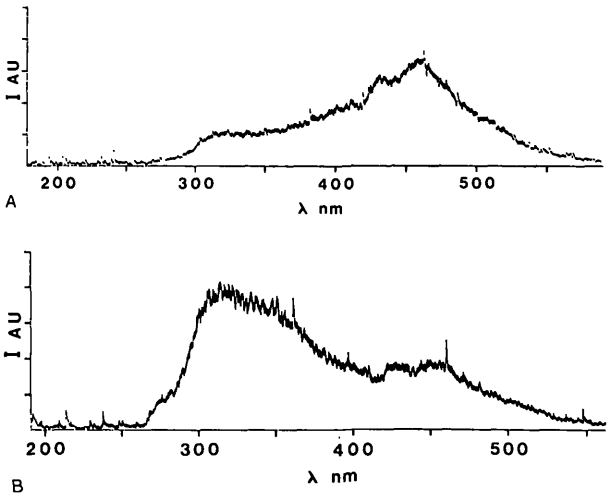

Fig. 3. Fluorescence spectra for human cornea irradiated with an ArF excimer laser at 2.5 mJ/cm^2. A lucite filter was used to remove the scattered laser radiation. (**A**) Epithelium. (**B**) Stroma.

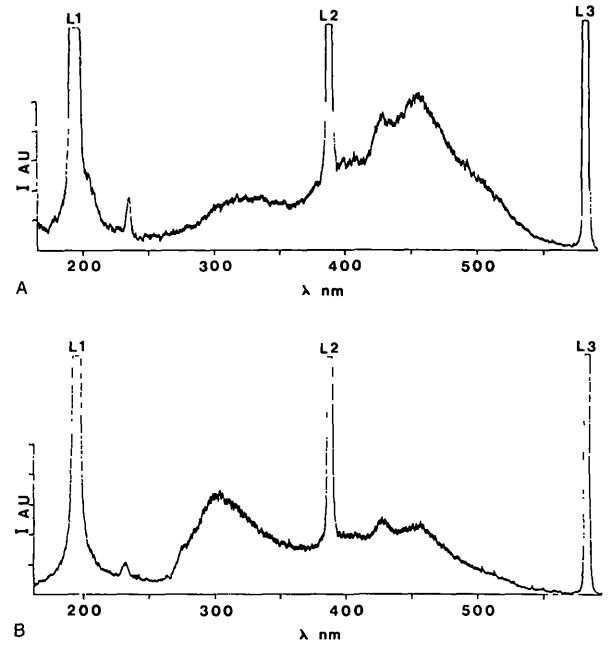

Fig. 4. Fluorescence spectra for human cornea irradiated with an ArF laser at 8 mJ/cm^2. (**A**) Epithelium. (**B**) Stroma.

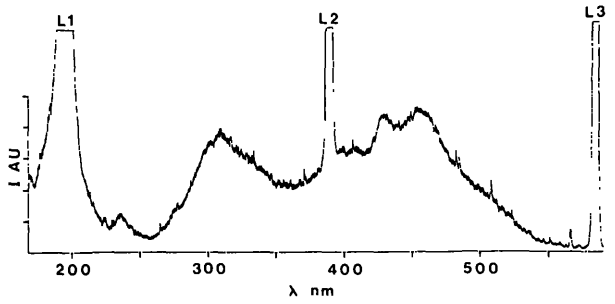

Fig. 5. Fluorescence spectra for human corneal stroma at close to the threshold for ablation (~52 mJ/cm^2).

illustrated by Figures 6A and 6B, showing the laser pulse recorded with the vacuum photodiode, and the fluorescence pulse recorded at 310 nm (within the spectral range of 0.6 nm) with the monochromator–photomultiplier detector. Both have durations of about 15 nsec (full width at half maximum). It also was ascertained by using the monochromator–photomultiplier to record both the fluorescence and laser pulses on separate shots, but with a common synchronization trigger for the oscilloscope, that these signals were essentially co-incident in time (to about 5-nsec uncertainty in synchronization). This behavior was confirmed at several wavelengths in the fluorescence spectrum, although a complete time-resolved analysis was not undertaken.

From a knowledge of the absolute spectral response and gain of the photomultiplier, the monochromator efficiency, and the geometry of the collecting optics, it was possible to estimate the quantum yield for fluorescence in the region around 300 nm. Thus, the radiation power (P_λ) entering the monochromator input slit and falling within the spectral resolution interval, $\Delta\lambda$, around the set wavelength λ, is calculated from:

$$P_\lambda = \frac{V_o}{GS(\lambda)RT(\lambda)}$$

Here V_o is the voltage output, G the photomultiplier gain, $S(\lambda)$ the absolute radiant sensitivity of the photocathode, R the load resistance, and $T(\lambda)$ the monochromator transmission. For the EMI 9813QB photomultiplier $S(\lambda' = 310\ nm) = 62\ mA\ W^{-1}$. The tube gain at the operating voltage of 1500 V is $G \simeq 5 \times 10^5$, so that with $R = 50\ \Omega$ and 60% transmission efficiency for the monochromator:

$$P_{\lambda'} \simeq 1 \times 10^6\ V_o\ W$$

where $\lambda' = 310$ nm.

For a peak output voltage of 300 mV (Fig 6B) at $\lambda' = 310$ nm, the power is 3.2×10^{-7} W, and for a signal duration of 12 nsec (full width at half maximum) the corresponding energy is 3.8×10^{-15} J. This is equivalent to $\sim 6 \times 10^3$ photons at 310 nm falling within the $\Delta\lambda = 0.6$ nm wavelength interval defined by the slit settings on the monochromator.

This signal must now be related to the total energy radiated by the illuminated disc on the cornea. Assuming the fluorescence is isotropic, the fraction, f, of the total power collected by the lens and transmitted through the input slit of the monochromator is:

$$f = \frac{\delta\Omega}{4\pi} \times \frac{4\delta x}{\pi d}$$

Here $\delta\Omega$ is the collection solid angle defined by the lens, and the factor $4\delta x/\pi d$ accounts for the fraction of radiation from the imaged disc of diameter d transmitted through the linear monochromator slit with width δx.

For the geometry used, with $\delta\Omega = 0.022$ sr, $d = 4$ mm, and $\delta x = 100\ \mu$m:

$$f = 5.5 \times 10^{-5}$$

This leads to an estimated total number of emitted photons of:

$$n_{\lambda'} \simeq 6 \times 10^3/f\Delta\lambda \simeq 1.8 \times 10^8\ \text{photons/nm}$$

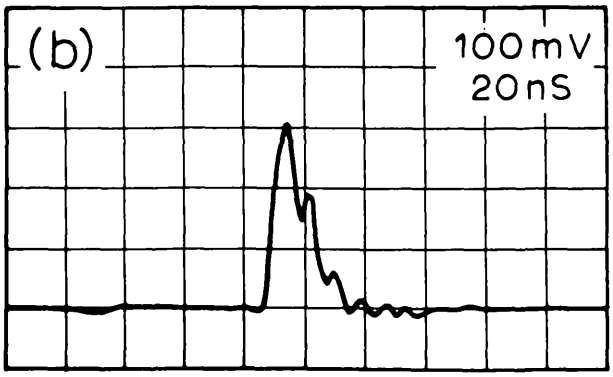

Fig. 6. (A) ArF laser pulse shape. (B) Fluorescence signal recorded at 310 nm with a photomultiplier detector.

The total number of photons radiated within the spectral interval 260–350 nm is then found from:

$$\int_{260\text{ nm}}^{350\text{ nm}} n_{\lambda'} \cdot \frac{I(\lambda)}{I(\lambda')} \cdot \frac{r(\lambda')}{r(\lambda)} d\lambda$$

where $I(\lambda)/I(\lambda')$ describes the recorded fluorescence spectrum suitably normalized at λ', and $r(\lambda)$ the relative spectral response of the detection system (Fig. 1). Approximate evaluation of this integral leads to a total emission of 9×10^9 photons. The number of incident ArF laser photons at 4.5 mJ/cm^2 (total energy 0.56 mJ) is 5.4×10^{14}, so that the net quantum yield is:

$$QY = \frac{\text{emitted photons (260–350 nm)}}{\text{incident ArF laser photons}} \simeq 10^{-5}$$

Consideration of the various uncertainties, particularly those in ascertaining the photomultiplier gain, make this estimate accurate to only one order of magnitude; it serves, however, as a useful baseline for evaluating possible damage effects of corneal fluorescence.

If one considers a 5-mm-diameter ablation of the cornea using 200 pulses at 200 mJ/cm^2 to be a clinically valid regime, an estimate may be made of the dose of fluorescence in the band 260–350 nm that is delivered to the cornea and lens. We calculate that a cell surviving at the very edge of the ablated zone would be exposed to a total fluorescent energy of about 10^{-4} J/cm^2, and that the energy passing through a 4-mm-diameter pupil to the anterior surface of the lens—if absorption by the cornea and aqueous, which would cut off radiation of less than 290 nm, is neglected—would be of the order of 5×10^{-6} J. These calculations, however, assume that the same quantum yield for fluorescence applies at the higher laser fluence level.

Discussion

We have limited this study to corneal ablation at 193 nm because this type of wound provides a suitable template for refractive surgery. The fluorescence associated with the ablation of corneal tissues with the ArF excimer laser has been examined previously by Loree and associates,[14] who noted an abrupt transition at 500 mJ/cm^2 due to the onset of laser-produced plasma. Our experiments have been restricted to much lower fluence levels (<50 mJ/cm^2), at which the ablative removal of tissue is very small, and thus the fluorescence spectra can be attributed to the irradiated tissue site rather than to luminous ablation products or laser-produced plasma.

The low-fluence (<10 mJ/cm^2) spectra for bovine and human cornea exhibit closely similar patterns, with emission as low as ~260 nm (Figs. 2–4). In both cases it has been found that there is a significant difference between stromal and epithelial tissue, which confirms the earlier observation that a difference in the color of the fluorescence may be used to monitor the removal of the epithelium.[4] Resolution of the fluorescence from this thin layer, without interference from the underlying stroma, is possible because of the short absorption depth for the 193-nm excitation wavelength.[2] The altered spectra obtained at higher fluence (Fig. 5) suggests that the fluorescence pattern may be fluence-dependent, possibly because of photoinduced changes in the deeper tissue produced by ultraviolet irradiation; a similar effect has been noted during radiation of arterial tissue.[17] Clearly this effect could be quite strong in the current experiments, since the tissue was exposed to many pulses in order to acquire the full spectral response. However, in practical terms, the spectra should be representative of those accompanying surgical irradiation, in which many pulse exposures would be used.

Time-resolved measurements show that the luminescence occurs in a short (~15 ns) pulse essentially co-incident with the laser. The promptness of this luminescence suggests its origin as singlet–singlet fluorescence as opposed to "phosphorescence" arising from forbidden triplet–singlet transitions, which would be expected to have a much longer time scale. This behavior can be contrasted with the results reported by Loree et al,[14] in which spectral features persisted for several microseconds under high-fluence irradiation. A possible origin of the ~310-nm peak is tyrosine, which exists at low concentrations in collagen and has a short radiative lifetime (~2.6 nsec).[18] Phenylalanine and tryptophan also may contribute in this spectral region. The ~460-nm peak has yet to be assigned.

It has been possible to estimate the quantum yield for UV fluorescence from our data. For the spectral interval 260–350 nm this is, to one order of magnitude, 10^{-5}. This low yield indicates that the principal pathways for the loss of photoexcited states will be internal conversion (to produce heating), intersystem crossing to nonradiative triplet states, or possibly photochemical rearrangements. There is some indirect evidence for the latter, from the change in fluorescence pattern that appears to occur in the transition from low-fluence to threshold-fluence irradiation, and in changes in the effective absorption coefficient at the laser wavelength between ablative and subablative conditions.

Predictions as to the long-term biologic effects of irradiation of tissue with an ArF excimer laser are

based on data gathered from experimental assays with both coherent and incoherent radiation. However, laser-generated photoproducts are not necessarily identical to those of incoherent irradiation; the high irradiance of laser radiation tends to produce single-strand breaks in DNA rather than pyrimidine dimerization.[19,20]

Isolated DNA strongly absorbs radiation at 190 nm,[21-24] and radiation from an ArF laser produces single-strand breaks in aqueous solutions of isolated DNA at a fluence of 50 mJ/cm^2.[25] However, for an intact cell it has been calculated that approximately 66–87% of incident radiation between 193 and 200 nm is absorbed by 1 μm of cytoplasm.[13,26] The shielding by cytoplasmic components, particularly aliphatic amino acids and peptides, attenuates the dose of photons to the centrally placed DNA target material until absorption at the cell surface disrupts the cell membrane and kills the cell.[26-28] The importance of cell size is demonstrated by the effect of synchrotron radiation at 190 nm, which is mutagenic to *Bacillus subtilus* spores[29] but not to yeast cells;[28] thus, the effect on the genetic material of cells larger than yeasts should be insignificant. In mammalian cell c ılture, a cellular interaction at the level of the chromosome (sister chromatid exchange) and a mutagenic effect at one genetic locus has been claimed,[9] but there has been no detectable mutagenic effect when different loci are studied.[8,9,13]

Although the effects of scattered radiation at 193 nm and secondary fluorescence have not been fully examined, yeast cells irradiated on an agar plate exhibit a zone of killing adjacent to the area of ablation.[10] This killing may be the result of heating of the substrate, photoacoustic damage,[16] or the generation of toxic photoproducts and free radicals.[30] However, the observation that some of the cells are photoreactiveable suggests that the site of part of the lethal injury is DNA damage.

The cornea begins to transmit radiation at approximately 295 nm, and there is a broad peak of lens absorption between 300 and 450 nm.[31,32] Wavelengths between 400 and 1400 nm pass to the retina. We note that the fluorescence spectra reported here contain wavelengths considered to be carcinogenic for mammals[33,34] and phototoxic to the lens and retina. However, although the minimum exposure to radiation at 300 nm that will produce transient lenticular opacities in the primate eye is believed to be as low as 0.12 J/cm^2,[35] this is a high figure in comparison to our estimate for the total fluorescence exposure from a clinical treatment. Recommendations for exposure limits to coherent and noncoherent UV radiation at the cornea for 300 nm are in the range 3.0 $\times$ 10^{-3}–1.0 $\times$ 10^{-2} J/cm^2, respectively,[36-39] and thus the phototoxic risk from the quantum yield of fluorescence from corneal ablation appears to be very slight.

Key words: cornea, excimer laser, fluorescence, epithelium, ultraviolet radiation

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