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Subsurface Femtosecond Tissue Alteration: Selectively Photobleaching Macular Degeneration Pigments in Near Retinal Contact

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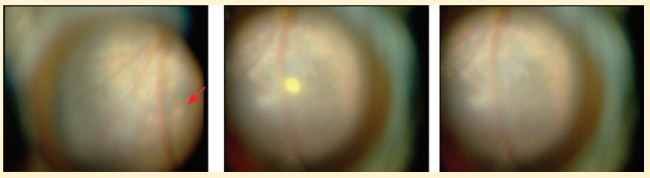
ABSTRACT: This paper uses advances in the ultrafast manipulation of light to address a general need in medicine for a clinical approach that can provide a solution to a variety of disorders requiring subsurface tissue manipulation with ultralow collateral damage. Examples are age-related macular degeneration (AMD), fungal infections, tumors surrounded by overlying tissue, cataracts, etc. Although lasers have revolutionized the use of light in clinical settings, most lasers employed in medicine cannot address such problems of depth-selective tissue manipulation. This arises from the fact that they are mostly based on one photon based laser tissue interactions that provide a cone of excitation where the energy density is sufficiently high to excite heat or fluorescence in the entire cone. Thus, it is difficult to excite a specific depth of a tissue without affecting the overlying surface. However, the advent of femtosecond (fs) lasers has caused a revolution in multiphoton microscopy (Zipfel et al. *Nat. Biotechnol.* **2003**, *21*, 1369−1377; Denk et al. *Science* **1990**, *248*, 73−76) and fabrication (Kawata et al. *Nature* **2001**, *412*, 697−698). With such lasers, the photon energy density is only high enough for multiphoton processes in the focal volume, and this opens a new direction to address subsurface tissue manipulation. Here we show in an AMD animal model, Ccr2 KO knockout mutant mice, noninvasive, selective fs two-photon photobleaching of pigments associated with AMD that accumulate under and in ultraclose proximity to the overlying retina. Pathological evidence is presented that indicates the lack of collateral damage to the overlying retina or other surrounding tissue.



1. INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in the Western World in the elderly, and the pigments that are universally associated with the disease accumulate in droplets called drusen that are behind the retina but in close proximity to it. Our results demonstrate that a femtosecond (fs) laser integrated with a slit lamp, a standard optical instrument widely used in ophthalmology, can be used to photoalter or bleach the pigmented structures called drusen in living Ccr2 KO knockout mutant aged mice exhibiting the hallmarks of the disease including drusen formation with its associated pigments.

Unlike past attempts to use lasers in AMD which have concentrated on the cw argon ion laser for irradiating drusen with collateral damage and associated neovascularization, the basis of our drusen bleaching methodology is the nonlinear optical interactions mediated by use of a fs laser. With such a laser, the energy density in the focal volume can be controlled to be sufficient so that only in the laser focus multiphoton interactions will be excited. Furthermore, the irradiation wavelength used in such multiphoton interactions is generally in the near-red (~800 nm), where, in terms of the perspective of the objectives of this paper, there is little retinal absorption.

The importance of this process was first realized in microscopy[1,2] and has even been used in medical diagnostics.[4] The important contribution of this realization in microscopy is clearly seen in Figure 1 where multiphoton excitation limits the fluorescence of a dye solution in a cuvette even with a low-powered lens to the focal volume, whereas cw laser excitation with the same lens excites fluorescence both above and below the point of focus. For fluorescence microscopy, this has been most beneficial since no bleaching of dye molecules occurs above and below the focus, and they only undergo bleaching in the region of the focal volume which is the region of interest in any laser scan to form an image. In diagnostics this is important since the interactions occur at a specific plane. However, the same principle of depth selectivity could be applied to clinical intervention, and the goal of the present paper is to further explore this potential in an animal model that has been developed for the disease AMD.

In earlier work, our laboratory realized the potential of this depth selectivity for porating cells under the surface of the epidermis of living mice for the effective introduction of naked DNA.[5] The nonlinear interaction in this case was simply selective dielectric breakdown at a specific tissue layer, and the temporary poration of cell membranes similar phenomena can

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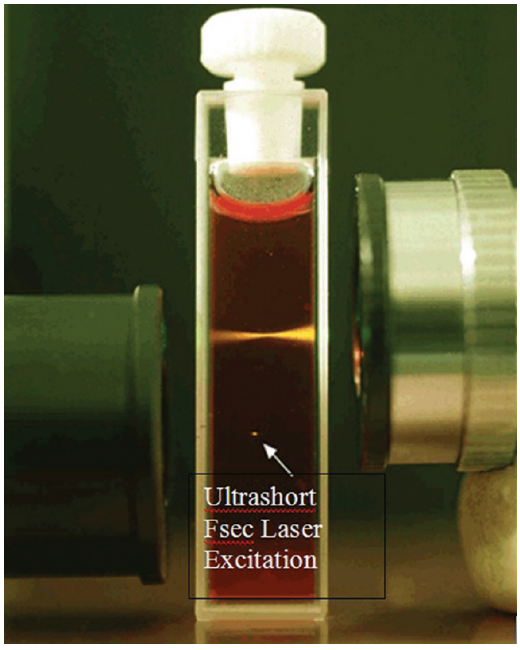

Figure 1. Comparison of "safranin 0" fluorescence emitted by 488 nm argon ion continuous wave (cw) laser excitation (top) with two-photon fluorescence of the same dye solution excited with an ultrashort fs laser (bottom) with a pulse width under 100 fs. Both lasers were focused through a similar lens [Image taken from M. K. Robinson, "Multiphoton microscopy expands its reach," Biophotonics International September/October Issue, 38–45 (1997)].

be used in cataract breakdown. Recently, the utility of this fundamental mechanism was demonstrated using two-photon excitation of newly synthesized two-photon optimized photodynamic therapy dyes to occlude blood vessels in nude mice using the nonlinear process of two-photon absorption.[6] More recently, we have also applied this approach to the elimination of fungal infections below the surface.[7]

The objective of the present study was to investigate whether an ultrafast 200 fs laser could be controlled sufficiently in terms of energy density to selectively bleach drusen in the focal volume of the fs laser without detectable pathological surrounding collateral damage to even closely opposed tissues. The objective was to cause such multiphoton absorption and subsequent bleaching only in the AMD associated fluorescent droplets while leaving the surrounding tissue untouched by the effects of the laser beam. Although the objective in this paper is clearly aimed at drusen bleaching, the results have the more general significance of selective tissue alteration below surfaces whether it be for photoexcitation or destruction, and the level of control gives hope that this technique could have wide implications in clinical medicine.

In terms of drusen, to understand the goals of this paper, one first has to understand the morphological distribution of these lipofuscin deposits. In an excellent review, Rattner and Nathans[8] lay out the structure of the retina and its associated tissues. As they note, the mammalian retina consists of three layers of neurons ~200 μm in thickness. The outermost layer (that is, the layer furthest from the lens of the eye) consists exclusively of photoreceptor cells with the light-absorbing retinal proteins, the visual pigments together with other phototransduction proteins associated within the region of the cell farthest away from the lens. This region of the photoreceptor cell is called the outer segment and is in close contact with a tissue called the retinal pigment epithelium (RPE), which is a monolayer of cells that are specialized to service the adjacent photoreceptors. The RPE, in turn, rests atop a specialized basement "membrane", referred to as Bruch's membrane. The word membrane is used here not as a biophysicist would refer to it but in the classic histological sense to mean an organized layering of extracellular matrix. This membrane in turn sits on a tissue known as the choroid which is at the back of the eye and has a rich complex of blood vessels. Drusen droplets which are always present in all forms of AMD develop in the back of the RPE in close proximity to Bruch's membrane. Thus, any laser intervention that selectively targets drusen and its associated pigments and proteins has to be transmitted through the retina and the RPE without collateral damage to these tissues.

A principal drusen pigment is called A2E. The structure of A2E is seen in Figure 2.[8] A2E is a distinctive molecule. It is a

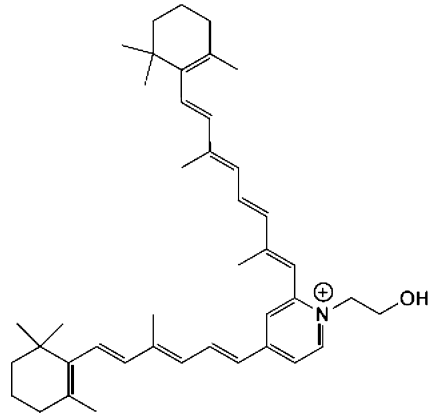

Figure 2. Chemical structure of the pigment bisretinal/ethanolamine adduct (A2E), the main light-absorbing chemical constituent of drusen.

bisretinal/ethanolamine adduct. This pigment is one minor and unavoidable byproduct of the biological retinoid cycle that regenerates part of the visual pigment pool in the retina each day. During this process, pigments that are byproducts of this process accumulate in the RPE in close proximity to Bruch's membrane and in ultra close proximity to the retina.

Although it is not by any means unequivocal that A2E leads to AMD and there is even evidence that in *advanced stages* of the development of the disease one-photon photobleaching of drusen, with its associated collateral damage, has little effect on further progress of the disease, one can say that there is no example of the disease AMD without drusen droplets containing A2E as one of the first visible stages of the disease. Furthermore, in the mutant mice investigated in this paper, it has been shown by laser desorption mass spectrometry that there is A2E in these mutants and not in wild type mice.[9]

In spite of the above, it is important to note that A2E is only one of the many factors that are associated with AMD, and thus our technology, which permits nondestructive and selective photobleaching of A2E in very early stages of AMD, may not be ultimately effective in retarding the disease but could only result in a tool to aid in investigating what such early photobleaching can accomplish in terms of the etiology of the disease.

What is known is that A2E does absorb light in the blue where two-photon excitation at 800 nm should be effective[10] to induce photobleaching of this characteristic yellow pigment.

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This results from the excitation process in the oxygen-rich environment of the eye which results in significant photochemistry that bleaches the natural yellow color of A2E. One scenario in the literature is that the normal light excitation in day to day vision could produce singlet oxygen which could destroy surrounding tissue leading to neovascularization and a wet, vision-destroying, form of AMD that is involved in the late stages of this disease.[11] Although any idea in the origins of AMD is a stimulant for controversy, it has been shown that one form of early onset AMD, called Stargardt's disease, is retarded by strategies to reduce a precursor of A2E.[8] Thus, the ultimate emulation of the results of this initial paper in this area could lead to some AMD relief.

2. EXPERIMENTAL PROCEDURES

All investigations involving animals conformed to the Associate of Research in Vision and Ophthalmology (ARVO) resolutions on the Use of Animals in Research. The animals were fed commercial diets and water ad libitum. Animals were kept in a 12 h light−dark cycle at constant temperature and humidity. The Institutional Animal Welfare Committee (NIH approval number OPRR-A01-5011) approved all animal experiments. All animal experiments were performed according to national regulations and institutional guidelines.

Wild-type C57BL/6 mice were purchased from Jackson Laboratories, and Ccl2−/− (Ccl2 KO) and Ccr2−/− (Ccr2 KO) strains, generated by William A. Kuziel and provided by his group, were backcrossed ten times to C57BL/6 mice and used in this study. All animals were kept in a pathogen-free (SPF) environment. During the experiments, the animals were anaesthetized by injecting imalgene 1000 (ketamine) (Rhone Merieux, 0.1 mL per 100 g of animal's weight, 100 mg/mL). For opening the diaphragm of the animal's eye during the procedure, one drop of mydramide (Fisher, sterile eye drops) and one drop of Efrin-10 (Fisher, sterile ophthalmic solution) were added to the animal's eye. Also, a contact lens was placed on the animal's eye to allow the viewing of the retina. A drop of methylcellulose 2% (oily eye drops) was put between the animal's cornea and the contact lens to improve contact between the lens and the cornea and to prevent drying and possible damage to the anterior tissues of the eye.

After the experiments, the animals were generally sacrificed in a week, and the eyes were taken for pathological examination in the Pathology Division of the Ophthalmology Department in the Hadassah Hebrew University Hospital. The eyes were fixed in 4% buffered formaldehyde for at least 48 h. The tissue was embedded in paraffin and processed. Sections of 3−6 μm thickness were produced and were stained with hematoxylin−eosin.

The fs infrared mode-locked Ti:Sapphire laser (Coherent, Mira 900) beam at 800 nm was delivered to the slit lamp (HAAG − Streit 900BM) via a standard articulated arm equipped with special mirrors designed for ultrashort infrared laser pulses (see photograph in Figure 3). An aiming green laser beam at 532 nm (Lambda Pro) was aligned with the femtosecond infrared laser beam to have a focus in the same point and to be at the same focus with the slit lamp. The aiming beam was focused under the retina of the mouse eye using a contact lens. The exposure time was controlled with a shutter (VS25SIT1) driven with a timer (T132 VNIBLITZ). A pedal switch on the shutter controller allowed for defined exposure parameters of the illuminated area with the infrared laser beam. The laser power was attenuated by a variable neutral density

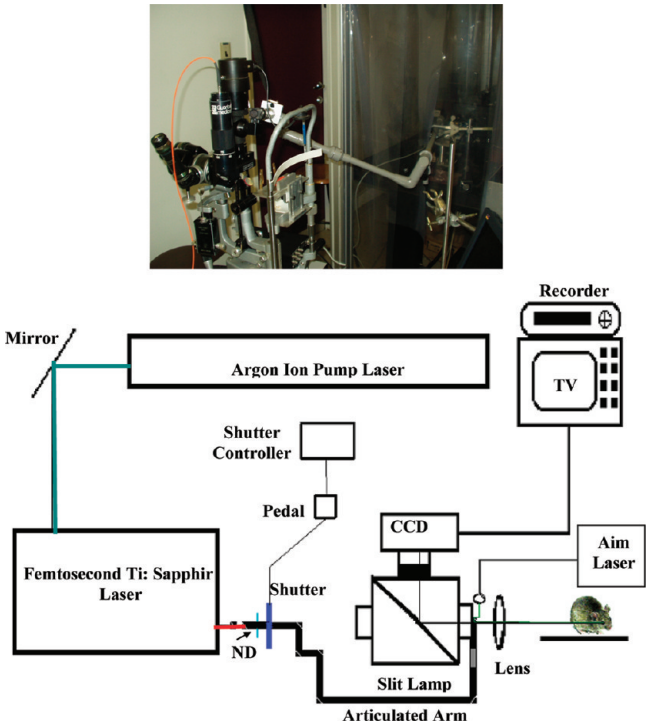

Figure 3. Photograph and diagrammatic representation of the slit lamp coupled femtosecond laser system. The photograph displayed shows the articulated arm for femtosecond beam delivery emanating from the tent built to keep the laser surrounding free of dust. The articulated arm is connected to the slit lamp as described. The red and green colors indicate the femtosecond laser and the argon ion laser, respectively. The acronym ND stands for neutral density filter.

filter. The whole process was monitored and recorded, using a CCD camera attached to the slit lamp and connected to the recording system (Figure 3).

One hundred and fifty mutant mice at the age of ∼10 months were irradiated in one eye using femtosecond laser illumination at an intensity of the laser of 20 mW. This was focused onto a spot on the retina through a slit lamp and a contact lens on the eye of the mouse. The combined optics resulted in an intensity on the retina of 12 mW in a focal spot ∼10 μm. The illumination time was 20 ms on the retinal tissue for the drusen bleaching process with a deposited energy of 0.240 mJ/cm^2. Approximately 50 μm from this irradiated spot, a region of the retina was intentionally damaged with the same intensity but with 5 times the irradiation time (i.e., 100 ms) or for a deposited energy of 1.2 mJ/cm^2. The object was to investigate whether, after such treatment of drusen, there was a loss of the characteristic A2E yellow color. This was observed online with fundus photography. The eye was checked after one week to verify that the drusen disappeared without observable retinal damage. The resulting mice were then sacrificed for pathological studies. As noted above, there were 150 mice that were irradiated, and there was an 80% success rate with the remaining 20% dying before they could be characterized.

3. RESULTS

To demonstrate whether our hypothesis that multiphoton fs photobleaching of drusen was possible without associated collateral damage to the retina, we focused on the first animal model discovered a few years ago which has all the hallmarks of AMD. This animal model was a strain of mice deficient in

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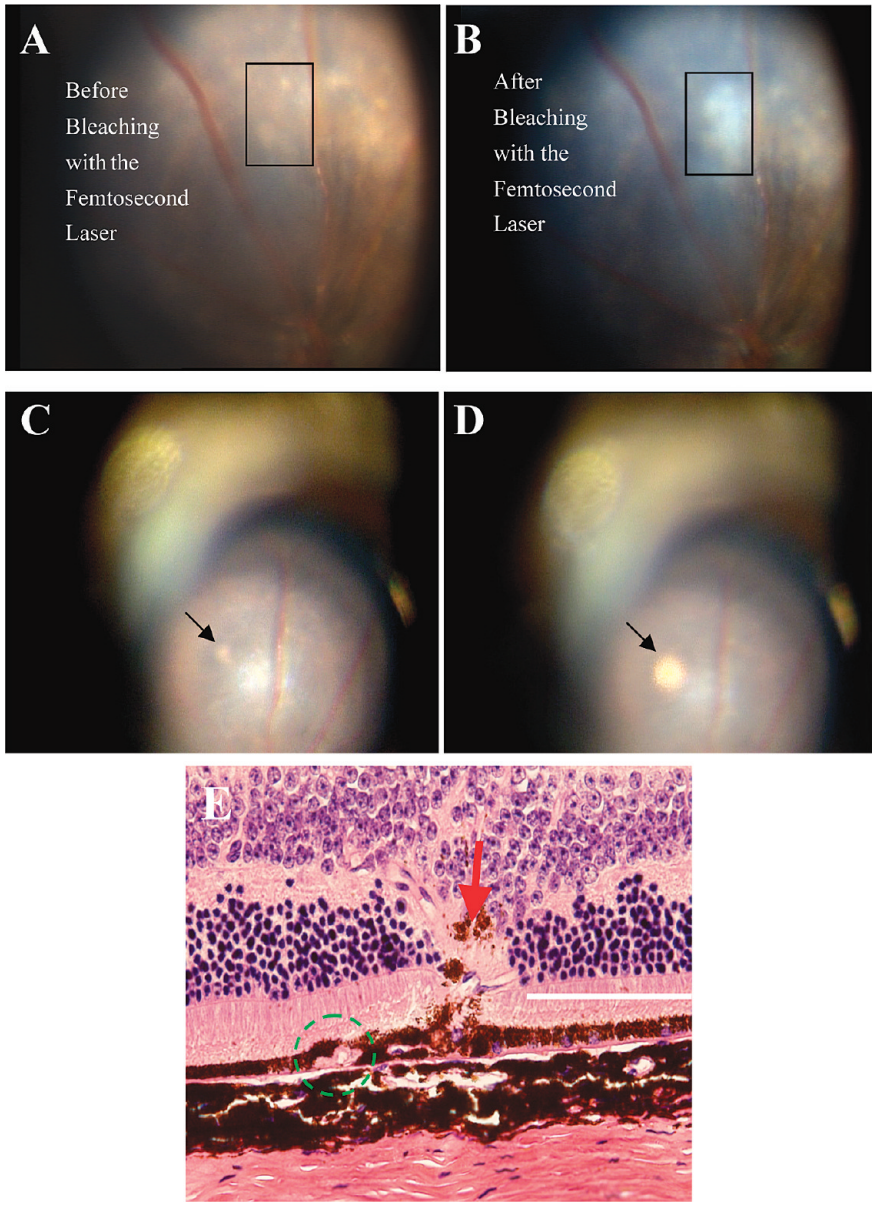

Figure 4. Multiphoton fs photobleaching of drusen. A Ccr2 KO mouse at the age of 10 months as seen with the CCD camera before (A) and after (B) the bleaching process; the bleaching of drusen with a laser wavelength of 800 nm (C) before and (D) during the laser illumination where the yellow emission characteristic of the A2E pigment in drusen is clearly visible. (E) Histological section of the retina of a Ccr2 KO mouse that was exposed with 20 mW for 20 ms at the green circle and 20 mW for 100 ms at the red arrow. In the region of the red arrow, the laser exposure was increased 5 times to cause the detectable damage that is clearly seen. (White scale bar in frame E is 40 μm.) This exposure was used as a mark to locate the region of drusen exposure as indicated by the green circle which corresponded to a drusen droplet [9].

either monocyte chemoattractant protein-1 (Ccl-2) or in its cognate C−C chemokine receptor-2 (Ccr-2).[9] This strain has been shown to accumulate drusen beneath the RPE. This drusen accumulation is especially apparent with age and when these mutant mice are kept in a well-lit environment. The laser system (Figure 3) and the protocols used for the experiments are fully described in the Experimental Procedures section.

Exemplary results obtained on these Ccr2 knockout mutant mice are shown in Figure 4A and 4B. The retinal blood vessels seen in these micrographs are out of focus in the images since the yellow drusen droplets that are to be bleached by the fs laser are just below the retina. Such bleaching is accomplished at a laser power that is an order of magnitude less than the threshold laser power that is required to cause retinal damage (see pathological results described below). As seen in Figure 4B, which was a frame from a video taken during the bleaching process, the yellow color of the drusen has clearly bleached, but edema (a slight swelling of the tissue changing its refractive index) is seen immediately after this irradiation in the regions where the bleaching of the drusen had occurred.

The drusen strongly absorbed the nearly invisible 800 nm light by two-photon absorption from the fs laser. During the irradiation process, a yellow emission was clearly seen from the drusen droplet. This is seen by comparing the results in Figure 4C which was a frame in the video before the laser was shone on the tissue and 4D where the laser is shining on the drusen droplet highlighted in Figure 4C. Notice that during this irradiation the drusen is emitting a yellow fluorescence which has been shown to be characteristic of the A2E pigment in drusen.[10] Of considerable importance is the observation that

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such multiphoton emission leads to photobleaching at a laser power that is an order of magnitude lower than the laser powers needed to affect damage to the overlying retina.

To prove the lack of retinal damage, histological studies were completed. The histology was performed on eyes where bleaching was evident (Figure 4B and 4D) and where, during the irradiation, a strong multiphoton induced photoemission of the A2E pigment was visible through the slit lamp as discussed above. An exemplary histological section is in Figure 4E below.

The green circle indicates the area of drusen bleaching that was accomplished with 5-fold less exposure than the region of the red arrow where the laser power was at a level that ablated the retina. At the red arrow, even the form of the laser beam coming into focus is seen, while in the region of drusen bleaching even the nuclei of the photoreceptors are unperturbed. An estimated number of cells destroyed in the damaged region based on the density of photoreceptor cells in this region is easily estimated by the nuclei of these cells and is in the range of tens of cells. This is further verified by the fact that we have the histological sectioning at a level of 3 μm per section, and this further supports the above number. Thus, the molecular bleaching process leaves no observable pathological collateral damage (certainly <1%) in the region of the bleached droplet in the photoreceptor layer above or in the underlying RPE based on this histological analysis.

In addition to the above, we have obtained a dose response curve for this bleaching process as shown in Figure 5. The form

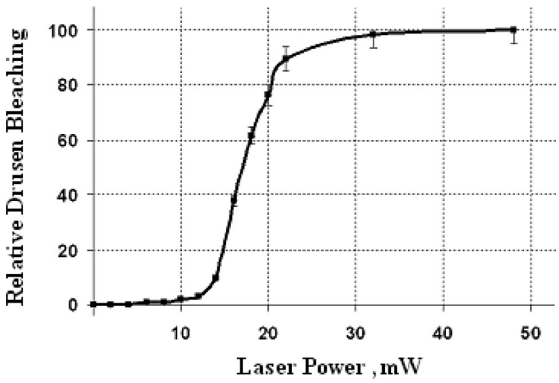

Figure 5. Dose response curve for the bleaching of drusen. 100 on the *y* axis corresponding to complete drusen photobleaching as estimated through observations with the slit lamp and the *x* axis is laser power in mW at the contact lens with a 20 ms exposure time.

of the bleaching as a function of the laser power for the same laser spot size follows a classic threshold process which is very characteristic of such nonlinear processes. There is a minimal threshold of approximately 10 mW where the first signs of drusen bleaching are seen, and then beyond 20 mW saturation is seen for the photobleaching process. At the level of 20 mW, an immediate bleaching was observed. These intensities are laser powers at the contact lens.

Furthermore, we have compared the alteration of a region where there was clearly drusen droplets close to the surface to a region where the concentration of drusen at the surface was significantly lower as visualized through the slit lamp. The results of such studies are shown in Figure 6 as a complete sequence of frames from a video of the bleaching process. To guide the reader, arrows indicating the various blood vessels in the region are marked. The red arrow is placed at the blood vessel closest to the top of the frame and the orange arrow on the blood vessel closest to the bottom of the frame. In Figure 6A, a yellow arrow indicates a region of accumulated drusen droplets as seen by the light of the slit lamp that preferentially highlights the drusen closest to the retina. The white arrow shows a region of lower drusen concentration as visualized with visible light from the slit lamp. The green beam is the aiming beam of a Nd:YAG cw laser.

In Figure 6B, the aiming beam and the laser pulse are irradiating a region just below the retina which is a region rich in drusen. In Figure 6C, this region is shown after bleaching, and one can notice the observed edema in the bleached area. Edema is generally due to a slight swelling of tissue, and this could have occurred due to the drusen absorption in this region depositing some heat. In Figure 6D the aiming beam and the laser pulse are shown on a region which is deficient in drusen. In Figure 6E the aiming green laser is the only laser on and has been moved in the direction of the lower blood vessel, and it is now clear that this drusen-deficient irradiated region shows little or no color change and no edema even though the same illumination powers and times were used. This further supports the assertion above that the edema could be due to a slight heating due to the drusen absorption of this laser in which two photons are exactly within the absorption of A2E.[10]

This sequence clearly indicates the distinction of drusen bleaching as a function of the extent of drusen droplets in the tissue. As indicated above, however, it should be noted that even in a region that is deficient in drusen the tissue is not completely clear of drusen in underlying layers. The near-infrared laser beam however effectively penetrates the tissue and elicits multiphoton emission from whatever pigments that may exist in the region, even those under the surface, and such multiphoton fluorescence emission from A2E is seen in Figure 6D. In this regard it should be indicated that the color change from the slit lamp is a one-photon process where the significant scattering does not allow the light to penetrate deeply to view the effect of the drusen that was bleached at larger distances from the retinal tissue. In fact, even after a five-pulse sequence this region of the eye, which is deficient in drusen, shows little or no color alteration.

4. DISCUSSION

We have shown that it is possible to cause effective photobleaching of drusen containing A2E pigments beneath the retina without causing histological changes in the retina itself and the surrounding tissue. This was indicated by producing a region of retinal damage at 5 times the total fluence within close proximity to the region where the drusen bleaching was affected. Thus, the histology in the photo-bleached area could be directly compared to the histology in the region where damage was intentionally caused. All such results clearly indicated that the laser intensities needed for drusen photobleaching were well below the values required for retinal tissue damage. The histological data (Figure 4E) shown in the Results section are only one example of many such similar results that were obtained on the numerous animals that were treated with this protocol, and as mentioned in the Experimental Procedures section, of the 150 animals treated all 80% of the living animals after the procedure were successful.

The steps of the bleaching process initially show a yellow light emission when the fs laser is irradiating the region where drusen is irradiated. Immediately after the bleaching process, retinal edema is observed in the area where the drusen was

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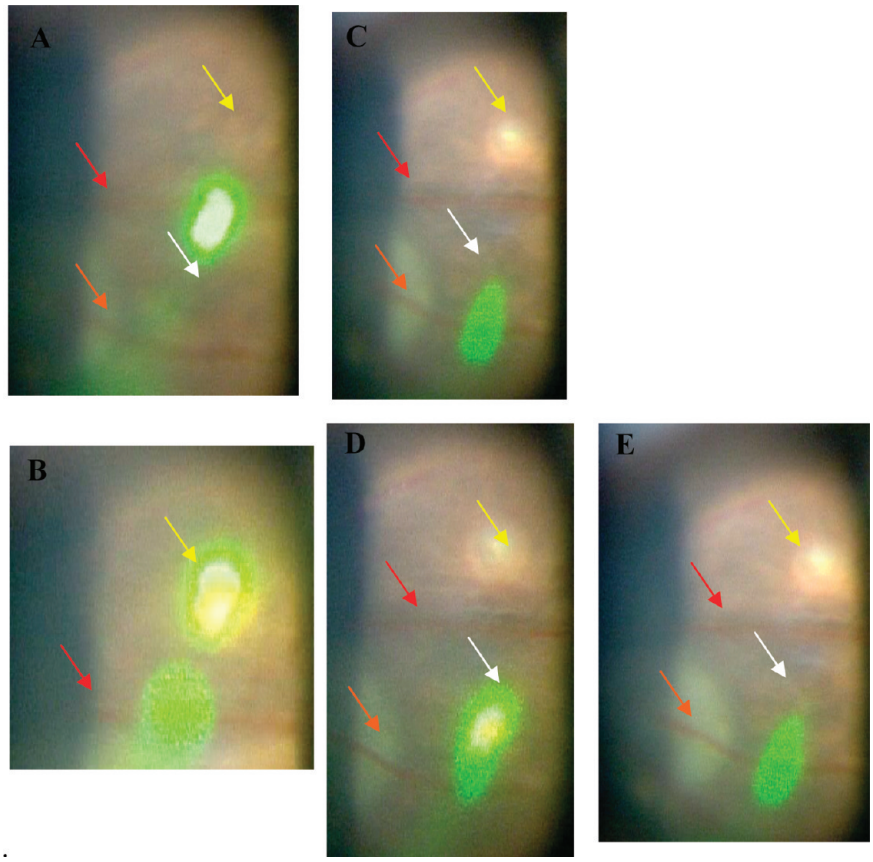

Figure 6. Sequence of frames from a video of the process of bleaching a region where there are drusen droplets as compared to a region where there is a reduced presence of drusen droplets. The red and orange arrows indicate two blood vessels, which will guide the discussion. The yellow arrow indicates the drusen region and the white arrow the region where there is reduced drusen. (A) A frame from the video before the bleaching of drusen. (B) A frame at the point when the fs pulse train was exposing the drusen droplets. (C) The change in color of the drusen after the fs laser exposure. (D) A laser pulse train exposing the region of the retina where there was no detected drusen through the slit lamp. (E) The region in D after the fs pulse train exposure.

bleached. This edema disappears after about one week, and no yellow pigment is seen. Such edema is generally seen only where drusen bleaching is detected. For comparison we have irradiated regions of the same retina with the same irradiation protocol where drusen was not present, and no such edema was detected.

Thus, the experiments reported in this paper suggest that one of the pigments associated with all forms of AMD, A2E, can be selectively photobleached without collateral damage to retinal tissue. Certainly these results cannot be taken to mean that this is in any form a treatment for this complicated disease, but the results do show the extreme precision of the fs laser in affecting such selective tissue alteration around highly sensitive retinal tissue. Further experiments will hopefully show the efficacy of such irradiation on the AMD progress. However, from a physical chemistry perspective we are aware that there are numerous ways to manipulate ultrashort pulses. Thus, this gives us great hope when we see that simply using the energy density of an ultrashort laser pulse one can impress such spatial selectively in tissue alteration. In essence, our results indicate that this form of ultrafast laser clinical intervention, which is virgin territory, should have a very bright future.

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Notes

The authors declare no competing financial interest.

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