Spatial and Spectral Imaging of Retinal Laser Photocoagulation Burns

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PURPOSE. To correlate in vivo spatial and spectral morphologic changes of short- to long-pulse 532 nm Nd:YAG retinal laser lesions using Fourier-domain optical coherence tomography (FD OCT), autofluorescence (AF), fluorescein angiography (FA), and multispectral imaging.

METHODS. Ten eyes with treatment-naive preproliferative or proliferative diabetic retinopathy were studied. A titration grid of laser burns at 20, 100, and 200 milliseconds was applied to the nasal retina and laser fluence titrated to produce four grades of laser lesion visibility: subvisible (SV), barely visible (BV, light-gray), threshold (TH, gray-white), and suprathreshold (ST, white). The AF, FA, FD-OCT, and multispectral imaging were performed 1 week before laser, and 1 hour, 4 weeks, and 3 and 6 months post-laser. Multispectral imaging measured relative tissue oxygen concentration.

RESULTS. Laser burn visibility and lesion size increased in a linear relationship according to fixed fluence levels. At fixed pulse durations, there was a semilogarithmic increase in lesion size over 6 months. At 20 milliseconds, all grades of laser lesion were reduced significantly in size after 6 months: SV, 51%; BV, 54%; TH, 49%; and ST, 50% (P < 0.001), with retinal pigment epithelial proliferation and photoreceptor infilling. At 20 milliseconds, there was healing of photoreceptor inner segment/outer segment junction layers compared with 100- and 200-millisecond lesions. Significant increases in mean tissue oxygenation (range, four to six units) within the laser titration area and in oxygen concentration across the laser lesions (P < 0.01) were detected at 6 months.

CONCLUSIONS. For patients undergoing therapeutic laser, there may be improved tissue oxygenation, higher predictability of burn morphology, and more spatial localization of healing responses of burns at 20 milliseconds compared with longer pulse durations over time. (Invest Ophthalmol Vis Sci. 2011; 52:994–1002) DOI:10.1167/iovs.10-6309

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Laser photocoagulation in diabetic retinopathy has been validated by the Early Treatment Diabetic Retinopathy Study (ETDRS) and the Diabetic Retinopathy Study.1,2 Conventional argon lasers used in clinical practice have included spot sizes 50–500 μm with a range of pulse durations from 20 to 500 milliseconds.2–4

The rationale behind retinal photocoagulation was based on numerous observations from animal laser studies, and histopathological features of argon laser lesions have been correlated with time-domain optical coherence tomography (OCT).5,6 In the last decade, laser burn thresholds have been reevaluated for argon therapy.4,7 To achieve a visible burn, the laser energy pulse initiated at the retinal pigment epithelium (RPE) level produces a thermal temperature rise within the neurosensory retina and change in natural retinal transparency, and hence the illumination light beam will scatter to produce a visible burn.8

The pattern scanning laser (Pascal, Optimédica, Santa Clara, CA) photocoagulator was introduced in 2006 for retinal photocoagulation.9 It semi-automates the procedure using a short-pulse duration (20–30 milliseconds) combined with rapid raster scanning of multiple spots. There may be less outer retina and RPE damage because of less collateral thermal diffusion.10

The extent of tissue damage that is required to generate a therapeutic outcome remains unknown. Marshall and coworkers11 reported histopathological correlations for suprathereshold laser in Macaca mulatta and the human retina that explain the thermal effects of laser at a cellular level. However, conventional laser parameters have been associated with burn expansion and tissue loss over time.12

Newer laser technology has been developed to target the RPE and minimize photoreceptor injury.13–15 Healing responses of short-pulse photocoagulation burns have been demonstrated in animal studies and have recently been shown in vivo for the human retina at different pulse durations and power levels.16,17 To identify a minimally traumatic laser treatment for therapeutic application, it is necessary to know whether laser lesions below the threshold visibility level reduce intraretinal tissue damage over time.

In our study, we used the pattern scanning laser (Pascal) system (532 nm) to examine laser titration burns applied to diabetic retinas. We used Fourier-domain OCT (FD-OCT) and fundus autofluorescence (AF) to noninvasively evaluate laser-tissue interactions at subclinical and low fluence levels. Multispectral imaging and fundus fluorescein angiography (FFA) tests were performed to evaluate alterations in tissue oxygenation and perfusion post-laser. The main objective of this study is to establish in vivo laser-tissue interactions for subvisible/subthreshold, barely visible, threshold, and suprathereshold photocoagulation burns by using spatial and spectral imaging techniques.
MATERIALS AND METHODS

The study protocol and informed consent forms were approved by the Research Ethics Committee. Data and safety monitoring were provided by an independent panel at the University of Manchester and Manchester Royal Eye Hospital. Patients referred by the South Manchester Diabetic Screening Service with PDR or pre-proliferative diabetic retinopathy (PPDR) were prospectively recruited. Informed consent was obtained from all patients before study entry. Ten eyes of seven consecutive patients were studied between September 1, 2009, and April 6, 2010. The study adhered to the tenets of the Declaration of Helsinki, and the study was registered at the Central Manchester University Hospitals NHS Foundation Trust.

All patients underwent slitlamp biomicroscopy and ultra-wide-field FFA (Optos, Dunfermline, Scotland) at baseline. Two masked retina specialists graded baseline angiography images for entry into the study, and additional inclusion criteria included patients older than 18 years of age, glycosylated hemoglobin (HbA1C) level ≤ 10%, no previous laser, intraocular drug therapy, or surgery to the study eye, blood pressure < 180/110, absence of any systemic medication known to be toxic to the retina, and no history of chronic renal failure or renal transplant for diabetic nephropathy.

Safety endpoints included all adverse events reported spontaneously by study participants, elicited by investigators, and observed by investigators. Adverse events were graded as mild, moderate, or severe and were assessed as being either related or unrelated to the laser treatment. As part of the ethical and good clinical practice, we recorded all serious adverse events whether or not they were deemed to be related to the treatment.

Pascal Laser Photocoagulation System

This is a frequency-doubled 532 nm Nd:YAG solid-state laser (Optimedica, Santa Clara, CA) that consists of a modified slit lamp and optical system, which telecentrically images the surface of a multimode step index optical fiber through a two-axis scanner.9,18,19 A graphical user interface allows clinical parameters including spot size, duration, and power output (10–2500 mW) to be controlled, and a foot pedal activates the laser.

LASER TITRATION GRID

A PRP lens (Mainster 165; Ocular Instruments Inc., Bellevue, WA) was used, with spot magnification factor 1.96 and theoretical retinal spot size 392 μm. An area of nasal retina, one disc diameter from the disc margin, was selected for grid placement with burn distribution greater than one-and-half burn widths. The clinical appearance of all laser lesions was graded by a single observer within 60 seconds of delivering the laser pulse by means of the following visual scale: no blanching (subvisible), light gray (barely visible), gray-white (threshold), and white with halo of edema (suprathreshold).

Starting at 100 mW, power was increased in 25 mW steps until a threshold burn was produced. A further 25 mW increment produced suprathreshold lesions. The threshold lesion power setting was then reduced by 25 mW (equivalent to fluence level of 3 J/cm²) to produce a barely visible lesion, and a further 25 mW reduction produced a subvisible lesion. The titration grid comprised three rows of graded intensity burns at 20, 100, and 200 milliseconds (Fig. 1).

Fundus Autofluorescence

Intensities of AF are determined by the quantity and distribution of lipofuscin.20 A fundus flash-camera system (TRC-50DX, type IA; Topcon Instruments, Newbury, UK) was used. The AF exciter filter placed within the illumination path has a central wavelength of 580 nm and bandwidth of 50 nm (60% transmission). The AF barrier filter, placed within the viewing path, has a central wavelength of 695 nm and bandwidth 40 nm. The AF image shows spatial distribution of autofluorescence.

Fourier-Domain Optical Coherence Tomography

Baseline FD-OCT (3D OCT-1000; Topcon Instruments) was performed in the week before treatment.21 The scans were 6 mm in length, and each ETDRS macular grid was placed nasal to the optic disc. A certified and masked ophthalmic photographer captured serial scans at each study visit.

Optos Wide-Field Imaging

The imaging (Optos) utilizes virtual point technology to create a “virtual” focal point located posterior to the patient’s iris plane that enables a panoramic 200° field of view of the retina (P200MA; Optos). Unlike full-spectrum white light used in conventional devices, this technology uses scanning low-powered lasers. This allows review of the retinal substructures in their individual laser separations: green laser (532nm) scans from the sensory retina to the pigment epithelial layers, and red laser (633nm) scans from the retinal pigment epithelium to the choroid. Certified and masked ophthalmic photographers undertook high-resolution color images and FFA (Optomap fx; Optos) of the titration grid.

Measurement of Laser Lesion Size

The greatest linear diameter (GLD) of a laser lesion was measured using horizontal FD-OCT images with Q-factor > 50 (Fig. 2). The lesion was aligned precisely using the fundus photograph on OCT and each section of the laser lesion scanned. The widest diameter passing through the center of the laser lesion was recorded as the GLD. The GLD was recorded for laser burns at 1 hour post-laser and compared with the GLD at 6 months post-laser. The morphology of laser burns was evaluated using FD-OCT scan grayscale image analysis of the titration grid.

Multispectral Imaging

Multispectral imaging was carried out with a modified digital fundus camera system (Dennis J, et al. IOVS 2010;51:ARVO E Abstract 2730) incorporating a 250 W lamp filtered by a fast tunable liquid crystal filter (Varispec VIS 07 to 20 STD; Cambridge Research Instrumentation, Cambridge, UK) and a low-noise peltier-cooled CCD array (Orca C4742-80-12AG; Hamamatsu Photonics, Hamamatsu, Japan) with a spatial resolution of 356 × 256 pixels (with 4 × 4 binning). Images were taken at eight different wavelengths selected according to the
absorption properties of blood components (range, 496–700 nm; total acquisition time, \( \approx 1.7 \) seconds). 22 Images are captured at two isobestic wavelengths (550 and 570 nm), at wavelengths where the ratio between light absorption by oxygenated and deoxygenated hemoglobin is maximal (560, 577, and 610 nm), and at a wavelength above 650 nm, where tissue scattering dominates.

Captured monochromatic images were aligned and corrected for systematic variations in spectral sensitivity. For each pixel in the aligned images of the laser titration area, relative oxygenation was calculated using a Beer-Lambert law model\(^2\) in technical computing language (MATLAB R2008a; Natick, MA). The relative oxygenation values calculated then formed the pixel “gray values” of the monochromatic oxygenation maps. In these oxygenation maps brighter areas (higher “gray values”) represent areas of greater relative oxygenation, and darker areas (lower “gray values”) represent areas of lower relative oxygenation, in arbitrary relative units of oxygenation.

Statistical Analysis

This was a pilot study of 120 laser lesions in 10 eyes. We performed statistical analyses (Statistica version 6; StatSoft Inc., Tulsa, OK). A 2-tailed \( t \)-test was used to explore differences in laser lesion GLD between all 3 groups at 6 months compared with 1 hour. The Mann-Whitney \( U \)-test and two-tailed \( t \)-test was used to evaluate changes in spatial oxygenation. The null hypothesis was rejected for \( P \) values < 0.05.

RESULTS

Eight eyes with PDR and two eyes with PPDR were studied. All patients were Caucasians with light retinal pigmentation. The baseline characteristics included a mean age of 42 years (range 27–63) and average HBA1C of 9.2% (SD 1.1). The laser parameters for all grades of laser lesions are shown in Table 1.

Laser Lesion Appearances at Specified Time Points

One Hour. On color scanning laser ophthalmoscopy (SLO) imaging, the 20-millisecond lesions showed increasing central gray-whitening without any halo effects (Fig. 1). At 100 milliseconds, subvisible and barely visible lesions produced increasing levels of retinal whitening, with a ring of edema above threshold levels. The 200-millisecond subvisible and barely visible lesions had an area of central whitening with a halo of translucent edema that increased above threshold. At threshold and suprathreshold levels, the lesions had a visible central retinal opaqueness and a larger ring of edema. There was a visible increase in grade of color and laser lesion size from subvisible 20-millisecond up to 200-millisecond suprathreshold lesions.

All laser lesions showed lack of AF corresponding to uptake of laser at the 12 laser burn positions (Fig. 3A). The level of hypo-AF increased from subvisible up to suprathreshold levels for all pulse durations. The subvisible laser lesions showed similar areas that lacked AF, and this confirmed outer retina laser uptake with intraretinal masking of the underlying AF signal.

With FD-OCT at 1 hour post-laser, the intensity and width of intraretinal reflectivity for each laser lesion increased according to laser burn visibility (Fig. 4).\(^{15}\) All the subvisible lesions produced a small column of hyper-reflectivity that confirmed laser-tissue interactions. The 100- to 200-millisecond threshold and suprathreshold lesions produced full-thickness intraretinal reflectivity at 1 hour, with no clinical signs of intraretinal or subretinal hemorrhage. All other laser lesions produced vertical bands of moderate to high reflectivity that extended from the internal aspect of the outer highly reflective layer to in-

<table>
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<tr>
<th>Visibility/Threshold</th>
<th>Pulse Duration</th>
<th>Power mW (SD)</th>
<th>Fluence J/cm(^2) (SD)</th>
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<tr>
<td></td>
<td>20 ms</td>
<td>100 ms</td>
<td>200 ms</td>
</tr>
<tr>
<td>Subvisible</td>
<td>370 (11)</td>
<td>125 (0)</td>
<td>100 (0)</td>
</tr>
<tr>
<td>Barely visible</td>
<td>395 (11)</td>
<td>150 (0)</td>
<td>125 (0)</td>
</tr>
<tr>
<td>Threshold</td>
<td>420 (11)</td>
<td>175 (0)</td>
<td>150 (0)</td>
</tr>
<tr>
<td>Supra-threshold</td>
<td>445 (11)</td>
<td>200 (0)</td>
<td>175 (0)</td>
</tr>
<tr>
<td>( P ) value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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* Significance level using two-tailed \( t \)-test.
FD-OCT at 3 months showed that the intraretinal reflectivity had incompletely resolved with reduced hyper-reflectivity persisting within the outer nuclear layer. The higher fluence levels of 100- and 200-millisecond lesions would account for the progressive thickening of the highly reflective layers on FD-OCT, with thermal disruption of the inner segment-outter segment junction of photoreceptors (IS-OS) and RPE layers. The 20-millisecond lesions all remained spatially confined with no signs of adjacent RPE disruption or choroidal window defects.

**Six Months.** At 6 months post-laser, color photography of the laser lesions showed two zones of intraretinal damage with central pigmentation and a surrounding ring of atrophy (Fig. 5A). Twenty millisecond lesions of subvisible, barely visible, and threshold intensity were barely visible on biomicroscopy. The 100- and 200-millisecond subvisible lesions were partially visualized on biomicroscopy. At 20 milliseconds, there was minimal central pigmentation and atrophy. However, all 100- and 200-millisecond lesions showed progressively larger areas of central pigmentation and atrophy (Fig. 5B). Using green-free SLO imaging, the extent and accumulation of central pigment could be accurately determined (Fig. 5B). The area of pigmentation appeared to increase from subvisible up to suprathreshold levels at all pulse durations. The amount of pigmentary changes was minimal ("hypotrophic") for 20-millisecond lesions, and maximal ("hypertrophic") for 200-millisecond lesions.

Over 6 months, the 100- and 200-millisecond lesions developed increasing size of the ring lacking AF that corresponded to photoreceptor/RPE atrophy (Figs. 3C, 3D). At 20 milliseconds, the subvisible and barely visible 20-millisecond lesions showed increased AF across the entire laser burn area, and there were no signs of AF loss at the edges of these lesions.

The intraretinal hyper-reflectivity was reduced in the outer nuclear layer for all lesion grades. At 100 and 200 milliseconds, there was disruption of the outer highly reflective layer at the base of laser lesions, with focal areas of hypo-reflectivity that corresponded to photoreceptor atrophy. The outer retinal layers were visible for all grades of 20-millisecond lesions, with no signs of intraretinal contracture.

**Three Months.** At 20 milliseconds, the subvisible and barely visible burns developed into small, well-localized, and partially pigmented laser lesions on color imaging. There was a reduction in the ratio of central hyper-AF and surrounding hypo-AF at 100 and 200 milliseconds (Figs. 3C, 3D). The 100- and 200-millisecond lesions showed a thick ring lacking AF corresponding to photoreceptor/RPE atrophy. The 20-millisecond threshold and suprathreshold burns developed a thinner outer ring lacking AF. Subvisible and barely visible 20-millisecond lesions remained hyper-AF with no areas lacking AF signal.

**Four Weeks.** The 20-millisecond lesions were partially visible on color imaging, in contrast to the pigmented laser lesions at 100 and 200 milliseconds. There was increased AF within the central part of the lesions, and this corresponded to the areas of pigmentation seen on color and green-free SLO imaging (Fig. 3B). There was a ring lacking AF centered on the lesions at 100 and 200 milliseconds, and this was less pronounced at 20 milliseconds. At 3 months (C) and 6 months (D), the burns at 100 and 200 milliseconds show a thick ring lacking AF with a large central area of hyper-AF. The 20-millisecond threshold and suprathreshold burns have a thinner outer ring lacking AF, with minimal central hyper-AF at 6 months (D).

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**Spatial and Spectral Imaging of Retinal Laser Burns**

**FIGURE 3.** AF photographs of the laser titration grid. At 1 hour (A) there are uniform spots lacking AF that correspond to each laser lesion at 20, 100, and 200 milliseconds. The four clinical grades of laser lesion according to burn visibility and threshold are SV, subvisible; BV, barely visible; TH, threshold; ST, suprathreshold. The SV lesions are barely detectable on AF at all pulse durations, with mild reduction of AF signals at the locations of SV burns. At 4 weeks (B), there are two defined zones of AF change for each laser lesion; there is increased AF within the central part of the lesions, and there is a ring lacking AF centered on the lesions at 100 and 200 milliseconds that is less pronounced at 20 milliseconds. At 3 months (C) and 6 months (D), the burns at 100 and 200 milliseconds show a thick ring lacking AF with a large central area of hyper-AF. The 20-millisecond threshold and suprathreshold burns have a thinner outer ring lacking AF, with minimal central hyper-AF at 6 months (D).

**FIGURE 4.** Horizontal Fourier-domain optical coherence tomography scans at 1 hour through three single rows of laser lesions within the nasal retina. Four vertical bands of increased optical reflectivity within the outer plexiform layer and outer nuclear layer, extending through the junction between the inner segment/outer segments of the photoreceptors (IS-OS) and into the apical surface of the RPE. The laser lesions at (A) 200 milliseconds, (B) 100 milliseconds, and (C) 20 milliseconds are shown. The four clinical grades of laser lesion are marked according to burn visibility and threshold: SV, subvisible; BV, barely visible; TH, threshold; ST, suprathreshold. The laser burns show increasing horizontal and vertical hyper-reflectivity changes ascending from SV to ST lesions. The lesions show a variety of similar reflectivity changes: increased reflectivity at the base of each lesion localized to the IS-OS and RPE of the outer highly reflective layer (HRL), hyporeflective splitting of the outer HRL with compression of the vertical band of reflectivity within the OPL/ONL, and/or a bleb of reduced optical reflectivity is present within the outer HRL, with elevation of the inner aspect and a triangular band of hyper-reflectivity within the OPL/ONL.
Suprathreshold levels at all pulse durations. The amount of pigmentary changes is minimal for 20-millisecond lesions and maximal for 200-millisecond lesions. The laser lesions showed two zones of damage with a central area of hyperpigmentation and a surrounding pale ring of atrophy. All grades of 20-millisecond lesion are partially visible, with minimal pigmentation and atrophy/pallor. A green-free SLO image (Optos) of the subject from (A) taken at 6 months. The area of pigmentation increases from subvisible up to suprathreshold levels at all pulse durations. The amount of pigmentary changes is minimal for 20-millisecond lesions and maximal for 200-millisecond lesions. (C) SLO fundus fluorescein angiogram photograph (Optos) of the subject from (A) is taken at 6 months. A central zone of hypofluorescence is present with a surrounding zone of hyperfluorescence. At 20 milliseconds, all grades of laser lesions showed mild increased fluorescence at the center with a thin ring of hyperfluorescence surrounding the lesions. At 100 and 200 milliseconds, there was a progressive increase in the size of the central hypofluorescence and increase in the size and area of the hyperfluorescent ring surrounding the lesions.

At 100 to 200 milliseconds, there was intraretinal contracture for all lesions and window defects of hyper-reflectivity at the edges of burns (Fig. 6). The window defects increased in signal from barely visible to suprathreshold intensity. These window defects corresponded to tissue atrophy and lack of AF seen at the edges of laser lesions. The central parts of the lesions at 100 to 200 milliseconds showed fusion of the OS and the apical RPE (Figs. 6A, 6B). At 6 months, there was diffuse RPE aggregation and photoreceptor atrophy at the locations of 100- to 200-millisecond laser lesions. There was a proportional increase in fluorescence for 100- to 200-millisecond lesions compared with 20 milliseconds, and this produced greater collateral tissue damage with intraretinal thermal disruption and rings of outer retinal atrophy around these laser lesions.

We observed the subvisible, barely visible, and threshold lesions at 20 milliseconds to remain spatially confined, with no axial or lateral spread to adjacent RPE cells, photoreceptors, or choroid at the 6-month examination (Fig. 6C). The 20-millisecond suprathreshold lesion showed a small central area of hyper-reflectivity localized to the IS-OS and apical RPE that represented RPE aggregation and photoreceptor in-filling. At all grades of 20-millisecond lesions, the outer retinal layers could be visualized. Low fluence levels for 20-millisecond lesions resulted in minimal thermal damage, with restoration and healing of layers. The 20-millisecond subvisible lesions showed complete restoration of the outer highly reflective layers. The RPE layer showed slight thickening at the central nidus of each laser lesion (barely visible, threshold, suprathreshold), with no disruption of the photoreceptor outer segments.

Fluorescein angiography was performed at 6 months post-laser. The laser lesions showed two distinct fluorescence patterns at 6 months compared with baseline (Fig. 5C). A central zone of hypofluorescence was present, with a surrounding zone of hyperfluorescence. At 20 milliseconds, all grades of laser lesions showed mild increased fluorescence at the center with a thin ring of hyperfluorescence. At 100 and 200 milliseconds, there was a progressive increase in the size of the central hypofluorescence and increase in the size of the surrounding hyperfluorescent ring.

The central hypofluorescence correlated with areas of increased pigmentation within outer retina that blocked the underlying fluorescence. The ring of hyperfluorescence corresponded to the atrophic rings on FD-OCT and color imaging and represented increased visualization of underlying fluorescence through atrophic window defects. The ring of hyperfluorescence increased in size from subvisible up to suprathreshold lesions for 100 to 200 milliseconds.

In this study, retinal ischemia was designated by areas of capillary dropout, vascular leakage and vessel wall staining.
multiple intraretinal hemorrhages, number of microaneurysms, and presence of cotton wool spots. The fluorescein angiography at 6 months compared with baseline within the laser-treated sector, demonstrated an overall reduction in extent of vascular leakage and vessel wall staining in seven of eight eyes (two eyes showed no vascular leakage at baseline), reduced numbers of intraretinal hemorrhages and microaneurysms in 9 of 10 eyes, and improved perfusion within areas of capillary dropout in five of six eyes (four eyes showed no capillary dropout in this area at baseline). The improvements in retinal ischemia reflect the increased tissue oxygenation demonstrated within the laser titration area. There were no visible changes of retinal vessel caliber or intravascular fluorescence at 6 months compared with baseline in the nasal quadrant. The expected arteriolar constriction post-laser was not demonstrated in this study, and it would be useful to image different points in the retina and study the change in oxygenation and retinal ischemia after therapeutic retinal laser photocoagulation in a randomized clinical trial.

Retinal Laser Lesion Size

The grade of laser burn visibility and laser lesion size increased in a linear relationship for both power and pulse duration (graph not shown). There was a semilogarithmic increase in lesion size at fixed pulse durations for the different grades of laser lesions (Fig. 7A). The change in laser lesion sizes is shown in Table 2.

After 6 months, the GLD of each laser lesion was evaluated compared with the GLD at 1 hour (Fig. 7B). At 20 milliseconds, all grades of laser lesion reduced significantly in size compared with 1 hour: subvisible, 51%; barely visible, 54%; threshold, 49%; and suprathreshold, 50%; P < 0.001. The threshold and suprathreshold lesions at 100 milliseconds (subvisible, 32%; barely visible, 25%; P < 0.001) and 200 milliseconds (subvisible, 9%; barely visible, 18%; P < 0.001) were reduced to a lesser extent compared with 20 milliseconds. At threshold levels, there was no significant changes in the sizes of 100- and 200-millisecond laser lesions at 6 months (P = 0.06 and 0.16, respectively). The GLDs of suprathreshold 100- and 200-millisecond laser lesions were significantly larger than the predetermined 392 μm spot size at 1 hour and 6 months (P < 0.001).

The "hypotrophic" subvisible 20-millisecond burns reduced in size by an average 51% to 96 μm at 6 months. The reduction in GLD for 20-millisecond laser lesions suggests a healing response as associated with RPE proliferation and restoration of IS-OS layers on FD-OCT. The reduction in sizes of 100- and 200-millisecond laser lesions did not show a healing response, because subvisible and barely visible lesions were associated with both greater photoreceptor atrophy and fusion of outer retinal layers on FD-OCT at 6 months.

Multispectral Imaging

Results are shown in Figure 8, which includes examples of the pixel-by-pixel maps of relative oxygenation concentration in the area of the laser. A significant increase in mean oxygenation of the tissue within the total laser titration area (range, four to six units) was found over the time period from 1 hour to 6 months post-laser (Mann-Whitney U test; P < 0.01). In one case, there was no significant change in tissue oxygenation. Between 1 hour and 6 months post-laser, there was a significant increase in oxygen concentration across the individual areas overlying the laser lesions compared with 1 hour (P < 0.01). No significant differences in change in oxygenation over this time were found between lesions at different pulse duration or visibility/threshold.

To assess within-subject measurement variability over this time period, we compared the relative oxygen saturation within the blood vessels, which we assume to be constant. The mean relative oxygen saturation within the blood vessels increased by two units from 1 hour to 6 months in three patients. After adjustment for this small intravascular measurement variability the changes in tissue oxygenation still remained significant (P < 0.01).

Discussion

This study has demonstrated significant healing responses with reduction in burn size at lower levels of clinical visibility using a 20-millisecond pulse laser. A novel observation was the complete realignment of outer highly reflective layer architecture for subvisible 20-millisecond lesions. Longer pulse duration produced increased disruption of IS-OS and RPE layers, greater perilesional photoreceptor atrophy, and variable changes in lesion size over time. The spatial tissue oxygenation increased significantly after laser photocoagulation of diabetic retinas, and we report an innovative method of noninvasively measuring spatial tissue oxygenation.
All clinical grades of 20-millisecond laser burns reduced on average 49%–54% in size from the 1-hour time point. This significant reduction in size was associated with greater RPE repopulation and photoreceptor in-filling. The 6-month FD-OCT appearances of subthreshold 20-millisecond burns appear similar to selective retinal therapy laser lesions. In this study, we have shown that increasing intensity of 100- and 200-millisecond pulse lesions have a different spatial and spectral natural history compared with 20-millisecond lesions. The 1-hour FD-OCT images of 20-, 100-, and 200-millisecond pulse burns appeared similar, but the temporal laser lesion-tissue interactions were determined by fluence and pulse duration. At 6 months, increasing levels of carbonization, as defined by hyper-pigmentation and hypertrophic tissue scars, were observed for threshold and suprathreshold 100- and 200-millisecond lesions. In contrast, threshold, barely visible, and subvisible lesions showed less carbonization and tissue remodeling.

### Table 2. Actual Values for Each Change in Lesion Size at 1 Hour Compared with 6 Months

<table>
<thead>
<tr>
<th>Pulse Duration</th>
<th>20 ms</th>
<th>100 ms</th>
<th>200 ms</th>
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<td></td>
<td>1 Hour</td>
<td>6 Months</td>
<td>1 Hour</td>
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<tr>
<td>Visibility/Threshold</td>
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<td></td>
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<tr>
<td>Subvisible</td>
<td>194 (11)</td>
<td>96 (11)</td>
<td>251 (7)</td>
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<tr>
<td>Barely visible</td>
<td>310 (10)</td>
<td>144 (9)</td>
<td>360 (6)</td>
</tr>
<tr>
<td>Threshold</td>
<td>345 (7)</td>
<td>176 (10)</td>
<td>408 (8)</td>
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<tr>
<td>Supra-threshold</td>
<td>393 (9)</td>
<td>195 (10)</td>
<td>471 (6)</td>
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</table>

* Significance level using two-tailed t-test.

![Image A](https://example.com/imageA.png)  
![Image B](https://example.com/imageB.png)

**Figure 8.** (A) The pixel-by-pixel maps of relative oxygen concentration in the area of laser at 1 hour (left) and 6 months (right) are shown. In these maps darker areas represent areas of lower oxygen concentration and brighter areas represent areas of higher oxygen concentration. The numbers under each lesion represent (in arbitrary units) the mean oxygen concentration within the region of the lesion. (B) The frequency distribution of relative oxygen concentrations for each pixel in the tissue outside of the lesions in both relative oxygen concentration maps, plotted against tissue oxygenation measured in arbitrary units. The histogram shows a shift in tissue oxygenation post-laser, with global increases in pixel frequencies and units of tissue oxygenation after laser photocoagulation.
ible and subvisible 20-millisecond burns produced sustained healing responses over the long term.

Sramek and coworkers have demonstrated a more even distribution of spatial heat/temperature changes within RPE using smaller spot laser lesions. Laser photocoagulation produces thermal destruction of photoreceptor inner segments that result in improved intraretinal oxygen delivery via increased diffusion of choroid-derived oxygen. The rise in tissue or preretinal PO$_2$ after laser photocoagulation has been demonstrated in animal studies. Subthreshold micropulse laser can produce increased intraretinal oxygen levels and reduced tissue oxygen consumption. During vitreous surgery, Stefansson and co-workers demonstrated significantly increased oxygen tensions over scatter laser-treated areas. In our study, we report for the first time in vivo the healing responses that occur with improved tissue oxygenation at both the location of laser burns and in surrounding retinal tissue using a 20-millisecond laser. The longer pulse durations produced increased spatial oxygenation but resulted in greater collateral tissue damage over time.

At present, there is no precise and quantitative method of noninvasively measuring tissue oxygenation of laser-treated areas in vivo. Our multispectral imager demonstrated an ability to detect small but significant improvements in retinal oxygenation at the locations of laser lesions and in the surrounding tissue over time. These improvements were still apparent after correcting for measurement variability, which suggests that this technique may be of use in monitoring changes in tissue oxygenation over time and post-treatment (laser, intravitreal drugs, vitrectomy). The multispectral findings could be correlated with improvements in angiographic retinal ischemia. Although we were unable to detect any significant differences in oxygenation between individual laser lesions, this may be explained by the small sample size. Another possible explanation could be that more than one process (fluence or pulse duration) has produced the increased oxygenation within the coagulated tissue. Regarding validation with previous animal studies, the shifts in spatial oxygenation observed using our multispectral camera system produced similar oxygenation patterns reported by Zuckerman and coworkers after laser photocoagulation.

The application of this technology to diabetic retinopathy may also include evaluation of tissue oxygenation levels within angiographic tissue infarction that could guide future laser management and perhaps also be used to quantify foveal oxygenation at the locations of laser lesions and in the surrounding tissue over time. Although the multispectral post data from this study are limited, we present the data here to demonstrate the potential of this technique in diabetic patients, and this investigation may have a role in larger therapeutic clinical trials.

For clinical practice, the laser photocoagulation parameters need to be carefully controlled. Laser fluence will determine the intraretinal temperature rise and energy reflected back from the RPE layers. Laser burns at 20 milliseconds have reduced fluence at all clinical grades of burn compared with 100 and 200 milliseconds. Despite lower fluence, all 20-millisecond laser lesions demonstrated effective uptake at the level of the RPE. In vivo, the shrinkage of 20-millisecond laser burns together with reparative structural changes may be explained by a potential heterogeneity in sensitivity of RPE cells to laser injury. At subvisible levels, the maximal reduction in laser lesion and restoration of cellular layers was observed, and this laser intensity was close to the minimum effective dose of laser irradiation used in our study.

A study limitation includes the low time points used for study, with analysis not performed at 1 day or 1 week after laser application. More short-term data may strengthen the observations of laser-tissue interactions. However, we previously studied the OCT and AF findings at 1 week and did not observe any significant differences in outer retina architecture between the 1-hour and 1-week time points, and this study aimed to address the laser-tissue interactions over the longer term. In a recent publication by Kriechbaum et al., the OCT features of laser lesions were analyzed at 1 day. In that study, the laser lesions appear similar in appearance to the 1-hour images we have demonstrated.

A further weakness of this work could be the possibility of an SLO-based AF imaging system producing greater interpretation of the AF results in this study. The current SLO-AF systems use excitation at ~488 nm and barrier filter at ~500 nm. However, the new modified flash system (Topcon TRC-50DX, type IA) wavelength bandwidths used for excitation and barrier filters are closer together, with excitation closer to yellow-orange. The barrier filter now has a longer wavelength bandwidth, compared with the SLO-AF, which straddles wavelengths from precursor fluorophores to improve the AF signal. It could be argued that potential variability exists in laser uptake between each laser burn because of inconsistent aiming beam focus. However, we used FD-OCT to visualize the burns at 1-hour post-treatment, and the visibility of burns correlated with the degree of hyper-reflectivity originating from the RPE layer through retinal layers.

In any eye, there will be variations in fundus pigmentation and melanosome populations. We used the retinal quadrant nasal to the disc margin, because this is recognized to have less variation in pigmentation and is less likely to be affected by difference in ocular axial length. In all 120 laser lesions evaluated, the laser ablation lesions produced similar morphologic and visible changes in retinal tissue using all our imaging modalities. Furthermore, the retinal area nasal to the optic disc did not show any significant alteration in ischemia on FFA, and this would have minimal impact on any oxygenation changes attributed directly to laser lesions. The changes in tissue oxygenation were also validated by comparison with the relative oxygen saturation within the blood vessels, and we did not detect any significant intravascular changes over time.

This study highlights the potential of multispectral imaging as a noninvasive tool to measure spatial tissue oxygenation in diabetic retina. For patients undergoing laser for ischemic and proliferative vascular retinopathy, there may be improved tissue oxygenation, higher predictability of burn morphology, and more spatial localization of healing responses of burns over time using reduced fluence 20-millisecond laser pulses compared with conventional laser pulses.

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References

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