Nanosecond Pulse Lasers for Retinal Applications

**Aim:** To determine the therapeutic range of a new, non-thermal, nanopulse laser and to produce greatest therapeutic range without causing collateral damage in porcine ocular explants.

**Summary Outcome:** Nanosecond pulses can provide a very wide therapeutic range of energies in which the RPE treatment can be performed. At energy levels more than 200 times less than conventional thermal laser produces therapeutic benefit without damage to the neuroretina. The therapeutic range of up to 6:1 produced by the speckle beam profile of the nanosecond laser is wider than previously reported.
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John P.M. Wood, DPhty, Malcolm Plunkett, Victor Previn, BEng, Glyn Chidlow, DPhty, and Robert J. Casson, DPhty, FRANZCO

1South Australian Institute of Ophthalmology, Ophthalmic Research Laboratories, Level 2 Hanson Institute, IMVS, Adelaide, South Australia, Australia
2University of Adelaide, School of Medical Sciences, Adelaide, South Australia, Australia
3Ellex R&D Pty Ltd, Adelaide, South Australia, Australia

INTRODUCTION

Retinal laser photoagulation is the currently accepted clinical strategy for treating such ocular conditions as proliferative diabetic retinopathy (PDR), diabetic macular edema (DME), and central serous retinopathy (CSR); typically utilizing 514 nm continuous wave (CW) argon or frequency doubled 532 nm Nd:YAG lasers with pulse durations in excess of 50 milliseconds [1–5]. The current lasers used for photoagulation are CW, long duration lasers resulting in thermal effect spreading with associated collateral damage. The mechanism of protective action of photoagulative laser treatment remains to be unequivocally elucidated, but suggestions have been made that its influence derives from a wound-healing response which is neuroretinal in origin [6–8]. Many researchers, however, believe that the retinal pigment epithelium (RPE) cell layer is the origin of the protective wound-healing response [9–26]. This is because of their intracellular pigmentary melanosomes which confer upon these cells the ability to absorb up to 50% of any incident laser energy [5]. Most of the laser energy which is absorbed by the melanosomes is converted to heat; often resulting in RPE destruction, and this, in turn, leads to the surrounding cells proliferating and migrating into the lesion sites [21].

It is believed that a reduced conductivity of metabolite flow across Bruch’s Membrane (BM), which separates the RPE from the vascular choroid, underlies several other retinal diseases such as DME, PDR, CSR, and age-related macular degeneration (AMD) [18,27]. The benefit of laser-induced treatment for such diseases is believed to result from a stimulation in production of matrix metalloproteinases (MMPs) from RPE cells, possibly arising from their proliferation; these enzymes are thought to (partially) digest Bruch’s deposits on BM and facilitate an

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*Corresponding to: John P.M. Wood, DPhty, Frome Road, Adelaide, South Australia 5000, Australia.
E-mail: john.wood@health.sa.gov.au
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increased trans-membrane conductivity [28]. Importantly, diseases which are purely neuroretinal in origin, such as retinal vascular occlusions or glaucoma would not benefit from this treatment modality.

Unfortunately, the close apposition of the retina and the RPE means that any thermal laser irradiation absorbed by the latter tissue will almost certainly cause significant photocoagulative damage to retinal photoreceptors via heat diffusion [9,29]. The loss of photoreceptors from thermal laser photocoagulation treatment and the resultant visual field defects, however, are considered to be an acceptable, if possibly undesirable, side-effect when the peripheral retina is treated [30]. With such collateral damage, however, the central retina (macula) cannot be treated. If the beneficial aspects of retinal laser photocoagulation could be harnessed in a more controlled manner, for example, by local and selective targeting of the RPE whilst confining energy to this cell monolayer, it could be possible to apply this therapy to central areas of the retina without collateral damage to other cells and subsequent loss of central vision (e.g., see Dorin [11]).

In 1983 Anderson and Parrish introduced the concept of selective photothermolysis which they defined as the confinement of damage by the employment of “brief laser pulses” [31]. This technique allowed pigmented structures, cells, and organelles to be selectively targeted, according to the laser pulse duration, whilst the actual energetic effects of such short laser pulses were spatially confined. This concept was later applied to retinal treatment by Roeder and Birngruber using a series of high nanosecond and microsecond duration laser pulses aimed at causing a lethal, focal insult to (small groups of) RPE cells with little external heat diffusion [32–37]. While this reduced the collateral damage caused by photocoagulation, the insult to the RPE was still thermal in nature, and very careful laser energy management was therefore required to avoid damaging apposed photoreceptors. An interesting perspective was further presented by Hansen and Fine [38], who concluded that with pulses longer than 4 microseconds, tissue injury would result mainly from thermal coagulation, whereas with shorter, nanosecond pulses, RPE cells would be predominantly injured by localized steam production around endogenous melanin granules. These conclusions were supported by Kelly and Lin [39], who applied 40 picosecond laser pulses (50 mJ/cm²) and were able to show porcine RPE cell death caused by transient intracellular bubble formation around melanosomes. They also showed that transient shock waves occurred around melanosomes, secondary to cavitation events.

These studies have shown that the concept of inducing an injury to the RPE, by transient intracellular microbubble formation resulting from low energy laser pulses, offers many potential advantages for retinal treatment. These include greatly reduced laser treatment fluence, increased treatment fluence range without retinal damage and a reduced dependence on variable fundus pigmentation. In the present study this approach to retinal laser treatment is explored to a much greater degree using 3-nanosecond duration laser pulses. The aim was therefore to determine which parameters of this laser could produce the greatest therapeutic range for RPE treatment without causing collateral damage to photoreceptors.

MATERIALS AND METHODS

Materials
Porcine eyes, from which ocular tissue explants were harvested, were obtained from Big River Pork (Murray Bridge, South Australia) and a local butcher. Petri dishes and 24-well culture plates were from Becton Dickinson (North Ryde, NSW, Australia). All laser systems together with their requisite slit-lamp microscope chin-rests plus microplate adaptor were kindly provided by Ellex Medical Lasers (Adelaide, Australia) and the calcein-AM viability dye was from the Invitrogen-based Molecular Probes Range (Melbourne, Australia). All other general chemicals were from Sigma Chemical Company (Castle Hill, NSW, Australia).

Tissue Harvesting
Tissue samples were prepared from enucleated porcine eyes approximately 2 hours post-mortem; minimum time 1.5 hours, maximum time 2.5 hours. The anterior portion of each eye was removed and the remaining posterior hemisphere, consisting of sclera, choroid, RPE, and retina, dissected into a "malt-seed-cross" formation centering on the optic nerve head region. Segments of approximately 5 mm² and still consisting of all major posterior ocular tissue layers (minus the lens and vitreous humor) were dissected from the region equidistant between the ocular equator and the optic nerve, in phosphate-buffered saline (PBS; 137 mM NaCl, 5.4 mM KCl, 1.28 mM NaH₂PO₄, 7 mM Na₂HPO₄; pH 7.4; 37°C). These "explants" as they were defined, were transferred to fresh, warmed PBS (500 µl) in 24-well culture plates for laser application. Porcine explants were used because the density and location of the fundus pigmentation was similar to that of the human eye.

Treatment of Explants
Culture plates (without covers) containing explants in PBS were placed onto a flat horizontally mounted adaptor platform which was attached to a slit-lamp microscope patient chin rest (see Fig. 1A). The laser light beams were directed and focused onto the surface of the RPE layer in explants in the wells via a 45° mirror attached above the platform on which the plates were set. The mirror also allowed viewing of the explant samples through the associated slit-lamp microscope. Laser pulse energy calibration was carried out using a Moleclectron Energy Max 500 energy meter placed in the laser beam path at the point where it exited from the microscope objective lens. Energy loss from the mirror used to reflect the laser pulse onto the horizontal samples was checked but found to be negligible.

Each explant was subsequently subjected to approximately 10–15 laser exposures. The neuroretina was left in
is hydrolyzed by endogenous esterases to yield fluorescent calcine ($\lambda_{ex}$: 495 nm; $\lambda_{em}$: 515 nm); thus, the presence of green fluorescence within the cytoplasm is a readily visible determination of the viability of the particular cell. Explant minus retina was incubated with calcine-AM dye for 30 minutes before being fresh-mounted without fixation; a standard proprietary "superglue" was used to affix the explant to a glass microscope slide in an attempt to render this tissue as flat in the horizontal plane as possible. The tissue was covered with anti-fade mounting medium (ProLong Gold; Invitrogen, Melbourne, Australia) and a coverslip and subsequently examined under a confocal-style fluorescence microscope. Explants tended not to be completely flat under microscopic examination: a slight undulation across a tissue when mounted meant that it was often difficult to keep a whole examined area uniformly even and focused. Thus, irradiated areas which showed an evenness in microscopic focus were selected for examination.

**Histological Analysis of Explants**

Explants to be subjected to histological analysis were fixed in 10% neutral-buffered formalin and processed for routine paraffin-embedded sections. Explants were orientated such that 5 $\mu$m transverse sections could be cut. Generally, sections were stained for routine histological analysis with hematoxylin and eosin (H&E) using a standard protocol. In some cases, however, sections were processed for basement membrane component labeling with the Gomori methamine silver (GMS) stain, again, using a standard protocol.

**Analysis of Laser-Induced Effects to Explants**

Each laser was tested at a range of energy settings. All irradiated areas on explants were examined and multiple images of representative laser exposure spots were captured for each laser and energy setting using a microscope-mounted digital camera; image dimensions were calibrated using ruler slides and an eyepiece graticule. The irradiated lasered regions of the RPE within the explant were analyzed for a number of parameters: (1) the minimum acute kill threshold, which was defined as the lowest laser energy exposure at which any RPE cell killing within the irradiated area could be discerned above background; (2) the 50% acute kill threshold, which was defined as the energy exposure at which approximately 50% of the RPE cells within the irradiated zone were killed; (3) the collateral kill threshold, defined as the energy level at which either RPE cells could be discerned to have been killed outside the diameter of the laser-irradiated region or at which photoreceptor effects could be noted; (4) the visual effect threshold (VET), defined as the lowest laser energy which elicited a visible effect through the slit lamp during laser irradiation (usually noted as bubble formation).

**Determination of Therapeutic Range Ratio Values**

The threshold values described were obtained as follows: each laser was tested at a range of energy settings. For
each treatment, on an individual explant, at least six images were obtained. Detailed analysis of RPE cells (absolute levels or decreases) in each explant after each laser irradiation was quantified and averaged across the six images for each energy setting. Each test setting for each laser was repeated at least three times on three separate days; overall threshold values were averaged to give the definitive values with which the requisite threshold effect could be observed. If the threshold effect occurred between tested energy intervals, an average value was calculated to define this parameter. When all threshold values had been determined, an "absolute therapeutic range" was determined for each laser; this equaled the difference in determined energy between the VET and the minimum detectable RPE kill threshold. Subsequent to this, the "therapeutic energy range ratio" was also reported for each laser; this was the respective ratio of the same two values.

Lasers Under Investigation

The following experimental frequency-doubled neodymium:yttrium-aluminum-garnet (Nd:YAG) lasers with 532 nm wavelengths (all provided by Ellex R&D Pty Ltd, Adelaide, South Australia) were used:

(1) Three nanoseconds pulse duration, 350 µm diameter spot, fine speckle-beam profile.
(2) Three nanoseconds pulse duration, 400 µm diameter spot, rough gaussian-beam profile.
(3) CW laser, 400 µm diameter spot, flat top beam profile; total beam exposure duration of 100 milliseconds.

Each laser was delivered coaxially along the viewing path of a slit-lamp microscope and neutral density filters were placed in front of the beam, in order to lower the energy level of the laser spot impinging on the cells by known increments if required.

RESULTS

Nanoscond Lasers

Two 3-nanosecond lasers were tested: the first had a fine speckle-beam profile (Fig. 2), and the second, a rough gaussian-beam profile (Fig. 6). The effect of the 3-nanosecond speckle-beam laser on RPE cells in explants and the relationship between the laser and the speckle-beam profile are demonstrated in Figure 2. Two radiant exposures were tested; at the minimum kill threshold (36 mJ/cm²) some RPE cell loss could be noted in the irradiated zone which was largely coincident with the speckle pattern peaks (Fig. 2A), indicating that at this exposure level only the highest speckle peaks were able to directly cause cell death. When the radiant exposure was at approximately twice the minimum kill threshold (69 mJ/cm²), all cells within the irradiated area were lost (Fig. 2B). This was the case notwithstanding the fact that not all individual speckle peaks were above this threshold value and many gaps existed between peaks. In addition, in neither case did any RPE cell death occur as a result of laser irradiation outside of the irradiated zone.

Irradiation of explants over a range of energies was carried out in order to ascertain the desired threshold values for defining use of the laser on cellular systems. Figure 3 shows the effects of the speckle-beam profile laser at a range of increasing energy settings. The normal appearance of the calcin-labeled RPE on the porcine ocular explant is shown in Figure 3A; a clear and relatively non-undulating and homogeneous monolayer of quasi-hexagonal cells was present; a few RPE cells were detected as dead in the preparation (black "gaps" in the RPE sheet; Fig. 3A). Application of laser irradiation at a radiant energy exposure of 27 mJ/cm² had little effect on the viability of cells (Fig. 3B), however, a clear loss of RPE labeling was discerned after applying the laser at 36 mJ/cm² (Fig. 3C), 45 mJ/cm² (Fig. 3D), and 130 mJ/cm² (VET; Fig. 3E). The first radiant energy level tested to show significant RPE killing compared with untreated cells was defined as the minimum acute kill threshold; this was defined to equal approximately 36 ± 0.8 mJ/cm² (Table 1). Furthermore, analysis of images obtained after laser treatment of explants enabled the 50% kill threshold to be defined as 45 ± 1.0 mJ/cm² (Table 1). Much higher
energies caused RPE damage outside of the irradiated zone. The collateral damage threshold value was defined as the energy level at which this phenomenon occurred. This was demarcated as 141 ± 4.5 mJ/cm² and an energy level greater than this is exemplified in Figure 3E (260 mJ/cm²). Photomicrographs shown in Figure 3 up to and including Figure 3E, moreover, demonstrated that at lower energies, even though cells were killed within the irradiated zone, they remained physically intact. Threshold data is summarized in Table 1.

The sequence of example images shown in Figure 4 show the RPE cell killing pattern at exposure levels above (141 mJ/cm²; Fig. 4A and B), below (76 mJ/cm²; Fig. 4E and F), and actually at the VET level (130 mJ/ cm²; Fig. 4C and D) for the 3-nanosecond speckle-beam profile laser. Also shown is the basic impact on the exposed underside of the retina, after peeling this tissue away. At exposure levels below the VET, little change to the retina could be detected (Fig. 4F), even though there was a loss of RPE viability. Furthermore, at the VET a faint but obviously more pronounced indication of the laser exposure area could be seen on the retina (Fig. 4D). However, when the radiant energy level was elevated above the VET (Fig. 4A), a discernible area of retinal damage was observed (Fig. 4B); it was also apparent that some retinal tissue had remained adhered to the RPE after separation of the retina from the explant (Fig. 4A), suggesting a possible thermal coagulative effect. Using these and other images, the collateral damage threshold relating to the retina for the 3-nanosecond laser was defined as 141 ± 4.5 mJ/cm², as documented in Table 1.

Tests were also carried out using a 3-nanosecond laser with a 2145 J/cm²/nm pulse beam profile; the effects of this laser on RPE cells in the explants, as well as the profile itself are shown in Figure 5. It is evident that this laser had a much broader peaked beam profile, with less spiking. This conferred a more homogeneous influence on RPE cells in the explants after irradiation. When the radiant exposure level was raised to just above the VET (240 mJ/cm²; Fig. 5), obvious RPE death could be seen. As with the speckle-beam profile laser, at this energy setting there was marked loss of the RPE cells within the irradiated zone in regions corresponding to the beam profile peak areas (kill threshold, KT; Fig. 6). The radiant exposure thresholds determined for each nanosecond laser at each of the parameters defined previously are summarized in Table 1.

Thermal Lasers

The CW laser was evaluated using an identical experimental technique as per the 3-nanosecond lasers, for accurate comparison. Although the CW laser had an essentially flat top beam profile, the size of the RPE kill area was found to be highly dependant on the radiant exposure level, as shown in Figure 6. The graph in Figure 6 shows the relationship between the observed RPE kill radius and the radiant exposure. As indicated in the graph, it is obvious that the RPE kill radius is absolutely dependent upon the radiant exposure received by the explant, above the minimum kill threshold (intersection of plot line with Y-axis; defined here as 7.958 mJ/cm²). Thus, there is little observed effect on RPE cells below the visible effect threshold, although the lesion size grows rapidly in direct proportion to the irradiation energy applied thereafter. The first visible effect was always a small white lesion in the center of the laser spot, which increased in diameter with higher exposure levels.

The image pairs of Figure 7 show the RPE cell killing pattern at different exposure levels and the impact on the
TABLE 1. Summary of Calculated Threshold Values and Therapeutic Range Ratios for Each Laser Tested

<table>
<thead>
<tr>
<th>Laser type</th>
<th>Beam profile</th>
<th>Pulse duration</th>
<th>Min. kill threshold</th>
<th>50% kill threshold</th>
<th>Collateral kill threshold</th>
<th>Visual effect threshold</th>
<th>Therapeutic range (absolute)</th>
<th>Therapeutic range (ratio)</th>
<th>Absolute safety range</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>Speckle</td>
<td>3 ns</td>
<td>36 ± 0.8</td>
<td>45 ± 1.0</td>
<td>141 ± 4.5</td>
<td>130 ± 2.8</td>
<td>94</td>
<td>3.6:1</td>
<td>11</td>
</tr>
<tr>
<td>NS</td>
<td>Gaussian</td>
<td>3 ns</td>
<td>89 ± 1.3</td>
<td>89 ± 2.0</td>
<td>218 ± 2.3</td>
<td>219 ± 3.6</td>
<td>130</td>
<td>2.5:1</td>
<td>—</td>
</tr>
<tr>
<td>CW</td>
<td>Flat-top</td>
<td>100 ms</td>
<td>7,958 ± 132</td>
<td>17,508 ± 325</td>
<td>20,691 ± 806</td>
<td>10,345 ± 449</td>
<td>2,388</td>
<td>1.3:1</td>
<td>10,345</td>
</tr>
</tbody>
</table>

NB, all values shown in mJ/cm²; NS, nanosecond laser; CW, continuous wave laser.

Therapeutic range ratio defined as minimum visible effect threshold value divided by the minimum detected kill threshold value.

Values obtained by analyzing images taken from laser-irradiated explants. Standard error of the mean (±SEM) is quoted; data harvesting was carried out by testing each laser at discrete energy points and not over a continuous scale of values (n = 8 for all values described).

Fig. 4. Effect of 3-nanosecond laser on porcine RPE explants and influence on aposed retina. These studies were carried out in order to attempt to define the collateral kill threshold, that is, the point at which the effects of the nanosecond laser on the RPE spliced over into an influence on the aposed neural retina. A, C, and E: Effect on RPE; (B, D, and F) effect on aposed retina. A and B: 142 mJ/cm², above the visible effect threshold. The image in (B) shows that at 142 mJ/cm², slight damage to the retina (defined thereafter as the collateral kill threshold) can be discerned; (C and D) 130 mJ/cm², at approximately the visible effect threshold, showing apparent minor effects to the retina; (E and F) 76 mJ/cm², below the visible effect threshold, showing no effect on the retina. Magnification ×200.

aposed underside of the retina, using the thermal CW laser. Below the minimum kill threshold (7,560 mJ/cm²; Fig. 7A for RPE and Fig. 7B for retina) there were no visible effects to either the retina or the RPE. Above the minimum kill threshold (8,277 mJ/cm²; Fig. 7C for RPE and Fig. 7D for retina), there was a central hole in the RPE layer. At this energy level, there was an impression of the laser zone clearly visible on the retina, which likely corresponded to the thermal wave generated by the irradiation. At the VET (10,345 mJ/cm²; Fig. 7E for the RPE and Fig. 7F for the retina) there was an increased demuded area within the RPE, central to the laser spot. Of note was that observation of the retina revealed that there was some apparent thermal fusion of RPE cells to their aposed retinal photoreceptors (Fig. 7F) in a region analogous to the irradiated RPE zone. This was also evidenced by an apparent tearing of RPE cells from BM (Fig. 7E).

Both of these effects were amplified at an energy level which was 50% greater than the VET (15,519 mJ/cm²; Fig. 7G for the RPE and Fig. 7H for the retina). At a much higher radiant exposure level (120% greater than the VET; 22,762 mJ/cm²), there was total RPE-retinal fusion (Fig. 7J), which was underlined by the fact that there were no longer any RPE, either dead or alive within the irradiated RPE layer (Fig. 7I).

Table 1 provides a summary of the lasers and the parameters employed along with the main threshold values experimentally determined and the requisite calculated therapeutic range ratios.

Histological Assessment of Explants After Lasing

Assessment of transverse sections through explants (Fig. 8), revealed that the CW and the 3-nanosecond lasers caused differential effects to the RPE cells. Compared with unlated explants (Fig. 8A and mainly, inset), CW-treated RPE cells were enlarged (Fig. 8B) and stretched in the apical–basal plane, and had been laterally separated from their neighboring cells. This was not the case after irradiation with the 3-nanosecond laser, where little difference could be discerned from the control section in terms of histological tissue alterations (Fig. 8C). There was no collateral damage to the retina after 3-nanosecond laser treatment (Fig. 8C), however,
after CW laser treatment, the retina was displaced away from the RPE and there was the appearance of swelling or edema in the subretinal space at the laser lesion site (Fig. 8C). Analysis of BM revealed that this structure remained intact at the histological level after treatment with either the CW (Fig. 8E) or the 3-nanosecond (Fig. 8F) lasers, as was the case in the unlaunched control tissues (Fig. 8D).

**DISCUSSION**

The present study describes the characterization of a short-pulse (3 nanoseconds), confined, non-conductive thermal, laser system, designed to apply a novel means of therapy to patients suffering relevant retinal disorders. The current lasers used in the clinic to induce photocoagulation are CW, long duration lasers resulting in thermal effects with associated collateral damage. The advantage of the novel 3-nanosecond laser system over the traditional thermal lasers is that the pulsed, very short duration laser effects can be titrated as spatially confined photodisruptors without resultant conductive thermal spread and therefore collateral damage. There is, therefore, far less likelihood of collateral damage to photoreceptors. This theoretically means that such lasers could even be applied within the central regions of the retina without causing direct damage.

Retinal phototoxicity studies have shown that thermal effects (and therefore damage) induced by irradiation with a 532 nm laser will predominate at pulse durations down to approximately 4–20 microseconds [38,40]. Furthermore, data for pulses shorter than 1 nanosecond have indicated that less energy is required for retinal damage than for longer pulses [40]. At pulse durations between approximately 1 nanosecond and 4–20 microseconds, however, the predominant effect will be micro gas bubble formation around melanosomes within RPE cells [41–44]. In these cells, the temporal confinement of energy provides spatial confinement of thermal effects and the resulting intracellular gas bubble production can induce a

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**Fig. 5.** RPE kill pattern after treatment of porcine retinal explants and typical measured beam profile of 3 nanoseconds rough gaussian-beam profile laser. Laser energy used at 240 mJ/cm², which was just above the visible effect threshold in this case (219 mJ/cm²). Magnification ×200.

**Fig. 6.** The relationship between RPE kill radius and radiant exposure for a thermal CW laser (pulse duration 100 milliseconds). Photomicrograph demonstrates the RPE kill pattern after irradiation at approximately the visible effect threshold; the 400 μm laser spot diameter is shown by the red circle and the kill radius at this energy setting as the red dashed line (radiant exposure for explant shown in image, 9,550 mJ/cm²). The graph shows the relationship between the observed RPE kill radius and the radiant exposure. The blue plot line shows the measured RPE kill radius and the purple line the observed visible effect threshold (VET) at the time of laser irradiation (for this laser, 10,346 mJ/cm²). It is obvious that the RPE kill radius is absolutely dependent upon the radiant exposure received by the explant, above the minimum acute kill threshold (intersection of plot line with Y-axis; defined here as 7.956 mJ/cm²). Thus, there is little observed effect on RPE cells below the visible effect threshold. Magnification ×200.
wound to the RPE without collateral damage. The 3-nanosecond pulses produced by lasers 1 and 2 in the present study are within the range required for intracellular micro gas bubble production around RPE melanosomes. Moreover, the speckle-beam profile of laser 1 adds an additional level of spatial confinement, as shown in Figure 2. Not only is confinement provided by the 3-nanosecond pulse duration but also by distributing the energy into a large number of discrete energy peaks, or speckles. This is similar in concept to selective photothermolysis which is applied to skin treatment regimes. The 3-nanosecond lasers utilized in the present study were capable of selectively affecting RPE cells under specific regimes. A similar effect was seen in the study of King et al. [41], who delineated that a nanosecond ND:YAG laser caused porcine RPE to have a decreased pigment content and mis-shaped, non-elliptical melanosomes which were striated and in various stages of maturity, in a manner suggesting that they were regenerating. The latter study demonstrated no collateral damage either, meaning that in both the latter and the current studies, RPE cells could be specifically targeted. The present data, in addition, are in approximate agreement with the studies of Roider and colleagues, who have used 8-nanosecond in comparison with microsecond pulse lasers to selectively ablate RPE cells [34,46,46]. These authors concluded, however, that their nanosecond pulses resulted in a reduced safety range compared with the microsecond lasers in terms of collateral photoreceptor damage; the more favorable outcomes described in the present study may result from the use of shorter 3 nanoseconds pulses, thereby applying reduced energy to the system.

In the present study, at higher energy settings a visible bubble could be observed at the laser-treated site. This is produced when the intracellular micro-bubbles are released and coalesce, with the concurrent production of cavitation forces and a shockwave [37,42,44,45]. The energy level at which bubbles were visible was defined as the visible effect threshold (VET): the setting at which the laser operator could first observe an actual effect. Just above the VET the bubbles often disappeared very quickly, while at higher fluence levels the bubbles often disappeared when the next laser pulse was applied in an adjacent area. It is likely to be possible to use the VET as a useful energy titration marker in the clinic for individual patients. By defining the VET energy level in the peripheral retina the operator would have a known effect versus energy value ratio for the individual patient. This is very important because this would take into account individual factors such as age and ethnicity as well as,.

Fig. 7. The relationship between radiant exposure energy and observed collateral damage to apposed photoreceptors after treatment with thermal CW laser (pulse duration 100 milliseconds). Laser effects to RPE cell layer and to apposed retina are demonstrated for five different energy level exposures. A and B: 7,650 mJ/cm²; at just below the minimum kill threshold (no change). C and D: 8,377 mJ/cm²; at approximately the minimum kill threshold, note loss of labeling but not structural integrity of RPE cells in center of irradiated area and wave imprint effect on corresponding area of the retina. E and F: 10,345 mJ/cm²; at the visible effect threshold, note increased RPE killing zone radius (E) and some apparent thermal fusion of RPE cells to photoreceptors (F). G and H: 15,519 mJ/cm²; at 50% energy above the visible effect threshold, showing approximately 100% RPE cell loss within irradiated region (G) and increased thermal fusion of RPE/retina (H). I and J: 22,762 mJ/cm²; energy level approximately 120% of the value of the visible effect threshold, note that at this energy level, not only have all the RPE cells within the irradiated area been thermally fused to the retinal photoreceptors, but cells outside of this region have also been similarly affected (collateral killing). Magnification ×200.
most importantly, pigment level of the fundus, which would likely be the greatest single factor likely to induce inter-patient variation. Thus, for an individual patient, the laser energy level could be set to the required value, based on the VET value determined in situ, for treatment of the central retina.

From the data obtained herein, a therapeutic range ratio was also calculated. This range was defined for potential clinical use: it represents the energy range over which there is RPE cell killing without surpassing the VET, which, as described, acts as a useful marker energy point above which collateral damage may occur. The greater the ratio value, then, the greater the potential clinical efficacy of the laser: this is obvious since there is a greater energy range at which cell death may be induced below the VET without minor energy adjustments having dramatic effects to the amount of RPE cell death. Furthermore, if an operator can clearly define a top energy value end-point for laser use in the clinic, then they can also have a much clearer understanding of the effect they will get if they reduce the energy below this point by known degrees.

When applying the 3-nanosecond laser (spike-beam), at an energy level just above the minimum kill threshold, there was a visibly incomplete RPE loss pattern (Fig. 2A): the cell loss which was evident corresponded to the laser speckle peaks, as suggested in the figure. Although the speckle peaks are likely to cause micro-bubble formation within corresponding RPE cells leading to their demise, it is also probable that the transient shock wave known to be associated with irradiation of cellular melanosomes and the subsequent cavitation events will cause the death of adjacent cells. When the energy level was elevated to approximately twice minimum kill threshold value there was complete RPE cell loss within the irradiated zone (Fig. 2B) indicating that at this energy level, shock waves accounted for the death of all RPE cells not associated with irradiation by the laser speckle peaks. These data illustrate that the RPE kill pattern within the irradiated zone can potentially vary enormously, depending both on the laser fluence level and the density/profile of the speckle pattern. This shock wave effect is also thought to cause the collateral kill of adjacent RPE cells positioned outside of the irradiated zone (as illustrated in Figs. 3 and 4), a phenomenon only seen when the energy was above the VET level. Note that there is, here, a relatively small safety margin between the VET level and the collateral kill level (see Table 1).

Figures 3 and 4 clearly demonstrate the ability of the 3-nanosecond laser to induce injury to the RPE without collateral damage to the tissue, particularly when combined with a spike-beam profile. Figures 4 and 8 also show that this laser produced little change to the opposed neuroretina below the VET, but at and above this level there
was a more marked effect, albeit without the signs of any gross or obvious tissue lesions. The 3-nanosecond pulse duration and speckle-beam profile can clearly spatially confine micro-bubble formation within the RPE cells, at or below the energy level defined by the VET, causing cell death without transmitting damaging heat to photoreceptors. Above the VET, it was obvious that the diameter of the collateral kill zone within the RPE cell layer increased in proportion to an increased fluence. Furthermore, this effect did not occur when the retina was removed from the explant before laser treatment, and it only occurred above the visible effect threshold, indicating that it was likely related to the cavitation-generated shock wave associated with visible bubble formation.

The rough gaussian beam profile, which was associated with one of the 3-nanosecond lasers relies on much of the applied energy being concentrated in a few large peaks. Figure 5 is a striking example of this effect: at the energy peaks there is obviously a significantly greater level of cell damage than between the energy peaks. This defines the fact that the 3-nanosecond laser used in conjunction with a rough gaussian beam profile is likely to have a far more limited clinical efficacy as compared to the laser with the speckle-beam profile; this is also confirmed in Table 1, where the defined therapeutic range ratio is lower for the former compared with the latter laser.

The effect of the 100 milliseconds laser pulses from the CW laser on the RPE and neuroretina were markedly different from the pulses delivered by the 3ns lasers. Spatial confinement of thermal effects within the RPE cells is not possible with 100 milliseconds pulses, making it very difficult to kill even minimal numbers of RPE cells without causing some thermal damage to the neuroretina. Although the CW laser had a flat top beam profile there are only a relatively small number of non-viable RPE cells at the center of the irradiated zone (Fig. 6) and this is because heat in the periphery has more time to diffuse away into the three-dimensional surrounding structure of the tissue when millisecond pulses are used. As the laser exposure level was increased, the kill diameter increased, as also shown in Figure 6. The consequent thermal build-up at the center of the irradiated area also began to coagulate the adjacent photoreceptors causing them to adhere in some areas to the RPE, in proportion to the amount of energy applied (Fig. 7); this resulted in a visible and permanent retinal lesion (also see Fig. 8). What is interesting is that the results in Figure 7 clearly show that photoagulative damage can occur even below the VET level; the coagulative bonding is sufficiently strong to cause RPE cells to be torn from the choroid, when the layers are separated, illustrating that thermal tissue adherence has resulted (also at the VET; Fig. 8). These data are in agreement with previous data published regarding the effect of thermal photoagulator lasers [6,21,29,32,47,48].

It is interesting to note that a recent study has described the attempt to use a similar laser to treat patients with geographic atrophy (GA) as a result of AMD [49]. The laser used in this previous study was a 527 nm Nd:YLF laser and like one of the 3-nanosecond lasers used in the present study, it had a rough gaussian-beam profile. However, this selective RPE laser treatment failed to slow down the progression of GA. It is possible that this was because of the greater pulse duration than in the present study; the former laser used had a pulse duration of 200 nanoseconds, some 66 times longer than the laser used herein. In addition, as the present data show, the use of the speckle-beam profile extends the range over which RPE cells can be killed without visible collateral damage. This offers the potential for a much greater clinical therapeutic range than gaussian-beam lasers such as the one reported by Praus et al. [49]. Interestingly, a 3-nanosecond speckle-beam laser similar to the one used in the present study has been tested in clinical trials for treatment of DME and it has been shown to be as effective at reducing edema as a CW laser, but at greatly reduced energy levels [50]. Although it is theorized that selective ablation of the RPE, in situ, could have a profound protective effect for other cells within the diseased retina, it must be stated that since this cellular monolayer does have an extremely important set of roles concerned with maintenance and support of the apoposed photoreceptors [51], then the absolute effect of loss of these cells on the retina cannot be absolutely stated as a positive influence, in a clinical setting. As discussed, it is believed that selective ablation of RPE cells can be positive in two ways: first, by actually terminating cells which are compromised by drusen or other basal laminar deposits; these cells being replaced by proliferating or dividing neighbor cells which may have either an increased relative membrane conductance or may have deposits diluted by cell division. Second, the release of factors, such as MMPs from neighboring cells which may help to improve trans-BM conductance, or other growth factors which may improve blood flow or act as trophic support for compromised retinal cells. Obviously, the disadvantages of RPE ablation reside in the loss of function caused by cell removal: for example, reduced maintenance of bi-directional retinal-choroidal nutrient/metabolite flow, loss of physical and trophic support of apoposed photoreceptors which would likely eventually become at least partially dysfunctional in the circumstances, or reduced secretion of extracellular membrane components into the subretinal space. More detailed studies on use of the 3-nanosecond laser for RPE ablation, in situ, would be required to fully delineate the effects on retinal tissue after extended periods of time.

In conclusion, this study has shown that nanosecond duration laser pulses, combined with a speckle-beam profile, produce a degree of spatial confinement for the laser effect within the RPE that cannot be achieved with traditional, thermal photo-coagulator lasers. With this technique it is possible to produce a discreet wound within the RPE which does not damage any adjacent structures, at greatly reduced laser fluence levels. Our definition of the therapeutic range and the relative safety range, for each laser (Table 1) clearly illustrates that the 3-nanosecond lasers produce a far greater useful range than the thermal lasers. Indeed, the 3-nanosecond laser with speckle-beam
profile was able to produce a therapeutic range, under certain conditions, of 3.6:1 (Table 1). Nanosecond pulse lasers may, then, be ideally suited to retinal treatment because of the low radiant exposures required, the lack of thermal damage to the apposed retina, their wide therapeutic range, and their reduced dependence on gross pigmentation.

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