Monitoring Intracellular Cavitation During Selective Laser Targeting of the Retinal Pigment Epithelium

by

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ABSTRACT

Selective destruction of the retinal pigment epithelium (RPE) has important applications in the treatment of a range of macular diseases such as diabetic retinopathy, diabetic macular edema or central serous retinopathy. Laser photocoagulation is the established therapeutic modality for treating these disorders. However, heat diffusion during the long exposure times results in an extended zone of thermal damage that includes the photoreceptors, the RPE, and the choroid layers. Shorter laser pulses can minimize heat diffusion to surrounding tissue by ensuring that the laser energy is thermally confined within the absorbing RPE cells. Our project aims to develop an in vivo irradiation system that employs high-speed scanning of a continuous wave laser beam, corresponding to equivalent µs exposure times, for selective RPE targeting with minimal damage to the overlying photoreceptors.

In order to achieve selectivity in a clinical application, the laser dosage must be precisely determined, since selective RPE lesions are not clinically visible. Rapidly heating the melanosomes creates microscopic bubbles within the RPE cells. This phenomenon, known as cavitation, has been proposed as the mechanism for laser-induced cell damage, and therefore determining the threshold for bubble formation in vivo will be critical. This work focuses on developing a probe system to monitor cavitation, by detecting the backscattered signal of the irradiation laser pulse to obtain information on bubble formation by the melanin microparticles. Laser pulse duration was varied between 3 µs to 50 µs. The fluence threshold for cell death was found to increase with increasing pulse duration (3 → 50 µs) and coincided with the threshold for bubble formation in the RPE for laser pulses up to 20 µs.

The proposed technique of selective laser targeting will be useful for treating RPE-related diseases without incurring the extensive damage associated with the application of conventional laser photoocoagulation.

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INTRODUCTION

Lasers were first used to treat diseases of the eye about 30 years ago when the first clinical devices employing Argon ion lasers were developed to photocoagulate the retina for therapeutic purposes. Laser photocoagulation in the eye has become one of the most widely used laser procedures in medicine and the established modality for the treatment of a variety of previously untreatable retinal disorders such as diabetic retinopathy, retinal detachment, diabetic macular edema and central serous retinopathy [6, 22, 29]. It is also used in the treatment of drusen in early age-related macular degeneration [1] and is also an option for a subset of patients with neovascular AMD [21]. For many patients, laser treatment can prevent vision loss if given in a timely fashion. However, significant peripheral destruction of retinal tissue at the treatment site is a serious side effect. The photocoagulation of the photoreceptors that inevitably results due to the heat diffusion of the laser energy across the retinal layers often leads to permanent vision loss and the creation of blind spots at the treatment sites [32]. Treatment near the fovea is especially dangerous since it can cause foveal burn and permanent loss of central vision. Furthermore, given the significant healing time needed to repair the peripheral damage, it usually takes several weeks before an assessment of successful treatment can be made and very often patients will need more than one treatment in order to prevent further loss of vision.

Certain macular diseases such as drusen in age-related macular degeneration, diabetic macular edema or central serous retinopathy are thought to be associated with a dysfunctional RPE layer. At the therapeutic wavelengths used, the retinal pigment epithelium (RPE) layer is the main absorption site in the retina. Thus, selectively targeting the RPE while sparing the photoreceptors offers considerable advantages over conventional photocoagulation. To minimize heat diffusion from the RPE and achieve selectivity, it is preferable to employ trains of short laser pulses [32, 35] or, alternatively, scan a continuous
wave (cw) beam across the diseased retina fast enough to effectively produce a train of short pulses at each exposed RPE cell [3, 4].

Our group aims to develop a method to selectively target the RPE, based on the scanning approach, with online control of treatment parameters to ensure minimal damage to the overlying photoreceptors. The work is based on the current hypothesis that controlled destruction of the diseased RPE by selective laser targeting will initiate a healing process and lead to closure of the defect by migration and proliferation of healthy neighboring cells and the formation of a new, functional RPE layer. Thus, the proposed technique will be useful for treating RPE-related diseases, including central serous retinopathy, diabetic macular edema, and drusen (in early age-related macular degeneration) without incurring the extensive damage associated with the application of conventional laser photocoagulation.
ANATOMY OF THE EYE

The human eye is a complex anatomical device that is able to refract light and produce a focused image that can stimulate neural responses and enable the sense of sight. The eye is essentially an opaque structure with a thin membrane at the front that makes a transparent opening known as the cornea. The cornea has the dual purpose of protecting the eye and refracting light as it enters the eye. Light from an object enters the eye first through the clear cornea and then through the pupil, the circular opening in the iris. Light that passes through the pupil opening will enter the crystalline lens. The lens is attached to the ciliary muscles which relax and contract in order to change the shape of the lens. By carefully adjusting the lens shape, the muscles assist the eye in the critical task of producing an image on the back of the eyeball. The light is converged by the crystalline lens at a nodal point immediately behind the lens and progresses through the gelatinous vitreous humor back to a clear focus on the retina. The retina contains the rods and cones (about 125 million rods and 6.5 million cones) which serve the task of detecting the intensity and the frequency of the incoming light. These rods and cones send nerve impulses to the brain when stimulated by light. The nerve impulses travel through a network of nerve cells that constitute up to a million neural pathways to the brain. This network of nerve cells is bundled together to form the optic nerve at the back of the eyeball. Electrical signals are then sent along this optic nerve and back to the posterior lobe of the brain, which interprets these electrical signals as visual images [37].
The retina

The retina lies between the vitreous body and the choroid layer at the back of the eye and is a complex network of photosensitive cells and various types of neurons. The ganglion cells, which are the output neurons of the retina, lie innermost in the retina closest to the lens and front of the eye, and the photoreceptors (the rods and cones) lie outermost in the retina against the retinal pigment epithelium and the choroid layer. Light must therefore travel through the thickness of the retina before striking and activating the rods and cones. Subsequently the absorption of photons by the visual pigment of the photoreceptors is translated into first a biochemical message and then an electrical message that can stimulate all the succeeding neurons of the retina. The retinal message corresponding to the light input and some preliminary organization of the visual image into several forms of sensation are transmitted to the brain from the spiking discharge pattern of the ganglion cells [18].

The macula, located in the center of the retina, is where most of the cone cells are located. The fovea, a small depression in the center of the macula, has the highest concentration of...
cone cells. The macula is responsible for central vision, seeing color, and distinguishing fine detail. The outer portion (peripheral retina) is the primary location of rod cells and allows for night vision and seeing movement and objects to the side (i.e., peripheral vision). The underlying epithelial layer of the neural retina is called the retinal pigment epithelium.

**The retinal pigment epithelium**

The retinal pigment epithelium (RPE), a monolayer of cells about 10 µm in size, lies between the sensory retina and the choroid and plays a central role in retinal physiology by forming the outer blood-retinal barrier and supporting the functions of the photoreceptors.

The RPE selectively transports oxygen and nutrients from the choroid to supply the outer third of the retina and removes the waste products of photoreceptor metabolism to be cleared by the choroidal circulation [39]. By virtue of its tight intercellular junctions and by selectively pumping metabolites, the RPE acts as a barrier, preventing access of larger or harmful chemicals to retinal tissue, thereby contributing to the maintenance of a stable and optimal retinal environment.

The RPE cells contain melanosomes, micrometer sized pigment particles, which absorb scattered and excess light that reaches the retina (for visible green wavelengths, up to 50% of all light entering the eye is absorbed by the RPE). It thus protects photoreceptors from photon injury and is thought to function to suppress production of photosensitized molecules, including singlet oxygen and free radicals [2, 8], and to detoxify peroxides [36].

One of the major functions of the RPE is the phagocytosis of the shed disk-membranes of the photoreceptor cells. The outer segment of a photoreceptor cell consists of a stack of such discs containing light-sensitive photopigment. These discs are being continually
formed by the inner segment, then move outwards in the outer segment towards the RPE where they are phagocytosed and their chemical components recycled [8].

The RPE also stores vitamin A, a precursor of the visual pigments, and thus participates in their regeneration as they are all bleached on exposure to light. Additionally, it syntheses glycosaminoglycans for the interphotoreceptor matrix i.e. the material lying between and separating the photoreceptors [27]. Lastly, one of the functions of the RPE is to respond to injury, by migration and proliferation, in order to initiate the wound healing process and the formation of scar tissue in the retina [9, 17, 32].

Figure 2. Light micrograph of a section of the retina
RPE-ASSOCIATED DISEASES OF THE RETINA

Those diseases of the retina which are characterized by leakage of fluid into the retina have been associated with a dysfunctional retinal pigment epithelium since this layer, together with Bruch’s membrane, forms the outer blood-retinal barrier. Disorders that exhibit this pathology include drusen in early age-related macular degeneration, diabetic macular edema and central serous retinopathy.

Age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of severe, irreversible vision loss in the Western world [1]. Up to 13 million Americans are afflicted with AMD. The disease is most commonly seen in people over age 50 and it’s prevalence increases dramatically with age [47]. AMD is a degenerative condition of the macula and as a result leads to deterioration of the central vision.

There are two basic types of age-related macular degeneration – dry (non-neovascular) and wet (neovascular). In the dry type of macular degeneration, the deterioration of the retina is associated with an abnormally functional retinal pigment epithelium and is characterized by the formation of small yellow subretinal pigment epithelial deposits, known as drusen. This phenomenon leads to a thinning and drying out of the macula. The amount of central vision loss is directly related to the location and amount of retinal thinning caused by the drusen.

In the wet type of macular degeneration, abnormal blood vessels (known as subretinal neovascularization) grow under the retina and macula. These new blood vessels may then bleed and leak fluid, thereby causing the macula to bulge or lift up, thus distorting or destroying central vision [1, 47].
Although the dry form of the disease is the most common, most irreversible vision loss is due to the wet form. Approximately 10-20% of patients (about 1.6 million people aged 50 and over in the U.S) develop the wet form which is responsible for approximately 90% of severe vision loss from AMD [1, 47]. Furthermore, a certain percentage of the dry type of macular degeneration turns to the more severe wet type with the passage of time [1].

**Diabetic macular edema**

Diabetic macular edema is encountered in a subset of patients suffering from diabetic retinopathy (both the non-proliferative and proliferative type). Diabetic retinopathy is the leading cause of irreversible visual loss in the working-age population. Up to 10% of all patients with diabetes will develop diabetic macular edema (DME) during their lifetime and up to 75,000 new cases are diagnosed each year. It is caused by the abnormal accumulation of fluid, or swelling, in the macula as a result of a disruption of the blood-retinal barrier. A disruption of the blood-retinal barrier (eg. due to a dysfunctional retinal pigment epithelium) can alter hydrostatic forces and pressure gradients across the barrier and lead to leakage of fluid into the retinal tissue [20, 41].

Up to 30% of patients with clinically significant macular edema (which is characterized by substantial thickening of the retina and formation of hard exudates in the center of the macula) will develop moderate visual loss [48].

**Central Serous Retinopathy**

Central serous retinopathy (CSR) occurs when a defect in the retinal pigment epithelium tight junction allows fluid from the choroid to leak into the subretinal space between Bruch’s membrane and the RPE, causing the retina to swell. Although the exact cause is
unknown, a high level of stress is a major risk factor [38, 46]. CSR typically affects adults between the ages of 20 to 45 and is much more common in men than in women. Although the retinal swelling reduces or distorts vision, the effects are usually temporary. Vision generally recovers without treatment within a few months of the onset of symptoms [12, 16]. However, recurrences are common and can affect 20 to 50 percent of people with CSR. Patients with frequent episodes may experience permanent vision problems, especially those with prolonged detachment or recurrent detachment of the macula (which can lead to permanent loss of central visual acuity). According to the results of a long term study of CSR, about 50% of patients eventually get the more severe form of the disease twelve years after initial clinical diagnosis [12].
CONVENTIONAL TREATMENT OF RPE-ASSOCIATED DISEASE –
ADVANTAGES OF PROPOSED TECHNIQUE

Conventional laser photocoagulation - limitations

Laser photocoagulation is a technique that has been employed by retinal surgeons to treat a variety of retinal conditions such as diabetic retinopathy, diabetic macular edema, retinal tear and detachment and age-related macular degeneration. The therapeutic targets of the laser treatment can vary with the disorder. In diabetic macular edema or drusen, the goal is the photocoagulation of the retinal epithelium layer and the restoration of the blood-retinal barrier through the formation of a new, healthy RPE layer \[10, 35\]. In the treatment of diabetic retinopathy, the therapeutic target is thought to be the oxygen-consuming photoreceptors \[43\]. It has been suggested that the photocoagulation of the RPE and the subsequent production of growth factors such as TGF-\(\beta\) might also play a role in the therapeutic process \[26, 45\]. In treating the wet form of age-related macular degeneration (as one of several experimental therapies), the laser targets the neovascularization and photocoagulates the abnormal blood vessels in an attempt to seal them and prevent further leakage in the retina \[13, 42\].

The pulse durations used in conventional laser photocoagulation are typically in the range 50-200 ms. Therapy is usually delivered with an Argon ion laser. At the wavelength employed (514 nm), up to 50% of the incident laser energy is absorbed by the retinal pigment epithelium (RPE). Significant heat diffusion away from the absorbing RPE during the long exposure time results in an extended zone of thermal denaturation (coagulation) that includes the photoreceptors, the RPE, and the choriocapillaris. The result is loss of vision in the treated area and visible, permanent damage to the retina. Laser treatment in close proximity of the fovea can cause foveal burn and permanent loss of central vision.
while the treatment may also increase chances of subretinal neovascularization and further loss of vision [15, 23]. In most treatments, it usually takes several weeks before knowing if laser surgery has been successful, and many patients will need more than one treatment to control their eye condition in order to prevent further loss of vision.

![Single pulse (100ms)](image)

Figure 3. In conventional laser photocoagulation, heat diffusion during the long pulse leads to damage of the neurosensory retina and the creation of blind spots

**Selective laser targeting – advantages**

Is damage to the sensory retina always necessary for successful treatment of retinal diseases? That is, can we improve treatment by selective laser targeting of the RPE without destroying the adjacent sensory retina?

When treating RPE-associated diseases such as drusen in dry-type age-related macular degeneration, diabetic macular edema or central serous retinopathy, selective targeting of the RPE layer can offer therapeutic advantages. Selectivity is achieved by employing short (μs) pulses that are comparable to the thermal relaxation time of the RPE cell, so that heat diffusion away from the target is minimized. Thermal relaxation time is defined for a spherical absorber as $t_r = d^2/27k$ (where $d$ is the sphere diameter and $k$ is the diffusivity) [5]. Values of $t_r$ are typically ~1μs for a 1μm melanosome and about ~5 μs for RPE cells.
If the duration of the irradiation pulse is of the order of magnitude of the thermal relaxation time of the absorbing RPE cell, then the laser energy is thermally confined within the target layer and damage to surrounding tissue (the sensory retina) can be avoided. It has been shown that by irradiating with a train of repetitive laser pulses that are short enough (on the order of a few $\mu$s), high peak temperatures can be selectively reached in the RPE [31, 32]. At the same time the temperature rise in the surrounding tissue is not significant, thus the photoreceptors are spared from photocoagulation and the retina retains its function [33, 34, 35].

![Repetitive µs-pulses](image)

Figure 4. In selective RPE targeting, employing a train of μs pulses that approximate the thermal relaxation time of the RPE minimizes heat diffusion and temperature increase in adjacent tissue.

A better approach to selective RPE photocoagulation that offers more flexibility than a pulsed laser is to scan a continuous wave laser beam across the diseased retina to effectively produce a train of short pulses at each exposed RPE cell. This approach combines the advantages of repetitive, pulsed irradiation with the practicality of a continuous wave laser source, provided that the equivalent exposure time is as short as the thermal relaxation time. Our group has developed an *in vivo* slit lamp - adapted laser scanner using a two dimensional acousto-optic deflector (AOD) to scan an irradiation beam across the retina. The irradiation beam was produced by a cw diode-pumped, solid-state 5 W Verdi laser that was delivered to the slit lamp by coupling it into a polarization-maintaining single mode
(PM) fiber. The linearly polarized output of the fiber was scanned by the AOD (in a pattern of separated lines or interlaced lines) and focused to a spot diameter of 18.5 μm (depth of focus ±200 μm) onto the eye samples. Selectivity of the scanning system was demonstrated \textit{in vitro} using calcein-stained RPE explants from porcine eyes and \textit{in vivo} using eyes of Dutch belted rabbits [3, 4].

Figure 5. Selectivity of the scanning system demonstrated \textit{in vitro} using calcein-stained porcine RPE explants. Alternating lines of live and dead cells correspond to the applied scanning irradiation pattern [4]

Figure 6. Histologies taken from eyes of Dutch belted rabbits irradiated with the scanning system show damage to the RPE layer but not to the adjacent photoreceptors above it [4]
Online monitoring and control of laser treatment

In conventional laser photocoagulation, treatment endpoint is determined by the appearance of grayish-white lesions in the retina - due to the coagulation of retinal tissue and the alteration of its scattering properties - which are ophthalmoscopically visible. Lesions produced by short laser pulses are not visible by ophthalmoscopic examination (Figure 7A) [4, 35]. Treatment success is currently assessed by fluorescein angiography one hour post-irradiation (Figure 7B). During fluorescein angiography, subjects are injected with a 10% fluorescein solution that can leak from the choroid and into the sub retinal space if the RPE blood-retinal barrier has been compromised, i.e. if the RPE cells at the treatment site have been destroyed [7, 31].

Figure 7. In vivo experiments on Dutch belted rabbit eyes using the scanning irradiation system. Scan patterns corresponding to damaged RPE layer are not ophthalmoscopically visible (A) but appear hyperfluorescent under fluorescein angiography (B) [4]

Monitoring changes in the autofluorescence of the RPE cells during selective cell targeting has been suggested as a possible online control of the treatment [35]. The fluorophore lipofuscin is the principal autofluorescent molecule in the RPE cells. It has been suggested that measuring the decay in autofluorescence of the RPE can provide information on laser-induced damage to the retinal epithelium layer. However, the variation in the
autofluorescence signal has been too high to provide a reliable information as to the viability of the RPE cells during the laser treatment.

Determining damage thresholds indirectly, by irradiating test sites at the periphery of the retina, prior to actual treatment, has been employed in a pilot study looking at selective RPE photocoagulation using a pulsed laser for the treatment of macular diseases [35]. This approach again requires the use of fluorescein angiography that can only be performed at least 1-2 hours following laser exposure. Even though damage thresholds in the macula are lower than in the peripheral retina, this method can provide an estimate of treatment parameters that should be followed to ensure that the photoreceptors are spared any damage. However, when treatment in the crucial macular area is desired, a more exact approach is warranted.

Thus, there is currently no reliable way to determine, during the application of the treatment, whether the intended target (the RPE) is being selectively destroyed or whether the laser parameters are enough to cause any cell death at all. By monitoring cell damage in real time, the parameters of the treatment (fluence, pulse duration/scanning speed) can be adjusted in order to ensure selectivity and efficiency in a clinical application as well as to avoid excessive damage to the photoreceptors.

**Monitoring intracellular cavitation as a way to assess RPE cell death**

Rapid heating of the melanosomes creates microscopic bubbles within the RPE cells that can induce highly localized damage. If the laser pulse duration is equal to, or less than, the thermal relaxation time of the target then the energy can be thermally confined within the target, causing rapid heating of the absorber itself. The extreme temperature rise can induce explosive vaporization of a thin layer of fluid in contact with the particle, raising the vapor
pressure high enough to overcome the surface tension of the fluid and create a bubble that expands rapidly [24]. As the bubble grows further, the vapor cools and condenses, its vapor pressure decreases and as result the bubble cannot sustain itself and collapses [40].

Cavitation, in individual melanosomes and in RPE cells has been observed by stroboscopic (high-speed) imaging [24]. Typical size of cavitation bubbles observed was of the order of a few micrometers, with a lifetime of about 100 ns to 1 μs [24].

![Figure 8. Cavitation in individual melanosomes irradiated with a 30 ps pulse (Bubble formation image taken 2 ns after laser pulse) [24]](image)

![Figure 9. Cavitation in an RPE cell irradiated with a 20 ns pulse (High-speed image of bubble formation taken 500 ns after laser pulse) [19]](image)

If bubble formation is the mechanism of RPE cell death in the microsecond regime, then detecting cavitation in the RPE can be utilized as a feedback control to monitor laser dosimetry in a clinical application. This feedback control will enable adjustment of laser exposure until bubble formation, which leads to cell death in the RPE, is detected and will provide a means to monitor the effectiveness of the treatment as it is delivered in patients.
DEVELOPMENT OF AN \textit{IN VITRO} SYSTEM FOR MONITORING INTRACELLULAR CAVITATION IN THE RPE

\textbf{Detecting bubble formation – Estimating scattering phase function}

Optical imaging of bubble formation in the RPE cannot be practically implemented \textit{in vivo} due to the lack of resolving power. Therefore indirect methods of obtaining the required information such as optoacoustic or interferometric monitoring have to be considered. Since the air-water interface at the edge of a bubble forming in water is expected to show increased optical scattering, it was decided that a scattering detection setup could be designed in order to attempt detection of laser-induced cavitation in the RPE. The scattering phase function for a growing bubble was calculated, based on Mie's scattering theory [28], to determine the fraction of an incident probe beam that is backscattered at the target tissue:

\[
\hat{f}_{\text{Mie}}(\theta) = \frac{S_1 S_1^* + S_2 S_2^*}{2\pi \int [S_1 S_1^* + S_2 S_2^*] \sin \theta d\theta},
\]

where

\[
S_1(\theta) = \sum_{n=1}^{\infty} \frac{2n+1}{n(n+1)} \left\{ a_n \frac{P_n^1(\cos \theta)}{\sin \theta} + b_n \frac{d}{d\theta} P_n^1(\cos \theta) \right\},
\]

\[
S_2(\theta) = \sum_{n=1}^{\infty} \frac{2n+1}{n(n+1)} \left\{ b_n \frac{P_n^1(\cos \theta)}{\sin \theta} + a_n \frac{d}{d\theta} P_n^1(\cos \theta) \right\}.
\]

\(P_n^1(\cos \theta)\) are the associated Legendre functions, \(a_n\) and \(b_n\) are the coefficients of the Mie series (which depend on the wavelength, \(\lambda\), the size of the scatterer, and the relative refractive index \(n\)). In the simulations, the following optical properties of the growing, non-absorbing bubble were used: \(\lambda = 532\ \text{nm}, n=1/1.33\), and the size of the bubble was varied from 1 \(\mu\text{m}\) to 5 \(\mu\text{m}\) (Figures 10 and 11).
Figure 10. Polar plot of the scattering phase function of a 10 μm probe beam (λ=532 nm) incident on a growing air bubble

Figure 11. As above, polar plot of scattering phase function (with expanded axes)
To estimate the fraction of incident light backscattered within the field of view of our detector, the following formula was used:

\[
\frac{S_{\text{backscat}}}{S_{\text{total}}} = \frac{A_{\text{bubble}}}{A_{\text{probe}}} \cdot Q_{\text{scat}}
\]

where \( A_{\text{bubble}} \) and \( A_{\text{probe}} \) are the cross sectional area of the bubble and the probe beam, respectively. \( Q_{\text{scat}} \) is the scattering coefficient, defined as the ratio of the scattering cross section to the geometric cross section. \( S_{\text{total}} \) is the total area under the \( f_{\text{Mie}}(\theta) \) curve and \( S_{\text{backscat}} \) the area under \( f_{\text{Mie}}(\theta) \) within the specified angles. Low numerical aperture (NA of 0.1 and 0.05) detection was assumed to more closely approximate conditions in the human eye. At \( \lambda = 532 \) nm, approximately 0.001% of a 10 \( \mu \)m laser beam incident on a 5 \( \mu \)m bubble can be sampled at the detector (Figure 12). Since this fraction is extremely low, it was decided to attempt detection of the backscattered signal from the higher power irradiation pulse itself, rather than from a separate, low power probe beam. An in vitro pump-probe system was previously explored in our laboratory that employed a separate diode laser probe beam to detect bubble formation in RPE cells irradiated by nanosecond (12 ns) and microsecond (6 \( \mu \)s) pulses [30]. However, since the ultimate goal of the project was to incorporate bubble detection in the in vivo scanning laser system, employing the backscattered signal from the irradiation beam itself would be preferable to using separate pump and probe (which would have to be scanned together with the moving irradiation beam). Furthermore, since the bubble forms before the end of the pulse, using the scattered signal from the pulse itself ensures that the cavitation event will not be missed by the probe.
Figure 12. Calculation, based on Mie scattering theory, of the fraction of light incident on a growing air bubble that is backscattered at NA=0.1 and 0.05 (f/5 and f/10 respectively)

**Experimental setup**

To initially study the feasibility of using the irradiation beam itself for online monitoring, as indicated by the Mie scattering calculations, a pulsed system was set up to detect bubble formation in RPE cells *in vitro* during irradiation with microsecond pulses.
Irradiation was performed by utilizing an acousto-optic modulator (AOM), coupled to a function generator, in order to chop microsecond pulses from a diode-pumped, solid state cw 10W Verdi laser ($\lambda=532$ nm). The diffracted first order beam was focused by a 10x infinity-corrected objective ($NA_{eff}=0.028$) to a 12 $\mu$m spot on the RPE sample so that one cell at a time could be irradiated with a single pulse. The low numerical aperture was used to match the optics of the eye and the \textit{in vivo} scanner setup.

The diameter of the irradiation beam at the focus was determined using the knife-edge method. This was achieved by mounting a 200 $\mu$m slit on a rotating wheel at the focal plane and measuring the rise time ($10\%$ - $90\%$) of the signal detected by a photodiode placed...
behind the wheel, as the slit cut through the irradiation beam. By multiplying the measured rise time with the known velocity of the slit edge, the $1/e^2$ spot diameter could be calculated:

\[
\frac{90\%}{10\%} = \tau \text{ (rise time)}
\]

The backscattered fraction of the irradiation beam incident on the RPE samples that was collected by the objective was confocally detected by a 10 MHz-bandwidth avalanche photodiode (APD) and the signal waveform was displayed on a 500 MHz digital oscilloscope.

Separation of the incident forward and the backscattered signal was achieved by placing a polarizing beam splitter cube and a quarter waveplate in the optical path between the AOM and the objective. The linearly polarized incident beam passing through the beam splitter cube was converted into circularly polarized light by the quarter waveplate and then focused by the objective on the RPE sample. The returning signal going back through the waveplate was converted back into linearly polarized light (at 90° to the incident polarization) enabling it to be deflected by the beam splitter cube to the photodiode. The signal was then focused by a 125 mm focal length lens onto the APD (detector diameter=1.5 mm). Confocal detection was needed in order to reject the bulk of the signal, which was mostly due to air-water interface above the tissue explant and not from the RPE layer itself. To check confocality of the system, a mirror was placed at the focal plane and moved along the z-axis while the signal reaching the detector was recorded (Figure 14).
The RPE explants used in the *in vitro* experiments were prepared from fresh bovine eyes. The eyes were cut equatorially and the vitreous humor was removed. Several explants were cut from each eye and then their retinal layer was carefully peeled off so that the RPE layer was exposed. The RPE explants were incubated for 20 minutes with calcein-AM (1-4 \( \mu \text{g/ml} \)) to stain viable cells and were then placed in a chamber containing buffer solution to be irradiated. The laser pulse duration was varied between 3 \( \mu \text{s} \) to 50 \( \mu \text{s} \) and for each pulse length the laser fluence was varied in order to determine the thresholds for cavitation and cell death.

A cw Argon ion laser at a wavelength of 488 nm was incorporated into the setup to excite calcein fluorescence that was used to assay viability of the RPE cells following treatment. RPE cells that remained viable appeared fluorescent when excited while cells that lost viability where identified by loss of calcein fluorescence. The fluorescence images taken before and after irradiation were collected by a CCD connected to a PC that was equipped with a frame-grabber board and were then displayed on a monitor. For every pulse
applied, the backscattered signal recorded on the oscilloscope and the fluorescent image captured by the CCD were matched and stored for analysis.

**In vitro results**

Representative examples of the backscattered signals are shown in Figures 15-17 together with images of calcein fluorescence in RPE cells before and after laser pulse. For each pulse duration, about 120-150 cells were analyzed. Thresholds for cavitation and cell death were determined by varying the laser fluence. The recorded backscattered signal and fluorescence images for each cell were correlated and verified independently by two observers. In the waveform images, baseline signal represents no pulse. The rectangular pedestal waveform is produced as the irradiation pulse is turned on and is back scattered by the RPE tissue. The spike occuring on the top of the pulse signal represents increased scattering due to formation of a bubble. At threshold levels, the bubble forms towards the end of the pulse as enough energy is eventually pumped into the RPE to initiate cavitation.

![Waveform image](image.png)

Figure 15. Backscattered signal from RPE cell irradiated with a 3 μs pulse, λ=532 nm, Fluence = 165 mJ/cm² (~10% above threshold).
Figure 16. Backscattered signal from RPE cell irradiated with a 3 μs pulse, $\lambda=532$ nm, Fluence = 180 mJ/cm$^2$ (~20% above threshold). At fluences much higher than threshold, bubble formation occurs earlier during the pulse.

Figure 17. Backscattered signal from RPE cell irradiated with a 50 μs pulse, $\lambda=532$ nm, Fluence = 550 mJ/cm$^2$ (0% above threshold)

As expected, the ED$_{50}$ threshold for bubble formation was found to increase with increasing pulse duration (3 -> 50 μs). As the pulse duration becomes greater than the thermal relaxation time of the RPE melanosomes (ie. >1 μs), there is increased thermal energy diffusion away from the microabsorbers and thus progressively higher fluences are needed to achieve the same peak temperatures and initiate the cavitation process. The ED$_{50}$
threshold for bubble formation at the 3 μs pulse was measured to be 155 mJ/cm² and varied up to 537 mJ/cm² for the longest pulse (50 μs) studied (Figure 18).

The threshold for cell lethality was found to be coincide with the threshold for bubble formation in the RPE for laser pulses up to 20 μs (Figure 19). That is, the fluence needed to initiate cavitation in the melanosome-rich RPE and the fluence necessary to cause RPE cell death are identical for the shorter pulse durations. This indicates that the mechanism of cell damage in this time regime is photomechanical in nature (cavitation) and not photothermal. As the pulse duration becomes greater than the thermal relaxation of the RPE melanosomes (~1 μs), heat diffusion away from the absorbing melanosomes becomes significant and thus the mechanism of cell damage undergoes a transition from a purely mechanical to a more thermal one [11]. At the longer pulses, enough thermal damage (denaturation) can be inflicted on irradiated RPE cells, which can lose viability without undergoing cavitation.

Figure 18. In vitro results - ED₅₀ threshold fluence as a function of pulse duration.
Figure 19. *In vitro* results - Comparing thresholds for cavitation and cell death in the RPE
Solid lines represent % of cell death as the fluence was varied for each pulse duration.
Broken lines represent % of cavitating cells. Error bars represent variation between eye
samples (RPE explants)

Since we could correlate cell death with the onset of cavitation in the RPE, at least for the
shorter pulse durations, it was decided that monitoring bubble formation in the RPE could
be implemented in an *in vivo* system to serve as an online control for selective RPE
targeting.
**IN VIVO SCANNING SYSTEM - MONITORING INTRACELLULAR CAVITATION IN THE RPE**

**Experimental setup – incorporating cavitation detection in the scanner system**

Scanning a cw laser beam across the diseased retina offers a better alternative in selective RPE targeting than a pulsed laser-based system. Combined with online monitoring of bubble formation as a means to assess cell damage, it is an attractive therapeutic option for a number of macular diseases.

The *in vivo* cavitation detection was implemented on the slit lamp-based scanner system that was developed in our laboratory employing a two dimensional acousto-optic deflector (AOD) for scanning the irradiation beam across the retina [3, 4]. A polarizing beam splitter cube and a quarter waveplate were placed in the optical path behind and in front of the AOD, respectively, in order to differentiate the incident forward and the signal that was back scattered at the sample. As in the *in vitro* setup, the linearly polarized incident beam passing through the beam splitter cube (from the output of the polarization-maintaining single-mode fiber) was converted into circularly polarized light by the quarter waveplate. The returning backscattered signal going through the waveplate was converted back into linearly polarized light (polarized perpendicular to the incident polarization), was descanned by the AOD and was reflected by the beam splitter cube to the photodiode. The signal was then focused by a 100 mm focal length lens onto a 10 MHz-bandwidth avalanche photodiode (APD) and the response displayed on a digital oscilloscope. However, the returning signal includes backscattered components from all layers in the eye sample, and not just the RPE, that are in the optical path of the beam (cornea, lens, retina and the choroid). Therefore, confocal detection was required, and a 15μm pinhole was placed in front of the photodiode, in order
to reject the scattered light from out-of-focus components and allow the weak signal from the RPE layer to be detected.

Figure 20. *In vivo* scanner setup incorporating the cavitation detection system.
Future experiments will investigate the applicability of this system as an online control system during selective RPE targeting \textit{in vivo}. To eventually monitor bubble formation in the RPE of treated animals using the \textit{in vivo} scanner, the variability in the reflectivity of the tissue as the beam is scanned across the retina must be taken into account. The "noise" in the signal from this variation might be high enough to mask potential bubble signals, especially at or near threshold levels. This challenge can be potentially simplified by incorporating online data acquisition and computation capabilities to the system so that acquired signals can be compared to a reference signal to reveal scattering changes due to bubble formation in the RPE.

Alternatively, the system can be used to calibrate treatment variables such as laser power and scan speed (which is related to pulse duration) in real time, so that optimum therapeutic parameters can be decided at the start of the treatment. The backscattered signal from single (stationary) points irradiated with single pulses of increasing laser power can be analyzed to determine the power levels at which bubble formation is initiated and adjust the laser.
dosimetry prior to the start of the actual treatment. This approach entails an understanding of the differences in the damage thresholds observed with the pulsed versus the scanning system. Comparisons of thresholds for RPE damage suggest higher thresholds with the scanning approach than with the pulsed laser approach, although studies have employed different experimental models and design parameters [4]. The existing scanner incorporating the online detection system can be utilized to study the relationship between the thresholds of the two approaches using the same experimental protocol.
DISCUSSION - FEASIBILITY OF PROPOSED TECHNIQUE

Although laser photocoagulation is widely used and is the established modality for the treatment of a range of retinal disorders, the significant risks to the retina that it entails cannot be overlooked. Treating disorders such as age-related macular degeneration or diabetic macular edema can halt the deterioration of a patient’s vision, however the extensive, and irreversible, destruction of the retinal tissue is not acceptable, especially when treating the macula. Better treatments are needed to alleviate these side effects.

Selectively targeting the RPE while sparing the photoreceptors offers considerable advantages over conventional photocoagulation. By employing trains of short (µs) laser pulses or scanning a cw beam across the diseased retina, heat diffusion from the absorbing RPE tissue can be minimized, thus avoiding the thermal denaturation of the photoreceptors and the creation of a blind spot at the treatment site. Damage to the RPE layer is subtle and is invisible to ophthalmoscopic examination. Selective destruction of the target can be observed by fluorescein angiography performed one to two hours post treatment.

The therapeutic effects of selective RPE targeting have been attributed to the migration of RPE cells at the edges of the laser lesion [14, 31] and the proliferation of new, healthy RPE [25] which lead to the restoration of a functional outer blood-retinal barrier.

Unlike conventional laser photocoagulation, localized damage to the RPE, without associated thermal destruction of the photoreceptors will allow for the possibility of repeating the treatment if the desired therapeutic response is not obtained in a single treatment.

A pulsed system for selective RPE targeting is currently in clinical trials in Europe. However, a scanning system with online feedback control should be a more flexible, effective and cheaper solution. A pulsed laser-based system is more difficult to implement.
in the clinic since pulsed lasers (with associated power supplies and water cooling systems) are usually too bulky and cumbersome for a compact clinical device. Pulsed laser systems such as the green Nd:YLF or Nd:YAG are also expensive to build. The market scope for such a consumer-driven clinical application would need to be expanded to the treatment of enough RPE-associated disorders to justify building such a high cost, low profit margin system. This significant barrier to entry into the market would be compounded by the fact that the high initial cost of a clinical device to selectively target the RPE would make it prohibitive for many smaller eye clinics. A scanning-based system will incorporate a low power, air cooled cw solid state laser that should be cheaper to purchase and run and is compact enough to be incorporated, together with the slit lamp and AOD, in a clinical system. Such a system, which can be competitively priced, can overcome these hurdles and become successful enough (in terms of procedures being performed in the clinic) to bring down prices so that it can become established in the field of eye surgery and the treatment of choice for RPE-associated disorders.

Selective RPE targeting using the scanning approach offers the flexibility of varying the scanning speed and irradiation pattern in the retina to optimize the therapeutic outcome. Variability of the pulse durations produced by a pulsed laser system is limited to a small range or a few fixed values, depending on the mode of operation. In a scanning-based system however, the scan speed can be more easily adjusted to produce the optimal effective pulse “duration” for the specific treatment. Furthermore, it has been shown that varying scan patterns (eg. separated versus interlaced lines) can lead to different thresholds [4], a parameter which can be eventually exploited in the design of the treatment.

It is evident that for successful selective RPE targeting, laser dosimetry is a crucial parameter that needs to be controlled during delivery of the therapy rather than post treatment. A scanning system that incorporates online feedback control would be well
received by clinic workers who will now be able to monitor and control the laser parameters online for optimum therapeutic results.
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