Selective targeting of pigmented retinal pigment epithelial (RPE) cells by a single pulsed laser irradiation: an in vitro study

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Abstract: This work describes the selective targeting of pigmented retinal pigment epithelial (RPE) cells by a single pulsed laser irradiation. We observed: (1) single pulsed laser irradiation caused cellular damages on pigmented, and not on non-pigmented RPE cells at laser radiant exposure up to 2550 mJ/cm^2; (2) in the mixture of pigmented and non-pigmented RPE cells, single pulsed laser-induced damage was confined to pigmented RPE cells. This study demonstrates that the pigmented RPE cells can be selectively damaged, using a single pulsed laser irradiation, without thermal coagulation to adjacent non-pigmented RPE cells.

OCIS codes: (140.0140) Lasers and laser optics; (170.0170) Medical optics and biotechnology.

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1. Introduction

The retinal pigment epithelium (RPE) is a monolayer of cuboidal cells located between the photoreceptors of neuroretina on the apical side, and the choriocapillaries of choroidal tissue on the basal side [1]. RPE cells perform many important functions essential to the visual process, such as the daily phagocytosis of rod and cone outer segment fragments; the metabolism of retinol (vitamin A) and some of its visual cycle intermediates (retinoids); the maintenance of the normal physiological functions of the retina-RPE-choroid complex [1]. Since the RPE monolayer is critical for the maintenance of photoreceptor health, its disruption causes irreparable damage to the overlaying photoreceptors, and is often accompanied by vision loss in the affected retina region. Disruption of the RPE monolayer has been implicated in the development of a number of retinal degenerative diseases, such as age-related macular degeneration [1].

The RPE cells in the retina contain numerous melanosomes and organelles enclosing the highly absorbing chromophore melanin. Thus, RPE cells are an ideal target for exclusive targeting via selective photothermolysis (SP) [2], even though it is located in a very sensitive cellular environment. For example, approximately 53% of the incident 514 nm light delivered by an argon laser is absorbed by the RPE, compared to less than 10% absorption by the photoreceptors [3]. However, the current techniques of retinal laser photocoagulation commonly use continuous wave (CW) lasers with pulse durations in the millisecond range or greater. At these pulse durations, irreversible coagulations of the neural retina and choroid occur due to heat diffusion, and typically result in laser scotomas.

In order to confine energy transfer to the RPE cell layer, the exposure duration must be comparable to or shorter than the thermal relaxation time of the target absorber. Thermal relaxation time defines the time required for the central temperature of a Gaussian temperature distribution with a width equal to the target's diameter to decrease by 37 percent [2]. This rapid deposition of radiant energy allows for minimal thermal diffusion and collateral damages. Melanin particles, the target chromophore in RPE cells, have a thermal relaxation time in the microsecond range [2]. It is worth noting that shorter duration exposure, and lower power settings were also successfully applied to diabetic retinopathy to limit the extent of collateral damages [4]. Previously, selective targeting of RPE has been achieved by repetitive laser pulses [5] or scanning CW lasers [6]. In this paper, we investigated if the selective targeting can be achieved using a single laser pulse to reduce the complexity required in the instrumentation and procedure of this approach.

The objectives of this study are to investigate the effects of single pulsed laser radiant exposure and melanin content on the viability of cultured RPE cells, and to demonstrate the selective killing of pigmented RPE cells by a single pulsed laser irradiation without thermal coagulation to adjacent non-pigmented RPE cells, while maintaining the cellular structural integrity in vitro. In addition, this study will also provide an in vitro system to further investigate the low power laser stimulation effects on pigmented RPE cells following a single pulse laser irradiation in vitro, and related biological mechanisms.

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2. Materials and methods

Dulbecco's Modified Eagle Medium/F12 (DMEM/F12), fetal bovine serum (FBS), phosphate-buffered saline (PBS), 100X penicillin-streptomycin stock solution, and 0.5% trypsin-0.02%EDTA stock solution were obtained from Invitrogen Life Technologies (Carlsbad, CA). F-12K Medium (Kaighn's Modification of Ham's F12) was purchased from ATCC (Manasses, VA). Sepia melanin was obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Fluorescent live/dead viability/cytotoxicity assay was obtained from Molecular Probes (Eugene, OR, U.S.A.).

2.1 *RPE cell culture and phagocytosis of melanin*

All experiments were performed using third, fourth and fifth passage human retinal pigment epithelial (ARPE-19) cells. ARPE-19 cells (obtained from ATCC) were cultured in Dulbecco's Modified Eagle's Medium/F12 (1:1) with 10% fetal bovine serum, 1% penicillin-streptomycin at 37°C, in a 5% CO_2 balance air atmosphere [7]. The cells were cultured in tissue culture treated culture dishes of 3.5 cm in diameter. The ARPE-19 cells are non-pigmented in their normal growth state, and served as the control non-pigmented RPE cells. To obtain pigmented ARPE-19 cells, confluent ARPE-19 cell cultures were incubated for 20 hrs with varying concentrations of sepia melanin as previously described [8]. Sepia melanin is relatively well characterized and easily accessible. It is often used as a model of melanin, especially in model systems. Prior to the incubation, sepia melanin was washed with RPE cell culture medium, and sonicated to obtain a uniform suspension. Just prior to the irradiation, the medium was replaced with PBS to avoid absorptions of laser energy by the medium, and the PBS was replaced with standard medium following laser irradiation.

2.2 *Spectrophotometric analysis of melanin*

Cultured cells were detached with 1ml of 0.25% trypsin with 0.02% EDTA (10 min at 37°C). An aliquot (50μl) was removed, and cells were counted using a hemocytometer. The remaining cell suspension was centrifuged, and the pellet was dissolved in 1N NaOH. The melanin concentration was determined by measurement of absorption at 475 nm, and then compared with a standard curve obtained using synthetic melanin [9].

2.3 *Experimental setup*

Irradiation of the RPE cells was performed using a pulsed dye laser (Palomar Medical Inc.) emitting at 590nm. The pulse energy was measured using an energy power meter (DigiRad, U.S.A., R-752 Universal Radiometer). ARPE-19 cells were irradiated at various radiant exposures (mJ/cm^2) using the experimental set-up shown in Fig. 1. A Helium-Neon laser with an output of 15mW, coupled into the optical path, was used for the alignment of laser beam. The pulsed laser was delivered through a 1 mm diameter, 2 m long optical fiber (0.22 NA) (Thorlabs, Inc. Newton, NJ, USA). The laser profile was characterized by MATLAB software (Fig. 2). Video images of the laser irradiation region were captured by a CCD camera through the slit lamp, digitized, and displayed on screen. The following parameters were used for laser irradiation: 5.0 mJ/cm^2-2550 mJ/cm^2 of laser radiant exposure and 1μs duration. Three sample dishes were irradiated per experimental condition.

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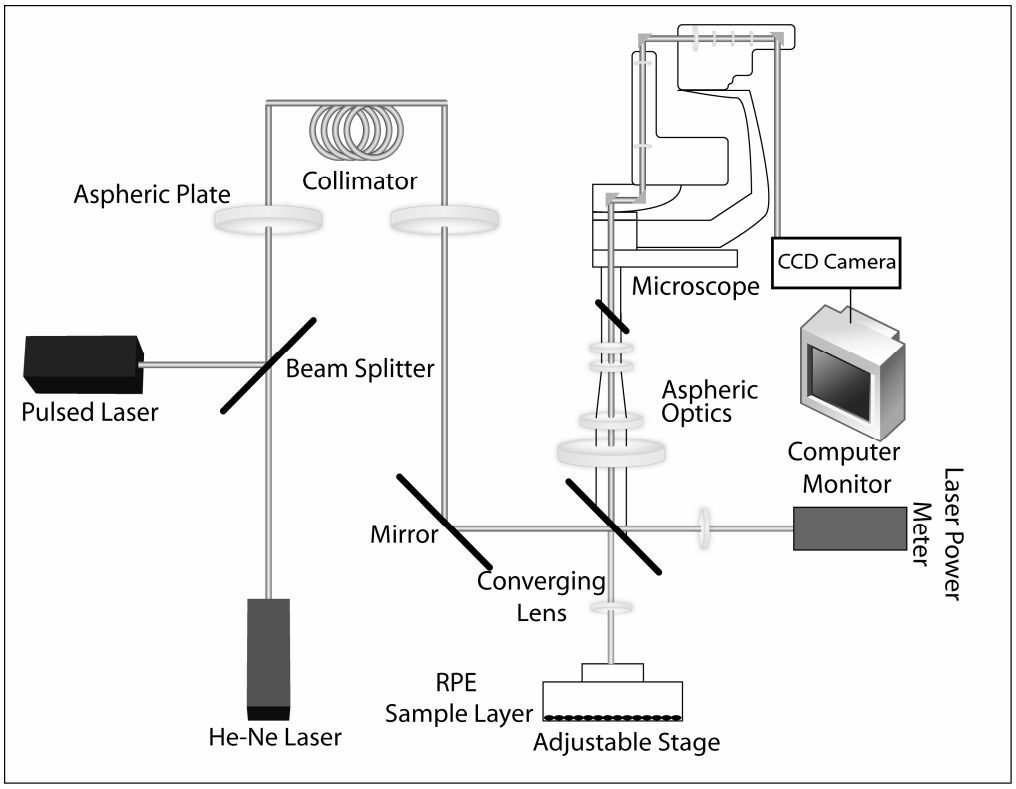

Fig. 1. Schematic representation of the experimental setup. Laser irradiation was reflected by a mirror to the stage where RPE cells were seated. The slit lamp was used to capture the video images of laser irradiation spots. Laser irradiation was performed under visual control.

2.4 *Analysis of laser spot size*

The analysis of the pulsed dye laser profile at the focal plane showed that the intensity distribution fitted the time-averaged intensity distribution of a Gaussian beam.

$$I(r,z) = \frac{|E(r,z)|^2}{2\eta} = I_0 \left(\frac{w_0}{w(z)} \right)^2 \exp\left(-\frac{2r^2}{w^2(z)} \right) \quad (1)$$

The parameter w_0, usually called the gaussian beam waist, was the radium at which the intensity has decreased to $1/e^2$ or 0.135 of its value on the axis [10]. By fitting the laser spot to a Gaussian profile, the beam waist of the pulsed dye laser was estimated to be 290±10 μm (Fig. 2).

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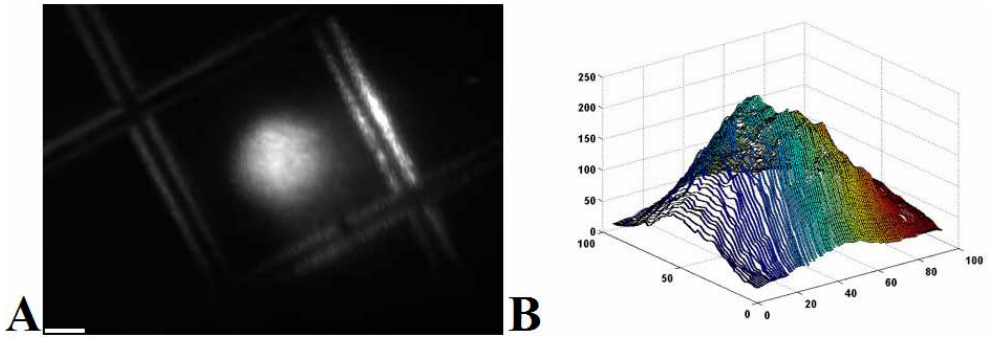

Fig. 2. Pulsed dye laser profile analysis. (a) Light micrograph of laser spot. Slight asymmetry is caused by imaging optics; (b) Gaussian laser intensity profile analyzed by MATLAB 6.5 (arbitrary units). Scale bar 500 μm.

2.5 *Cellular viability of pigmented RPE cells following laser irradiation*

The viability of pigmented ARPE-19 cells was evaluated using a fluorescent live/dead viability/cytotoxicity assay. The fluorescent live/dead viability/cytotoxicity assay utilizes calcein-AM and ethidium homodimer (EthD-1), which localizes to live and dead cells, respectively. The assay solutions were prepared according to the manufacturer's recommended protocol. Briefly, 200 μl of the solution (2.0 μM calcein-AM and 4.0 μM EthD-1 in PBS) was applied to each cell culture dish. The culture was incubated for 20 min at 37°C, and then analyzed using fluorescence microscopy (Zeiss Axiovert 200M, Carl Zeiss MicroImaging, Inc.) Live cells were distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein-AM to the intensely fluorescent calcein. The polyanionic calcein dye is well retained within live cells, producing an intense uniform green fluorescence (excitation/emission ~495 nm/515 nm). EthD-1 enters the cells with damaged membranes, and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing the bright red fluorescence in dead cells (excitation/emission ~495 nm/635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. Cellular viability was measured as an average of N irradiated spots divided by the same number N of non-irradiated spots (in this case, N=3) with the spot size of w_0 on the same dish. Images were taken at the identical gain and exposure settings.

2.6 *Selective targeting of pigmented RPE cells: mixed non-pigmented and pigmented ARPE-19 cells*

Human ARPE-19 cells were grown in two matching flasks (75 cm^2) to confluence, where there was minimum cell proliferation, as previously described [11]. One of the flasks was fed with melanin for 20 hrs, the other one was not, and subsequently washed with PBS. The ARPE-19 cell cultures (non-pigmented and pigmented) were then trypsinized (0.5% trypsin-0.02%EDTA) for 10 min. The ARPE-19 cell suspensions were recovered by centrifugation (500 rpm for 5 min), and re-suspended in medium. The two cell suspensions were combined and thoroughly mixed, yielding a suspension of a 1:1 mixture of pigmented and non-pigmented cells. The mixed cell suspension was immediately replated in a 35 mm dish at the confluent density, incubated for 24 hrs to allow the formation of a continuous cell sheet, and then irradiated.

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3. Results

3.1 *Melanin phagocytosis by RPE cells*

Cellular phagocytosis of melanin by human RPE cells at different melanin concentrations is shown in Fig. 3. The distribution of melanin was perinuclear and the amount of phagocytized melanin increased with the increasing concentrations of melanin. An incubation time of 20 hrs provided a qualitative difference in the amount of ingested melanin as a function of concentration, and was therefore subsequently used to evaluate the effect of the level of RPE cell pigmentation on viability following laser irradiation. The melanin concentrations normalized by the total protein concentrations were in the range of 0.03 mg/mg protein to 0.05 mg/mg protein in this study.

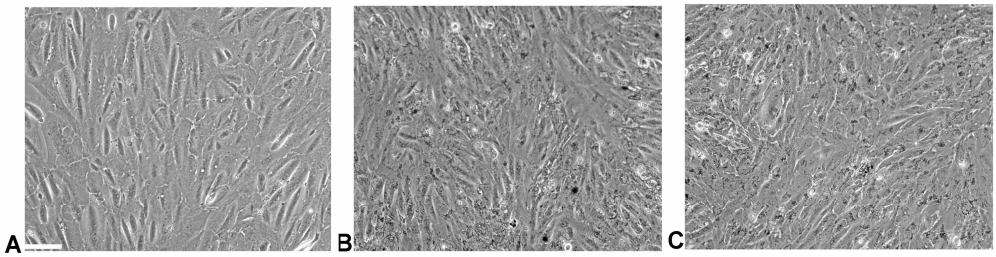

Fig. 3. Phase contrast microscope images of confluent ARPE-19 cells following 20 hrs of incubation with melanin. (a) 0.0 mg melanin/mg protein. (b) 0.03 mg melanin/mg protein. (c) 0.05 mg melanin/mg protein. Scale bar is 100μm.

3.2 *Viability of pigmented RPE cells following laser irradiation*

Figure 4 shows qualitatively the effects of laser irradiation on the viability of pigmented RPE

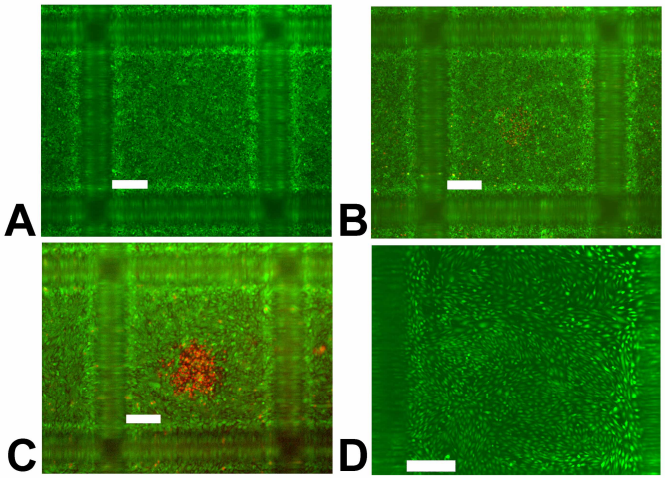

Fig. 4. Effects of laser irradiation on viability of pigmented RPE cells. Cells were stained with the viable dye calcein (green) and dead stain ethidium homodimer-1 (EthD-1) (red). A) Laser radiant exposure at 5 mJ/cm^2. B) Laser radiant exposure at 55 mJ/cm^2. C) Laser radiant exposure at 700 mJ/cm^2. Melanin content is 0.05mg/mg protein. Scale bar is 400 μm. The light and dark brown spots are the reflection of grid edges. D) Effects of laser irradiation on viability of non-pigmented RPE cells at laser radiant exposure of 2550 mJ/cm^2. Scale bar is 640 μm.

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cells. Figure 4(a) corresponds to a laser radiant exposure of 5 mJ/cm^2. At this laser intensity, the green cytoplasmic staining demonstrated that there was no obvious cellular injury on pigmented RPE cells. When the laser radiant exposure was increased to 55 mJ/cm^2 (Fig. 4(b)), some dead cells with the red nuclear staining appeared. With the increasing laser radiant exposure, more dead cells with red nuclear staining were observed (Fig. 4(c)). For the sham controls, consisting of irradiated cells without melanin, the fluorescent cytotoxicity assay showed no evidence of cellular injury using radiant exposure even at 2550 mJ/cm^2 (Fig. 4(d)).

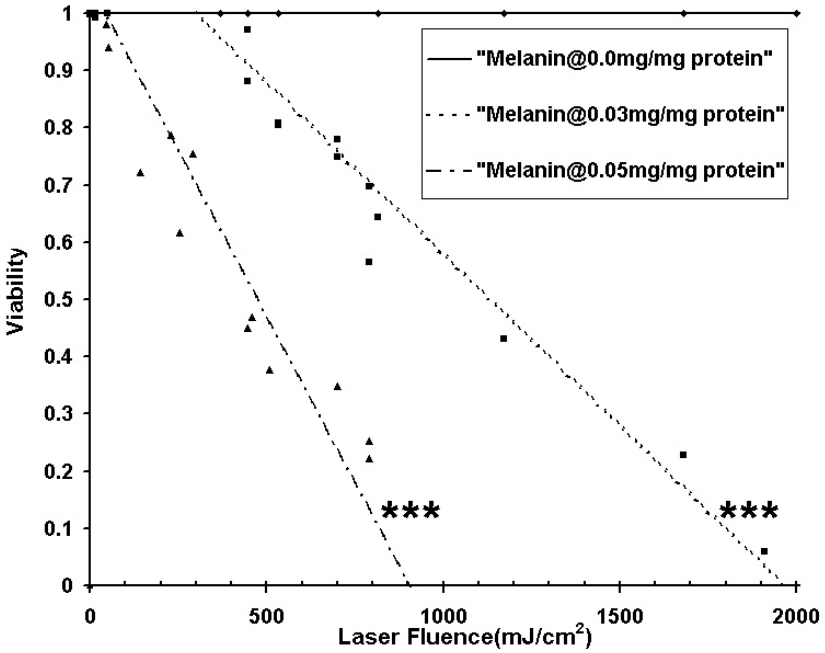

Fig. 5. Viability of pigmented RPE cells as a function of laser radiant exposure and melanin content. The lines represent the least-square linear fits of the experimental data with the following regression parameters (a=slope; b=ordinate; threshold=intersection with 100% viability line; r=regression coefficient). Melanin content 0.03 mg/mg protein: a=0.99; b=-9.8×10^{-4}; r=0.98; threshold=1140 mJ/cm^2 (Corresponding to 50% viability); Melanin content 0.05mg/mg protein: a=1.05; b=-1.1×10^{-3}; r=0.98; threshold=460 mJ/cm^2 (Corresponding to 50% viability). P values shown are comparisons with non-pigmented cells.***: p<0.001. The meanings of symbols are as following. Solid circle: experimental data for 0.0 mg/mg protein melanin content; solid square: experimental data for 0.03 mg/mg protein melanin content; solid triangle: experimental data for 0.05 mg/mg protein melanin content.

For the viability quantification of the pigmented RPE cells following laser irradiation, the objective area within a radius of $1\times w_0$ was used to count the live /dead cells in order to evaluate cellular viability as a function of laser radiant exposure as well as melanin content. The viability of pigmented ARPE-19 cells was defined as the normalized green fluorescent intensity of calcein-AM, calculated as the green fluorescent intensity within the laser spot size after laser irradiation, divided by that within a similar spot size without laser irradiation. The cellular viability as a function of laser radiant exposure and melanin content of the RPE cells is displayed in Fig. 5. The viability curve shows that there is a critical value for the laser radiant exposure, below which the laser irradiation has no obvious cellular injury effects on pigmented RPE cells. When the laser radiant exposure is above this critical value, the laser irradiation starts to produce cellular injury effects on pigmented RPE cells. The higher the laser radiant exposure, the more pigmented cells were targeted. The higher the melanin

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content, the lower the critical value of laser radiant exposure. Therefore, the melanin content played a significant role during laser radiation.

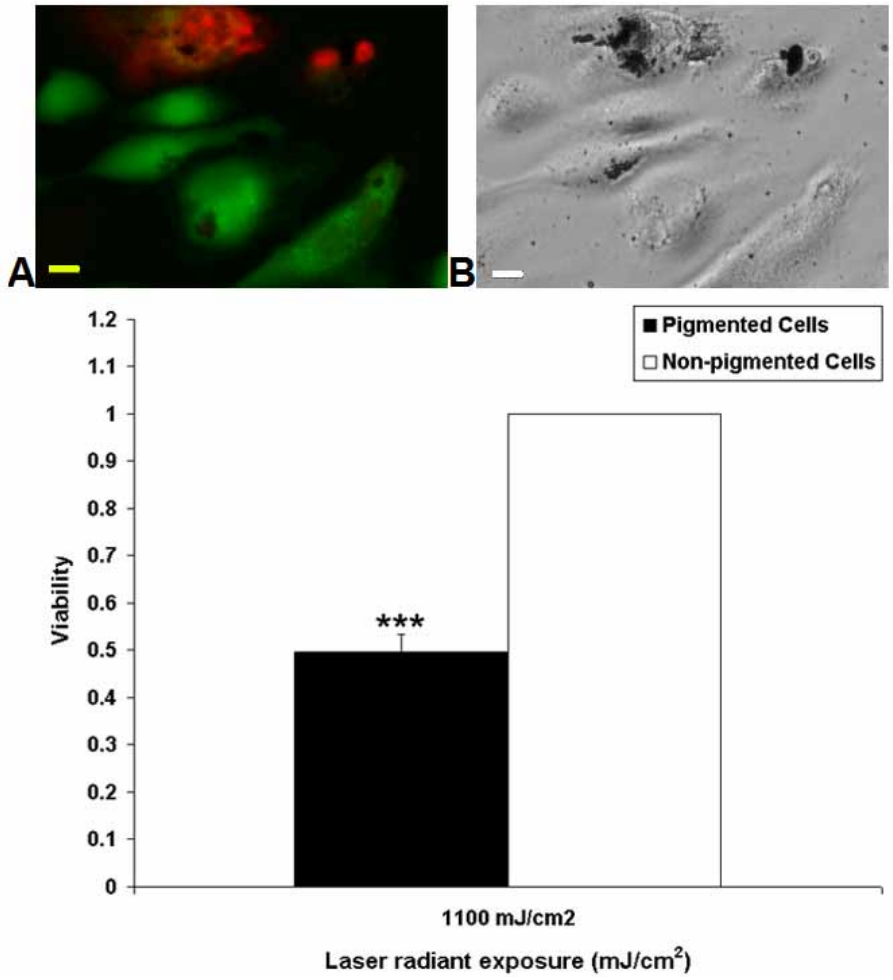

Fig. 6. Selective targeting of pigmented retinal pigment epithelium cells at a laser radiant exposure of 1100 mJ/cm^2. (a) Fluorescence image (red nuclear staining represents dead cells, green cytoplasmic staining represents live cells); (b) Phase contrast image. Scale bar is 10 μm; (c) Viability of pigmented and non-pigmented RPE cells. ***: $p<0.001$. N=6.

3.3 *Selective targeting of pigmented cells*

Although non-pigmented cells appear not to absorb the laser irradiation (Fig. 6), thermal energy absorbed by pigmented cells could potentially damage their neighboring non-pigmented cells. To assess the selectivity and confinement of the laser treatment, its effects on mixed cell populations of pigmented and non-pigmented ARPE-19 cells were analyzed using a high laser radiant exposure (1100 mJ/cm^2). This laser radiant exposure corresponds to 50% viability for RPE cells preincubated in 0.03 mg/mg protein melanin content. Figure 6 shows the selective targeting results of pigmented ARPE-19 cells without any associated damage to their adjacent non-pigmented cells. Only the ARPE-19 cells within the irradiation zone containing melanin were selectively killed as demonstrated by the red nuclear staining, whereas the adjacent non-pigmented ARPE-19 cells showed no evidence of cellular damage demonstrated by the green cytoplasmic staining. The viability of pigmented ARPE-19 cells

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was around 50%, which was significantly lower than that of non-pigmented ARPE-19 cells, which was 100% (***:$p<0.001$, N=6). Immediately following the laser irradiation, the damage to the pigmented ARPE-19 cells was so subtle that by phase contrast microscopy alone, it was difficult to morphologically differentiate affected from non-affected cells. This suggests that melanin containing ARPE-19 cells can be selectively targeted without significant collateral damage to the adjacent non-pigmented ARPE-19 cells at the laser radiant exposure levels of 1100 mJ/cm^2.

4. Discussion

The action of laser light on a biological sample gives rises to three different phenomena, depending on the characteristics of the radiation: photothermal effects, photomechanical effects, and photochemical effects. Which mechanism prevails at the retina depends on pulse wavelength, duration, intensity, and repetition rate. In general, for a long exposure times, (longer than 1 ms) thermal effects are the most relevant [12]. If pulse durations are on the order of ps or fs, the pure mechanical effects like damage produced by shock waves prevails [13]. The transition pulse duration from thermal to mechanical origin of damage is between 10 and 50 μs, and pulse duration below 10 μs leads to mechanical related damage. Lee et al [14] recently confirmed this conclusion. In the case of RPE cells, approximately 50% of the incident light will be absorbed by their melanosomes [3]. If the laser radiation is sufficiently intense, the RPE cells may be damaged or killed. In this study, the pulse duration is of 1 μs, so the dominating damage mechanism is mechanical.

It was demonstrated in this study that pigmented RPE cells could be selectively targeted using a single pulsed laser irradiation with pulse duration of 1 μs. The experimental set up is shown in Fig. 1, and the typical laser spot is shown in Fig. 2. This technique relies on the preferential absorption of an appropriate wavelength by the target chromophore, and on an exposure duration which is comparable to or shorter than the thermal relaxation time of the absorber. One distinguishing characteristic of selective photothermolysis is the specific targeting of pigmented cells without the need to focus the laser to achieve specificity. That is, all cells with chromophore within the irradiation field will be affected, while cells without chromophore will not be affected [2]. The advantage of targeting multiple cells by selective photothermolysis is that large irradiation zones can be treated, and yet the target selectivity can still be maintained [2] without the challenging technological complication associated with the single-cell focusing [6]. Fig. 4(a-c) shows the effects of laser irradiation on pigmented RPE cells at different laser radiant exposures. There is no obvious cellular injury on pigmented RPE cells at low laser radiant exposures. With the increase of laser radiant exposure, more and more cells are killed by laser irradiation. Figure 4(d) shows the effect of laser irradiation on non-pigmented RPE cells at laser radiant exposure of 2550 mJ/cm^2. There is no obvious cellular injury on non-pigmented RPE cells at laser radiant exposure of 2550 mJ/cm^2. Figure 5 shows the viability of these pigmented cells as a function of laser radiant exposure and melanin contents. When the laser radiant exposure is below a critical value, laser irradiation has no obvious injury effects on pigmented RPE cells. When laser radiant exposure is above a critical value, laser irradiation exerted cellular injury effects on pigmented RPE cells. The higher the laser radiant exposure, the more cells are killed. In order to avoid thermal damage to adjacent cells, laser exposure needs to be shorter than the thermal relaxation time of the target, confining heat to the target. In this study, target diameter (melanin grain) is in the order magnitude of micrometer, thermal relaxation time is approximately about 1 μs. Therefore, at pulse durations shorter than 1 μs, energy is deposited within the target more rapidly than it diffused away. The target temperature becomes much higher than that of the surroundings, and thermal diffusion to the surrounding non-pigmented cells is minimized. Experimental results of this study strongly support this point as non-pigmented cells appear unscathed even when pigmented cells a few micrometers away are killed by selective photodamage. When the RPE pigmentation is higher, less laser energy is

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needed to target pigmented RPE cells. The threshold of laser radiant exposure at a melanin content of 0.05mg/mg protein is 500 mJ/cm^2. This value agrees with angiographically determined threshold radiant exposure of 500 mJ/cm^2 per pulse published by Brickmann and co-workers [6]. The temperature increase in the RPE cells following the selective photocoagulation was estimated [15] as 80 K for a radiant exposure of just 100 mJ/cm^2 for a close mesh. Based on above estimation, the temperature at the surface of RPE cells is estimated as about 64°C for this study. Such a temperature increase will induce expressions of heat shock proteins, and secretions of growth factors but not necessarily thermal necrosis. This temperature increase indicates that selective photocoagulation with low power energy may potentially induce stimulation effects on pigmented RPE cells, which will be investigated in future work.

Melanin concentration range was investigated in the pigmented epithelium of 61 postmortem normal human eyes from donors of ages ranging from 14 to 97. The content of soluble melanin in the pigment epithelium declined with age from the highest values of 0.095 mg/mg in the 14-50 years old group to the lowest values of 0.022 mg/mg dry weight in the group of over 70 years old [16]. The principle of extraction of total protein from ARPE-19 cells in this research is similar to that of pigment epithelium preparation in Schmidt's work. The melanin concentrations in the present study were in the range from 0.03 mg/mg protein to 0.05 mg/mg protein.

The in vitro study was done with artificially pigmented ARPE-19 cells. However, the validity of selective photocoagulation has also been demonstrated in vivo [17]. As reported, RPE defects in patients were proven angiographically by fluorescein or ICG-leakage in the range of 450-800 mJ/cm^2 per pulse. With the laser parameters used in this research, neither bleeding nor scotoma was observed as proved by microperimetry, which indicated no adverse effects to the choroid and the photoreceptors, respectively. During and after irradiation, it has been shown that irradiated locations were ophthalmoscopically invisible, since effects were very limited and confined.

While selective RPE cell death will probably prevent loss of vision, sublethal laser irradiation could potentially induce RPE recovery. Since wavelengths used to perform selective RPE targeting are readily transmitted by the cornea, selective RPE cell targeting could be performed clinically using a gonioscopic approach with a slit-lamp laser delivery system. The ability to confine laser irradiation to the RPE layer of the retina suggests a potential avenue for treatment of macular degenerations.

In the literature, two techniques for "selective RPE targeting" have been described. First one is high repetition rate photocoagulation [5] and second is a scanning technique using a CW laser [6]. In true experimental homogeneous systems, repetitive pulsing may be of some benefits. Given the heterogeneity of RPE and melanosome packing within the RPE cell, the advantage of a high repetition rate technique will be substantially minimized and presumably be no better than a single-pulsed technique. The scanning approach also may have some advantages compared to high-repetition rate technique. However, there are several important disadvantages. First, it is very technically difficult to perform very localized scanning of the retina, especially close to the macula, without extremely sophisticated eye tracking systems. Second, it is unlikely in the real human eye, that one will be able to maintain a 10 μm beam spot size given the large scattering properties within the eye, especially those with disease such as cataracts. Third, scanning may result in targeting of an extensive area of the retina which may actually worsen the disease process.

The goal of treatment for diseased RPE is to stimulate its repair rather than to ablate it. The systematical studies of low power laser stimulations on pigmented RPE cells are extremely lacking in the literature. Herewith, a procedure was developed to achieve the selective targeting of pigmented RPE cells, and this in vitro system will be used further to investigate the low power laser stimulation effects and related biological mechanisms in future work.

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In conclusion, it has been demonstrated that pigmented RPE cells can be killed and most importantly can be selectively targeted using a 1 μs laser pulse without associated thermal damage to neighboring non-pigmented cells, while maintaining cellular structural integrity. This technique will simplify the complexity required in the instrumentation and procedure in the repetitive laser pulses or scanning CW lasers while providing both adequate safeties for selective RPE targeting as well as an appropriate biological response. In addition, this system will be used to systematically investigate if low power laser energies can induce significant stimulation effects, such as promoting cell regeneration and wound healing, on pigmented RPE cells without the loss of viability in future work.

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