Quantitative Evaluation of Retinal Response to Laser Photocoagulation Using Dual-Wavelength Fundus Autofluorescence Imaging in a Small Animal Model

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PURPOSE. To demonstrate the usefulness of dual-wavelength fundus autofluorescence (FAF) imaging for noninvasive, quantitative monitoring of dynamic changes associated with healing of retinal photocoagulation lesions in a small animal model.

METHODS. Brown Norway rats, exhibiting substantial age-dependent lipofuscin autofluorescence, were used to characterize the kinetics of FAF recovery after retinal photocoagulation. An argon laser with a beam diameter of 100 μm, exposure duration of 0.1 seconds, and a range of laser powers (8–22 mW) were used to create subthreshold, threshold, and suprathreshold lesions. A modified retinal angiograph was used to obtain dual-wavelength FAF images at 488 and 514 nm to quantify and monitor changes in retinal fluorescence up to 6 months.

RESULTS. Compared to white light funduscopy, the FAF images exhibited heightened definition and clarity of lesion boundaries immediately after laser exposure. No significant reduction in FAF was measured at or below laser powers of 8 mW. Furthermore, a linear, dose-dependent decrease in FAF ($R^2 = 0.9605$) was observed immediately after laser exposures of 13 to 22 mW. Complete recovery of baseline FAF was observed 0.9605) was observed immediately after laser exposures of 13 to 22 mW. Complete recovery of baseline FAF was observed for 13.5 and 16 mW exposures at 3 weeks and 4 months, respectively. However, retinal damage was still evident at 6 months after suprathreshold exposure induced using 22 mW laser power.


Fundus autofluorescence, derived primarily from the accumulation of lipofuscin within the retinal pigment epithelium (RPE),1,2 has become a prominent fixture in retinal imaging for the diagnosis and assessment of inherited retinal diseases such as Best disease, Batten disease, and Stargardt disease.3–4 Fundus autofluorescence (FAF) imaging has also been used in the evaluation of late-onset diseases such as age-related macular degeneration5,6 and proliferative diabetic retinopathy.7 The latter afflictions, along with numerous others, are commonly treated by means of photocoagulation to target areas of geographic atrophy or vascular complications. Multiple laser therapy methods are available; however, conventional retinal photocoagulation is achieved using a continuous wave laser operating at either 514 or 532 nm, spot sizes ranging from 100 to 500 μm, exposure durations of 50 to 150 ms, and powers from 100 to 500 mW. The precise dosimetry generally depends on the severity and location of the condition and the pigmentation of the individual being treated.

The inherent value, and often necessity, of laser exposure for therapeutic or diagnostic purposes in the field of ophthalmology has prompted substantial interest in developing a better understanding of damage mechanisms and identifying distinctive biomarkers of laser damage in the eye. Previous studies have explored a wide range of exposure parameters, animal models, and grading metrics in an effort to characterize laser damage in the retina. Based on the exposure parameters, laser damage in the eye is divided into three principal groups: photochemical, photomechanical, and photothermal and this has typically been shown with well established methods.8–10

Fluorescein angiography, near infrared reflectance, and white light funduscopy techniques have frequently been used to characterize damage in animal models of retinal laser exposure.11–14 Histologic evaluations15–22 and electrotetroretinography23–24 are also commonly used to determine structural features and functional outcomes. Typical laser exposure studies and photoocoagulation therapies rely on gross morphologic changes for the assessment of retinal damage. The visible end points observed using conventional imaging modalities, after photothermal damage, occur as a result of the protein denaturation caused by a local increase of at least 10°C in the temperature of the RPE.2 The ensuing thermal diffusion often destroys the adjacent neural retina and can lead to the progressive expansion of atrophy. Some of the higher powered laser exposures are also capable of rupturing Bruch’s membrane, which may ultimately promote choroidal neovascularization. The potential for inducing these complications creates a difficult dilemma from a therapeutic standpoint.

Greater understanding of the biochemical composition and spectral properties of endogenous fluorescent pigments within the retina has provided a new perspective for monitoring changes in the RPE.25–30 Multiple fluorophores are present in the retina, providing an emission spectrum that encompasses a broad band from 500 to 800 nm with optimal excitation from 480 to 520 nm.1,25 Extensive research has already demonstrated the quantification, spatial distribution, and clinical relevance of macular pigment density measurements with regard to retinal pathologies using the dual-wavelength approach.31–34 Among the emerging imaging techniques that
may be used for the characterization of laser-induced retinal injury, the dual-wavelength method of FAF imaging, originally propounded by Delori et al., provides a means of quantitatively assessing changes in FAF after laser exposure. This technique capitalizes on the abundance of a native contrast agent to provide the level of sensitivity required to distinguish between minimal differences in laser exposure powers. These subtle changes may differentiate laser exposure powers that result in permanent changes in RPE autofluorescence properties compared with transient modifications in the lipofuscin density that are ultimately capable of full recovery. Other groups have already demonstrated the usefulness of single-wavelength autofluorescence by measuring changes in the retina as a result of photochemical damage. Given the degree of sensitivity of FAF imaging with the confocal scanning laser ophthalmoscope, we propose using the dual-wavelength method to acquire autofluorescence images and to quantitatively monitor changes in the rodent retina after the induction of photothermal damage.

**Materials and Methods**

**Animal Model/Animal Preparation**

All experimental methods were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch and are in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retired breeder Brown Norway Rats (Charles River Laboratories, Wilmington, MA) at least 9 months of age were used in this study. Aged rats were integral to the study because they exhibited the necessary lipofuscin accumulation required for AF imaging.

All animals were housed according to standard 12-hour light/12-hour dark cycles under white fluorescent light. Before imaging, animals were anesthetized with isoflurane (1.0%-2.0%), pupils were dilated with tropicamide (1%), and eyelid retractors were used to hold the eye open. Corneal hydration was maintained throughout imaging by applying 0.5% hypromellose lubricating eye drops (Alcon Laboratories, Fort Worth, TX) every 2 to 3 minutes. An adjustable, heated platform was used to maintain body temperature and allow appropriate positioning of the rodent eye for imaging and photocoagulation.

**Laser Exposure**

An argon laser (Ultima 2000; Coherent, Santa Clara, CA) operating at 514 nm in conjunction with a slit lamp (model 30 SL-M; Zeiss, Thornwood, NY) was used to deliver laser radiation and induce lesions. Pre-exposure images were taken to evaluate changes in white light funduscopy, infrared reflectance, and autofluorescence. White light funduscopy images were obtained by incorporating a digital SLR camera (D70s; Nikon Inc., Melville, NY) into the slit lamp. A fixed exposure time of 0.1 second and a laser spot size of 0.10 mm at the cornea were used in all experiments to induce retinal photocoagulation. A range of powers from 5 mW to 22 mW were used to determine threshold limits of minimal visible lesions (MVLs) in the Brown Norway rat model. A minimum visible lesion was defined as the lowest exposure at which a visible blanching of the retina was observed with a slit lamp during a comprehensive white light fundus examination within the first 30 minutes after laser exposure. A total of 21 rats were used to monitor the changes associated with laser exposure for up to 6 months. A subset of six rats was used to conduct dosimetry studies for MVL exposure and provide autofluorescence measurements immediately after laser exposure. The remaining 15 rats were divided into three exposure categories: group 1 (n = 3) multiple lesions at laser exposure of 8, 13.5, 16, and 22 mW were created in each subject; group 2 (n = 6) multiple exposures at 16 and 22 mW; group 3 (n = 6) multiple exposures at 20 and 22 mW. Animals were imaged daily in the first week after laser exposure to assess the acute response, weekly during the first month, and then monthly to ascertain long-term AF recovery.

**Fluorescein Angiography**

Fluorescein angiography was conducted after obtaining autofluorescence images in the dosimetry group of animals to establish disruption of the integrity of the RPE as a result of laser exposure. A 1% solution of fluorescein sodium was administered by intraperitoneal injection after laser exposure at 24 hours, 1 week, and 2 weeks.

**Autofluorescence Imaging and Lipofuscin Density Measurements**

A modified confocal scanning laser ophthalmoscope (Heidelberg Retina Angiograph I; Heidelberg Engineering, Heidelberg, Germany) was used to obtain images of the fundus by either infrared reflectance or autofluorescence using excitation wavelengths of 488 and 514 nm. This custom hardware configuration and analytical software package was originally designed by Heidelberg Engineering to measure macular pigment optical density and lipofuscin. Given that rodents lack a macula, this instrument was used primarily to acquire lipofuscin autofluorescence changes in response to photothermal injury.

All images were acquired in the high-resolution mode (512 \times 512 pixels) over a 30° \times 30° field of view (FOV). The detector sensitivity was also maintained at a fixed value for all subjects to avoid changes in the dynamic range and errant values caused by pixel saturation. The system scan rate was set at 12 images per second, and nonnormalized averages of 16 images acquired at each wavelength were used for autofluorescence quantification. The sequential imaging mode on the system allowed for a seamless transition between the 488-nm and 514-nm sources, resulting in an overall acquisition time of <3 seconds per wavelength once the animal was correctly positioned. The images were aligned based on morphologic features of the retina (i.e., blood vessel patterns). If extensive eye movement prevented colocalization between the averaged autofluorescence images in the software, the imaging procedure was repeated. Additionally, images from each time point were aligned to ensure the same regions were evaluated on a pixel-by-pixel basis. To prevent the possible induction of photochemical damage during fluorescence imaging of the retina, typical experimental conditions and geometric values derived from the Hughes schematic eye for the rat were used to calculate the total radiant exposure. These values are presented in Table 1.

Retinal autofluorescence values were calculated by measuring the differences in the optical densities using fixed extinction coefficients for the blue and green light that were used as excitation sources. Figure 1 depicts typical images obtained from the system that were used for image analysis presented as 488 nm excitation autofluorescence, 514 nm excitation autofluorescence, and an autofluorescence density map. The principle behind this dual-wavelength method is based on the difference in the emission profiles of the retinal fluorophores (predominantly lipofuscin) in relation to the excitation wavelength. The ratio of optical densities at each region of interest is obtained and then normalized to the baseline dual-wavelength autofluorescence ratio. Variables in retinal illumination, such as pupil size and optical axis, are compensated by evaluating the ratio of the two excitation images given that each wavelength is equally affected. Therefore, this ratiometric approach using the dual-wavelength configuration for the autofluorescence excitation enabled us to quantify

**Table 1. Light Exposure Parameters Used during Image Acquisition**

<table>
<thead>
<tr>
<th>Light Source</th>
<th>Wavelength (nm)</th>
<th>Output Power (μW)</th>
<th>Radiant Exposure* (J/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diode laser</td>
<td>830</td>
<td>60</td>
<td>0.11</td>
</tr>
<tr>
<td>Argon laser</td>
<td>514</td>
<td>290</td>
<td>0.03</td>
</tr>
<tr>
<td>Argon laser</td>
<td>488</td>
<td>300</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Exposure durations of 60 seconds (typical) were assumed in the calculation of the radiant exposure for NIR-reflectance and 3 seconds for each argon laser source.
only the changes in FAF caused by the laser-induced damage. This normalized dual-wavelength autofluorescence ratio enabled us to group multiple subjects and to analyze observed trends in the dynamic FAF profile. Autofluorescence ratios were averaged for multiple lesions across no fewer than three age-matched animals.

RESULTS

Laser Dosimetry

Initially, white light funduscopy was used to determine the MVL threshold in the aged Brown Norway rat model. As shown in Figure 2, a range of exposure powers was used in a subset of animals (n = 6) to determine the laser parameters for creating an MVL. A comprehensive slit lamp evaluation was conducted within 30 minutes of laser exposure. Multiple laser lesions were assessed in each animal. In each subject, the characteristic gray lesion was barely visible at exposure powers of 16 mW and 22 mW, whereas lesions made using lower laser powers were not detectable during a standard slit lamp evaluation. This determination established the criterion for the MVL threshold level at 16 mW. Laser powers greater than 22 mW were deemed suprathreshold and therefore were not used to assess the lower limits of our autofluorescence detection capabilities. Our threshold determination was consistent with previously published results by Lund et al.39 This correlation helped validate the use of the Brown Norway rat laser exposure model with regard to established exposure thresholds. Corresponding autofluorescence images, taken immediately after placement of the lesions, revealed heightened definition of the MVL morphology and exhibited detectable changes as the result of subthreshold exposure at 13.5 mW. Laser exposures at 8 mW did not produce any noticeable changes in either white light or autofluorescence images. Similarly, powers lower than 8 mW were evaluated but exhibited no visible changes with either imaging modality.

Quantification of Changes in Autofluorescence Immediately after Laser Exposure

The heightened levels of lipofuscin in the aged rodent model enabled us to acquire baseline FAF levels and to quantify the immediate reduction in autofluorescence as a function of laser power. Laser lesions created using 8, 13.5, 16, 20, and 22 mW were assessed to establish the sensitivity of our imaging technique in the differentiation of a narrow, low-dose range incorporating both subthreshold and MVLs. This dose-dependent reduction in autofluorescence is plotted in Figure 3, and the accompanying data (Table 2) present the number of lesions measured at each exposure power, radiant exposure, mean dual-wavelength autofluorescence ratio, and corresponding SE. Laser powers greater than 8 mW resulted in a significant decrease in the autofluorescence ratio relative to the magnitude of the laser power (ANOVA, P < 0.001; observed power = 1). No significant changes in autofluorescence were detectable at 8 mW (paired t-test, P = 0.867). Regression analysis was performed to establish the linear dose-dependent decrease in autofluorescence ($R^2 = 0.9605$) over the measured range of laser powers.

Short-Term White Light Funduscopy and Autofluorescence Evaluation

The time course assessment of two imaging modalities, white light funduscopy and autofluorescence, shown in Figure 4 depict the acute-phase changes observed during the first week after laser exposure. The MVLs at 16 and 22 mW were selected because they exhibit the distinctive gray appearance in the white light fundus images immediately after laser exposure. Our initial short-term examinations of the laser lesions revealed pronounced differences in the dynamic appearance of the affected areas based on the dual-wavelength FAF imaging method. Observations of the gray lesions using white light funduscopy showed a gradual fading at follow-up imaging time.

FIGURE 1. Image acquisition for lipofuscin quantification based on dual-wavelength excitation and subtraction. (A) 30° FOV autofluorescence image of the rat retina using a 488-nm source for excitation. (B) 30° FOV autofluorescence image of the rat retina using a 514-nm source for excitation. (C) Lipofuscin density map based on optical density measurements taken with each excitation wavelength.

FIGURE 2. Dosimetry to establish the clinically defined MVL threshold using our laser exposure configuration and the parameters already described. (A) Composite white light image exhibiting the characteristic blanching immediately after laser exposure at powers of at least 16 mW. (B) Autofluorescence images (488-nm excitation shown) exhibit a higher degree of definition and disruption of the retina as a result of the photothermal insult.
points of 24 and 48 hours. Ultimately, the lesions were no longer distinguishable at 72 hours after laser exposure or at any other subsequent imaging time point greater than 3 days.

Laser lesions in the FAF images appeared as hypoautofluorescent spots immediately after laser exposure when compared to the undamaged RPE. At 24 hours, there was a shift toward hyperautofluorescence in both the 16- and 22-mW lesions, with the brightest hyperautofluorescent areas observed in the central regions corresponding to the 22-mW exposures. At 48 hours, the 16-mW lesions appeared to be amorphous, hyperautofluorescent regions as multiple lesion boundaries began to overlap. The 22-mW lesions also exhibited increased hyperautofluorescence but maintained their general circular shape. One week after laser exposure, the 16-mW lesions showed a decrease in hyperautofluorescence trending toward preexposure levels, whereas the 22-mW lesions maintained heightened hyperautofluorescence.

Quantification of Dynamic Changes in Autofluorescence after Laser Exposure

As shown in Figure 5, the changes in autofluorescence observed over 4 months revealed further information regarding threshold limits of laser exposure. Two-way ANOVA was conducted on the data to establish statistical significance of the observed changes in autofluorescence based on the two conditions shown, time after exposure ($P = 0.002$, observed power $= 0.931$) and laser power ($P = 0.04$, observed power $= 0.496$).

Complete recovery of the baseline autofluorescence levels were observed at 3 weeks and 4 months for the 13.5- and 16-mW lesions, respectively. At these two time points, the autofluorescence measurements did not exhibit a statistical difference after exposure to 13.5 mW (paired $t$-test, $P = 0.601$) or 16 mW (paired $t$-test, $P = 0.937$) compared with preexposure autofluorescence. Finally, half the animals ($n = 3$) from group 3, receiving exposures of 20 and 22 mW, were evaluated at 6 months and still exhibited hyperautofluorescence. A summary of the observed long-term autofluorescence recovery is presented in Table 3.

**Fluorescein Angiography Assessment**

Fluorescein angiography, conducted within 1 hour after laser exposure, was used to independently verify the laser lesion location and to confirm the disruption of retinal continuity. Each laser exposure revealed the expected disruption of the RPE, shown by the characteristic “halos” around the lesions; however, these features were not visible during follow-up imaging at 1 and 2 weeks. Furthermore, no indications of choroidal neovascularization were present during subsequent imaging sessions. Moreover, histologic evaluation of the eyes, on completion of the study, did not reveal evidence of damage using standard hematoxylin and eosin staining techniques at the subthreshold lesions where FAF recovery was observed.

**DISCUSSION**

The primary objective of this study was to explore the use of dual-wavelength FAF imaging as a sensitive means of noninvasively monitoring changes in the RPE as the result of photothermal damage. The heightened definition of the laser lesion boundaries using FAF underscores the benefits of monitoring lipofuscin density as a biomarker for retinal damage compared...
with other conventional imaging techniques. Moreover, we were able to demonstrate that measurable changes to the RPE were detectable below the conventional MVL threshold by using dual-wavelength FAF imaging. The reduction in FAF intensity, measured immediately after exposure, also provided a highly sensitive marker for retinal laser injury as a function of light dose.

Although the origin of all the changes resulting in the immediate decrease of the FAF signal are not clear, the temporary hypoautofluorescence may be partially attributed to an increase in blue light attenuation by denatured proteins shortly after photothermal injury. This may explain the relatively rapid transition from hypoautofluorescence to hyperautofluorescence observed during the acute-phase response. Similar observations have been reported in clinical studies using standard AF imaging techniques.40,41 It is also possible that some of the changes measured in FAF were caused by additional contributing factors, such as photobleaching and decreased absorption of melanin. The early hypoautofluorescent signal may be partially attributed to the photobleaching of melanosomes, which have been shown to bleach in a time-dependent manner in response to intense visible light sources.42 Furthermore,
Long-Term Autofluorescence Recovery

<table>
<thead>
<tr>
<th>Power (mW)</th>
<th>No. Lesions Evaluated</th>
<th>Recovery of Autofluorescence</th>
</tr>
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<tbody>
<tr>
<td>8</td>
<td>5</td>
<td>N/A*</td>
</tr>
<tr>
<td>13.5</td>
<td>4</td>
<td>3 wk after exposure</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>4 mo after exposure</td>
</tr>
<tr>
<td>20</td>
<td>9</td>
<td>Retinal damage still evident at 6 mo</td>
</tr>
<tr>
<td>22</td>
<td>9</td>
<td>Retinal damage still evident at 6 mo</td>
</tr>
</tbody>
</table>

* No significant change in autofluorescence signal was detectable after laser exposure.

photooxidation of RPE melanin has been shown to reduce the ability of the RPE cells to perform antioxidant functions, which may impede the wound healing process and contribute to the long-term hyperautofluorescence observed after suprathreshold laser exposures.

In addition to detecting short-term changes in RPE autofluorescence, we were able to monitor dynamic changes in the laser lesions for up to 6 months in our small animal model to show autofluorescence recovery in relation to the radiant exposure levels. We measured a complete recovery of baseline autofluorescence after laser exposures of 13.5 and 16 mW at 3 weeks and 4 months, respectively. This does not preclude the possibility that underlying permanent retinal damage is present, but the return to baseline autofluorescence levels may provide some indication of RPE recovery. We also observed a trend toward baseline AF levels after 20- and 22-mW exposures (Fig. 5), although complete recovery of AF was still not present 6 months after exposure. There have been reports of photoreceptor migration that appear to fill in deficits left after UV-A exposure, which may account for the apparent decrease in the autofluorescence ratio after retinal photocoagulation. The acute and permanent autofluorescence changes in a dose-dependent manner. Interestingly, we observed a nearly identical decrease in FAF immediately after exposure for subthreshold laser powers at 13.5 mW. However, our suprathreshold lesions (radiant exposures ~25–30 J/cm²) exhibited signs of permanent damage 6 months after the initial photothermal insult, whereas the Morgan group did not observe any permanent damage with comparable photochemical exposures.

Short pulsed lasers have also been used for retinal therapy to create photomechanical damage through the formation of microbubbles within RPE cells during microsecond exposures as the result of thermal confinement.

The relationships between changes in autofluorescence and retinal damage mechanisms associated with light exposure are not well understood. Based on our findings, the initial photothermal insult results in the expected denaturation of proteins, but the subsequent changes in the autofluorescence profile measured at follow-up imaging time points appear to be reminiscent of photochemical damage. Radiant exposures from the imaging light sources (488 and 514 nm) used in our experiments are well below the ANSI safety standards, indicating there would be a negligible contribution to the change in the lipofuscin density from photochemical damage. However, we cannot rule out the possibility of a secondary, latent effect in the marginal areas of the laser lesions, which undergo considerable stress during thermal diffusion and equilibration.

Although the initial damage mechanism is different, both the photothermal and the photochemical lesions exhibit comparable features with regard to the changes measured in the autofluorescence profile over time. Other groups have shown similar trends in immediate autofluorescence reduction using low-dose exposure powers for longer durations, but with radiant exposures similar to those in our present study, to induce photochemical damage. Morgan et al. observed transient and permanent autofluorescence changes in a dose-dependent manner. Interestingly, we observed a nearly identical decrease in FAF immediately after exposure for subthreshold laser powers at 13.5 mW. However, our suprathreshold lesions (radiant exposures ~25–30 J/cm²) exhibited signs of permanent damage 6 months after the initial photothermal insult, whereas the Morgan group did not observe any permanent damage with comparable photochemical exposures.

Dual-wavelength FAF imaging may be used in the evaluation of microbubbles within RPE cells during microsecond exposures as the result of thermal confinement. Although characterization of this type of laser tissue interaction was beyond the scope of our present study, it would presumably result in a similar, quantifiable reduction in FAF using the dual-wavelength method.

The 30° × 30° field of view used in this study does not provide the high degree of spatial resolution that is required for RPE mosaic imaging at the cellular level, as has been recently demonstrated by combining adaptive optics and fluorescence imaging modalities. However, the damage incurred during photocoagulation still results in detectable changes in retinal autofluorescence at relatively low radiant exposures. This further exemplifies the potential for dual-wavelength autofluorescence imaging in a clinical setting to evaluate both the short-term and the long-term changes associated with retinal damage. Moreover, as the field of molecular imaging is developed, dual-wavelength FAF imaging may be used in the evaluation of the RPE response at a molecular level, leading to an enhanced understanding of subtle changes associated with retinal injury and pathology.

The dual-wavelength FAF images acquired with our system provide numerous benefits over both white light funduscopy and near infrared reflectance, which have traditionally been used in focal laser lesion placement and assessment. The contrast provided by lipofuscin not only reveals a more detailed picture of the lesion morphology immediately after the photothermal insult but also allows for time-course imaging to monitor wound healing and biochemical changes in the RPE. The dual-wavelength approach also eliminates many of the factors that can complicate FAF imaging such as pupil diameter, nonuniform illumination, and nonuniform pigment/flurosphore distribution. This is particularly problematic with retinal imaging in rodents, in which the geometry and optical power of the eye can magnify these issues, increasing the variability of FAF measurements. Furthermore, noninvasive quantification of retinal changes enables us to detect significant trends using smaller populations through follow-up imaging measurements at the precise sites of damage over extended periods of time.

In conclusion, our investigations, designed to assess the capabilities of dual-wavelength autofluorescence imaging as a highly sensitive tool for the detection of retinal injury, have demonstrated that this novel application may be used to quantitatively monitor time-course changes in the dual-wavelength autofluorescence ratio after retinal photocoagulation. The ac-
cumulation of lipofuscin in the aged Brown Norway rat makes this model an ideal subject with which to characterize laser-induced retinal phototoxicity. Multiwavelength FAF could potentially be used as a screening tool to determine the severity of retinal damage after laser therapy and as an indicator of RPE health based on the extent of autofluorescence recovery.

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References


