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Objective Area Measurement Technique for Choroidal Neovascularization from Fluorescein Angiography

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Abstract

The purpose of this study was to develop a non-biased method of quantitatively measuring choroidal neovascularization (CNV) areas based on late-phase fluorescein angiography (FA) images. Experimental CNV was induced in Long Evans rats by laser disruption of the Bruch's membrane. FA was performed weekly for 5 weeks. Multi-Otsu thresholding (MOT) was used to quantify CNV in late-phase FA images from both experimental rodent CNV and wet age-related macular degeneration patients (wAMD). Images were automatically thresholded into three levels based on the image histogram, with the highest level containing CNV. To determine the technique's ability to quantify CNV areas, rats were given either triamcinolone acetonide or dexamethasone sodium phosphate to treat CNV and compared to untreated rats. The rat CNV lesion areas measured from 5-week histology sections from each treatment group were compared to areas measured from the corresponding FA images. MOT was able to detect statistical decreases in rodent CNV area in the treatment groups versus control from weeks 3 through 5. The ratio of CNV area measured from histology to area measured from FA images was not statistically different between groups. Finally, to determine the usefulness of MOT on pathological morphologies of CNV, MOT was performed on late-phase FA images from patients with classic and diffuse CNV. The technique was able to segment classical CNV in wAMD patients, but performed poorly with diffuse CNV. MOT provides a robust, objective, and quantifiable area measurement of CNV lesion area in both experimentally-induced and pathological CNV. The results indicate that MOT could be a useful research tool in helping evaluate the effects of therapeutics on CNV growth.

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INTRODUCTION

Choroidal neovascularization (CNV) secondary to age-related macular degeneration (AMD) is the most common cause of severe vision loss in elderly patients (Green and Wilson, 1986; Klein et al., 2007). By the year 2025, it is expected that the incidence of CNV secondary to AMD in elderly patients in the U.S. will reach over 12% (Klein et al., 2007). Recent advances in anti-vascular endothelial growth factor (VEGF) therapeutics show much promise in addressing the need to effectively treat CNV (Brown et al., 2009; Rosenfeld et al., 2006). As more therapeutics are developed and evaluated in animal models, an objective and simple method to quantify the extent of CNV lesion area *in vivo* is needed.

Animal models of CNV fall into three categories: laser- and light-induced, surgically induced, and transgenic and knockout mouse models. The laser-induced rat model of CNV involves rupture of the Bruch's membrane by a laser beam (Dobi et al., 1989; Frank et al., 1989). This model allows for observation of CNV progression with imaging techniques such as fluorescein angiography (FA) and remains the standard for most CNV treatment evaluation experiments as it is relatively simple to create, inexpensive, and reproducible (Grossniklaus et al., 2010).

Several methods exist for quantification of CNV lesion area in animal models. One method requires multiple retinal specialists to manually outline and measure lesion areas based on FA images. This method is time-consuming, subjective, and the measured areas may vary among specialists. Often, a tiered grading scale is used (ranging from no leakage to severe leakage/hyperfluorescence) comparing early- to late-phase FA images that are analyzed by masked retinal specialists, averaged, and compared (Giani et al., 2011; Krzystolik et al., 2002; Takahashi et al., 2004; Yu et al., 2008). However, this method is also subjective, time-consuming, and can be easily influenced by image properties (e.g., brightness and contrast) that may vary between time points. More quantitative methods for monitoring CNV include flat-mount preparations, immunohistochemistry with serial reproductions, and optical coherence tomography (OCT) (Giani et al., 2011; Miller et al., 1990). Although flat mount and immunohistochemistry techniques yield a quantitative measurement of the lesion size, they require animals to be sacrificed at specified time points, leading to the use of numerous animals and the inability to track the same lesion over time. OCT allows for the lesions of the same animal to be viewed at multiple time points. The latest generations, such as Heidelberg Enhanced Depth Imaging (EDI), can image choroidal lesions in detail.

CNV grading systems based on fluorescence leakage can also be misleading and their results contradictory. Ciulla et al. (2001) demonstrated intravitreal injection of triamcinolone acetonide (TA) as a potent inhibitor of CNV in a laser-induced rat model. However, this conclusion was only supported through histological evaluation as they could not detect difference between control and TA treated lesions using their leakage grading system. Kim and Toma (2010) used an FA area measurement technique performed by a masked retina specialist manually outlining CNV lesions and confirmed TA to be a potent inhibitor of CNV.

To address the issues of objectivity, ease of use, and cost-effectiveness, a new technique has been developed based on FA images and Otsu thresholding. In Otsu thresholding, a threshold is found which maximizes inter-class variance of the pixel levels on each side of the threshold. This separates a grayscale image into foreground and background (Otsu, 1979). Liao et al. (2001) proposed an algorithm improving the efficiency of computation of inter-class variance and generalized Otsu's method to determine thresholds for multiple classes within a single image (multi-Otsu thresholding).

This study applied the multi-Otsu thresholding (MOT) technique to objectively determine CNV lesion areas from FA images in a laser-induced model of CNV. The results were compared to corresponding histological sections of the lesions seen in the FA images for three different treatment groups: control (no treatment), TA intravitreal injection, and intravitreal injection of thermo-responsive hydrogel containing a suspension of dexamethasone sodium phosphate loaded nanospheres. FA images of CNV from wet AMD (wAMD) patients were also analyzed with MOT to determine the technique's usefulness in other morphologies of CNV.

METHODS

Animal Preparation

All animal procedures were in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Illinois Institute of Technology, and with the principles embodied in the statement on the use of animals in ophthalmic and vision research adopted by the Association for Research in Vision and Ophthalmology. Long-Evans male rats (1–3 months, 300g–350g) were purchased from Harlan Laboratories (Indianapolis, IN). Animals were anesthetized using 80 mg/kg of ketamine hydrochloride (Fort Dodge Animal Health, Fort Dodge, IA) and 10 mg/kg xylazine (AnaSed® Injection, Akorn, Inc., Decatur, IL) via intraperitoneal (IP) injection. Corneas were anesthetized using proparacaine drops (Bausch and Lomb, Rochester, NY) and dilated using phenylephrine (Bausch and Lomb) and atropine drops (Bausch and Lomb). Heart rate and blood oxygen saturation were monitored with a PulseOximeter (8500AV; Nonin Medical Inc., Plymouth, MN). Animals were placed on a heated stage and monitored to maintain a core body temperature of 37°C.

Laser-Induced Model of CNV

Laser photocoagulation was performed using an argon-green laser (AKC-8000, NIDEK, Inc., Fremont, CA) attached to a slit lamp, with laser power of 400 mW, duration of 100 ms, and spot diameter of 50µm. A 90-diopter lens was used to view to the posterior pole of the eye and focus the laser beam. Five to six lesions per eye were induced two to three disc diameters from, and centered on, the optic disc. Laser-induced disruption of Bruch's membrane was identified by the appearance of a bubble at the site of photocoagulation. Laser spots that did not result in the formation of a bubble were excluded.

Experimental Groups

Animals were separated into three groups to determine the ability of CNV quantification by MOT across multiple treatments. For analysis purposes, CNV lesions were considered independent within each treatment group. A control group of 11 rats received a 5 µl intraocular injection of 1x PBS. A second group of three rats received a 5 µl injection of 40 mg/ml triamcinolone acetonide (TA) (Kenalog®-40, Bristol-Myers Squibb, New York, NY). Intravitreal injections of TA have been shown to play a role in improving CNV in both humans (Challa et al., 1998; Rechtman et al., 2003) and laser-induced rat models of CNV (Ciulla et al., 2001; Kim & Toma, 2010). A third group of five rats received a 5 µl injection of thermo-responsive hydrogel containing a suspension of dexamethasone sodium phosphate (DSP)-loaded nanospheres. Intravitreal injection of dexamethasone has been shown to improve CNV in humans when combined with other therapies (Augustin et al., 2007) and as a single treatment in laser-induced rat models of CNV (Edelman & Castro, 2000). All injections were made through a 30-gauge needle.

The thermoresponsive hydrogel, developed by Tutturro et al. (2011), induces no long-term effects on retinal function and does not alter the release characteristics of the DSP-loaded

nanospheres once suspended within the hydrogel (Kang-Mieler et al., 2012). DSP-loaded nanospheres, described by Kang-Mieler et al. (2011), were prepared using a standard double-emulsion, solvent evaporation technique using PLGA (50:50, Mw 7–17 kDa) and had an encapsulation efficiency of 39%. DSP released from the nanospheres suspended in the hydrogel within 24 hours, with the equivalent of a 1.2 mg/ml dose. The release profile of the DSP-loaded nanospheres within the thermoresponsive hydrogel can be seen in Figure 1.

Fluorescein Angiography (FA)

Fluorescein angiography (FA) was performed weekly for five weeks after laser photocoagulation using a confocal scanning laser ophthalmoscope (cSLO) system (Heidelberg Retina Angiograph (HRA), Heidelberg Engineering, Germany). Images were captured at 1–2 min, 10 min and 20 min after intraperitoneal injection of 0.5 mL of 20% fluorescein dye (Sigma-Aldrich, St. Louis, MO).

Human AMD Images

To determine the efficacy of MOT on other morphologies of CNV beyond laser-induced CNV, late-phase FA images were obtained from patients with classic and diffuse CNV. Late-phase FA images were acquired using a Zeiss FF4 Fundus Camera (Carl Zeiss Meditec, Oberkochen, Germany) mounted on a WinStation 5000™ (Ophthalmic Imaging Systems Inc., Sacramento, CA). All images were collected retrospectively.

Quantification of CNV Lesion Area

Multi-Otsu Thresholding—An implementation of the multilevel Otsu threshold described by Liao et al. (2001) is available as a plug-in for ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>; plug-in available at <http://rsbweb.nih.gov/ij/plugins/multi-otsu-threshold.html>). The plug-in allows for a grayscale image to be segmented into a maximum of five levels. An example of image processing is presented in Figure 2. The use of late phase (20 min) images allows time for the fluorescein to clear the normal vasculature, preventing vessel hyperfluorescence from interfering with area measurements. For the purposes of this investigation, three levels, or “regions,” are defined: background (Fig. 2B), diffuse leakage (non-hyperfluorescent leakage; Fig. 2C), and CNV (hyperfluorescent leakage; Fig. 2D). The threshold within ImageJ is adjusted to include all pixels within the “Region 2” (CNV) plug-in output. The CNV lesion was then loosely outlined by the user to avoid vessels and other non-lesion pixels (Fig. 2E). The measured number of pixels was then converted into area using a scaling factor based on the optical properties of the rat eye and field of view of the image using the following equation:

$$SF = \frac{\pi}{180} \times N'F' \times \frac{FOV}{RES}$$

where SF is the scaling factor in micrometers per pixel, $N'F'$ is the distance from the second nodal point of the eye to the retina in micrometers, FOV is the field of view in degrees, and RES is the length of the image in pixels (Patel et al., 2011).

Only images that were centered around the lesion, well focused on the choroid and non-saturated with even illumination were used. All areas are reported as average $\pm$ standard error. MOT was performed on FA images from week 2 onward. Kuroki et al. (2002) reported that laser-induced CNV in rats did not fully form in many lesions until two weeks following laser induction. At week 2, some lesions still had strong wound staining and leakage that caused the measured lesion areas to be highly variable. However, by week 3 the

lesions had fully formed across the wound and became well defined, allowing for clear differences to be seen between treatment groups.

Histology—Serial histological sections were made of the lesions from the control, TA-treated, and DSP nanosphere gel-treated groups. Animals were sacrificed by sodium pentobarbital overdose five weeks after induction of CNV. Eyes were enucleated, fixed in Davidson's solution, stained with hematoxylin and eosin, and embedded in paraffin. Serial sections of 5 μm width were made every 50 μm through the target lesions. To compare histology to the area measurements from the FA images, the width of the lesions in each slice was multiplied by the distance between slices (50 μm) to give an approximation of the surface area covered by the lesion.

The extent of the lesion in each slice was determined by measuring the relative thickness across the lesion. Relative thickness is defined as the ratio of the thickness from the bottom of the pigmented choroid to the top of the CNV lesion to the thickness of adjacent, normal choroid (Ming et al., 2004). Sections of lesions with relative thickness larger or smaller than one were included in the area calculations. Choroid substantially thicker than normal (relative thickness > 1.1) was defined as fibrovascular growth. The lesions from the treated groups were often observed to have little fibrovascular growth. The choroid around the lesion site was very thin and de-pigmented, with the retina prolapsed into the wound. These lesions were defined as wound lesions and the extent of the wound lesion was defined as choroid that was substantially thinner than normal (relative thickness < 0.9). Three complete lesions from each treatment group were analyzed.

Statistical Analysis

Each CNV lesion was considered independent within a treatment group. Average areas of the total number of lesions from each treatment group at each time point were compared between treatment groups using one-way ANOVA. Bonferroni's post-hoc test was used if ANOVA was significant for $p < 0.05$. The ratio of the lesion area measured from the FA images to the corresponding lesion area calculated from histology was also compared between groups using one-way ANOVA.

RESULTS

Experimental CNV

The MOT technique effectively delineated the CNV lesion from the surrounding retina in the experimental laser model, allowing for quantification of CNV lesion areas. As seen in Figure 2B, the "background" threshold output contains only the typical background fluorescence. The "diffuse leakage" (Fig. 2C) output contains the non-hyperfluorescent leakage around the CNV lesion and from out-of-focus vessels. The "CNV" output (Fig. 2D) includes the hyperfluorescent central region of the CNV lesion. Figure 3 shows representative lesions from the treatment groups and the CNV output from MOT. It can be seen that the control lesion is larger and more spread out than the treated lesions, with treated lesions being more contained around the initial wound site as they did not exhibit as much fibrovascular growth (Fig. 5).

The areas for all lesions were measured using MOT for weeks 2 through 5 after laser-induction (Fig. 4). As expected, lesion areas were not significantly different between treatment groups at week 2 ($p=0.51$) due to strong wound staining and leakage that caused the measured lesion areas to be highly variable (Kuroki et al., 2002). As the weeks progressed, clear FA images focused on the choroid were more difficult to acquire; hence, the reduced number of lesions by week 5 for all treatments. Repeated testing and exposure

to anesthesia can cause the cornea to decrease in transparency (Turturro et al., 2011) and hindered clear FA images.

CNV lesion areas from control animals were as follows: week 2, $0.043 \pm 0.004 \text{ mm}^2$ (n=43); week 3, $0.053 \pm 0.006 \text{ mm}^2$ (n=53); week 4, $0.049 \pm 0.006 \text{ mm}^2$ (n=49); week 5, $0.046 \pm 0.004 \text{ mm}^2$ (n=33). Control lesion areas did not significantly change throughout the experimental timeframe ($p=0.94$). Histology confirmed considerable CNV lesions in control animals at week 5. Retinal detachment was present above the fibrotic lesions with multiple layers of RPE cells beginning to envelope both the inner and outer surfaces of the CNV membrane (Fig. 5A). Substantial fibrovascular proliferation into the retina was observed. CNV lesions produced in the control animals are consistent with previous studies (Dobi et al., 1989; Giani et al., 2011; Krzystolik et al., 2002).

TA-treated lesions were well defined throughout the five weeks with small hyperfluorescence surrounding the lesion (e.g., Fig. 3B). CNV lesion areas from TA-treated animals were as follows: week 2, $0.029 \pm 0.017 \text{ mm}^2$ (n=4); week 3, $0.016 \pm 0.002 \text{ mm}^2$ (n=11); week 4, $0.019 \pm 0.002 \text{ mm}^2$ (n=11); week 5, $0.024 \pm 0.004 \text{ mm}^2$ (n=4). TA-treated lesion areas were significantly smaller than control at week 3 ($p=0.005$) and week 4 ($p=0.021$). TA-treated lesion areas appear to be half the size of control areas at week 5, though the difference is not significant ($p=0.17$). This is likely due to the reduced number of TA-treated lesions available for measurement. Histology confirmed minimal CNV lesions in TA-treated eyes at week 5 (Fig. 5B). Fibrovascular proliferation was confined to the perimeter of the wound with minimal vessel growth into the retinal space. Histological results were consistent with previous studies for TA-treated lesions 5 weeks post-induction of CNV (Ciulla et al., 2001, 2003).

CNV lesion areas from DSP-treated animals were as follows: week 2, $0.037 \pm 0.006 \text{ mm}^2$ (n=28); week 3, $0.028 \pm 0.005 \text{ mm}^2$ (n=25); week 4, $0.021 \pm 0.004 \text{ mm}^2$ (n=25); week 5, $0.027 \pm 0.006 \text{ mm}^2$ (n=19). DSP-treated CNV lesion areas were also significantly smaller than control lesions at week 3 ($p=0.024$), week 4 ($p=0.002$), and week 5 ($p=0.016$). Histology showed minimal CNV growth similar to TA-treated eyes after week 5, with fibrovascular proliferation confined to the perimeter of the lesion and no substantial fibrovascular proliferation into the retinal space (Fig. 5C).

DSP- and TA-treated lesions exhibited a similar course of changes throughout the five weeks. There was no significant difference in CNV lesion area between these two groups at any time point (Fig. 4). This suggests that controlled release of a low dose of DSP released over 24 hours is as effective as bolus injection of TA.

Histology was performed on three lesions from each treatment group to compare the areas determined by MOT to histological areas. The ratio between MOT and histology indicates how much of the actual lesion area was apparent from FA. For area comparisons based on MOT to be valid, the MOT areas for all treatment groups need to have the same direct proportionality to histological lesion size (i.e., the same ratio). There was no significant difference between treatment groups in regard to the area ratios for the MOT technique compared to histological lesion areas ($p=0.51$). Table 1 shows the area ratio (MOT vs. histology) for each treatment group. The average area ratio among all treatments for MOT compared to histological lesion areas was $64.3\% \pm 2.3\%$. This lack of a significant difference between treatment groups suggests MOT as an effective technique in quantifying CNV lesion areas.

Pathological Morphologies of CNV

FA images from human wAMD patients were used to test the effectiveness of MOT in segmenting pathological morphologies of CNV. MOT performed on late-phase FA images from these patients is shown in Figure 6. Figure 6A and B are examples of classic CNV, displaying hyperfluorescent and localized FA staining similar to the experimental CNV. The output of MOT was able to distinctly segment the CNV and not include any irrelevant features such as retinal blood vessels, producing similar results to the experimental CNV. Figures 6C and D show examples of diffuse CNV, displaying mottled and moderate fluorescence spread throughout the image. The diminished fluorescence causes the MOT output to include retinal blood vessels in addition to the CNV. The low level of fluorescence of the diffuse CNV relative to the background is a particular problem in Figure 6D. The low level of fluorescence causes MOT to identify more of the diffuse fluorescence in the space between CNV lesions, preventing the edges of the lesions from being sharply delineated and merging closely spaced lesions. The results show that MOT works well on classical or localized CNV but performs poorly on cases of diffuse CNV with low levels of fluorescence relative to background.

DISCUSSION

MOT detected clear differences in CNV lesion areas between the treatment groups in the animal experiments. The area measured by MOT was approximately two-thirds of the CNV lesion determined by histology. This may be explained by both avascular fibrous tissue being included in the histological lesion area which would not have fluoresced in FA images (Dobi et al., 1989), as well as the likely overestimation of histological areas due to the rectangular area approximation of the limited number of histology sections per lesion. However, the ratio of areas measured with MOT versus histology was not significantly different between the treatment groups, suggesting that the CNV area measured by MOT from FA images reliably correspond with CNV lesion size determined by histology.

Areas measured from FA images did not differ significantly among treatment groups at week 2 due to CNV not being completely formed. As mentioned above, Kuroki et al. (2002) reported that laser-induced CNV in rats did not fully form in many lesions until two weeks following laser induction. The high variability of lesion areas determined by MOT suggests that at week 2, strong wound-staining and leakage prevented significant differences in lesion areas to be observed. However, by week 3, the lesions had fully formed across the wound and became well defined, allowing for clear differences to be seen between treatment groups.

Controlled release of a low dose of DSP over 24 hours resulted in significantly reduced CNV lesion areas compared to control during weeks 3–5. This reduction in CNV lesion area was comparable to TA-treated lesions as there were no significant differences between these two groups throughout the duration of the experiment. In the human vitreous, DSP has a short half-life of approximately 3.5 hours (Herrero-Vanrell et al., 2011) while TA is much less soluble and has been reported to have a half-life of approximately 19 days (Beer et al., 2003). The significantly reduced CNV lesion areas suggest that controlled release of DSP can compensate for a short half-life of DSP and can be an effective way to deliver DSP.

MOT was able to segment CNV in both experimental and pathological CNV imaged with cSLO or fundus camera. The optimal condition for MOT is to have a uniformly illuminated FA image. Images must not be saturated and must be in grayscale. The best results are obtained with late-phase (20 min) FA when the fluorescein has largely cleared from the vessels. In healthy retinal vasculature, fluorescein does not leak out of the vessels due to the blood-ocular barrier (e.g., Lund-Andersen, 2002). In late-phase FA (20 minutes after the

injection of fluorescein), fluorescein has largely cleared from the normal, healthy vasculature. However, in CNV patients, this is not the case. Due to breakdown of the Bruch's membrane (and blood-ocular barrier), the fluorescein can escape the vasculature via the leaky vessels and localize within the lesion. Hence, pooled fluorescein in CNV lesion can be visualized clearly in late-phase of FA. If FA images are taken before the fluorescein has mostly cleared from the vessels, MOT becomes less sensitive and not as objective. The user is then required to minimize the contribution of retinal vessels to the area measurement by carefully drawing a polygonal measuring box around the lesion so that the vessels are excluded from measurement.

The main advantages of MOT over other measurement techniques are that it provides a fast and objective measurement of CNV using open-source software (ImageJ) and that it can be employed by non-retinal specialists. MOT is based on pixel variation; thus, it is not as sensitive to image brightness so long as the original FA image is evenly illuminated. This is advantageous over tiered grading scales that are dependent on the level of hyperfluorescence (i.e., absolute brightness) of the lesion. With grading scales, overall image brightness may influence one's perception as to the extent of lesion hyperfluorescence. The automatic and adaptive nature of MOT removes the subjective nature of grading scales based on FA.

MOT is a fast algorithm that does not require much computational power to determine the thresholds that define each region's output. It also provides a useful tool to basic researchers without access to OCT with EDI, which allows for a more detailed view of the choroid. Though OCT with EDI can provide a detailed view of the choroid, there are no simple algorithms to automatically quantify fibrovascular growth in these scans. MOT is a simple algorithm that can be quickly implemented to observe CNV over a wide field of view.

Toma et al. (2010) previously evaluated several edge detection techniques for measuring experimental CNV area from FA images using commercial software. The techniques had some level of automation; however, retinal specialists were still needed to outline the lesion or the user was required to manually adjust the threshold level for each image until the irrelevant microvasculature surrounding the lesion disappeared. The authors also stated that these techniques were subjective in nature. MOT relies on adaptive thresholding, allowing for a more objective quantification of CNV that does not require the user to re-adjust the threshold for each image. Additionally, MOT has a stronger physical basis than edge detection. It divides the image into three regions based on the expected types of fluorescence observed in FA images (background, diffuse leakage, and hyperfluorescence). Edge detection, in contrast, requires the user to modify various parameters until an "appropriate-looking" level of segmentation is achieved. Both MOT and edge detection are useful for quantifying classical or localized CNV lesions; however, they both perform poorly when used for detecting diffuse lesions due to the lower brightness levels of the lesions. Low brightness results in a low threshold for edge detection causing the technique to be susceptible to noise and the detection of false edges and irrelevant features (Faghieh and Smith, 2002).

In conclusion, MOT provides a simple and objective method of quantifying localized CNV from late phase FA images. MOT was able to detect clear differences between treatment groups in laser-induced rat model of CNV. MOT was also able to segment classic CNV lesions from wAMD patients, but performed poorly in cases of diffuse CNV. The technique has similar performance to edge detection methods but does not require the user to adjust parameters for each image, making it more objective when comparing CNV areas across multiple time points.

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Highlights

- Developed a non-biased method of quantifying choroidal neovascularization (CNV)
- Method detected difference between experimental treatment groups
- Method was able to also segment pathological CNV

Figure 1. Release profile of DSP from DSP-loaded nanospheres suspended within thermo-responsive hydrogel. Nearly all (92%) of the encapsulated DSP is released within 24 hours. (n=3; error bars represent S.E.M.)

Figure 2.

Lesion area quantification is performed using the “Multi-Otsu Threshold” plug-in for ImageJ. **A)** The original image is opened in ImageJ and three levels are selected. After MOT, three images are produced: **B)** Region 0 (Background), **C)** Region 1 (Diffuse Leakage), and **D)** Region 2 (CNV). With Region 2 selected, the threshold is adjusted to include all pixels within the image (shown as red in **E)**). The lesion is outlined (yellow square in **E)**) and the number of pixels within the lesion is measured, which is then converted into an area using the scaling factor described by Patel et al. (2011). Note the example image has been cropped for demonstration purposes.

Figure 3. MOT applied to FA images from laser-induced CNV in rats. FA images are on the left side and the output of thresholding on the right. The lesions are representative of their treatment groups 3 weeks post-lasering: control (**A**), TA (**B**), and DSP (**C**). The optic disc can be seen in bottom of (**B**, **C**). Scale bar (200 μ m) shows scale for all images.

Figure 4.

CNV lesion areas for Control, TA, and DSP treatments determined using MOT. No significant difference is seen among treatments at week 2. CNV areas for both TA and DSP treatments are significantly less than non-treated areas ($p < 0.05$) at weeks 3 and 4. By week 5, a significant difference is only seen between control and DSP treatments. (Error bars represent S.E.M.)

Figure 5.

Representative H&E sections of lesions from each treatment group at week 5. In control eyes (A) the retina remained detached from the site of injury and RPE cells began to migrate over the CNV membrane (black arrows). Note the fibrovascular proliferation arising through the disrupted RPE and Bruch's membrane and infiltrating the retina. In both TA-treated (B) and DSP-treated (C) eyes, the retina is drawn into the defect and did not detach. Minimal FVP is seen in both TA- and DSP-treated eyes with fibrovascular proliferation limited to the edges of the lesions (white arrows). (Scale bar is 50 μ m.)

Figure 6. MOT technique applied to FA images of CNV from human AMD patients. The method was tested on both classic (**A**, **B**), mixed (**C**), and diffuse (**D**) CNV. As seen by the isolated lesions after MOT, the technique is able to segment out CNV. In the cases of (**C**) and (**D**), retinal vessels are also segmented due to the large areas of diffuse CNV.

Table 1

Area ratios of multi-Otsu thresholding compared to histology in the laser-induced rat model of CNV.

Treatment	Multi-Otsu Threshold (mm²)	Histology (mm²)	Ratio
<i>Control</i>	$3.13 \pm 0.85 (\times 10^{-2})$	4.7 ± 1.17	$66.5\% \pm 2.3\%$
<i>TA</i>	2.66 ± 0.45	4.14 ± 0.87	$65.2\% \pm 2.4\%$
<i>DSP</i>	1.76 ± 0.47	2.87 ± 0.8	$61.4\% \pm 2.2\%$
<i>Average</i>			$64.3\% \pm 2.3\%$