A Novel Imaging Technique for Experimental Choroidal Neovascularization

Mercedes Campos,1,2 Juan Amaral,2,3 S. Patricia Becerra,5 and Robert N. Fariss1

PURPOSE. Choroidal neovascularization (CNV) is the end point of several ocular diseases that lead to blindness. The authors developed an imaging technique for visualizing and quantifying morphologic changes associated with experimental laser-induced CNV.

METHODS. CNV was induced using laser energy to disrupt Bruch’s membrane. Rats were euthanatized immediately after laser injury and at 1, 2, 3, 4, 7, 14, and 60 days. Nonlasered eyes were used as the control. Eyes were enucleated and fixed, and the posterior eye cups were fluorescently labeled with markers for nuclei (DAPI; 4',6'-diamino-2-phenylindole), endothelial cells (isolectin IB4), microglia (CD11b), and filamentous actin (phalloidin). FITC-dextran perfusion was compared with our technique. A confocal microscope was used to evaluate flat-mounted specimens. Computer software generated three-dimensional reconstructions for qualitative and quantitative analysis of confocal image stacks.

RESULTS. In nonlasered areas, RPE cells were visualized as a uniform hexagonal array. Immediately after laser exposure, a circular area devoid of fluorescent labeling was observed, indicating disruption of the choroid–Bruch’s membrane–RPE complex. One day after laser exposure, cellular debris and fragmented nuclei were present, and an autofluorescent ring was visible at the site of Bruch’s membrane disruption. The ring correlated with bubble formation and CNV induction. Three days after laser injury, phalloidin-labeled RPE cells and isolectin-labeled endothelial cells increased significantly, reflecting cell proliferation and migration. By day 4, isolectin-positive cells forming vascular tubes were visualized. The volume of CNV vessels increased exponentially during the next 3 days. By 7 days, a well-defined isolectin-labeled CNV network was present, and its volume was preserved for several weeks. CNV volumes calculated on the basis of FITC-dextran perfusion were significantly lower than volumes obtained using lectin-labeled samples.

CONCLUSIONS. A novel imaging technique was developed that allows a three-dimensional reconstruction and measurement of laser-induced CNV lesions in rat choroid/RPE flatmounts. This technique provides excellent morphologic detail and facilitates the study of critical early events in CNV, including the rupture of Bruch’s membrane and the formation of endothelial clusters before vessel formation. CNV complexes are labeled at an earlier stage and more reproducibly than with FITC-dextran perfusion, providing a more accurate preclinical evaluation of antiangiogenic molecules. (Invest Ophthalmol Vis Sci. 2006; 47:5163–5170) DOI:10.1167/iovs.06-0156

Choroidal neovascularization (CNV) is a pathologic process involving the formation of new blood vessels that are continuous with choroidal vessels. These vessels grow through breaks in Bruch’s membrane, invading the sub-RPE and/or subretinal space.6 Left unchecked, CNV leads to severe, often irreversible, visual loss.

CNV is associated with a variety of ocular diseases, including macular degeneration, myopia, histoplasmosis, angiod streaks, tumors, and traumatic and idiopathic conditions, all of which have in common the presence of breaks in Bruch’s membrane.7 The recent resurgence in interest in CNV has been driven by the discovery of molecular mechanisms guiding this process and by the recognition that treatments targeting neovascularization will be a central strategy for treating the wet form of age-related macular degeneration (AMD). AMD is the leading cause of irreversible vision loss in the elderly population in the United States and other Western nations.8 CNV in the macula was first described in 1929 by Holloway and Verhoeff,9 but its importance was not recognized until 1967 when Gass,10 evaluating patients with fluorescein angiography, suggested that CNV may play an important role in the pathogenesis of macular diseases. However, the pathogenesis of AMD remains unclear; 4 to 10 million Americans are estimated to have some form of the disease. Although an estimated 80% of AMD patients have the nonexudative form, the exudative form (CNV) may be responsible for almost 90% of the severe visual loss (20/200 or worse) due to AMD.6 Given that the appearance of CNV has devastating implications for the progression of AMD and other macular diseases and that current treatments have limited effectiveness, there is great interest in identifying clinically relevant inhibitors of ocular neovascularization and in the development of effective treatments targeting CNV.

Evaluation of potentially relevant angiogenesis inhibitors and testing the effectiveness of new treatments in animal models rely on methods for the assessment and quantification of experimental CNV. Laser injury of the RPE–Bruch’s membrane–choroid complex is a widely used technique for inducing CNV in primates11,12 and rodents.11,12 These laser-induced wounds are morphologically complex, three-dimensional (3-D) structures that undergo dynamic reorganization before and during neovascularization.

Several methodologies have been developed to evaluate experimental CNV. Classic histologic cross sections provide excellent morphology, but allow only the measurement of two-dimensional structures. To analyze the CNV complex in its entirety, serial reconstructions are needed, making the analysis difficult and time consuming.13,14 Corrosion casts preserve the 3-D architecture of the neovascular complex. However, this method is not widely used, because it is technically challenging and requires the destruction of the tissues associated with the vessels.15,16 Many studies with CNV models employ vascular perfusion of high-molecular-weight fluorescein-isothiocyanate

From the 1Biological Imaging Core and 3Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, Bethesda, Maryland. 2Contributed equally to the work and therefore should be considered equivalent authors.

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Corresponding author: Robert N. Fariss, Bldg. 7, NEI-NIH, Rm. 204, 7 Memorial Drive MSC 0703, Bethesda, MD 20892-0703; farissrn@nei.nih.gov.

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(FITC) dextran in choroid/RPE flatmount preparations.17–19 In contrast to cross sections, flatmounts allow complete visualization of intact laser-induced lesions. They are analyzed by fluorescent microscopy for measurement of lesion area, and by confocal microscopy serial sections for volume measurements.20,21 Because this technique permits visualization of patent vessels, unperfused vessels cannot be visualized. Several studies have reported that CNV vessels must be 7 to 10 days old before well defined perfused vessels can be visualized. Therefore, FITC-dextran perfusion will not adequately identify newly forming vessel complexes at early stages of CNV, limiting information regarding early changes.18,20

We developed a method to visualize and quantify the morphologic changes of experimental CNV, which provides advantages over conventional methodologies. This technique utilizes confocal microscopy to image fluorescently labeled cellular components of CNV complexes. The full thickness of these laser wounds can be analyzed by collecting serial optical sections. The fluorescently labeled RPE-chorioid preparations retained their complex 3-D structure allowing a qualitative and quantitative reproducible evaluation of the entire CNV lesion.

**Materials and Methods**

**Laser-Induced CNV**

All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Sixty Brown Norway male rats (Charles River Laboratories, Rockville, MD) weighing between 300 and 350g were used in this study. Rats were anesthetized with an intraperitoneal injection of a 40 to 80 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 10 to 12 mg/kg xylazine (Ben Venue Laboratories, Bedford, OH) mixture. Topical 0.5% proparacaine was applied and pupils were dilated with a mixture of 1% tropicamide and 2.5% phenylephrine (Alcon Fort Worth, TX). Hot pads maintained the body temperature while rats were placed in front of a slit lamp. Four to eight shots surrounding the optic nerve were placed with a Nd:YAG 532-nm laser (Alcon) using a 5.4-mm contact fundus laser lens (Ocular Instruments, Bellevue, WA), a spot size of 50 μm, power between 80 and 90 mW, and 0.100 seconds of exposure time. The end point ‘bubble formation’ assures breakage of Bruch’s membrane.11 The animals were euthanatized by CO2 exposure immediately after laser injury and at 1, 2, 3, 4, 7, 14, and 60 days after injury. Two animals were used as nonlasered control subjects for visualizing normal choroid-RPE complexes.

**Flatmount Technique**

With the nictitans membrane (nasal) used for orientation, eyes were enucleated and fixed for 1 hour in 4% paraformaldehyde (EM Grade; Polysciences, Inc. Warrington, PA) in phosphate-buffered saline (PBS; 9 g/L NaCl, 0.232 g/L KH2PO4, 0.703 g/L Na2HPO4 [pH 7.3]) for 1 hour. Under a dissecting microscope, the anterior segment and crystalline lens were removed, and the retinas were detached and separated from the optic nerve head with fine curved scissors. The remaining eye cups were washed with cold ICC buffer (0.5% BSA, 0.2% Tween 20, 0.05% sodium azide) in PBS. A 1:1000 dilution of a 10 mg solution of 4,6-diamidino-2-phenylindole (DAPI), a 1:100 dilution of a 1 μg/mL solution of isoelectric IB4 conjugated with Alexa Fluor 568, and a 1:100 dilution of a 0.2 units/μL solution of phalloidin conjugated with Alexa Fluor 488 (Invitrogen-Molecular Probes, Eugene, OR) were prepared in ICC buffer and centrifuged for 1 minute at 5000 rpm. Alternatively, CD11b (MCA275R; Serotec, Oxford, UK) conjugated with Alexa Fluor 488, an antibody that labels microglia in retina and brain, was used at dilutions of 1:200 to identify retinal microglia. A humidified chamber was prepared, the eye cups were covered with fluorescent dyes prepared as described earlier, incubated at 4°C with gentle rotation for 4 hours, and washed with cold ICC buffer. Radial cuts were made toward the optic nerve head, and the sclera-choroid/RPE complexes were flatmounted (Gel-mount; Biomedia Corp. Foster City, CA), covered, and sealed.

**FITC-Dextran Perfusion**

High-molecular-weight FITC-dextran (MW 2 × 106; Sigma-Aldrich, St. Louis, MO) was used in six rats for vascular perfusions, using a method similar to previously published methods.17–19 Briefly, eyes were enucleated and fixed for 1 hour in 4% paraformaldehyde in PBS, and finally 5 mL of 50 mg/mL FITC-dextran. Immediately afterward, eyes were enucleated and rinsed with 1 mL of 4% paraformaldehyde in PBS. The anterior segments were removed, the retinas detached, and the posterior eye cups labeled with isoelectin IB4, as previously described, flatmounted, and covered. The confocal images obtained were evaluated with commercial image-analysis software (Volocity; Improvision Inc. Lexington, MA), and the results were compared with our fluorescent labeling technique.

**Confocal Microscopy**

Multiplane z-series were collected with a confocal microscope (SP2; Leica, Exton, PA) and a 40X, 1.25 numerical aperture, oil-immersion objective. All images were collected at a 1024 × 1024-pixel resolution and a depth of 8 bits per channel. Voxel dimensions were 0.3662 μm for the x- and y-axes and 0.4884 μm for the z-axis. Fluorescent signals for DAPI (400–500 nm), Alexa Fluor 488, and FITC (500–550 nm) and Alexa Fluor 568 (580–675 nm) were collected by using a sequential scan mode to reduce bleed-through. For each experimental time point in this study, multiple lesions were imaged and evaluated (sample size for each time point shown in parentheses): control/nonlasered (4), 5 minutes (5), 1 day (23), 3 days (7), 4 days (11), 7 days (51), 14 days (6), and 60 days (10).

**Lesion Evaluation and Quantification**

Confocal microscope z-series collected as TIFF images were analyzed with the image-analysis software (Volocity; Improvision Inc.). An image sequence was generated, and a visualization module (Volocity Visualization; Improvision Inc.) was used to build animated 3-D reconstructions of the CNV complex. The navigation palette was used to turn the green (phalloidin, RPE) and blue (DAPI, nucleus) channels off. CNV complexes were identified using the red channel (isolectin), and their volumes in cubic micrometers quantified (Volocity Classification module; Improvision Inc.) at different time points after laser treatment.

**Results**

**Temporal and Spatial Changes in New Vessel Formation**

The use of confocal microscopic z-series and specific cellular markers conjugated with fluorescent dyes allowed visualization of different layers of the choroid-RPE complex. Phalloidin is a toxin with specific affinity for the F-actin cytoskeleton. Phalloidin conjugated to the fluorochrome Alexa Fluor 488 was used to label the RPE cells. The phalloidin label was visualized in green in the present study. Isolectin IB4, a lectin derived from the seeds of *Griffonia simplicifolia*, has specific affinity for α-β-galactosyl residues and has been used for specifically labeling endothelial cells and microglia in a number of species. It was conjugated with the fluorochrome Alexa Fluor 568, to outline the vessels in red. DAPI, a nuclear stain with specific affinity for double-stranded DNA, was used to visualize the nuclei in blue. The normal morphology of the choroid-RPE complex was visualized in nonlasered rat eyes by confocal microscopy. Phalloidin-label identified the actin bundles of the RPE cells forming a tightly packed, uniform hexagonal monolayer (Fig. 1A, top left). No lectin-labeled endothelial cells were visualized in the RPE layer (Fig. 1A, top middle). DAPI labeling
identified the nuclei of the RPE cells in a merged image (Fig. 1A, top right). Early morphologic changes preceding new vessel formation were visualized within 3 days after laser injury. Immediately after laser treatment, a tightly circumscribed circular region was visible at the site of injury, correlating with the disruption of the normal morphology of the choroid, Bruch’s membrane, and RPE cells. Within this circular zone, phalloidin and isolectin labeling were abolished, whereas some DAPI-positive nuclear fragments were visible (Fig. 1A, second row), consistent with the disruption of the RPE cells. One day after laser treatment, the circular lesion contained an extended area of phalloidin-labeled cellular elements, as well as DAPI-positive nuclei of diverse sizes and shapes and few isolectin-positive cells, consistent with cell proliferation and mobilization of inflammatory cells. An autofluorescent ring was detected in the layer between the RPE and the choriocapillaris (Fig. 1A, third row). Cryosections from the same flatmount correlated the ring with the borders of the laser-induced break in Bruch’s membrane (Supplementary Fig. S1; all supplementary files are online at http://www iovs.org/cgi/content/full/47/12/5163/DC1). Three days after laser exposure, phalloidin-labeled RPE cells were detected closer to the center of the laser lesion, reducing the diameter of the defect and suggesting that RPE cells had spread and/or migrated from the margins of the lesion. A densely packed mass of lectin-positive cells filled the center of the circular defect, but no lectin-labeled vascular tubes were detected at this time point (Fig. 1A, fourth row).

Four days after laser exposure, morphologic changes associated with new vessel formation were visualized. A confocal microscopic z-series allowed the visualization of isolectin-positive tubelike structures that originated beneath the choriocapillaris and traversed Bruch’s membrane and the RPE cells. This was the earliest time point at which new vessels were made visible in the injured area by isolectin labeling (Movie 1). Vascular tubes were visible in the lesion 4 days after laser injury (arrowheads; see also Movie 1). In more advanced lesions (14 and 60 days) several feeder vessels are visible (arrowheads; see also Movie 2). Scale bar, 100 μm.

**FIGURE 1.** Laser-induced CNV. (A) Early morphologic changes preceding new vessel formation. Rat RPE-choroid flatmount preparations from normal and laser-injured regions were fluorescently labeled with the F-actin-specific marker phalloidin (left, green channel), the endothelial and microglial cell marker isolectin IB4 (center, red channel), and the nuclear marker DAPI (right, blue channel; merged images). The right is an overlay of the three channels. Representative images of flatmount preparations before laser injury (n = 4) and 5 minutes (n = 5), 1 day (n = 23), and 3 days (n = 7) after are shown. An autofluorescent circular defect in Bruch’s membrane was visible 1 day after laser injury (arrowhead). See also Supplementary Fig. S1. (B) Morphologic changes associated with new vessel formation. Representative images of flatmount preparations fluorescently labeled as in (A). The evolution of new vessel formation at 4 (n = 11), 7 (n = 51), 14 (n = 6), and 60 (n = 10) days is shown. Vascular tubes were visible in the lesion 4 days after laser injury (arrowheads; see also Movie 1). In more advanced lesions (14 and 60 days) several feeder vessels are visible (arrowheads; see also Movie 2). Scale bar, 100 μm.

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vessel complex remained visible, and the recovery of the phalloidin-labeled RPE cells within this zone was visualized (Fig. 1B, bottom).

**Morphology and Localization of Endothelial Cells and Microglia**

Isolectin IB₄ labels microglia, as well as endothelial cells. To distinguish these two cell types, we used the microglial marker CD11b in combination with isolectin IB₄, and evaluated both the morphology and localization of the labeled cells. As shown in Figure 2, both fluorescent-labeled cell types can be distinguished in the neovascular complex. In contrast to endothelial cells that form tube-like structures, microglia exhibited a dendritic morphology with no tube formation and were localized superficial to the new vessels (Movie 3).

**Bruch’s Membrane Disruption, Bubble Formation, and CNV Induction**

The term “bubble formation” is used to describe the appearance of a subretinal bubble at the moment of laser injury to the retina. Laser-exposed rats were evaluated funduscopically, to correlate the bubble formation with disruption of Bruch’s membrane and CNV induction. The minimal energy necessary to induce bubble formation was used, to produce more uniform CNV complexes.

A fluorescent ring was visible during the first 24 hours in eyes with bubble formation. This ring, which possessed broad-spectrum autofluorescence (500–750 nm), was visible in unlabeled samples and correlated with the location of the disruption of Bruch’s membrane (Fig. 3A). Twenty-four hours after laser injury, 21 of 23 lesions with bubble formation had a visible autofluorescent ring. Lesions with bubble formation generated a neovascular response as seen in those animals in which the laser lesion was allowed to evolve. However, those laser lesions with no bubble formation showed very poor induction of CNV, and the presence of hemorrhage within the bubble invariably generated a more intense neovascular response (Fig. 3B).

**3-D Reconstructions of Choroid–RPE Flatmounts for Quantification of Vessel Volume**

To measure the volume of the neovascular complex, confocal z-series were collected and image analysis (Volocity software; Improvision, Inc.) was used to create dynamic 3-D reconstructions of the lesions. New vessel volume was quantified only in isolectin IB₄-positive new vessels with bubble formation; lesions with no bubble or bubble with hemorrhage were excluded. The isolectin IB₄-labeled choriocapillaris and microglia were subtracted from the 3-D reconstructions. The choriocapillaris was identified as a lobular network lying in a single plane.

![Figure 2. Isolectin IB₄, CD11b labeling of the CNV complex. Seven days after laser exposure, flatmounted retinas double labeled with isolectin IB₄ (red) and CD11b (green; n = 15) are shown. Right: isolectin IB₄ labeling of new vessels is shown. Left: CD11b, an antibody marker for microglia is shown. Middle: superimposed image with both channels. Arrowheads: location of microglial cells (see also Movie 3). Scale bar, 100 μm.](image)

**Figure 3.** Laser-induced disruption of Bruch’s membrane and CNV formation. Lesions were labeled with markers for phalloidin (green), isolectin IB₄ (red), and DAPI (blue). (A) In 1-day lesions with bubble formation (n = 16), an autofluorescent ring is visible deep to the RPE. In fluorescently labeled samples (top left), the ring can be selectively visualized at far-red wavelengths (top right). Bottom: similar area with no bubble formation. (B) Correlation between bubble formation and CNV induction (total n = 30). Representative 7-day lesions collected from the same animal are shown. Top: lesion with no bubble formation; middle: vessel growth after bubble formation; bottom: consequences of bubble formation with associated hemorrhage. Left: the three channels superimposed; right: isolectin-labeled endothelial cells. Scale bar, 100 μm.
new vessel volumes of 57 lesions (21 rats) derived from bubble injury were calculated. Each dot represents one unit (37.6 μm³).

**FIGURE 4.** Reconstruction and quantification of vessel volume from confocal z-series. (A) Confocal z-series of fluorescently labeled flat-mount specimens were collected at various time points to generate dynamic 3-D reconstructions of the lesions. The volume of these structures was calculated with image-analysis software (Volocity, Improvision, Inc.). Choriocapillaris and microglia were excluded from measurements and vessels volumes in lesions 4 to 60 days after laser injury were calculated. Arrows: x (green), y (red), and z (blue) axes. Each square represents one unit (37.6 μm). (B) New vessel volumes in lesions (N = 57) with bubble and no hemorrhage 7 days after laser injury. Each dot represents 1 lesion.

below the RPE cells. In contrast to the choriocapillaris, new vessels were identified as a tortuous irregular vascular complex that originates in the choroidal layer and grows through Bruch’s membrane toward the RPE cells. Because microglia localize mainly superficially to new vessels, the apical (superficial) confocal optical sections were excluded, thus excluding the majority of microglia for quantification purposes. During the time period between 4 and 7 days after laser exposure, the new vessel volume (isolectin IB₄-label) in the CNV complex increased exponentially. After 7 days and up to 60 days (the longest time point analyzed) after laser injury, no statistically significant changes in volume were observed (Fig. 4A). The new vessel volumes of 57 lesions (21 rats) derived from bubble formation and without hemorrhage were quantified at 7 days after laser injury. As seen in Figure 4B, a base 10 logarithmic transformation was applied. The mean and its 95% confidence intervals were calculated from the transformed data (5.179 ± 1.96), and these statistics were then back-transformed to the original scale. The geometric mean volume was 151,180 μm³ with a 95% confidence interval of 132,831 to 172,062 μm³. Variability among lesion volumes within the same animal as well as between animals was observed.

**Comparison between Isolectin IB₄ Labeling and FITC-Dextran Perfusion in New Vessel Identification**

We directly compared isolectin IB₄-labeling of endothelial cells and FITC-dextran vascular perfusion for identifying and quantifying new vessels associated with CNV. Isolectin IB₄-labeled endothelial cells were visualized as early as 3 days after laser exposure, and vascular tubes were detected by day 4. By 7 and 14 days, a well-defined neovascular complex was invariably present (Fig. 1B). In contrast to isolectin IB₄, FITC-dextran identified only perfused vessels. Even though the retinal vessels were consistently well perfused (Fig. 5A, insets), detection of new choroidal vessels by FITC-dextran in early time points was always difficult. Although FITC-dextran-perfused vessels were detected with greater efficiency at later time points (7–14 days), the estimated vessel volume was significantly lower than with isolectin labeling. When both channels were superimposed, isolectin IB₄ labeling of the vessel wall and FITC-dextran labeling of the vessel lumen were visualized (Fig. 5A). A graph representing the median values in volume measurement between the two methods at 4, 7, and 14 days after laser injury shows a four- to sixfold difference in calculated volume between FITC and isolectin at all time points (Fig. 5B). These results demonstrate that isolectin IB₄ labeling permitted earlier and more complete visualization and quantification of new vessels than FITC-dextran perfusion.

**DISCUSSION**

We have described a technique for the evaluation and quantification of experimental laser-induced CNV. Choroid–RPE flat-mounts labeled with specific endothelial and RPE markers are used to generate quantitative–qualitative 3-D reconstructions of the CNV complex for the assessment of morphologic changes associated with CNV. The use of flatmounts and confocal microscopy allows volumetric measurements of newly formed vessels during the earliest phases of neovascularization, providing advantages over previous methods.

The use of cell-specific markers is critical for characterizing the identity of various cellular components associated with the CNV complex. We evaluated several vascular markers used for the identification of new vessels. In our preparations, isolectin IB₄, a lectin widely used as a vascular marker, labels endothelial cells more discretely than either PECAM-1 or von Willebrand factor (factor VIII) antibodies. Microglial cells are also labeled with isolectin IB₄ but are easily distinguished from endothelial cells, based on their dendritic morphology and superficial localization relative to new vessels (Fig. 2). By excluding the superficial optical sections the isolectin IB₄-labeled microglia can be subtracted to quantify only the neovascular complex. Quantification of the CNV complexes was reproducible and was obtained as early as 4 days after laser injury. Furthermore, our technique provides additional information regarding microglial activity during CNV development and the role of the RPE, macrophages, and other specific changes that occur after laser injury.
Laser injury produced a wound composed of several concentric circular areas: (1) A central area where the highest energy was delivered and all the cells were destroyed, (2) a rim surrounding the center that corresponded to the area were the energy dissipates and produced substantial cellular damage, and (3) an outer area where cell disruption was minimal and the healing process began. Each area has specific features that can be analyzed with fluorescent markers. Phalloidin labeling permitted us to observe morphologic changes in RPE cells resembling those described by Korte et al. using a chemical ablation model. These included the appearance of several morphologically distinct classes of RPE cells located at the edge of the regenerating epithelial monolayer that proliferate and migrate toward the center of the lesion. Isolectin IB₄ labeling allows study of the mobilization of endothelial cells toward the lesion to form new vessels, and DAPI demonstrates cell distribution throughout the lesion. In addition, because the morphology of the tissue is preserved, histologic cross sections may be prepared, increasing the amount of information that could be obtained from each lesion.

In agreement with previous studies, there is a correlation between Bruch’s membrane rupture, bubble formation, and new vessel formation. FITC-dextran perfusion and isolectin IB₄ labeling. The median results of measurements of three lesions per time point and per labeling technique are shown.
and induction of CNV. Laser injuries without bubble formation show very poor CNV induction, and lesions with bubble formation associated with hemorrhage invariably generated an intense neovascular response. Restricting volumetric analysis to lesions with bubble formation and no hemorrhage minimized variability in new vessel quantification. In contrast to Ryan,2 who reported that the occurrence of hemorrhage at the time of laser photocoagulation does not play an important role in the development of neovascularization, we invariably found stronger neovascular responses in lesions with bubble formation when hemorrhage was also present. Secondary hemorrhage associated with bubble formation is minimized by using the lowest energy necessary for rupture of Bruch’s membrane. These differences could be due to species variations (e.g., monkey versus rat), the laser technique used, or the type of laser used, since both techniques and equipment have evolved. We also found variability in the extent of the neovascular response among lesions in the same animal and between animals (Fig. 4B). Similar variability is also reported in patients with CNV.10 To our knowledge, this is the first time that Bruch’s membrane rupture was correlated in flatmount preparations with an autofluorescent ring visualized with fluorescent microscopy. Bruch’s membrane is a pentalaminar structure containing elastin and collagen layers; these components normally exhibit weak autofluorescence.26 The laser energy vaporizes Bruch’s membrane, causing collagen and elastin fibers to contract to the borders of the lesion. Within the first 24 hours after laser exposure, the border of the defect was visible at the level of Bruch’s membrane as an autofluorescent ring. Afterward, the defect was obscured by the cell proliferation process. Both bubble formation and the autofluorescent ring are manifestations of the same phenomenon.

In this study, we provide a comparison between our technique and other widely used methods for analyzing experimental CNV. FITC-dextran perfusion was originally developed to visualize retinal vasculature17 and has been used successfully to evaluate fully developed CNV lesions18–22; however, this technique does not permit consistent visualization of vessels during the early phase of new vessel formation.16,20 The fact that choroidal vessels are not always well perfused, even with good retinal perfusion, indicates that this technique (FITC-dextran) may yield variable results when used for the measurement of CNV in laser-induced models.20 We note that the volumes obtained in this study with FITC-dextran perfusion are comparable with the ones reported by Apte et al.22 in 7-day laser injuries using confocal microscopy on choroid-RPE flatmounts. Because labeling is limited to perfused vessels, FITC-dextran perfusion provides lower volume estimates than those obtained with our technique. However, the combination of FITC-dextran perfusion and isoelectric IB4 labeling is useful for visualization of perfused and unperfused new vessels and could allow a better understanding of experimental CNV development. CNV is a dynamic process involving the growth, remodeling, and maturation of nascent vessels. With time, unperfused vessels in a CNV complex become perfused vessels. The use of isoelectric labeling permits the visualization of this process (vessel formation) from its inception, while avoiding the variability associated with FITC-dextran perfusion.

Sakurai et al.23 estimated CNV vessel volume at a single time point by summing two-dimensional areas from confocal images collected at 1-μm intervals. By significantly reducing the interval between image planes (0.4884 μm), we have obtained substantially greater axial resolution and more precise estimates of vessel volumes, including those of newly formed vessels. The ability to quantify volumes from surface-rendered 3-D representations has been invaluable in our studies of these dynamic structures.

In conclusion, we have described a reproducible and reliable method for the quantitative and qualitative evaluation of experimental CNV that can be used to evaluate further the morphologic changes and the expression of specific markers in different areas of the lesion, allowing not only a better understanding of the processes involved in CNV formation and development but also a more accurate preclinical evaluation of new antiangiogenic molecules.

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References


**ERRATUM**


The numbering for the atrd3 mutation given is incorrect. The atrd3 point mutation in Pde6b is A1814G causing N605S and not A1817G causing N606S as stated in the paper. In Table 1 the entry for atrd3 in the DNA Change column should read A1814G and the entry in the Protein Change column should read Asn605Ser. In Figure 1 the label for the atrd3 mutation should read “N605S” and the accompanying arrow should point to N605.

Under Discussion, paragraph 2, the second sentence should read, “Two have missense mutations (His620Gln and Asn605Ser) and the third a 5’ splice site mutation (IVS11+5G→A).” The seventh sentence should read, “The only other missense mutation, Pde6b^{atrd3} is Asn605Ser, situated nearby in the putative catalytic domain, and is more detrimental to protein function based on the phenotype analysis.” The ninth sentence should read, “The affected asparagine residue is highly conserved in mammalian phosphodiesterases (Fig. 1), and the phenotypic consequence of the substitution indicates the importance of this residue in protein function.”

In paragraph 3, the eighth sentence should read, “The impact on protein function of the Asn605Ser mutation in atrd3 is less readily assessable.”