INTRODUCTION

Alteration in blood supply to the retina occurs in most retinal pathologies, such as glaucoma,1,2 or diabetic retinopathy.3,5 An understanding of the hemodynamic mechanisms generated during retinal damage is essential for the development of new therapeutic avenues. Laser Doppler flowmetry (LDF) is used to assess ocular blood flow in the clinical setting. LDF is attractive because it is noninvasive, it enables continuous assessment of the blood flow changes, and can be used at near infrared wavelength so that it does not influence retinal activity. It is based on the Doppler effect, which describes the frequency shift that a photon undergoes when scattered from a moving particle (such as a red blood cell) whereas light scattered by stationary cells remains un-shifted.7,8 When a laser beam illuminates a number of red blood cells moving through a network of capillaries at various velocities and in different directions, the light scattered by the red blood cells consists of a summation of waves with various frequency shifts. The difference between frequency of the emitted light and the spectrum of backscattered light is directly proportional to the mean velocity of the blood and the amplitude is proportional to the number of cells; the product of both gives a measure of the tissue perfusion.9 When directly apposed to the tissue, the temporal resolution of the probe is in the tenth of second range and the spatial resolution is in the range of 0.1–1 mm.8,10–12 LDF was previously used in...
a number of animal models notably rabbits and cats to assess choroidal blood flow, or optic nerve head blood flow.

Rodents are used to model common pathological conditions that affect ocular blood flow in humans. To date the ability to measure retinal blood flow in rodent models has relied on terminal techniques such as microspheres trapping or autoradiography. Different LDF set-ups have been tested to investigate blood flow changes in the fundus of rat in order to provide a follow-up of the pathological changes in retinal disease models but none of them are ideal. The Heidelberg retinal flowmeter provides good spatial resolution since the laser beam can scan the eye fundus, although it is not considered appropriate for measuring pure retinal microcirculation in rats. Its use is moreover not well adapted for small animals. LDF settings using LDF needle probes (containing one light-emitting optical fiber and 1–2 collecting optical fibers) are more affordable in terms of costs. They have been used to assess retinal/choroidal blood flow in rats after removing the lens to allow proximity to the retina and localized measurement. This model, however, does not reproduce in vivo intraocular pressure conditions and thereby retinal perfusion pressure. Recently, a new LDF probe design has been developed in which the Moor instruments DP7a type probe possesses eight collecting fibers positioned in a ring around the central light delivery fiber which increases the collecting light area approximately three times more than a standard probe composed of 1–2 collecting fibers. Moreover, the DP7a is an opaque 6-mm plastic cylinder that covers the diameter of the rat eye, which avoids light exposure of the retina whilst enabling easy positioning of the probe. For these reasons, we assumed that this design could represent an affordable technique to assess retinal/choroidal blood flow in rodent models and vasoactivity to endogenously and exogenously administered molecules in the posterior segment.

In the current study, the use of the DP7a probe was tested for its sensitivity to assess blood perfusion changes in the posterior segment of the rat eye when placed at the corneal surface. The vasoactivity was measured in rats under various paradigms, i.e. hypercapnia, hyperoxia or intravitreous injection of saline, adenosine, sodium nitroprusside or Et-1 or laser photocoagulation of the retinal arteries. The DP3 probe was used in rats during hypercapnia to compare the signals delivered by DP3 or DP7a probe in this condition. Adenosine reactivity was also tested in the C56BL/6 mice using the DP3 probe (more adapted to the diameter of the mouse eye) to test a pharmacological challenge for further use of LDF in this species.

**MATERIALS AND METHODS**

**Animals**

Albinos male Wistar rats (200–250 g) and C56BL/6 mice (25–30 g, n = 5) from Charles River (St-Constant, QC, Canada) were used. The animals were housed individually and placed in a room at 23°C with a 12h light/dark photoperiod, with food and water provided ad libitum. All experimental methods and animal care procedures were approved by the local institutional Animal Care Committee at the Université de Montréal, under the auspices of the Canadian Council on Animal Care. The ocular blood flow was measured in rats using the DP7a probe during hypercapnia (n = 15), hyperoxia (n = 9), intravitreous injection of saline (n = 9), adenosine (n = 12), sodium nitroprusside (n = 8) or Et-1 (n = 6) or laser photocoagulation of the retinal arteries (n = 6). The DP3 probe was used in rats during hypercapnia to compare the signals delivered by DP3 or DP7a probe in this condition. Adenosine reactivity was also tested in the C56BL/6 mice using the DP3 probe (more adapted to the diameter of the mouse eye) to test a pharmacological challenge for further use of LDF in this species.

**Animal Preparation**

Rats were anesthetized with urethane (1.2 g/kg, i.p.) (Sigma Chemicals, St. Louis, MO, USA). Urethane was used for the design of this study because multiple measures were made and it provides a stable anesthesia over time, however, ketamine-xylazine anaesthesia has also been tested and is adequate for a shorter experiment (data not shown). During the entire extent of the experimental period, body temperature was monitored with a rectal thermometer and maintained at 37°C by a heating pad (FHC, Bowdoinham, ME, USA). Both blood pressure and heart rate were monitored from the tail using a noninvasive blood pressure cuff system (BP1000, Kent Scientific Corporation, Torrington, CT, USA). Once the animal was anesthetized, polyurethane catheters were inserted into the femoral artery (Tygon Micro Bore, I.D. 0.01000, O.D. 0.03000, Small Parts, Miramar, FL, USA) to further monitor the arterial blood gases. Before and immediately after the surgery, the topical anesthetic lidocaine hydrochloride 2% (AstraZeneca, Mississauga, ON, Canada) was applied on the skin incision to minimize pain. The animal was placed in a stereotaxic frame (David Kopf instruments, Tujunga, CA, USA) to immobilize the head and the superior and inferior eyelids were sutured open. The pupil was dilated with one drop of atropine sulfate 1% (Isopto Atropine solution, Alcon, Toronto, On, Canada) and the corneal hydration was maintained over the measurement period with the regular topical administration of 0.9% saline solution. Local ocular anesthesia was provided by regular topical administration of proparacaine 2.5% (Alcaine, Alcon)
to prevent pain. In this condition, no obvious ocular movements were apparent under surgical microscope observation. A cannula (30 G, Small Parts, USA), linked to a 10 µL Hamilton syringe by tubing was inserted into the vitreous body just behind the limbus with an angle of approximately 45° from the equator.

Laser Doppler Flowmetry

The basis of LDF is the Doppler effect, which describes the frequency shift that a photon undergoes when scattered from a moving particle (such as a red blood cell). This shift, \( \Delta f \), is proportional to the velocity of the moving red blood cell. \( \Delta f \) is extremely small but can be detected using optical mixing spectroscopy. When a laser beam illuminates a number of red blood cells moving through a network of capillaries at various velocities and in different directions, the light scattered by the red blood cells consists of a summation of waves with various \( \Delta f's \). It can be generally noted that most of the light emerging from a tissue has been scattered solely by static structural components of the tissue. This non-shifted light acts as a reference signal that is detected together with the Doppler-shifted light scattered by the moving red blood cells. The output current of the detector contains only the components oscillating at the various \( \Delta f's \), not the original laser frequency. A plot of the power of the photocurrent as a function of \( \Delta f \) constitutes the Doppler shift power spectrum, from which the mean velocity of the red blood cells, the number and the flux of these cells, all expressed in perfusion units (PU) can be determined. The DP7a probe (eight collecting fibers positioned in a ring around the central light delivery fiber, 1-mm separation, 6-mm external diameter, numerical aperture of the optic fibers: 0.22, Moor Instruments) was tested in rats. The DP3 probe (one emitting fiber, one collecting fiber, 0.5-mm separation, 0.8-mm external diameter, Moor Instruments) was also used in rat in order to compare the LDF signal in function of the fibers separation and further tested in mice. The LDF probe was fixed on a probe holder adapted to the stereotaxic frame (Kopf instruments, Tujunga, CA, USA) and was stereotaxically placed in contact with the center of the cornea in parallel to the optic axis without exerting any pressure on the eye bulb (Figure 1). The beam divergence from the LDF probe, in air, is about 12 degrees but when refractive index matching at the cornea and the focusing effect of the lens are taken into account, this leads to sampling a circular area with a diameter of about 0.5 mm at the fundus. It should be mentioned that this volume is an estimate since the beam could not be visualized on the fundus. The same optics applies to the collecting fibers as to the emitting fiber. This means that the maximal signal is detected from the overlap between the diverging laser beam of the emitting fiber and the field of view of the collecting fibers, i.e. the probe would not ‘see’ much larger than the illuminated spot. It is possible that diffuse multiscattered photons could reach the probe but it has been shown that the power of diffuse intraocular scatter is \( 10^{-3} \) weaker compared to the scattered laser light seen by the probe. So even if diffuse multiscattered photons reach the probe, their influence in the LDF signal (calculated from the spectrum of the backscattered light) is weak if not negligible. The tissue perfusion was continuously monitored noninvasively with the blood flow monitoring system (laser wavelength: 780 ± 10 nm, output power: 1.6 mW, DRT4, Moor instruments Ltd., Axminster, Devon, UK). The probe was positioned so that a stable signal around 600–700 PU (assuming absence of significant large vessels) was recorded continuously. Each probe was calibrated against a standard reference (Brownian motion of polystyrene microspheres in water) provided by the manufacturer so the baseline value was identical from one probe to the other. Zones providing a saturated signal were avoided (which might correspond to orientation of the laser beam on large vessels). Baseline perfusion was recorded for a period of 10 min to ascertain the stability of the signal. The baseline and the maximal effect of treatment were obtained by the average flux values of 90 recordings over a 3-min period.

Hypercapnia and Hyperoxia

A small mask was placed on the nose and mouth of the rats 15 min before administration of the CO\(_2\). Rats were allowed to breath a gentle flow of medical air (3 L/min; 21% O\(_2\), 79% N\(_2\)). Hypercapnia or hyperoxia was then induced by inhalation of 8% CO\(_2\) in medical air or 100% O\(_2\), respectively, for 3 min. Before and during the inhalation period, partial pressure of the blood gases was measured using a veterinarian clinical blood gases and electrolytes analyzer (I-stat, HESKA, Fort Collins, CO), from arterial blood samples collected via the arterial catheter.

Intravitreal Injections

The vasoactive agents tested were chosen for their vasodilatory properties (adenosine, sodium nitroprusside) or vasoconstrictive effects (Et-1) and their preferential effect on endothelial or smooth muscles cells. These agents all play a significant role in the physiological regulation of blood flow in the retina. The nucleoside adenosine is a potent neuromodulator that induces vasodilation in various organs, including the brain and the eye. Sodium nitroprusside, a vasodilator, is a nitric oxide donor. Sodium nitroprusside acts independently of the vascular endothelium and causes vasodilation by increasing cGMP in vascular smooth muscle cells. Et-1 is a potent vasoconstrictor peptide secreted by the endothelial cells. The
concentration of the drugs was adapted from previous studies taking into account the dilution and slow diffusion of the drugs into the vitreous: 2 mM adenosine (Sigma Chemicals, St. Louis, MO, USA), 3810 µM sodium nitroprusside33,39 or 40 μM Et-1 (Peninsula Laboratories Inc., Torrence, CA, USA).40 The pharmacological agents (5 µL in 0.9% saline) were injected at a constant rate over 30 s through the cannula both in the rat and mice eyes.

Retinal Arteries Photocoagulation

Three rats were anesthetized with a mixture of ketamine (85 mg/kg), acepromazine (2.5 mg/kg) and xylazine (15 mg/kg) i.p. The head was stabilized with a stereotaxic frame (Kopf instruments) and the superior eyelid was sutured. The pupil was dilated with one drop of topical atropine sulfate 1% (Isopto Atropine solution, Alcon) and the mean baseline blood flow value was obtained as previously described (see above). Photocoagulation of the retinal arteries19,20 of the right eye was then made with a red laser light (300 mW, 300 ms) using a photocoagulator mounted on a binocular ophthalmoscope (Novus Varia, Lumenis, Santa Clara, CA, USA). Using a 20X Volks lens, the laser beam was focussed successively on each branch of the central retinal artery at the level of the optic disk, so 5–6 vessels were coagulated for each eye. According to visual examination, the coagulation was not total because of immediate spontaneous reperfusion of retinal collaterals by vascular shunt. Rat showing an hemorrhage in the retina were discarded. The LDF probe was replaced at the same coordinates and angle to measure the tissue perfusion immediately after the lesion (Figure 1). The left eye was kept intact. Two days later, the rats were sacrificed and the eyes enucleated. The lens was removed and the eye cup immersed in a solution of 4% paraformaldehyde for postfixation. After a 1-h fixation, retinas were removed from the eye cup, dissected into four quadrants, whole-mounted and stained with cresyl violet for visualization of the arterial lesions.

Statistical Analysis

Results are expressed as means ± SEM of values. Statistical differences between drug or gas inhalation effect on LDF signal and baseline values were evaluated with Student’s t-test on paired samples using the SPSS 15.0 software (SPSS inc. Chicago Illinois). Statistical differences between gas inhalation effect on physiological parameters (Table 1) and baseline values were evaluated with a nonparametric Wilcoxon test (n = 4 for each group). Probability values less than 0.05 were considered to be statistically significant.

RESULTS

Recording of Blood Flow

The design of the DP7a LDF probe (same diameter as the rat eye bulb, plan surface) allowed its easy positioning tangent to the eye bulb and centered on the external diameter of the eye (Figure 1). In this position, a stable tissue perfusion signal of 600–700 PU was recorded (Figure 2). The signal recorded was not affected by a voluntary variation of a 1–2° angle of the probe from the optic axis or a slight translation of the probe of 0.1–0.2 mm from the center of the eye. The signal did not show periodic fluctuation that could be assimilated to heart rate or arterial pulsation. These characteristics of the signal and location suggest that the measurements were made within the same region across rat fundus and most probably assessed microcirculation (Figure 1). Other positioning of the probe could result in a rapid rise in and saturation of the signal amplitude which might correspond to the positioning of the probe over large vessels. The intravitreal injection of saline did not
affect the signal baseline, except an artefact at the beginning of the injection (Figure 3E).

**Hypercapnia and Hyperoxia**

The arterial pCO$_2$ was significantly increased ($p = 0.029$) and the arterial pH significantly decreased ($p = 0.028$) during the hypercapnia compared to the prehypercapnic values, as commonly found in hypercapnia studies.$^{41,42}$ Blood pressure, heart rate, arterial pO$_2$, arterial HCO$_3$, and hematocrit were not affected by hypercapnia ($p > 0.05$) (Table 1). A rapid and strong augmentation of LDF signal using the DP7a LDF probe was recorded upon the inhalation of 8% CO$_2$ (Figures 2 and 3C). This was maintained over the period of administration of CO$_2$. The values of the tissue perfusion obtained with the LDF showed a significant augmentation of 18% ± 11 ($p = 0.001$) using the DP7a compared to baseline (Figure 2A and 2B). As well, a rapid decrease of LDF signal using the DP7a LDF probe was recorded upon the initiation of 100% O$_2$ inhalation (Figure 3D). This was maintained over the period of administration of O$_2$. The values of the tissue perfusion obtained with the LDF showed a significant decrease of 8% ± 4 ($p = 0.001$) using the DP7a compared to baseline (Figure 3D). For both CO$_2$ and O$_2$ experiments, the basal level was recovered at the end of the inhalation period, although consecutive regulatory changes of tissue perfusion could be observed postinhalation in some animals (Figure 3C and 3D).

**Intravitreal Administration of Drugs**

Adenosine as well as sodium nitroprusside induced a significant 14–16% increase of perfusion (Figure 3A, 3F, 3G) compared to baseline (adenosine, $p < 0.0001$; sodium nitroprusside, $p = 0.002$) measured by the DP7a probe. Et-1 induced a significant decrease in the tissue perfusion ($-11\%$, $p = 0.016$, Figure 3H), which was maintained for about 5 min before declining. The changes observed were evoked with a slow onset, corresponding to the diffusion of the agent. The response was maintained for more than 10 min before declining. However, the recording was usually stopped before basal level was recovered. The slow decline of LDF signal after drug injection might be due to slow washout of the drugs injected within the vitreous.

**Retinal Vessels Photocoagulation**

The values of posterior segment perfusion obtained with the DP7a LDF probe after photocoagulation of the central retinal arteries showed a significant drop of 45% ($p = 0.038$) from the mean baseline (Figure 4). Whole-mount lesioned retina stained with cresyl violet showed a strong cell and vessels damage – as detected by the presence of shrunk and atrophied cells and vessels and blood clots – and compared to the contralateral intact eye (Figure 4). However, the ischemia was not total since reperfusion of retinal collaterals by vascular shunt was observed immediately after photocoagulation.

**Comparison with Measurements Obtained with the DP3 Probe**

Although the main objective of this study was to test the sensitivity of the DP7a probe, comparison with the measurements obtained with DP3 probe was made. DP3 (1.5 mm external diameter) is composed of only one receiving fiber which is less distant to the emitting
fiber than in the DP7a. These complementary experiments were performed first to evaluate the consequence of a 0.5 mm (DP3) distance between emitting and receiving fibers rather than 1 mm (DP7a) on the LDF signal, and second to test the possibility to use the DP3 probe for mice research (in which DP7a probe diameter would be too large for the eye globe). Similar to the DP7a, the basal signal recorded by DP3 ranged
from 600 to 700 PU but the baseline signal showed greater fluctuation amplitude over the recording period with the DP3 (DP3: 22% fluctuation compared to average baseline vs 14% for the DP7a, Figure 2). Hypercapnia induced similar vasoreactivity, i.e. blood flow increase (20% ± 10, p = 0.03). In the mice, intra-vitreal injection of adenosine induced a significant increase of the tissue perfusion compared with the baseline (57% ± 42, p = 0.04) using the DP3 LDF probe.

**DISCUSSION**

In the present study, we tested a noninvasive and sensitive LDF method able to assess the hemodynamic variations in rodent posterior segment under the local influence of various pharmacological or metabolic agents. Intravitreal vasoactive agents induced a modulation of blood flow detected by DP7a LDF probe that was reproducible and stable, which is relevant for repeated measurement over days to test pathological conditions and the effectiveness of new medicine. Whether these changes could be attributed to retina or choroid circulation is discussed below. Moreover, this study shows that use of a needle probe (DP3) could be beneficial for mice experiments.

All pharmacological agents injected intravitreally induced significant blood flow changes compared to baseline, i.e. vasodilation for hypercapnia, adenosine, sodium nitroprusside and vasoconstriction for hyperoxia and Et-1. Endothelium-dependent vasodilation was assessed by hypercapnia and direct action on smooth muscles was assessed by sodium nitroprusside. Adenosine has a more complex action which could involve endothelium through the prostaglandin pathway, smooth muscle cells and pericytes in capillaries through K-ATP channels and A2 receptors. Et-1 mostly exerts its physiological response through the activation of two G protein-coupled receptors, named ET-A and ET-B located on vascular smooth muscles cells. This suggests that the LDF technique using the DP7a probe is sensitive enough to assess relative retinal/choroidal blood flow changes by drugs or endogenous factors acting at the level of endothelium, smooth muscle cells or pericytes.

A striking feature of our findings is the quite modest amplitude (10–19%), of the significant changes measured using the DP7a probe in the rat eye. The observed hypercapnic response (18% increase) was lower than measured previously in retina using autoradiography (30% increase) or microsphere techniques (80% increase). The intravitreal injection of adenosine (2 mM) induced a significant augmentation of posterior segment perfusion (14%) which was also lower than previous results using microspheres trapping in rabbits or microsphere techniques. The intravitreal injection of adenosine (2 mM) induced a significant augmentation of posterior segment perfusion (14%) which was also lower than previous results using microspheres trapping in rabbits or using fluorescein angiography in newborn pigs (5 nM adenosine, 79%; 50 nM, 323%). However, the effect of adenosine on mice eye was stronger (57%). The vasodilation observed after intravitreal administration of sodium nitroprusside (16%) was consistent with other studies, although lower (103% at 15 nM sodium nitroprusside). The difference in amplitude amongst studies may be attributable to experimental set-up, like the concentration and mode of administration of the drug, the animal model and the anesthetics used. Moreover, the vascular bed assessed by the microsphere or angiography techniques – mostly 20–25 µm microvessels – is different from the one assessed by LDF (microcirculation). The larger vessels embedded by smooth muscles are more reactive to pharmacological,
metabolic or shear stress than microcirculation composed with endothelial cells and pericytes. Alternatively, the long light path from the LDF probe to the fundus implied divergence of the beam resulting in increased probability that (i) an incident photon hits a larger vessel which will affect the Doppler shift spectrum and (ii) photons undergo multiple scattering. This would consequently affect the flow value, however, would not obliterate the validity of the results. Indeed, it was previously demonstrated that the Doppler signal varies reasonably linearly with the blood flow within larger vessels or tubes.

Another important factor that can explain the modest changes in blood flow recorded with this technique compared to other which measured more purely retinal blood flow is the contribution of the choroidal blood flow to the LDF signal. As the choroid is highly perfused and blood flow not regulated the same way as the retina, the portion of the LDF signal derived from the choroid might dominate the recorded LDF signal. The absence of pigment in the albino rat eye and the near infrared wavelength used are in favor of a good penetration of the laser beam through the eye. The distance between the two emitting or receiving optic fibers seemed however to have little effect on the depth of the volume sampled in the present set-up since the results obtained with DP3 or DP7a probe were similar both in perfusion unit and percentage of change – the depth of the sampled volume is theoretically proportional to the distance between the illuminating fiber and the fibers detecting the scattered light. Because the probes were not in contact with the retina, there was most likely overlap between the area illuminated and collected. However, the photocoagulation of the retinal arteries experiment suggested a contribution of the choroidal circulation to the LDF signal. The exact proportion of this contribution could not be quantified because of residual blood supply via collateral vessels of the retina. Our results however suggest that the signal arising from the choroid might lead to an underestimation of the changes in retinal perfusion. Thus, the simultaneous assessment of unaltered choroidal blood flow and regulated retinal blood flow using our LDF set-up would result in low amplitude changes. This has been actually shown in the hyperoxia and possibly in the pharmacological experiments. It has been shown that the hyperoxia does not affect choroidal blood flow, justifying that the changes measured here might be attributed to retina blood flow regulation. Moreover, as drugs were injected intravitreously, it could reasonably be hypothesized that they principally target the internal retina vascular bed rather than the external choroid, leading to a possible lack of response of the choroid. Many pharmaceuticals like anti-VEGF molecules are not able to diffuse throughout the retina to the choroid, which could also be the case in our study. For example, sodium nitroprusside did not increase choroidal blood flow in humans after intravenous injection. Thus, although it has been shown that the choroid was vasoreactive to intravenous adenosine or endothelin, the volume and concentration of the drugs used in the present study might not be sufficient to diffuse to the choroid and induce changes in the choroidal blood flow. The weak effect of hypercapnia is more puzzling since it usually induces increase in choroidal blood flow in most species. However, some recent studies using microspheres and magnetic resonance imaging showed that the choroidal blood flow was not significantly affected by hypercapnia, supporting the possibility that choroid might mask to some point the retinal signal. In addition, the increase of blood flow induced by adenosine was stronger in mice than in rats, which could be due to the slower response of the choroid blood flow to the LDF signal due to absorption of the photon by the pigmented epithelium (which is absent in the albino rat). Thus, the relative blood flow changes recorded with the current LDF method matched better previous data of blood flow regulation measured in the retina rather than in the choroid. It could be claimed that the changes measured here are attributable mainly to perfusion changes within the internal part of the retina. The amplitude of these changes is however difficult to quantify due to an unknown contribution of the highly perfused choroid to the LDF signal.

CONCLUSION

Notwithstanding the limitation that the LDF signal could not evaluate with accuracy the portion of retinal or choroidal circulations, this method can assess the variation of retinal/choroidal blood fluxes under a variety of local pharmacological agents or metabolic challenges. The LDF signal was stable over a long period of time and was sensitive enough to detect blood flow changes over several minutes after the administration of the agents. The relatively noninvasive technique presented here allows continuous assessment of ocular blood flow likely useful to assess hemodynamic changes under pathological conditions. This LDF set-up is an affordable alternative to complex techniques under development such as confocal or OCT arrangement of the laser delivery.

ACKNOWLEDGEMENTS

The authors would like to thank Charles Riva (Medical School, University of Lausanne, Switzerland) and Rodney Gush (Moor Instruments, UK) for a careful reading and discussion of the manuscript, Florence Dotigny for her technical assistance and Denis Latendresse for the graphic work. SH and MP are recipients of a Studentship from the Vision Research Network (FRSQ) and the Foundation Fighting Blindness, respectively. This work was presented at the ARVO Meeting, Fort GLasses.

Declaration of interest: This study was supported by the Vision Research Network (FRSQ).

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Assessment of Rat Retinal/Choroidal Blood Flow Using LDF


