Improved Interpretation of Flow Maps Obtained by Scanning Laser Doppler Flowmetry Using a Rat Model of Retinal Artery Occlusion

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PURPOSE. To improve the interpretation of Heidelberg Retina Flowmeter (HRF; Heidelberg Engineering GmbH, Dossenheim, Germany) flow maps by examining the influence of specific vascular structures and focus depth in the presence and absence of retinal blood flow.

METHODS. HRF flow maps were recorded from the inferior retina of anesthetized Brown Norway rats over a wide range of focus levels, before and after laser occlusion of the retinal circulation. Analysis of the resultant flow maps showed that the sample window was positioned on a retinal artery, arteriole, or vein, or in a retinal capillary area, with or without a visible underlying choroidal vessel. The relationship between HRF-measured flow (arbitrary units) and focus depth was determined for each location. At the conclusion of each experiment, the effect of reduction of systemic blood pressure on the choroidal circulation and the level of background signal in the HRF flow map with no ocular blood flow were assessed.

RESULTS. The strongest flow signals came from the retinal arteries, veins, and arterioles and were reduced to choroidal background level after occlusion of the central retinal artery. Larger choroidal vessels also contributed strong flow signals. In contrast, the flow signal from the retinal capillary area was weak and unaffected by retinal artery occlusion. Changing the depth of focus significantly altered the contribution from the major retinal arteries, arterioles, and veins, but no significant depth effect was seen for retinal capillaries or choroidal vessels. The HRF flow signal remaining when systemic blood pressure was reduced to zero was not significantly different from the capillary sampling location when the eye was normally perfused.

CONCLUSIONS. In the pigmented rat eye, the HRF signal from retinal capillaries is not significantly different from the background noise unrelated to blood flow. Strong flow signals can be obtained from the retinal arteries, arterioles, retinal veins, and choroidal vessels. Current HRF flow maps in the rat therefore reflect blood flow in the larger elements of the microvasculature rather than the capillary network.

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Supported by the National Health and Medical Research Council of Australia and by Canadian Institutes of Health Research Grant MOP-57851.

Submitted for publication July 1, 2004; revised August 19 and September 22, 2004; accepted September 24, 2004.

Disclosure: D.-Y. Yu, None; R. Townsend, None; S.J. Cringle, None; B.C. Chauhan, None; W.H. Morgan, None

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The energy demand for phototransduction and visual processing in the retina is high. The retina is served by two distinct circulations, separated by the avascular photoreceptor region. The retinal vessels are distributed within the inner two thirds of the retina, whereas the outer retina is mainly nourished from the choroidal circulation. Noninvasive measurement of blood flow in the eye has many applications in clinical ophthalmology. One commercially available instrument for this purpose is the Heidelberg Retinal Flowmeter (HRF; Heidelberg Engineering GmbH, Dossenheim, Germany). Although this instrument is being used in clinical research, there is ongoing debate as to the reliability of HRF flow measurements in a practical setting.1,2 Much of the difficulty in interpreting the flow maps stems from the complex architecture of the retinal and choroidal vasculature and the extent to which the signals from different vascular structures are intermixed. This is one of the reasons that the HRF instrument does not purport to measure absolute blood flow. Measurements are always reported in arbitrary units (AU), and great care must be exercised to standardize recording conditions as much as possible, to reduce instrument or environment related influences on the HRF flow measurement.

Our purpose in the present study was to improve the interpretation of HRF flow maps by examining the influence of specific vascular structures and focus depth, both in the presence and absence of retinal blood flow and in the complete absence of ocular blood flow. Studies in animal models allow independent modulation of retinal and choroidal blood flow while the effect on the HRF-measured flow values is determined. We chose to use pigmented rats as our animal model. The retinal vascular distribution and level of pigmentation is not unlike that in humans, and the same questions regarding the mix of retinal and choroidal signals and background noise can be addressed. The central retinal artery in Brown Norway rats also offers the advantage of a high degree of pigmentation where the central retinal artery branches to form the retinal circulation, making the entire retinal arterial system amenable to reliable laser occlusion. Choroidal blood flow can also be manipulated by reducing systemic blood pressure by exsanguination of arterial blood.

METHODS

General Preparation

Six pigmented Brown Norway rats were used in the study. Animals were anesthetized with an intraperitoneal injection of 100 mg/kg 5-ethyl-5-(1’-methylpropyl)-2-thiobarbiturate (Inactin, Sigma-Aldrich, St. Louis, MO). Atropine sulfate (20 μg) was administered intramuscularly to minimize salivation. The trachea was cannulated for mechanical ventilation, the left jugular vein for venous infusion of supplemental anesthetic or a bolus dose of rose bengal, and the femoral artery for...
continuous blood pressure monitoring and occasional aspiration of arterial blood (60 μL) for blood gas analysis (model 238; Ciba-Corning, Helsted, Essex, UK). The rat was then mounted prone in a modified stereotaxic apparatus (model 51400; Stoelting, Chicago, IL) and the head fixed in position. The rat was artificially respirated (rodent respirator, model 683; Harvard Apparatus, Holliston, MA) with a ventilation rate of 90 breaths/min and a tidal volume that ensured normal arterial pCO2 levels. Rectal temperature was monitored and maintained at 37.5°C by a homeothermic blanket (Harvard Apparatus). Experiments usually lasted 5 hours, after which the rat was killed with an anesthetic overdose. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Ocular Surgery and Laser-Induced Central Retinal Artery Occlusion

The left eye was used for all flow measurements. The pupil was dilated with 1% tropicamide (Mydriacyl; Alcon, Frenchs Forest, New South Wales, Australia). An eye ring was sutured to the conjunctiva at the limbus and fixed to the stereotaxic framework of a custom-built ultra-microsurgical system. A plano-concave contact lens was placed on the cornea to allow the vitreous and the fundus to be visualized using an operating microscope (model OPMI6-5F; Carl Zeiss Meditec, Oberkochen, Germany) during laser-induced central artery occlusion, or with the HRF device during flow measurements. An additional stereotaxic framework was specifically manufactured for mounting the HRF camera. This device allowed centralization of the HRF laser through the pupil and the adjustment of camera position from the eye. The camera head could be removed and replaced in the same position, thereby virtually eliminating any instrument-eye alignment errors and allowing the same region of fundus to be imaged before and after laser occlusion of the retinal circulation.

To occlude the central retinal artery, a bolus injection of 0.1 mL of rose bengal (Chauvin Pharmaceutical Ltd., Essex, UK) into the jugular vein was immediately followed by a single 490 mW shot of laser light (532 nm diode laser; model GI; Iridex, Mountain View, CA) at the center of the central retina. Pulse duration was 300 ms and the spot size was 300 μm delivered via a device (Iridex) mounted on the operating microscope.

Image Acquisition

Retinal blood flow was measured using the HRF, a confocal scanning laser Doppler flowmeter (software version 1.04 W; Heidelberg Engineering). The technique relies on measuring time-related intensity variations of backscattered light from an illuminated spot on the fundus. These intensity variations are due to interference between back-scattered light from stationary structures, such as tissue and vessel walls, and from moving blood particles. The intensity variation measurements are subjected to a fast Fourier transform to obtain the power spectrum of the frequency-shifted components. Thereafter, three hemodynamic variables: velocity, volume, and flow are computed from the power spectrum in arbitrary units. The instrument and its operation have been detailed elsewhere. Briefly, a diode laser (wavelength 780 nm) is used to scan the retina at the selected focal plane (for example, the retinal surface). The image resolution is 256 × 64 picture elements (pixels). Each of the 64 horizontal lines is scanned 128 times with a line-repetition rate of 4 kHz. The 128 intensity measurements at each location are made by a photodiode behind a confocal pinhole. The result of each processed scan is a two-dimensional map of the imaged area. The measurements derived with this technique have been shown to have a linear relationship to actual flow rates in experimental bench model systems. However, there are still gaps between the theoretical principles of the technique and practical experience, because blood velocities an order of magnitude greater than the predicted limit for the instrument have been reported.

The HRF objective lens used in our studies was specially designed and manufactured by Heidelberg Engineering GmbH, for imaging the rat retina. Increments of focus depth were modified to 0.125 D per step instead of 0.25 D on the standard instrument. Scan locations were selected approximately two disc diameters away from the optic disc along a vascular arcade with at least one artery or vein with some visible branches on the HRF flow map. Flow maps were recorded at different focus depths. The camera head was positioned so that the superficial retinal artery and vein were in sharp focus with the diopter adjustment set to zero. The focus depth was then adjusted to start from +1.5 D in the vitreous through the full thickness of the retina and choroid as far as −3.5 D. Sharper focus of the large choroidal vessels occurred at approximately −1.5 D. The smallest step of 0.125 D was used in the range from 0.5 to −2.0 D and a step of 0.25 D was used for the remainder of the focus range studied. The HRF flow maps were measured from various locations, including a retinal artery, vein, arteriole, and visible choroidal vessels, before and after laser-induced central retinal artery occlusion. The relationship between HRF-measured flow and focus depth was determined. The effects of alteration of systemic blood pressure on HRF-measured choroidal flow after central retinal artery occlusion were also recorded. The systemic blood pressure was reduced in a stepwise fashion by exsanguination, and, at each blood pressure level, an HRF image was acquired. Preliminary experiments demonstrated that −1 minute was an adequate time for the systemic pressure and flow values (measured by HRF) to stabilize at each flow rate. The same blood pressure increments were produced in every case so the systemic blood pressure values were combined into discrete ranges for the averaged data.

Fluorescence Angiographic Technique

To define the detailed architecture of the choroidal vessels and confirm the occlusion of the central retinal artery, we performed fluorescein angiography. After the final HRF image was acquired, the red blood cells were flushed out of the retinal and choroidal vasculature with Krebs, followed by injection of a bolus of 100 μL of 5% FITC/3% gelatin, warmed Krebs solution. The eye was then enucleated and immersed in chilled buffered formaldehyde. After 24 hours of immersion fixation the retina and choroid with sclera were dissected from the eye and wholemounted for analysis with fluorescence microscopy.

Data Analysis

Various locations of the HRF flow map were selected for quantitative analysis in a 10 × 10- or 4 × 4-pixel window. Measurements on the HRF flow map were performed in the following locations: (1) retinal artery, (2) retinal vein, (3) retinal arteriole, (4) a capillary area without visible retinal and choroidal vessels, and (5) a capillary area overlying a visible choroidal vessel. Only HRF flow data were analyzed. When selecting capillary locations and areas containing visible choroidal vessels, we took care to avoid the presence of any visible retinal vessels at the artery, vein, or arteriole level. Alignment of the window of measurement for sequential images was achieved by tracing the vessel outlines on an acetate film overlying the HRF flow image. Only one sample of retinal artery, arteriole, or vein was used, but two samples of capillary areas and choroidal vessels were used when available.

The HRF data from images captured at different focus depths was graphed to study the relationship between focus depth and HRF flow. Bidirectional SE bars were used when a range of systemic blood pressures were grouped. All statistical testing was performed on computer (SigmaStat; SPSS Scientific, Chicago, IL). One-way repeated-measured ANOVA with a significance acceptance level of P < 0.05 for the F value was performed to determine any significant differences in measured HRF flow at different focus depth planes and two-way ANOVA with an acceptance level of P < 0.05 was used to determine any significant differences in measured HRF flow, before and after central retinal artery occlusion, and the influence of changes in focus depth.

When appropriate, Student’s t test was used. All mean data are expressed as the mean ± SE, and all error bars on graphs also represent the standard error.

Interpretation of HRF Flow Maps

The result of each processed scan is a two-dimensional map of the imaged area. The measurements derived with this technique have been shown to have a linear relationship to actual flow rates in experimental bench model systems. However, there are still gaps between the theoretical principles of the technique and practical experience, because blood velocities an order of magnitude greater than the predicted limit for the instrument have been reported. The HRF objective lens used in our studies was specially designed and manufactured by Heidelberg Engineering GmbH, for imaging the rat retina. Increments of focus depth were modified to 0.125 D per
RESULTS

Fundus Photographs before and after Laser-Induced Central Retinal Artery Occlusion

Figure 1 shows fundus photographs of a Brown Norway rat taken with a plano contact lens and an operation microscope. Figure 1A was taken before central retinal artery occlusion and shows the radial distribution of retinal arteries (A) and veins (V). The fundus was heavily pigmented and only a few choroidal vessels (Ch) were identified. The pigment deposit at the head of the central retinal artery was clearly visible. Figure 1B is a fundus photograph taken after central retinal artery occlusion. The optic disc had a whitish appearance, and the retinal arteries were slightly narrowed and darker and the veins uneven in caliber and darker, indicating blockage of the retinal circulation. Choroidal vessels were still difficult to identify.

Fluorescein-Labeled Retinal Wholmount after Retinal Artery Occlusion

Figure 2 shows microscope photographs of a fluorescein-perfused retina that had the central retinal artery coagulated before fluorescein perfusion. Figure 2A shows the flatmounted retina where it is evident that there is no fluorescein staining of the retinal vasculature or the retina itself in regions away from the disc. Figure 2B shows a section of choroid with the retina removed. The staining pattern reflects the distribution of the choroidal vasculature. The choriocapillaris can be recognized as a dense intervening network. Large choroidal vessels behind the choriocapillaris were embedded in the dark pigment granula. Unlike our experience in albino rats, the pattern and edges of large choroidal vessels could not be identified easily (our unpublished data, 2004).

HRF Flow Map at Different Focus Levels before and after Central Retinal Artery Occlusion

A set of representative HRF flow maps recorded at different focus levels at the same location in a pigmented rat eye before and after central retinal artery occlusion is shown in Figure 3. This demonstrates a clear relationship between the focus level and the measured HRF flow map. At all focus levels in the preocclusion images, some contribution from the major retinal and choroidal vessels was visible. However, the HRF flow in the retinal vessels appeared distinctly sharper when the focal plane was near that of the superficial retina, reaching peaks of 2277 ± 206 and 4006 ± 455 AU, which were significantly higher than that of the choroidal side for the 10 × 10 (P < 0.001) and 4 × 4 (P < 0.001) sample windows, respectively. The HRF flow measured in the 4 × 4-pixel sample window was significantly higher than that in 10 × 10-pixel sample window (P < 0.001). After retinal artery occlusion, the measured HRF flow rates decreased significantly at all focal planes (P < 0.001) and to 158 ± 42 and 166 ± 63 AU for the 10 × 10- and 4 × 4-pixel windows at 0 D. At focal planes deeper into the choroid, the HRF flow rates were only slightly higher than those of the retinal side (P < 0.05). Similar results were seen for the retinal vein. Figure 6 shows the average HRF flow results as a function of focal plane for 10 × 10- and 4 × 4-pixel windows. At all focal planes, the HRF flow was >1000 AU. Measured flow rates at 0 D were significantly greater, reaching 2085 ± 158 and 3596 ± 166 AU, which were significantly higher than those of the choroidal side for the 10 × 10 (P < 0.001) and 4 × 4 (P < 0.001) windows, respectively. The HRF flow values measured in the 4 × 4-pixel sample window were significantly higher than those measured in the 10 × 10-pixel sample window (P < 0.001). After occlusion of the central retinal artery the measured flow rates in retinal veins was significantly reduced (P < 0.001) at all focal planes, declining to 170 ± 36 and 149 ± 31 AU at 0 D for the 10 × 10- and 4 × 4-pixel windows, respectively. When an area containing a retinal arteriole was selected for analysis (Fig. 7), the measured HRF flow rates were lower than those in the major retinal vessels. The flow rates at 0 D were not significantly higher than those in the deeper focal planes (P = 0.823) in the 10 × 10-pixel window, but were significantly higher than those in the deeper focal planes (P < 0.001) in the 4 × 4-pixel window. At 0 D, the average HRF flows in the 10 × 10- and 4 × 4-pixel windows were 694 ± 61 and 1050 ± 158 AU, respectively. After occlusion of the central retinal artery, there was a significant decrease in measured HRF flow in all the focal planes (P < 0.001). At 0 D the flow rates were 274 ± 26 and 264 ± 42 AU in the 10 × 10- and 4 × 4-pixel windows respectively. The results when selecting a retinal capillary area free from any visible underlying choroidal vessels are shown in Figure 8. There was a statistically significant tendency for HRF-measured flow to increase at deeper focal planes (P < 0.05). At 0 D the flow rates were 222 ± 37 and 240 ± 46 AU in the 10 × 10- and 4 × 4-pixel windows, respectively. There was no significant reduction in HRF-measured flow after occlusion of the central retinal artery (P = 0.825). At 0 D the flow rates were 239 ± 20 and 245 ± 33 AU in the 10 × 10- and 4 × 4-pixel windows respectively. The results from a sample region containing a visible choroidal vessel are shown in Figure 9. The focal plane had little bearing on the measured flow rate. The HRF flow measured from choroidal vessels was not significantly affected by occlusion of the central retinal artery in either the 10 × 10-pixel (P = 0.066) or the 4 × 4-pixel (P = 0.146) windows. Before occlusion, the measured flow rates at 0 D were 735 ± 91 and 825 ± 104 AU for the 10 × 10- and 4 × 4-pixel windows.

Relationship between the HRF Flow Rates and the Focus Depth

The averaged results across all animals of the retinal and choroidal flow rates at different focus levels were measured from various locations, including the retinal artery, vein, arteriole, capillary bed, and choroidal vessels, before and after laser-induced central retinal artery occlusion. Data are presented for both 10 × 10- and 4 × 4-pixel sample windows. Figure 5 shows that, when the sampling window overlaid a retinal artery, the HRF flow rate was >1300 AU, regardless of the focal plane setting. The flow value increased significantly when the focal plane was near that of the superficial retina, reaching peaks of 2277 ± 206 and 4006 ± 455 AU, which were significantly higher than that of the choroidal side for the 10 × 10 (P < 0.001) and 4 × 4 (P < 0.001) sample windows, respectively. The HRF flow measured in the 4 × 4-pixel sample window was significantly higher than that in 10 × 10-pixel sample window (P < 0.001). After retinal artery occlusion, the measured HRF flow rates decreased significantly at all focal planes (P < 0.001) and to 158 ± 42 and 166 ± 63 AU for the 10 × 10- and 4 × 4-pixel windows at 0 D. At focal planes deeper into the choroid, the HRF flow rates were only slightly higher than those of the retinal side (P < 0.05). Similar results were seen for the retinal vein. Figure 6 shows the average HRF flow results as a function of focal plane for 10 × 10- and 4 × 4-pixel windows. At all focal planes, the HRF flow was >1000 AU. Measured flow rates at 0 D were significantly greater, reaching 2085 ± 158 and 3596 ± 166 AU, which were significantly higher than those of the choroidal side for the 10 × 10 (P < 0.001) and 4 × 4 (P < 0.001) windows, respectively. The HRF flow values measured in the 4 × 4-pixel sample window were significantly higher than those measured in the 10 × 10-pixel sample window (P < 0.001). After occlusion of the central retinal artery the measured flow rates in retinal veins was significantly reduced (P < 0.001) at all focal planes, declining to 170 ± 36 and 149 ± 31 AU at 0 D for the 10 × 10- and 4 × 4-pixel windows, respectively. When an area containing a retinal arteriole was selected for analysis (Fig. 7), the measured HRF flow rates were lower than those in the major retinal vessels. The flow rates at 0 D were not significantly higher than those in the deeper focal planes (P = 0.823) in the 10 × 10-pixel window, but were significantly higher than those in the deeper focal planes (P < 0.001) in the 4 × 4-pixel window. At 0 D, the average HRF flows in the 10 × 10- and 4 × 4-pixel windows were 694 ± 61 and 1050 ± 158 AU, respectively. After occlusion of the central retinal artery, there was a significant decrease in measured HRF flow in all the focal planes (P < 0.001). At 0 D the flow rates were 274 ± 26 and 264 ± 42 AU in the 10 × 10- and 4 × 4-pixel windows respectively. The results when selecting a retinal capillary area free from any visible underlying choroidal vessels are shown in Figure 8. There was a statistically significant tendency for HRF-measured flow to increase at deeper focal planes (P < 0.05). At 0 D the flow rates were 222 ± 37 and 240 ± 46 AU in the 10 × 10- and 4 × 4-pixel windows, respectively. There was no significant reduction in HRF-measured flow after occlusion of the central retinal artery (P = 0.825). At 0 D the flow rates were 239 ± 20 and 245 ± 33 AU in the 10 × 10- and 4 × 4-pixel windows respectively. The results from a sample region containing a visible choroidal vessel are shown in Figure 9. The focal plane had little bearing on the measured flow rate. The HRF flow measured from choroidal vessels was not significantly affected by occlusion of the central retinal artery in either the 10 × 10-pixel (P = 0.066) or the 4 × 4-pixel (P = 0.146) windows. Before occlusion, the measured flow rates at 0 D were 735 ± 91 and 825 ± 104 AU for the 10 × 10- and 4 × 4-pixel windows,
respectively. After occlusion the corresponding figures were 583 ± 82 and 547 ± 65 AU, respectively.

As an aid to comparing the magnitude of the HRF flow signals from different vascular elements, the results from each selected location are shown superimposed in Figure 10. The largest flow signals were clearly obtained from the major retinal arteries and veins. The smaller sample window (4 × 4 pixels) gave significantly higher flow readings than the 10 × 10-pixel window when positioned on a retinal artery or vein and focused near the plane of the retinal vessels. To a lesser extent, the same was true of the retinal arteriole. By far the smallest HRF flow signal was obtained when an area of retinal capillaries was selected that was free of any visible underlying choroidal vessel. The same comparison is shown in Figure 11.

FIGURE 1. Fundus images from a Brown Norway rat before (A) and after (B) laser occlusion of the central retinal artery. (A) Radial distribution of retinal arteries (A) and veins (V), showing a highly pigmented zone at the head of the central retinal artery (arrow). Relatively few choroidal vessels (Ch) are visible. After laser occlusion, there was stasis of blood in the retinal vessels. The disc had a pale appearance and the fundus generally appeared paler.

FIGURE 2. Microscope photographs of sections of an eye perfused with fluorescein after laser occlusion of the central retinal artery. (A) Wholemounted retina. The retinal artery (A) and vein (V) were free of fluorescein label at all locations away from the optic disc (open arrow). (B) A view of a section of choroid after the retina had been removed. Staining of the choroidal vasculature was evident. The choriocapillaris was recognizable as a dense, intervening network. Large choroidal vessels behind the choriocapillaris were embedded in the dark pigment granula. The pattern and edges of large choroidal vessels could not be identified easily (arrows).
for the postocclusion state. After occlusion of the retinal circulation, the only region maintaining higher measured flow rates than the background noise was the region containing a visible choroidal vessel. There was no significant difference between flow rates measured from the occluded retinal artery, retinal vein, or retinal capillary area.

**HRF Flow Rates from the Choroid and Retinal Capillaries as a Function of Systemic Blood Pressure**

Figure 12 shows a graph of measured HRF flow rates as a function of systemic blood pressure when the retinal circulation was occluded. The data were collected from five eyes of five animals and two samples each taken from locations with or without a visible choroidal vessel. When an area was selected with a visible choroidal vessel, there was a clear relationship between systemic blood pressure and measured HRF flow. In an area free of any visible choroidal vessels, there was no such relationship between HRF flow and systemic blood pressure. These data also provide measurement of the background noise in a no-flow situation. When systemic blood pressure was zero (and presumably ocular blood flow had ceased), the residual HRF flow rate averaged $187 \pm 8$ AU in regions containing a choroidal vessel and $194 \pm 10$ AU in areas free of choroidal vessels. These HRF flow rates were not significantly different. Combining these data gave an average residual HRF flow rate of $190 \pm 7$ AU.

**DISCUSSION**

The HRF is a confocal scanning laser Doppler device introduced approximately 10 years ago and used mostly in clinical ophthalmology research for the noninvasive measurement of retinal blood flow. Valuable information has been provided from previous studies of the validity of HRF in bench experiments, in animals in vivo and in clinical studies. However, many important questions about the interpretation, reliability and applicability of HRF measurements remain. For example, we do not know (1) the true meaning of the arbitrary units reported by the HRF, (2) the optimum way to sample...
retinal blood flow values from a HRF flow map, (3) the reliability of retinal blood flow measurements in different regions of retinal vasculature, and (4) the degree of influence of potential confounding factors such as alignment and the optical properties of the ocular media and fundus.7,12–15

The purpose of this study was to improve our understanding of HRF flow maps in terms of relative contribution from different vascular structures and to estimate the background noise unrelated to blood flow. We wanted to determine (1) the ability of the confocal scanning laser Doppler device to differentiate between retinal and choroidal blood flow, (2) the effect of the selective occlusion of the retinal circulation on the HRF flow measurements from different sampling locations at different focus levels, and (3) the ability of the confocal scanning laser Doppler device to detect choroidal blood flow.

It is inevitable that the HRF flow map will contain some noise unrelated to retinal blood flow. Using the standard noise estimation and correction algorithm, the background noise level in the present study was 190 ± 7 AU after central retinal occlusion and zero systemic blood pressure. This is comparable to that previously reported in rabbits (150–300 AU) under zero-flow conditions (van Heuven WAJ, et al. IOVS 1996;37: ARVO Abstract 4424) and also that in our recent studies in an isolated pig eye preparation (171.9 ± 44.7 AU), in which the perfusate flow was readily stopped (our unpublished data, 2004). A knowledge of the flow-unrelated background noise level is critical for assessment of whether measurements from the HRF flow map represent true blood flow–induced signals. If measured results from a particular sampling location at a
given perfusion rate were not significantly different from the results at zero flow, it would indicate that the HRF-measured results were dominated by noise, thereby the signal-to-noise ratio (SNR) would be small, and no meaningful flow information could be extracted. It is critical to determine the ability and reliability of the HRF flow measurements based on the magnitude of the SNR. When reviewing the literature of HRF usage, it is important to be aware that the background offset calculation has changed in some software revisions, and several users have developed their own methods of reducing background noise. For example, Wang et al.\(^{16}\) reported an average background flow rate of \(~110\) AU at the margin of the disc after occlusion of the central retinal artery in monkeys. However, their analysis was custom designed to eliminate under- or overexposed pixels, making comparison between the level of background noise in our study inappropriate.

In understanding HRF-measured blood flow maps, it is important to take into account the frequency range over which Doppler-shifted signals can be detected. The standard instrument rejects Doppler-shifted frequencies lower than 125 Hz (to eliminate movement artifacts), and frequencies \(>2\) kHz average background flow rate of \(~110\) AU at the margin of the disc after occlusion of the central retinal artery in monkeys. However, their analysis was custom designed to eliminate under- or overexposed pixels, making comparison between the level of background noise in our study inappropriate.

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(see the sampling rate of 4 kHz). An upper frequency limit of 2 kHz means that center line velocities in the larger retinal vessels may be beyond the detection rate of the instrument. However, it seems likely that the flow-related signals that we have observed in the major retinal vessels originate from the slower moving RBCs nearer the wall of the vessels. Another possible explanation for the recordable signals from retinal vessels is that the degree of light scattering may not be sufficient to randomize the angle of collision with moving RBCs. Under such circumstances, the angle between the optical axis of the HRF and the plane of the vessel may become a factor, significantly reducing the apparent velocity vector in the direction of the HRF instrument. In either case, while not an accurate analysis of the flow, the HRF-measured signals provide information that reflects flow in these vessels.

A previous study of HRF-measured flow in pigmented rats reported changes in retinal blood flow during oxygen inhalation. However, the sampled area was described as “away from large visible vessels in the retina.” This may not rule out the presence of smaller arterioles and venules that we have shown to contribute significant flow signals. It is also possible that choroidal vessels contribute to any oxygen-induced changes measured from the chosen area of retina. Although it is generally thought that the choroid in some species (including humans) is unresponsive to oxygen inhalation, it has been shown to contribute to blood flow in the choroid. The HRF flow signal from the choroidal vessel is the dominant source of flow signal.

(due to the sampling rate of 4 kHz). An upper frequency limit of 2 kHz means that center line velocities in the larger retinal vessels may be beyond the detection rate of the instrument. However, it seems likely that the flow-related signals that we have observed in the major retinal vessels originate from the slower moving RBCs nearer the wall of the vessels. Another possible explanation for the recordable signals from retinal vessels is that the degree of light scattering may not be sufficient to randomize the angle of collision with moving RBCs. Under such circumstances, the angle between the optical axis of the HRF and the plane of the vessel may become a factor, significantly reducing the apparent velocity vector in the direction of the HRF instrument. In either case, while not an accurate measurement, the HRF-measured signals provide information that reflects flow in these vessels.

A previous study of HRF-measured flow in pigmented rats reported changes in retinal blood flow during oxygen inhalation. However, the sampled area was described as “away from large visible vessels in the retina.” This may not rule out the presence of smaller arterioles and venules that we have shown to contribute significant flow signals. It is also possible that choroidal vessels contribute to any oxygen-induced changes measured from the chosen area of retina. Although it is generally thought that the choroid in some species (including humans) is unresponsive to oxygen inhalation, it has been shown to contribute to blood flow in the choroid. The HRF flow signal from the choroidal vessel is the dominant source of flow signal.

An important question that must be addressed is the degree to which the rat eye may be similar to or different from the human eye. There are clearly differences in the size of the eyes, the optics, and the vascular distribution, and there may be potential differences in the scattering properties of the retinal tissue. This, however, is true to some degree of all animal models. In the present study, our rats possessed a similar pigmentation level in the retinal pigment epithelium as judged by the presence of smaller arterioles and venules that we have shown to contribute significant flow signals. It is also possible that choroidal vessels contribute to any oxygen-induced changes measured from the chosen area of retina. Although it is generally thought that the choroid in some species (including humans) is unresponsive to oxygen inhalation, it has been shown to contribute to blood flow in the choroid. The HRF flow signal from the choroidal vessel is the dominant source of flow signal.

Our studies of HRF flow signals at different focal depths in regions containing different vascular elements allow us to draw several conclusions. First, despite the confocal nature of the instrument, the major retinal vessels contribute to the measured HRF signal over a wide range of focal depths, perhaps due in part to scattering from the blood-filled vessel restricting the penetration depth of the incident light into the remaining retina. However, because the influence of the choroidal circulation during retinal occlusion can still be seen when the focal plane is set to the innermost retina, some integration of the measured area is clearly evident in the more transparent areas of retina. This result is perhaps to be expected, given that the theoretical sampling area of the measurement includes a cone in front of and behind the focal plane, and, in the human eye, the total depth of these Rayleigh cones is approximately 400 μm. However, many users of the instrument appear to place great emphasis on the ability to extract HRF flow signals from specific retinal depths. The peak HRF flow from the retinal artery and vein sampling locations was many times higher than the background noise level (SNR > 10–20) and the SNR for retinal arterioles was approximately 3 to 5. Second, although blood flow in the choroid is higher than that in the retina, the HRF flow measured from the choroidal vessels in the rat was much lower than that from the retinal artery and vein sampling locations and only two to three times higher than background noise level. This unexpectedly low HRF flow from the choroidal vessels may be caused by attenuation of the HRF flow signal, which could be produced by absorption by the retinal pigment epithelium and the choroidal melanocytes. Third, and perhaps most surprising, the HRF flow signal measured from the capillary sampling location was weak and unaffected by retinal artery occlusion. Our finding that retinal capillary blood flow made very little contribution to HRF-measured flow is in contrast to several clinical and theoretical studies, but may well account for the difficulties that many observers have found in trying to obtain consistent results from HRF flow measurements at the retinal capillary level.

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evident over a wide range of focal planes. Our results have been obtained from the standard HRF instrument adapted for use in the rat eye. Comparison with other HRF studies should take account of species differences and the specific analysis techniques used.

As mentioned before, laser Doppler flowmetry is a method for noninvasive measuring of tissue blood flow using the Doppler shift of laser light as the information carrier. Tissue is a scattering and absorbing medium that has a higher refractive index than air. Scattering of laser light in any medium is related to the heterogeneity of the medium. Tissue is composed of molecules containing discrete electrical charges, electrons, and protons. If a molecule or a cluster of molecules in a cell membrane is illuminated by laser light, the electrical charges in the molecules are set into oscillatory motion by the electric field of the incident wave. Moving or vibrating charges radiate electromagnetic energy in all directions. The complexity of interaction between laser light and tissue should never be underestimated, and so laser Doppler flowmetry is sometimes referred to as an “art.” Such an expression indicates that a combination of factors must be considered to use the technique optimally and generate reliable results. One difficulty is the absence of a reliable means of calibration of the instrument in terms of absolute flow values. The absence of a gold-standard technique for measuring the heterogenous distribution of retinal and choroidal blood flow precludes a simple in situ calibration.25

In conclusion, the Brown Norway rat is a useful model to aid in the interpretation and validation of HRF flow measurements. Our results have confirmed that there is a significant amount of flow-related information in the HRF map obtained from the current instrument. Careful selection of measurement site is clearly important in influencing the noise immunity of the HRF flow measurement. Flow-dependent HRF signals can be recorded from the larger elements of the retinal microvasculature; however, the light-scattering properties of the larger retinal vessels may not fit well with the Bonner and Nossal4 theory of multiple scattering used by the HRF to analyze the Doppler spectrum. In addition, the HRF does not have the frequency range needed to detect the full range of RBC velocities in larger vessels. The manufacturer therefore does not support the use of the HRF instrument for measurement of blood flow in larger vessels.24 In addition, further technical improvements in the HRF seem to be needed, to obtain reliable flow signals at the capillary level.

Acknowledgments

The authors thank Dean Darcey, Paula Yu, Judi Granger, and Megan Dallas for their invaluable assistance and the Heidelberg Engineering for loan of the HRF and for technical support.

References