Confocal Scanning Laser Doppler Flowmetry in the Rat Retina

Origin of Flow Signals and Dependence on Scan Depth

Balwantray C. Chauhan, PhD; Paula K. Yu, PhD; Stephen J. Cringle, PhD; Dao-Yi Yu, MD, PhD

Objective: To investigate the origin of signals from scanning laser Doppler flowmetry (SLDF) and the influence of axial scan depth on the measurement of blood flow in the rat retina.

Methods: We performed SLDF in 5 adult Sprague-Dawley rats using a specially modified Heidelberg retina flowmeter. Axial scans were obtained from +2 diopters (D) to −3 D (in steps of 0.25 D) or from +1 D to −2 D (in steps of 0.125 D) relative to the retinal surface. Fluorescein isothiocyanate–dextran angiograms were obtained in whole-mounted retinas to visualize the angiography and identify measurement locations in the SLDF flow maps. Axial SLDF flow profiles were obtained in an artery, vein, arteriole, venule, and capillary bed using the mean blood flow values in 2 × 2–, 4 × 4–, and 10 × 10–pixel measurement windows.

Results: The SLDF images showed good correspondence with the angiograms and resolution to third-order arterioles and venules; however, neither the superficial nor deep capillary circulations were visualized. Flow was imaged from large choroidal vessels. Measured flow from capillaries was independent of depth and indistinguishable from background levels.

Conclusion: The technique of SLDF images blood flow in larger retinal vessels but not in capillaries.

Clinical Relevance: Scanning laser Doppler flowmetry may not reliably measure capillary blood flow.

Arch Ophthalmol. 2006;124:397-402

MEASUREMENT OF RETINAL blood flow can provide valuable clinical information in the management of patients with diabetic retinopathy, age-related macular degeneration, and other retinopathies. Angiographic techniques using conventional optical1-3 or scanning laser image4 acquisition with conversion of angiographic data to flow values have been described5; however, these methods have not been fully validated using experimental or clinical models. Laser Doppler techniques6-8 are noninvasive and therefore offer an advantage over angiographic techniques, which require an intravenous injection of fluorescent dye. Considerable controversy exists about the origin of the laser Doppler signal,9,10 specifically regarding the caliber of contributing vessels and whether they are situated superficially or deeper in the sampled tissue.

In this study, we used SLDF for noninvasive retinal blood flow measurement in rats. The effect of changing the focus depth of the incident laser light was investigated on flow measurements, whereas the origin of the signals in individual retinas was investigated by mapping the measured flow values with the retinal vasculature labeled with fluorescein isothiocyanate conjugated (FITC)–dextran.
RESULTS

A total of 5 animals were used in this study. In 2 animals, the axial SLDF images were obtained in increments of 0.25 D, whereas in the remaining 3 they were obtained in increments of 0.125 D.

The pattern of the retinal vasculature visualized with the SLDF images showed good spatial correspondence relative to the 0-D setting. This procedure was repeated for the other 2 locations. Hence, 3 sets of images, with each containing a depth or axial series at each location, were obtained. After animal preparation, all the images were obtained in approximately 45 to 60 minutes.

SCANNING LASER DOPPLER FLOWMETRY

Retinal blood flow was measured using the Heidelberg Retina Flowmeter (HRF; Heidelberg Engineering GmbH, Dossenheim, Germany), a confocal scanning laser Doppler flowmeter. 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 backscattered 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 multiple frequency shift components. Thereafter, 3 hemodynamic variables, velocity, volume, and flow, are computed from the frequency shift components. Thereafter, 3 hemodynamic variables, velocity, volume, and flow, are computed from the

DATA ANALYSIS

The raw SLDF data from each image were processed using the HRF software (version 1.04W) to obtain the flow images. The image quality of the flow maps in each of the 3 sets of images was carefully examined for exposure and detail of the perfusion pattern. One set of images was then selected for analysis based on image quality. Using the pattern of vessels from the FITC-dextran angiograms, 5 locations in the SLDF images were carefully chosen for obtaining flow measurements in an artery, vein, arteriole, venule, and capillary bed (identified in the angiograms). The Cartesian coordinates of the location were noted and sequential depth measurements were always made at this location, since there was no eye or HRF movement during the axial scans for a given location. Using the software, average measurements in a 2 × 2-, 4 × 4-, and 10 × 10-pixel window centered on the given location were made. These flow values were digitally recorded using the software.

We carefully compared the pattern of the vasculature from the axial series of SLDF flow images to the pattern of the retinal vasculature with the axial angiograms. To supplement this qualitative visual analysis, we determined whether the measured flow values for different locations obtained as described above changed as a function of retinal depth to address the possibility of extracting flow-related information in SLDF images that do not show obvious flow patterns that resemble the vasculature. For each animal and location, flow profiles (in axial depth) obtained for each of the 3 measurement windows were plotted.

METHODS

GENERAL PREPARATION

Adult Sprague-Dawley rats were anesthetized with an intraperitoneal injection of 100 mg/kg of 5-ethyl-5-(1'-methylpropyl)-2-thiobarbiturate (Inactin; Sigma Chemical Co, St Louis, Mo). Atropine sulfate (20 µg) was administered intramuscularly to minimize salivation. Body temperature was monitored and maintained at 37.5°C using a rectal thermometer and a homeostatic blanket (Harvard Apparatus, Holliston, Mass). Animals were killed with an anesthetic overdose. All procedures complied with guidelines from the institutional committee on the care of laboratory animals.

SCANNING LASER DOPPLER FLOWMETRY

Retinal blood flow was measured using the Heidelberg Retina Flowmeter (HRF; Heidelberg Engineering GmbH, Dossenheim, Germany), a confocal scanning laser Doppler flowmeter. 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 backscattered 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 multiple frequency shift components. Thereafter, 3 hemodynamic variables, velocity, volume, and flow, are computed from the frequency shift components. Thereafter, 3 hemodynamic variables, velocity, volume, and flow, are computed from the

FITC-dextran angiograms, 5 locations in the SLDF images were carefully chosen for obtaining flow measurements in an artery, vein, arteriole, venule, and capillary bed (identified in the angiograms). The Cartesian coordinates of the location were noted and sequential depth measurements were always made at this location, since there was no eye or HRF movement during the axial scans for a given location. Using the software, average measurements in a 2 × 2-, 4 × 4-, and 10 × 10-pixel window centered on the given location were made. These flow values were digitally recorded using the software.

We carefully compared the pattern of the vasculature from the axial series of SLDF flow images to the pattern of the retinal vasculature with the axial angiograms. To supplement this qualitative visual analysis, we determined whether the measured flow values for different locations obtained as described above changed as a function of retinal depth to address the possibility of extracting flow-related information in SLDF images that do not show obvious flow patterns that resemble the vasculature. For each animal and location, flow profiles (in axial depth) obtained for each of the 3 measurement windows were plotted.

DATA ANALYSIS

The raw SLDF data from each image were processed using the HRF software (version 1.04W) to obtain the flow images. The image quality of the flow maps in each of the 3 sets of images was carefully examined for exposure and detail of the perfusion pattern. One set of images was then selected for analysis based on image quality. Using the pattern of vessels from the FITC-dextran angiograms, 5 locations in the SLDF images were carefully chosen for obtaining flow measurements in an artery, vein, arteriole, venule, and capillary bed (identified in the angiograms). The Cartesian coordinates of the location were noted and sequential depth measurements were always made at this location, since there was no eye or HRF movement during the axial scans for a given location. Using the software, average measurements in a 2 × 2-, 4 × 4-, and 10 × 10-pixel window centered on the given location were made. These flow values were digitally recorded using the software.

We carefully compared the pattern of the vasculature from the axial series of SLDF flow images to the pattern of the retinal vasculature with the axial angiograms. To supplement this qualitative visual analysis, we determined whether the measured flow values for different locations obtained as described above changed as a function of retinal depth to address the possibility of extracting flow-related information in SLDF images that do not show obvious flow patterns that resemble the vasculature. For each animal and location, flow profiles (in axial depth) obtained for each of the 3 measurement windows were plotted.
with the FITC-dextran angiograms for the larger vessels. In many cases, the SLDF images showed resolution to third-order arterioles (Figure 1); however, some of the arterioles, often of first or second order, shown in the angiograms were not apparent in the flow images. Venules draining the deep capillary circulation were also clearly resolved by the SLDF images. However, neither the superficial nor deep capillary circulations could be visualized (Figure 1) in any of the axial SLDF images of any animal. At deeper locations, flow information from apparently large vessels that did not correspond to vessels in the angiograms was also visualized. Since only the retina was dissected and whole mounted for obtaining the angiograms, it was concluded that this information likely originated from choroidal vessels. Careful examination of superficial SLDF images also showed blood flow information from relatively large vessels in the choroid (Figure 1).

Representative flow profiles of one animal for all locations and 3 measurement windows are shown in Figure 2. The data for measurements in artery, vein, arteriole, and venule showed that the flow values depended on axial depth, indicating that the highest flow values were obtained when the focal plane corresponded to the axial location of the vessel. The flow profiles obtained from the capillary bed did not seem dependent on axial depth, confirming the visual observation that the SLDF images do not contain information from the capillaries. The fact that the flow values at these locations were low and did not change with axial depth suggests that flow information from the capillaries cannot be distinguished from the background noise level.

The mean axial blood flow data obtained from the 10×10–pixel measurement window for all 5 measurement locations in all animals are shown in Figure 3. The data for the 2×2– and 4×4–pixel measurement window were similar and are not shown. The peak flow values in the arteries varied from 2000 to 7000 AU and in veins from 3500 to 5500 AU. In all cases, flow measurement values in the artery and vein showed dependence on scan depth with a single peak. However, in one animal (R-387), measurements in the vein yielded 2 peaks because of interference from a choroidal vessel. Although measurements from the arteriole and venule locations also depended on scan depth, the flow values were lower and peaks not as distinct, especially for the venules. Measurements from the capillary locations were generally very low and not systematically dependent on scan depth. In 2 cases (R-348 and R-386), flow values decreased slightly with scan depth, whereas in 1 case the opposite (R-387) was noted.

**COMMENT**

Techniques for measuring blood flow in the retina and optic nerve head have the potential to provide important clues about the pathophysiology of many retinal and optic nerve disorders. They can also play an important role in the diagnosis and management of these diseases.
Validation studies of clinical devices for measuring blood flow can be problematic, since gold standard methods are either unavailable or invasive. Because the retina contains multilayered vascular beds, it is impossible in human studies to determine the contribution of each layer to the final measurements obtained with a technique such as SLDF. Although studies in experimental animals may have limited direct relevance to clinical measurements, experimental studies provide a powerful start to understanding the validity and limitations of blood flow measurements for translation into clinical practice.

Our study demonstrates that SLDF is capable of measuring signals from larger retinal vessels, down to second- or third-order arterioles and venules. This was evidenced by both the FITC-dextran angiograms and the quantitative measurements performed at these locations. It was notable that not all arterioles and venules that were shown in the angiograms produced a detectable flow signal in the SLDF images. There was no obvious trend that the ability to detect SLDF flow signals was related to vessel size, because similarly sized vessels located in close proximity (as shown by the angiograms) were often not all detected with SLDF. It is possible that the flow signals are critically dependent on image quality and that the relatively high curvature of the rat retina may have caused some optical distortion and made the Doppler signal difficult to detect. Alternatively, it is possible that these vessels were not perfused at a level to have a detectable flow measurement at the time of the imaging session.

Many authors have used SLDF with the assumption that flow measurements originating from capillaries can be reliably detected. However, evidence from this and a recent study strongly suggest that SLDF cannot measure reliably in capillaries. First, in the present study we were unable to visualize any capillaries as localized in the angiograms in either the superficial or deep capillary layers in the respective SLDF images. In the HRF analyses, the Doppler frequency shifts between 125 and 2000 Hz, which correspond to a detection range in the velocity vector parallel to the detector of 0.05 to 0.78 mm/s. Since the plane of larger retinal vessel is nearly perpendicular to the detector, substantially higher velocities can theoretically be detected.

However, because the angle between the velocity vector and the detector axis likely varies, even along an individual vessel, and since the Doppler shift near 90° for a given velocity changes rapidly with small variations in this angle, it is probably not meaningful to compare flow values among different locations in a flow map. Comparing the same location before and after an intervention or over time may be more meaningful, providing the properties of the static scatterers have not changed. The possibility exists that the velocity in capillaries is too low and produces Doppler shifts that escape detection with a lower cutoff frequency of 125 Hz. The SLDF flow maps were recomputed in some animals with a lower cutoff of 31 Hz to address this issue (G. Zins, PhD, oral communication, January 2005). In all cases the recalculated flow maps failed to reveal capillary flow; hence, we do not believe that the frequency bandpass of the HRF is the reason for not detecting capillary flow. Second, we were unable to show any focus depth-dependent changes in flow when measurements were
made in locations devoid of retinal vessels except capillaries, as indicated in the angiogram. Additionally, flow measurements in capillaries were very low and close to noise levels. Finally, in a recent study, we showed that there were no changes in SLDF-measured flow in locations that contained capillaries immediately before and after laser occlusion of the retinal circulation, whereas significant decreases, down to background levels, were noted in all other retinal vessels.20

The SLDF images showed signals that originated from vessels that were not detected in the angiograms and were therefore highly likely to be choroidal in origin. These signals were detected even at relatively superficial retinal locations (Figure 1). In clinical studies, SLDF measurements are usually made at one focal plane. Axial flow profiles are not recorded due to acquisition time, eye movements both during and between axial scans, and the current inability to accurately register axial SLDF images. It is therefore difficult to rule out the interference of choroidal signals in the measurement of retinal blood flow on the basis of a single axial flow image, even when the focus is set to the superficial retina. Because the axial resolution of confocal ophthalmoscopy in the human eye is estimated to be approximately 300 µm,21 it is likely that despite confocal optics, flow signals from at least the anterior choroid can be detected when the imaging focal plane is set at the superficial retina. Indeed, SLDF measured blood flow in areas of retina affected by a branch vein occlusion where absence of blood flow was confirmed by fluorescein angiography.22 Thus, clinical studies have also provided evidence that the choroidal circulation influences SLDF-measured flow in the retina. Based on the ocular constants in the rat eye23 and the optical setup of the HRF, the axial resolution is estimated to be similar to that in the human eye (G. Zinser, PhD, oral communication, January 2005). With the modified HRF setup for use in rats, a 1-D change in focus is equivalent to approximately 110 µm (G. Zinser, PhD, oral communication, January 2005). Examination of the axial SLDF images (Figure 1) shows that the flow imaged in the large vessels at deeper locations are choroidal given that the rat retina is approximately 325 to 350 µm thick.24

Our study was necessarily descriptive because it is not meaningful to pool data across animals, since measurement locations were chosen according to the respective angiograms and not location, size, and depth of vessels. Therefore, computing means to examine the effect of focus depth on flow measurements across all animals would have been inaccurate since the respective vessels may have been located in different axial locations. This was particularly the case with venules, because they emerged superficially at a steep gradient from the deep capillary bed. We believed it was more appropriate to present the data from each animal separately. Examining the correspondence between the angiograms and SLDF images was qualitative and subjective, because an objective and quantitative analysis is not readily feasible with this form of data. Although the rat and human retina and its circulation are not completely equivalent, an experimental study such as this can offer many advantages over clinical studies in understanding the applications and limitations of SLDF. Because the eye was stabilized and the
HRF fixed to the stereotaxic frame, no motion occurred during image acquisition, ensuring high-quality images. In clinical studies, motion artifacts are virtually impossible to eliminate. Additionally, we were able to obtain axial scans at the same transverse location, allowing us to generate axial flow profiles that help us understand the contributions of the different sources of blood flow to the measured values. Such studies would be difficult to replicate clinically given the current hardware and software limitations. Correlating the angiarchitecture obtained with the FITC-dextran angiograms to the SLDF images is a powerful method to identify the blood vessels that contribute to the SLDF measured flow.

In summary, based on angiograms and axial SLDF flow images, we showed that SLDF can image blood flow in arteries, veins, and some arterioles and venules. We were unable to demonstrate that capillary flow could be reliably imaged. Finally, some choroidal vessels can exert an influence on blood flow measurements obtained from retinal locations.

Submitted for Publication: February 14, 2005; final revision received June 28, 2005; accepted June 30, 2005.

Correspondence: Balwantray C. Chauhan, PhD, Department of Ophthalmology and Visual Sciences, Dalhousie University, Second Floor, Centennial Building, Queen Elizabeth II Health Sciences Centre, Halifax, Nova Scotia, Canada B3H 2Y9 (bal@dal.ca).

Author Contributions: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Disclosure: None.

Funding/Support: This study was supported by grant MOP-57851 from the Canadian Institutes of Health Research, Ottawa, Ontario (Dr Chauhan), and the National Health and Medical Research Council of Australia, Canberra (Drs D.-Y. Yu and Cringle).

Acknowledgment: We are grateful to Gerhard Zinser, PhD, Heidelberg Engineering, for modifying the HRF for use in rats, computing the axial resolution of the HRF in the rat eye, and recalculating the SLDF flow maps at the lower-frequency cutoff.

REFERENCES