We investigated the effect of localized visual stimulation on human retinal blood velocity using an adaptive optics scanning laser ophthalmoscope (AOSLO). To measure the blood velocity response, the AOSLO scanning raster was moved over the target arteries and red blood cell velocity was measured. Localized visual stimuli were delivered by projecting flicker patterns inside or outside the target artery’s downstream region. The blood velocity increased in the presence of a flicker stimulus in the downstream region but not when outside the downstream region. The blood velocity increased more with larger area of stimulation. This increase was significant even when the stimulus was smaller than $600 \mu m \cdot 600 \mu m$. These findings suggest that when the retina regulates its blood flow to metabolic demands, it regulates blood velocity in the vascular system selectively, according to activity of neurons within its field of influence.

Keywords: functional imaging, ganglion cells, retina


Introduction

Neural activity in the brain results in rapid changes in cerebral blood flow. This relation between neural activity and blood flow has been called neurovascular coupling. Neurovascular coupling in the brain was first reported more than 100 years ago by Roy and Sherrington (1890) and led to a large body of later brain research (Haydon & Carmignoto, 2006; Metea & Newman, 2006). The existence of neurovascular coupling, together with the utilization of $O_2$ by active tissue, is the major basis for functional brain imaging and has opened a window for researchers to understand brain activities and functions (Villringer & Dirnagl, 1995) as well as brain pathology (Girouard & Iadecola, 2006).

The retina is also part of the central nervous system and has the advantage that direct, noninvasive optical assessment is possible. Neurovascular coupling has previously been documented to occur in the retina using techniques such as laser Doppler flowmetry (Falsini, Riva, & Logean, 2002; Riva, Falsini, & Logean, 2001; Riva, Logean, & Falsini, 2004), laser Doppler velocimetry (Garhofer et al., 2004), Doppler optical coherence tomography (Wang, Fawzi, Tan, Zhang, & Huang, 2011), pulsed Doppler sonography (Michelson, Patzelt, & Harazny, 2002), scanning laser Doppler flowmetry (Michelson et al., 2002), fluorescein angiography (Kiryu, Asrani, Shahidi, Mori, & Zeimer, 1995), and blue field entoptic phenomenon (Scheiner, Riva, Kazahaya, & Petrig, 1994). Retinal neurovascular coupling suggests that the retina can adapt its blood flow to metabolic demands. However, it is not clear whether such blood flow response is locally determined, even though it is expected to be. This has in part been a methodological issue since most techniques are limited to making measurements either in large vessels, which
feed very large areas of the retina, or to capillaries, which are very local, and these studies have often used full-field flicker stimulation. Investigations using local flicker stimulation are also more difficult because it is necessary to coordinate the location of the blood flow measurement to the stimulus presentation.

In the current study we used an adaptive optics scanning laser ophthalmoscope (AOSLO) that includes localized high resolution imaging, a wide-field low resolution view, and a programmable stimulus generator to study the retinal blood velocity response in midsize retinal arteries while varying the size and location of visual stimuli. This is possible because the AOSLO can provide in vivo retinal images with near-diffraction limited lateral resolution (Chui, Song, & Burns, 2008; Geng et al., 2009; Song, Zhao, Chui, & Burns, 2008; Tam, Martin, & Roorda, 2010; Zou, Qi, & Burns, 2011). Because our technique is based on direct imaging of the light backscattered by erythrocytes, it does not require injection of any contrast dye. In this study, the AOSLO scanning raster was moved over the target artery to measure blood velocity while localized visual stimulation was delivered by presenting luminance flicker elsewhere, inside or outside the target artery’s downstream feeding region.

**Methods**

**AOSLO imaging system**

Blood velocity measurements were obtained using the Indiana AOSLO system described by Ferguson and colleagues (2010). In brief, a supercontinuum laser (Fianium, Beverly, MA) with an 840-nm filter (12 nm bandwidth) was used as the imaging source. Wavefront errors were measured with a Shack Hartmann wavefront sensor and corrected using two deformable mirrors (Mirao 52e DM, Imagine Eyes, Orsay, France; Multi-DM, Boston Micromachines Corporation, Cambridge, MA) in a Woofer-Tweeter control system (Zou, Qi, & Burns, 2008). The spatial resolution of this AOSLO system is from 2 to 2.5 μm at the retina for a dilated pupil (6.5–7.6 mm) (Zou et al., 2011). Subjects’ head movements were minimized using a chin rest. A 30° imaging system (Physical Sciences Inc., Andover, MA) provided a simultaneous en face image (Hammer, 2003).

This AOSLO has a wide-field steering design that allows high resolution imaging within ±15° on the retina without need for fixation change. A field steering mirror is conjugate to the eye’s pupil and allows the experimenter to steer the high resolution imaging field and navigate to the target blood vessels.

**Localized stimulus delivery**

A mini liquid crystal display (LCD) screen with optical assembly was placed behind a beam splitter. The optics were arranged so that the LCD could be focused on the retina, and the pupil of the stimulus delivery system was maintained conjugate to the eye’s pupil. The luminance flicker was generated on the LCD screen using MATLAB (Mathworks, Natick, MA). The full area of the LCD screen subtended 27° x 21° on the retina. The LCD was back-illuminated by a light-emitting diode that could be varied in intensity.

The luminance flicker stimuli were square-wave modulated, between a minimum illuminance of 0.6 lux and a maximum illuminance of 6.6 lux, similar to the illuminance used in previous studies for blood flow changes in response to flicker (Falsini et al., 2002; Riva et al., 2001; Riva, Logean, & Falsini, 2005). The LCD area outside the flicker stimulus, or the entire LCD screen when not flickering, was maintained at a constant illuminance of 3.6 lux, as was the surrounding region. The flicker frequency was 10 Hz, which is a midrange frequency optimum to induce large blood flow increase (Riva et al., 2001, 2005).

**Blood velocity measurement using AOSLO**

The blood velocity was measured using the line scan mode of the Indiana AOSLO system, which has been described in detail elsewhere (Zhong, Petrig, Qi, & Burns, 2008; Zhong, Song, Chui, Petrig, & Burns, 2011). The beam scanning was performed by a 15-kHz resonant scanner and a programmable vertical scanner. To increase the sampling rate, both directions of each line scan were captured and treated as two separate lines by the frame grabber. The vertical scan was programmed to pause briefly on top of the target blood vessel, allowing 30-kHz sampling of the slightly angled intersection between the horizontal scan and the blood vessel. Erythrocytes crossing the scan line are visualized as diagonal streaks whose slopes represent the erythrocyte velocity along the scan direction. The erythrocyte velocity along the vessel axis is then calculated by taking into account the angle between the scanning direction and the vessel axis.

Typically, the time required for a single cell’s velocity measurement is less than 2 ms, related to the number of selected image lines in the line scan image. This high sampling frequency minimizes the influence of eye movements. To further eliminate eye movement-based artifacts we monitored details of the scans. During the line scan portion of each frame we can detect motion of the eye based on the fact that stationary structures such as the vessel wall should appear as vertical straight lines. If there is a horizontal component of eye
movement, then these lines deviate from the vertical. If there is a vertical component of eye movement, and the stationary structures are small, then the scan line will be moved off the structure and the vertical lines will be interrupted. We can also detect vertical motion between frames. We restricted data analysis to measurements taken when there were no eye movements. The sampling rate of our blood velocity measurement (the frame rate) was 56 Hz, which is sufficient to track the cardiac pulsatility (i.e., 45 velocity measurements per heart beat for a resting heart rate of 75 beats per minute).

Human subjects

Two subjects were tested. The typical imaging session duration was about 1 hour. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study. Subjects were dilated with 0.5% tropicamide. When the vertical scan was running, the exposure of the imaging beam (280 μW, 840 nm) and the wavefront sensing beacon (50 μW, 740 nm) were more than 10 times below the American National Standards Institute (ANSI) safe exposure level for continuous viewing at this field size (780 μm × 540 μm). When the vertical scan was stopped, the same optical power was spread on a smaller field size (according to the calculations for the standard, 780 μm × 25 μm), but the exposure was still safe for more than 2 hours of continuous imaging according to the ANSI standards (Delori, Webb, & Slaney, 2007). The vertical scan motion was programmed to return every frame (it was never stationary for longer than 18 ms), which ensured safety and comfort. The research was approved by the Indiana University Institutional Review Board and met the requirements of the Declaration of Helsinki.

Procedure

In experiment 1, we studied the influence of flicker location. Centerline blood velocity was measured in retinal arteries under four different flicker conditions. In condition 1, the blood velocity was measured before any flicker stimulation (baseline). In condition 2, it was measured with the luminance flicker activated outside the downstream feeding region of the artery. In condition 3, the flicker pattern (size equal to that in condition 2) was activated inside the downstream arterial feeding region, while the centerline blood velocity was continuously measured. Finally, in condition 4, the blood velocity was measured with the whole LCD screen flickering to deliver wide-field stimulation for comparison.

In experiment 2, we investigated the relation of stimulus area to blood velocity within the downstream region of the measurement vessel. The flicker stimulus was placed in the downstream region of target arteries, and the size of flicker was changed from zero to 10° × 10° in 2° increments. Centerline blood velocity was measured without any flicker first, and then for each flicker size setting.

We used separate systems for imaging and stimulation and they were not fully synchronized. As a result we did not measure the rise time of the blood velocity response to visual stimulation. The rise time has been reported to occur within about 10 s of flicker onset and to decrease with a similar time course after stimulus offset (Riva, Logean, et al., 2004). We therefore allowed the blood velocity response to reach its maximal response by ensuring that velocity measurements were obtained at least 1 min after stimulus onset. Between different stimulus conditions, the subjects were given at least a 2-min break to allow blood velocity to return to baseline. During the break, the subjects kept on looking at the nonflickering LCD screen to maintain their adaption to the average illuminance.

Results

Blood velocity response to flicker presented at different locations (experiment 1)

Centerline blood velocity across an entire cardiac cycle was measured for a superior retinal artery (72 μm diameter) feeding the fovea. The measurements for each condition are shown in panel B of Figure 1. The measured velocity, integrated across the heart cycle, depended on the nature of the stimulus presentation. If a 10° × 10° region of retina outside the downstream area was stimulated (condition 2), blood velocity was similar to that measured for no stimulation (condition 1), with the integrated velocity being 15% higher in condition 2. If a 10° × 10° region was flickered in a portion of the downstream retina supplied by the artery (condition 3), the integrated blood velocity increased by 48%. With equal flicker area, the average velocity increment under condition 3 is more than three times higher than that under condition 2. When the stimulus size was further increased to the entire region subtended by the LCD screen (27° × 21°, condition 4), the total velocity increased slightly further to a value 65% larger than baseline.

The results can also be expressed in terms of the change in maximum velocity (systolic velocity) during a cardiac cycle. Table 1 shows five systolic peak centerline velocity measurements for condition 2 and
condition 3. The systolic peak centerline velocities under condition 3 are significantly higher than those under condition 2 ($p < 0.01$; one-tailed $t$-test with 95% confidence interval).

Blood velocity response to downstream flicker of different areas (experiment 2)

The blood velocity was measured for various sizes of the focused flickering stimulus within the downstream region of the measured vessel. Since collecting entire cardiac cycles was difficult due to small eye movements, in experiment 2 the peak velocity during a cardiac cycle was used as a measure of blood velocity. Two subjects participated in this experiment. For both subjects blood velocity was first measured for a uniform field (no flicker) and then measured for each flicker size setting from $2^\circ \times 2^\circ$ through $10^\circ \times 10^\circ$ in $2^\circ$ increments. For each condition, after the subject was fixating on the target and they had adapted to at least 1 min of the stimulus condition, the first four systolic peak centerline velocity measurements were averaged (Figure 2). The systolic centerline velocity increased with stimulus size for both subjects. The relation is not proportional to the total area being stimulated.

Discussion

We have shown that blood velocity increases during visual stimulation, confirming previous studies. Presumably, the blood velocity increase results from increased local metabolic demand by the retinal tissue within the downstream region of the artery. By projecting localized stimuli onto different locations of retina, this technique has the potential to probe the local retinal neuron functionality in detail. Since a localized flicker stimulus as small as $2^\circ \times 2^\circ$ produces a significant change in blood velocity, our technique is sensitive to the local changes in neural activity. It is important to note that although we did not measure smaller areas, the response from the $2^\circ \times 2^\circ$ flicker falls totally outside of the range of steady-state velocities for both subjects, implying that with optimized stimulus presentation, responses to smaller areas may be measurable. This also suggests that as retinal disorders

<table>
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<tr>
<th>Systolic peak centerline velocity (mm/s)</th>
<th>Cardiac cycle</th>
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<td>1</td>
<td>46</td>
<td>48</td>
<td>45</td>
<td>46</td>
<td>46.2</td>
<td>1.1</td>
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<tr>
<td>Condition 3</td>
<td>4</td>
<td>46</td>
<td>46</td>
<td>58</td>
<td>58</td>
<td>60.2</td>
<td>2.3</td>
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Table 1. Systolic peak centerline velocity measurements for conditions 2 and 3.
such as glaucoma interrupt normal neural activity as well as neurovascular coupling, sensitive measures of altered blood velocity response may be helpful in understanding the progression of such retinal diseases. Similarly, it has been reported that diabetics have problems regulating retinal blood flow (Patel, Rassam, Newsom, Wiek, & Kohner, 1992), and our approach may allow more precise and sensitive measures of the regulatory ability of the vascular system in diabetes.

Other investigators have examined area summation effects on blood flow in retina. Some groups studied functional signals, which are associated with local blood flow changes. By using hemifield stimulation during functional magnetic resonance imaging (fMRI), Duong, Ngan, Ugurbil, and Kim (2002) were able to identify localized fMRI signal changes related blood flow changes in cat retina. Schallek and Ts’o (2011) later reported localized reflectance changes in cat retinal images due to patterned visual stimuli. By injecting dyes into the blood circulation, they demonstrated such reflectance changes were due to local blood volume changes. In the domain of blood flow measurement, Riva, Petrig, Falsini, and Logean (2000) showed that retinal blood flow is proportional to the area of the retina stimulated by diffuse luminance at the optic nerve head. By placing the diffuse flicker field at the macular region and moving the measurement site in a circle around the optic disc, this group was also able to identify certain sites with larger responses compared with others (Riva et al., 2005). Since their studies focused on the capillary meshwork near the optic disc, which is beyond the optical resolution of their instrument, the exact local feeding relationship between the flicker region and the blood vessel is unknown. The most likely source of their change is the need for the small capillaries of the optic nerve head to support the unmyelinated ganglion cell axons as they course toward and through the nerve head. Thus, the initial results of Riva et al. (2005) provide indirect evidence of the spatial summation properties of blood flow response. By measuring in the arteries supplying specific areas we can provide a complementary measurement of the demands arising at the ganglion cell bodies.

Although our imaging source is at 840 nm, the subject can still see the scanning raster as a dim rectangle. Since the raster region is illuminated only intermittently because of scanning, the scanning raster itself is flickering at the frame rate (56 Hz). However, we expect the effect of this flicker to be small. First, the rectangle is dim and drives a limited number of ganglion cells. Second, the raster has a width of 780 μm and a height of less than 540 μm. The small size of our raster also limits its flicker influence. Third, it has been reported that the flicker influence attenuates quickly when the frequency is above 20 Hz (Falsini et al., 2002). What’s more, the raster flicker is present both at baseline and during LCD flicker (i.e., every time we acquire data, and with equal retinal illuminance each time). Even if this 56-Hz flicker acts as an additional stimulus over and above the one from the LCD, it is expected to have the same effect on both baseline and after-flicker measurements. So the underestimation of the LCD flicker influence, if any, happens...
only when we demonstrate the influence as a percentage increase.

Measurements of volumetric blood flow in retinal vessels depend on blood velocity as well as vessel diameter. In fact, the vessel radius enters into the equation by the square, and therefore, changes in vessel caliber have a stronger influence on changes in blood flow than changes in velocity. Since, in the retina, major retinal arteries increase in diameter by 2 to 5% during visual stimulations (Formaz, Riva, & Geiser, 1997; Garhofer et al., 2004; Polak, Schmetterer, & Riva, 2002; Wang et al., 2011), these changes go in the same direction as the changes in velocity we found in our study. Therefore, the local velocity changes we observed in a single artery are expected to underestimate the associated blood flow changes in that vessel and, in turn, to the entire region supplied by it. Based on the previous findings, the contribution of vessel diameter increase is expected to add 4 to 14% to the increase in blood flow over and above the velocity changes we observed.

The wide-field flicker (condition 4 in Figure 1) did induce the largest blood velocity response, presumably because its flicker area included almost the entire downstream region for the artery shown. This is consistent with the data from experiment 2. As seen in Figure 2, the blood velocity response increased with downstream flicker size. The exact form of this rise is not linear with stimulus size or area. This is presumably because of the distribution of cells in the retina. The increase in blood velocity at small sizes presumably occurs because of the high concentration of inner retinal neurons near the fovea. For visual stimulation, it is the density of inputs that is important. Thus, while the ganglion cells are concentrated in an elliptical ring at about 0.4 to 2 mm from the foveal center (Curcio & Allen, 1990), the input to those cells comes from the fovea and parafovea. Thus, our 4° × 4° stimulus is most likely to stimulate proportionally more cells than the 2° stimulus, but further increases in stimulus area would have a decreasing impact, as is seen in the results. The saturation of the velocity response at our largest field size presumably arises because the target is extending beyond the region supplied by the measured artery.

As we have observed, a small stimulus outside the downstream feeding region still induces a small velocity response compared with baseline. One possible explanation might be that the temporal modulation of luminance on one area of the retina could cross-talk to some extent into adjacent retinal areas simply due to scattering of the stimulus light, introducing a small amount of secondary flicker stimulation elsewhere.

The current technique does have some limitations. While the high scanning frequency minimizes the influence of eye movements for individual velocity measurements, the need to maintain the position to a few microns over time makes it difficult to monitor continuous changes. In fact, obtaining sufficient numbers of complete heart cycles to average was difficult and explains why in experiment 2 we presented data only for the maximum blood velocity. While image tracking can help, the precision of our current tracking technique (Ferguson et al., 2004) is not fine enough to meet this very strict constraint. It is especially difficult for longer periods required for time course studies because of the need to blink and maintain a relaxed accommodation. In principle, however, because we obtain the data in bursts that are never more than 4 s, we could measure the time course by multiple sampling even if blinks or eye movements cause some periods of information loss. Doing this will also require precise synchronization of the stimulus and measurements cycles, which was not available for this initial study. However, the complete time course of the velocity response can be obtained in anaesthetized animal models. Another limitation with the current technique, as mentioned previously, is that the stimulation control and the image acquisition were not fully synchronized. After the onset of the stimulus, the subject typically needs some time (10 to 60 s) to stabilize fixation. As a result, the exact stimulation time cannot be accurately controlled. As an alternative we kept the stimulation time much longer (at least 1 min) than the needed time (10 s). The velocity measurements under different stimulation conditions are thus obtained after different onset times. However, the blood velocity typically remains at the raised level after about 10 s (Riva, Logean, et al., 2004; Riva et al., 2005).

Disrupted neurovascular coupling and vascular dysfunction was also reported in several ocular diseases (e.g., ocular hypertension and glaucoma) using full-field flicker (Grieshaber, Mozaffarieh, & Flammer, 2007; Riva, Salgarello, et al., 2004). Under those pathological conditions, the flicker-induced hemodynamic response was reduced. The localized flicker and blood velocity measurements of our technique offer the potential to investigate the progress of such diseases at different retinal eccentricities.

**Conclusion**

Flicker stimulation increases retinal blood velocity in a manner that is spatially related to the location and size of the flicker stimulus. Our findings that blood velocity in a blood vessel depends on whether cells in the downstream region were stimulated or not suggest that the retina adapts its blood flow to local metabolic demands by regulating blood flow selectively, according to the spatial relationship between the metabolically activated region and the specific associated vessel.
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