Direct and Noninvasive Assessment of Parafoveal Capillary Leukocyte Velocity

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Purpose: Alterations in leukocyte velocity have been implicated in many retinal disease processes. However, direct and objective assessment of leukocyte velocity in retinal capillaries has been limited by a reliance on invasive contrast dyes that allow leukocyte visualization only for a short time span. The recent application of adaptive optics in a scanning laser ophthalmoscope (AOSLO) has made long-term imaging of parafoveal leukocyte movement possible without contrast dyes. In this study, using the AOSLO, we demonstrate a new method to investigate retinal parafoveal capillary leukocyte velocity.

Design: Experimental study.

Participants: Six normal healthy subjects ranging from 25 to 35 years of age with clear ocular media.

Methods: The parafoveal zone of the retina was imaged in all subjects using an AOSLO.

Main Outcome Measures: Leukocyte velocity was determined in the parafoveal capillaries including the foveal avascular zone border. Leukocyte velocity was measured directly from movie segments in which the leukocytes were clearly visible.

Results: The mean parafoveal leukocyte velocity for 6 subjects was 1.37 mm/second, ranging from 0.77 to 2.10 mm/second. Leukocytes were not visible in all parafoveal capillaries.

Conclusions: Parafoveal capillary leukocyte velocity can be directly and noninvasively measured without the use of contrast dyes using an AOSLO. *Ophthalmology 2005;112:2219–2224 © 2005 by the American Academy of Ophthalmology.*

Due to their visibility through the optics of the eye, retinal vessels provide researchers a unique opportunity to study hemodynamics noninvasively. To attest to the importance of measuring retinal hemodynamics, many innovative techniques and instruments have been used to assess blood flow characteristics of varying size retinal vessels including laser Doppler velocimetry and flowmetry, Doppler optical coherence tomography, laser speckle phenomenon, blue field entoptic phenomenon, dye dilution curves, and scanning laser ophthalmoscope (SLO) with contrast agents.[1–9] However, all of the currently developed techniques have some limitations, and to our knowledge, there is still no gold standard for studying retinal hemodynamics.

Understanding hemodynamics in parafoveal capillaries is extremely important. This is due to their position adjacent to and surrounding the fovea, which is responsible for providing central vision. However, the few methods that are capable of studying parafoveal capillary hemodynamics are limited in repeatability due to the use of fluorescent contrast agents, are unable to measure blood velocity directly, are subjective, or may have highly variable hemodynamic measurements.[1,4,7,10,11] This inability to measure retinal microvessel blood velocity directly, objectively, and noninvasively has made it difficult to perform research involving comparisons across different subjects and longitudinal parafoveal capillary hemodynamic studies.

The image quality from direct retinal imaging techniques in subjects without significant ocular opacities and having minimal scatter has always been limited by the inability to correct for the blur caused by the presence of high-order ocular monochromatic aberrations. But, in the last decade, the application of adaptive optics has pushed the quality of retinal images from living human eyes close to the diffraction limit. Adaptive optics involves the measurement of ocular aberrations with a wavefront sensor followed by a correction of the aberrations using a deformable mirror.[12,13] Adaptive optics has been applied to 2 retinal imaging techniques: (1) flood illuminated fundus photography[12] and (2) confocal adaptive optics scanning laser ophthalmoscopy.[13]

The confocal adoptive optics scanning laser ophthalmoscope (AOSLO) takes high-contrast, high-resolution videos of the retina at 30 frames per second, and we have visualized individual leukocyte cells moving through the parafoveal capillaries without using contrast agents (Invest Ophthalmol Vis Sci 443[suppl]:3628, 2003) (Am Acad Optom 80[12 suppl]:8, 2003). To our knowledge, the AOSLO is the only instrument that allows direct and continuous recording of leukocyte movement in parafoveal capillaries without the use of contrast agents (videos 1–3 [available at http://aaojournal.org]). (The videos demonstrate the capabilities of the AOSLO for imaging microscopic structures of the retina, including leukocytes traveling through parafoveal capillaries and the underlying photoreceptors. Leukocyte movement is best seen in real-time video segments.) Our purpose in this study was to apply the newly developed capabilities of the AOSLO for direct, noninvasive, and objective measurement of parafoveal capillary leukocyte velocity.

Materials and Methods

This research followed the tenets of the World Medical Association Declaration of Helsinki. Informed consent was obtained from the subjects after we explained the nature and possible complications of the study. Our experiment was approved by the University of Houston Committee for the Protection of Human Subjects.

Subjects

Six subjects participated in this study. Subject ages ranged from 25 to 35 years. All subjects were healthy and had no systemic or ocular disorders. All subjects were dilated before imaging with 1 to 2 sets of tropicamide (1%) and phenylephrine HCl (2.5%). All subjects were imaged in the left eye using the AOSLO.

The videos that were used in this article were selected from a data set of videos that were collected for another research experiment. The other research experiments required numerous videos per subject and were gathered during imaging sessions that lasted up to 2 hours. Imaging sessions in which the intention is to record videos of blood flow movement were shorter and lasted approximately 30 minutes, excluding subject dilation time.

Adaptive Optics Scanning Laser Ophthalmoscope Imaging System

Before the adaptive optics correction was made, the subject's refractive error was corrected to the nearest 0.25 diopter with spherical and cylindrical trial lenses placed at the subject's spectacle plane. Then, the AOSLO system used a custom-built Shack-Hartmann wavefront sensor[14] and a 37-channel deformable mirror (Xinetics, Inc, Devens, MA) to measure and correct the subject's residual ocular monochromatic aberrations in a closed-loop feedback system. The AOSLO imaging system used in this study (Fig 1) is described in greater detail by Roorda et al.[13] A bite bar was used for each subject to aid in stabilization. The imaging wavelength was 660 nm and the field of view was 1.4 × 1.5°. Videos were recorded at a frame rate of 30 Hz. Images were recorded using a maximum of 30 microwatts of 660 nm light (measured at the cornea), which continuously scanned in a raster on the retina. With this exposure level, we are approximately 100× lower than the maximum permissible exposure suggested by the American National Standards Institute.[15]
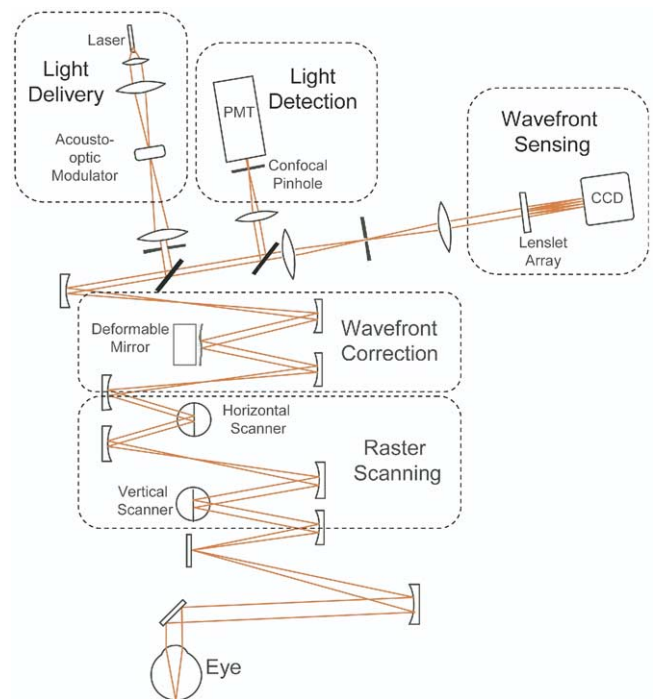

Figure 1. Schematic of the adaptive optics scanning laser ophthalmoscope (AOSLO). The 5 main elements are (1) light delivery, which is a laser light source emitted from a single-mode optical fiber; (2) light detection, which is a photomultiplier tube (PMT) behind a confocal pinhole; (3) wavefront sensing, which is a Shack–Hartmann wavefront sensor; (4) wavefront correction, which is a 37-channel deformable mirror (Xinetics Inc., Devens, MA); and (5) a raster scanner, which is a 16-kHz resonant scanning coupled with a 30-Hz galvanometer scanner. The system integrates adaptive optics into the path to allow for continuous monitoring and updating of the aberration correction. Aberrations are corrected on the way into the eye to form a sharp focused spot at the retina, and aberrations are corrected on the way out of the eye to refocus a compact image of the spot on the retina at the confocal pinhole. CCD = charge-coupled device.

Leukocyte Velocity Measurement

Parafoveal capillary leukocyte velocity was measured directly from AOSLO video segments where leukocyte movement was visible. Leukocyte movement was visible for all 6 subjects in this study. Video clips 1 to 3 (available at http://aaojournal.org) show example video segments that were recorded using the AOSLO. Leukocytes were visible in retinal capillaries, including those bordering the foveal avascular zone. Figure 2 demonstrates the parafoveal capillary region from which leukocyte velocities were measured in all subjects.

Direct measurements of leukocyte velocity were performed as follows. First, the location of an individual leukocyte was determined in a succession of frames (Fig 3). Next, the total distance traveled by each leukocyte was measured directly from a single correlated image of successive frames. The total distance traveled per leukocyte was measured using custom image measurement programs that were written using the Matlab software package (The Mathworks, Natick, MA). Axial length measurements were based on 3 repeated measurements with either a Sonomed A5500 A-Scan Ultrasound (Latham and Phillips Ophthalmic Products, Inc, Grove City, OH) or Carl Zeiss IOL Master (Carl Zeiss Meditech, Inc, Dublin, CA). Conversion from retinal field angle to
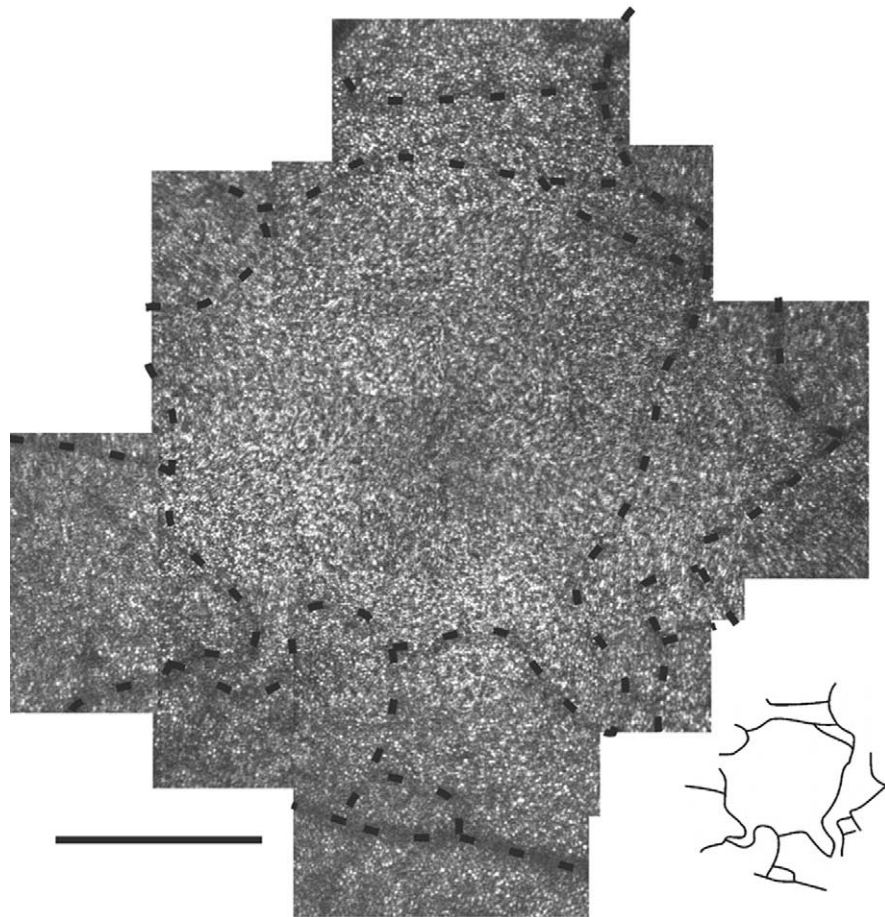
Figure 2. Composite of several correlated images of subject E showing the foveal avascular zone border where leukocyte movement is visible. A dark dashed line is drawn over the capillaries to assist the reader in this visualization. The inset shows a more detailed line drawing of the capillary network. The scale bar is 1° (302 μm).

true retinal size was performed based on the subject's axial length as described in Bennett et al.[16] To reduce error in the direct measurements, the velocity of a single leukocyte was based on an average of 5 repeated measurements. All measurements were performed by one operator. We studied leukocyte velocity in 2 individual capillaries per subject. For each individual capillary, the velocities of 5 different leukocytes were measured.

Results

By examining the differences between 5 separate measurements of a single leukocyte, we computed the sensitivity of the method to changes in leukocyte velocity. For the 5 repeated velocity measurements of an individual leukocyte the standard error of the mean was 0.023 mm/second, which means that the direct imaging method used here is sensitive to changes in velocity as small as 0.076 mm/second (95% confidence level).

Ten leukocyte velocity measurements were made for each of the 6 subjects, for a total of 60 measurements. The mean leukocyte velocity for all subjects ranged from 0.77 to 2.10 mm/second, and the overall mean was 1.37mm/second. Table 1 shows the average, minimum, and maximum leukocyte velocity values for each subject's selected capillaries.

Discussion

This study demonstrates that the AOSLO can accurately measure leukocyte velocity in the parafoveal capillaries, including the smaller capillaries that line the foveal avascular zone. Our overall mean leukocyte velocity for all subjects of 1.37 mm/second compares well with Yang et al[8] who obtained a mean leukocyte velocity of 1.37±0.35 mm/second, but was lower than Arend et al's[17] who found a mean leukocyte velocity of 2.68±0.3 mm/second. Both studies used an SLO with fluorescein angiography. Our mean leukocyte velocity is within the range of mean leukocyte velocities, 0.25 to 1.46 mm/second that have been reported by other researchers that used the blue-field entoptic phenomenon.[7,17–21]

Advantages

There are several major advantages to using this new technique for measuring retinal capillary leukocyte velocity. The ability to measure retinal capillary blood velocity non-invasively, directly, and repeatedly without the use of contrast agents has potential for allowing longitudinal retinal
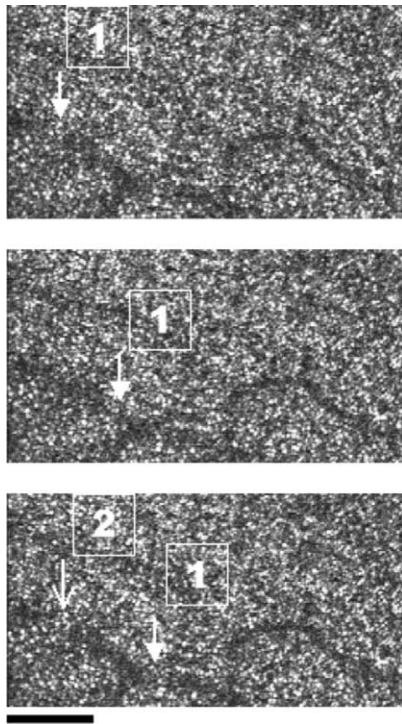
Figure 3. Three successive frames demonstrate a single leukocyte's (1) change in position from left to right in each frame. A second leukocyte (2) has just come into view in the last frame. Because it is difficult to identify single leukocytes in still images it is recommended that readers view the videos 1–3 (available at http://aaojournal.org). Scale bar, 100 μm.

the level of disease progression could also be studied non-invasively using this technique.

Limitations

Because relatively clear ocular media is currently necessary for the adaptive optics to perform well, this technique could not be used on some subjects with poorer ocular clarity. This might include subjects with severe cataracts or corneal surface and tear film irregularities.

We currently use a bite bar to aid in stabilizing the patient while imaging, which could not be used in a large clinical study. In future work, we plan to use a pupil tracker with the AOSLO in place of a bite bar.

The field size of the AOSLO is small compared with conventional imaging systems. We keep the field size small to maximize the pixel density of the image and to increase our sensitivity to leukocyte motion (i.e., we increase the number of pixels traveled per second by reducing the field size). Although the field of view is small, we find that we can easily go back to the same capillaries on repeated imaging sessions. The real-time video feedback to the operator helps to align the patient and subject fixation is very reliable as long as they are given a proper fixation target.

Leukocyte velocity calculations assume that the capillaries are traveling tangential to the retinal plane. This is a fair assumption because capillaries in the retina are known to be stratified. Parafoveal capillary beds go from a single layer of capillaries at the terminal capillary ring bordering the foveal avascular zone to a multilayer stratified arrangement.[22,23] Some capillaries do travel out of planes tangential to the retinal surface, but because our system is confocal (i.e., images are limited to light from axial planes), we are less likely to visualize the full extent of those capillaries and include them in the analysis.

During this study we used a 660-nm red wavelength laser for imaging leukocyte movement. We believe that our current method may be improved by imaging with a green laser

capillary hemodynamic studies. The AOSLO imaging system is very sensitive to small changes in leukocyte velocity, which is important for studying retinal velocity alterations. This method could be used in pharmaceutical studies involving the effects of medications on retinal capillary velocity. Changes in retinal blood-flow velocity in relation to

Table 1. Leukocyte Velocity Statistics

Subject	Capillary	Minimum Velocity (mm/sec)	Maximum Velocity (mm/sec)	Mean Velocity (mm/sec)	Standard Deviation
A	1	0.93	1.53	1.17	0.24
A	2	1.36	1.85	1.58	0.19
B	1	1.68	2.10	1.80	0.17
B	2	1.75	2.07	1.95	0.12
C	1	0.77	1.08	0.94	0.12
C	2	1.03	1.35	1.16	0.13
D	1	0.95	1.39	1.12	0.16
D	2	1.21	1.44	1.36	0.09
E	1	1.33	1.58	1.44	0.11
E	2	1.26	1.46	1.38	0.09
F	1	1.06	1.53	1.33	0.18
F	2	1.12	1.47	1.23	0.16

Shows the mean, maximum, and minimum leukocyte velocities in each individual capillary of 6 subjects, who are labeled A through F, respectively. Each capillary's mean leukocyte velocity is based on the mean velocities of 5 independent leukocytes. The standard deviation of each capillary represents the variability in mean velocities between 5 independent leukocytes.

versus a red laser. We are currently investigating the benefits of imaging leukocyte movement and retinal capillaries with a 532-nm green wavelength laser (Am Acad Optom 80[12 suppl]:8, 2003). Because the erythrocyte component (i.e., oxygenated hemoglobin) has relatively more absorption for green versus red wavelengths, the contrast between erythrocytes and leukocytes can be increased when imaging is done with a green laser. This improvement in contrast would increase detection of leukocytes and might allow for automation of blood velocity measurement with custom software.

Does This Method Measure Leukocyte Velocity?

We believe that the features that can be seen flowing through the capillaries are leukocytes for several reasons. Zwick et al[24] observed leukocytes directly, without the use of a contrast agent, in a garter snake using a commercial SLO. As in our case, the leukocytes appeared with positive contrast against the dark capillaries. Direct imaging without adaptive optics was possible in the garter snake because of their exceptional optics. They confirmed that the cells were indeed leukocytes by observing the same behavior of cell movement using acridine orange contrast agent, which is a fluorescent stain that is specific for leukocytes. Another study demonstrated that the fluorescent dots seen against the darker capillaries with an SLO were indeed leukocytes by removing the plasma and erythrocytes from a fluorescent–labeled blood sample, injecting the fluorescent–labeled leukocytes into the subject, and finding that the fluorescent leukocytes showed the same behavior when imaged, compared with conventional SLO fluorescein angiography.[8] In all cases of previous imaging of leukocytes, the leukocytes showed the same intermittent behavior as the cells we see with the AOSLO (Invest Ophthalmol Vis Sci;38(4):(s84), 1997. The frequency of leukocytes, which is <1% of the cells in blood,[25] is also consistent with the cells we are imaging. Leukocytes are much larger than red blood cells[25], and are therefore easier to resolve with the AOSLO. Erythrocytes are expected to be relatively transparent with our red imaging light source (660 nm), based on their absorption characteristics.

Although we cannot image erythrocyte rouleaux movement with the AOSLO, the anatomical dimensions of the blood cells and the parafoveal capillaries limit the transit of cells to a single file. This arises because the inner diameter of the smaller retinal capillaries near the fovea are approximately 5 microns, and both leukocytes and erythrocytes are larger in diameter than the parafoveal capillary diameters.[26–29] Therefore, the velocity that is being measured, based on leukocyte movement, represents the blood velocity of all blood cells. However, we cannot measure the blood velocity during the periods when there is no leukocyte within the capillary.

In conclusion, this article demonstrates a new method for direct and noninvasive measurement of parafoveal capillary leukocyte velocity without the use of contrast dyes by using the AOSLO imaging system.

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