Measurement of Retinal Blood Vessel Width Using Computerized Image Analysis

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We report a new method for quantitating retinal blood vessel width from fluorescein angiogram negatives using a computerized image analyser. Interuser and intrauser variability were 3 to 4 times lower than reported with older methods. In addition, model blood vessels were used to test the accuracy of our method under four clinically relevant conditions: variations in vessel width, fluorescein concentration, flash intensity and background fluorescence. Although computed measurements were affected by vessel width, fluorescein concentration and flash intensity, the linearity of the actual versus computed width was maintained during wide variations in all four conditions ($r = 0.95, P < 0.0001$). Accuracy was best achieved at a flash intensity of 150 W/sec and a fluorescein concentration of 45 µg/ml. The results of this study provide a better understanding of factors affecting the apparent width of retinal blood vessels in fluorescein angiograms. This technique should be useful in rapidly obtaining accurate measurements of blood vessel width from fluorescein angiograms. Invest Ophthalmol Vis Sci 29:1258-1264, 1988

The relationship between chronic changes in retinal blood flow and the development of diabetic retinopathy is presently unknown. Elaborate techniques have, therefore, been developed for measuring retinal blood velocity. These include laser doppler velocimetry,1-5 slit-lamp fluorophotometry,6 two-point fluorophotometry,7 blue field entoptic technique,8 and image analysis.9 Since retinal blood vessel diameter affects blood flow measurements by a power of two (blood flow ($F$) = $r^2 \times v$, where $r$ = radius and $v$ = mean velocity), accurate measurements of width are needed if accurate retinal blood flow determinations are to be realized. Vessel caliber measurement techniques provide only relative values10 and their accuracies have never been tested.

Existing techniques for measuring blood vessel width consist of two steps which may both affect the apparent blood vessel width. Step one is the acquisition of the vessel image and step two is the measurement of the vessel width. Acquisition techniques include fundus5-16 and monochromatic photographs,17,18 fluorescein angiograms,9,19-23 and video tapes.8,16,24 Measurement of the vessel width is performed by visually or densitometrically locating the vessel edge from the background. Visual techniques, the most common, include the use of a measuring microscope,15,24 a microscope with a screw micrometer eyepiece,11,19 or a micrometer scale in one eyepiece,4 an image splitter,15,16,25 and image projection with measurement by calipers.5,12,14 Densitometric techniques include scanning densitometry12,15,19,20 and image analysis.7,8,9,23

Fluorescein angiograms were selected for image acquisition in the present study because they are widely used, provide superior edge definition, and most importantly, include the plasma zone. Other techniques (not using fluorescein angiograms), measure changes in the red blood cell column which may not be proportional to changes in the internal diameter.26-28 Image analysis was chosen for measurement of blood vessel width because visual techniques are less reproducible and more time consuming and because recent advances in computer technology allow the use of an inexpensive minicomputer.

Model blood vessels were used as standards to detect possible systematic sources of error. Systematic error sources include: (1) vessel width; (2) fluorescein dye concentration; (3) photographic flash intensity; and (4) background fluorescence. Model vessels of different diameters were compared to determine if the measured width of one size changed disproportionately from the others under varying conditions. Different dye concentrations were compared and the concentration at which the width was measured was also determined. Flash intensity was varied to determine the flash setting at...
which the computed and measured widths were the same. Different backgrounds were compared because variations in the pigmentation of the retinal pigment epithelium between subjects or in choroidal fluorescence during fluorescein angiography might introduce error into the computer width.

Materials and Methods

Image Analysis

The negative to be analyzed was placed on a fluorescent light box (Ladd Research Industries, Inc., Burlington, VT) and held flat by a weighted glass microscope slide. The negative was magnified ×2.5 (Zeiss Stereomicroscope, Model IV b, Carl Zeiss, 7082 Oberkochen, West Germany), inverted electronically to a positive by a Vidicon camera (Model NC-68D, Dage MTI, Inc., Michigan City, IN), and converted to a digital image by a LeMont Image Analyzer. The LeMont OASYS System consisted of the Vidicon camera, IP-512 frame buffer-analog processor-arithmetic logic unit (Imaging Technology, Waltham, MA), DEC LSI 11/73 minicomputer with 1024K core memory, RT-11 Operating System (Digital Equipment Corp., Maynard, ME), Sony Trinitron color video monitor (Model PVM-1271Q), eight inch floppy drive, and a 10 megabyte fixed Winchester hard disk. A digital tape transport (Model 9000, Kennedy Company, Monrovia, CA) provided image storage. The density range of the scanner, which scanned in a linear mode, was manually adjusted to the 0.30–2.3 density range of the film. A precision ruler was used to calibrate the magnification of the system.

A well-focused portion of the negative was selected for analysis and the vessel to be measured was positioned parallel to either the x or y axis (Fig. 1). The optical density of the selected area was averaged twice and recorded in memory as a 480 × 512 byte pixel matrix. Each of the pixels registered a gray level value from 0 to 255 that was proportional to the film density (Fig. 2). Once in memory, the portion of the vessel to be measured was selected with a joybox (Fig. 1).

The digital pixel readout of a vessel obtained from this technique (Fig. 2) is similar to that obtained from a densitometer (Fig. 3). At the point where the vessel edge is encountered, the pixel values are substantially higher than the background. The width of the vessel was taken as the full width at one-half of the maximal value of the vessel profile curve (Fig. 3). The one-half maximal value was determined separately for each side of the curve. This compensated for variations in background intensity. To minimize the effect of noise, the maximal and minimal values were taken as the average of the second and third maximal or minimal values, respectively. If the curve crossed the half-maximal value more than twice, as frequently occurred if there was a branch vessel, it was discarded. Each vessel was measured at 32 points, 15 to 18 μm apart, and the recorded width was taken as the average of the nondiscarded measurements. The vessel width, the standard deviation of the measurement, and the number of nondiscarded curves were printed or recorded on disk.

Analysis of Preselected Vessels by Multiple Users

Areas with one or more vessels on the analyzed negatives were selected as described above and stored on disk. The location of the vessel(s) section(s) to be measured by the other two users was defined by recording the command sequences used to position the

Fig. 1. Magnified image of cat retinal vasculature from a 35 mm fluorescein angiogram. Note the indistinct edges. A joybox is used to select vessel segment for measurement.

Fig. 2. Pixel map showing density variations across a retinal blood vessel.
joybox over the vessel(s) (Fig. 1). The recorded image was displayed on the monitor, the film negative was placed under the microscope/video camera combination, and its image was superimposed on the recorded image and manually aligned. The aligned image was then recorded in memory. Between one and three vessels were analyzed on each negative.

Photography

All photographs were taken with a Topcon variable angle retinal fundus camera (Model TRC-50V/50VT, Topcon Instruments Corporation of America, Paramus, NJ). Lens to object distance was 10.5 cm for all studies. A 50° angle was used for the model blood vessel experiments, and a 20° angle for the animal experiments, yielding magnifications of ×1.7 and ×3.7, respectively (manufacturer’s specifications). Fuji gelatin excitation and barrier filters supplied with the camera were used. At all flash levels, the flash intensity did not vary when measured on three occasions with a Minolta Flash Meter III. Kodak 35 mm Tri-X pan film was used and developed in Kodak Duraflo developer at 78°F at a speed of 8.5 ft/min using a Hope processor to minimize variations in negative density. At flash intensities above 36 Joule, densities of the photographic negative fell on the linear portion of the characteristic film curve.

Model Blood Vessels

Model blood vessels were made by mixing 2 ml of aqueous fluorescein into 20 gm of preactivated Mercox®. The concentrations used were 0.5, 1.5 and 4.5g/ml. After activation, the Mercox (Ladd Research Industries, Inc., Burlington, VT) was injected into plastic tubes of three different diameters. For this purpose, two polyethylene tubes (PE50, PE90) and the plastic tubing from a 19 gauge butterfly needle were used. The Mercox-filled tubes were left to harden overnight, and the tubing was removed the following day. The resultant casts were measured five times with a caliper accurate to within ±5 μm. Their external diameters were 550 ± 10, 785 ± 20, and 1240 ± 20 μm, respectively (mean ± 2 STD). Each set of casts was photographed together at each of the seven different flash intensities (18, 36, 75, 100, 150, 200 and 300 Joule) available on the fundus camera. Different levels of background fluorescence were achieved by photographing the model blood vessels in front of a tank filled with varying concentrations of fluorescein (0, 10, 30 and 90 pg/ml). These are referred to as background 0, 1, 2 and 3, respectively. Concentrations were selected to provide a range of fluorescence up to, but not exceeding that of the lowest concentration model blood vessel. At the beginning of each roll of film a Schaedler precision ruler (John Schaedler, Inc., New York, NY) was placed perpendicular to the casts and photographed using a green filter for calibration of the image analyzer. The width of the model vessel images on the film was similar to that of cat retinal blood vessels from fluorescein angiograms on the film. The differences between the widths of the model vessels and cat retinal blood vessels result from the use of different calibration scales, not from differences in the widths of the vessel images on the film.

Animal Experiments

Fluorescein angiograms from 20 cat retinas were used to determine the reproducibility of the image analysis measurement technique. The animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research. The animals were anesthetized with ketamine HCL (30 mg/kg IM), and the head secured in a stereotaxic device. The lids were retracted and the fundus camera carefully focused on the inferior temporal vasculature. Aqueous fluorescein (10%, 0.1 ml/kg) was administered intravenously and photographs were taken at frequent intervals for 5 min.

Reproducibility

To evaluate intrauser and interuser variability, 40 blood vessels from 20 fluorescein angiograms of cat retina were measured by three users on three different days. One user was selected with no prior knowledge of computers or ophthalmology to determine if these were prerequisites for reproducible measurements. To determine the intrauser variability in measuring multiple frames from a fluorescein angiogram, three users analyzed the 40 cat retinal blood vessels three times on the third day. Before inclusion in the study, each user was instructed on the use of the analyzer and given one day to practice. The studies will be defined as: Study A = day 1; Study B = day 2; Study
The effect of model blood vessel size on computed width

Table 1. The effect of model blood vessel size on computed width

<table>
<thead>
<tr>
<th>Actual diameter ± SD (μm)</th>
<th>Computed width* (μm)</th>
<th>SD of the measurement* (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>550 ± 5</td>
<td>582.3 a</td>
<td>19.1 D</td>
</tr>
<tr>
<td>785 ± 10</td>
<td>773.3 b</td>
<td>21.6 D</td>
</tr>
<tr>
<td>1240 ± 10</td>
<td>1116.8 c</td>
<td>17.4 D</td>
</tr>
</tbody>
</table>

* Means with different letters are significantly different at P < 0.05. Significance was determined using the Ryan-Einot-Welsch Multiple Range Test (SAS).

The values were obtained by averaging the computer measurements of the vessel diameter and the standard deviation of the measurements for the given sizes at all flash intensities, backgrounds, and model blood vessel concentrations.

C = day 3, first analysis; Study D = day 3, second analysis; and Study E = day 3, third analysis.

Analysis of Data

To test if differences between model blood vessel measurements were statistically significant at a P < 0.05 at each level of the four clinically relevant variables, the Ryan-Einot-Gabriel-Welsch multiple range test was used. Data from a flash intensity of 18 Joules was not included in the analysis because the model blood vessels were not discernible visually by our analysis technique in many of the negatives.

Results

Model Blood Vessel Measurements

Fluorescent-cylindrical Mercox casts were used to evaluate the effect of four pertinent clinical variables on computed blood vessel width. Cast diameter had a statistically significant effect on the computed diameter width, but not on the standard deviation of the measurement (Table 1). The linearity of the system is reflected in the strong association between cast size and computed diameter under all the conditions tested (r = 0.9514, P < 0.0001). The absence of a relationship between the standard deviation of the measurement and the size reflects the fixed magnitude of the error of the system. As a result, the coefficient of variation is larger in the smaller vessels.

Fluorescein concentration had a statistically significant effect on both the computed diameter and the standard deviation of the measurement (Table 2). The higher the concentration, the larger the computed diameter (r = 0.3828, P < 0.0001), and the smaller the standard deviation of the measurement (r = 0.1108, P < 0.0001). The most likely reason for these differences is that the light emitted from the edge of the lower concentration casts is not sufficient to exceed the threshold of the photographic film, whereas it is sufficient in the higher concentration casts. Accuracy was best at the intermediate Mercox cast fluorescein concentration (1.5 g/ml). These results suggest that a gradual decrease will occur in measured retinal blood vessel width during the course of a fluorescein angiogram as fluorescein concentration decreases.

Flash intensity had a statistically significant effect on the measured width and the standard deviation of the computed width (Table 3). High flash intensities were associated with significantly larger vessel measurements and a smaller standard deviation (r = 0.3074, P < 0.0001). The most likely reason for this is that at low flash intensities the number of fluorescein particles excited at the edge, where the cross-sectional area is smallest, does not exceed the threshold sensitivity of the film. This would result in a smaller measured width and a larger standard deviation. Accuracy, as assessed by the point at which the measured width was closest to the actual width, was best at a flash intensity of 150 Joules.

Different backgrounds did not have a statistically significant effect on computed width (Table 2). Accuracy was best at the intermediate Mercox concentration at all flash intensities, backgrounds, and sizes.

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Different backgrounds did not have a statistically significant effect on computed width (Table 2). Accuracy was best at the intermediate Mercox concentration at all flash intensities, backgrounds, and sizes.
Table 4. The effect of background fluorescence on the computed model blood vessel width

<table>
<thead>
<tr>
<th>Background fluorescence</th>
<th>Computed width* (μm)</th>
<th>SD of the measurement* (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>825.6^a</td>
<td>16.7^n</td>
</tr>
<tr>
<td>1</td>
<td>828.6^a</td>
<td>17.1^b</td>
</tr>
<tr>
<td>2</td>
<td>814.2^b</td>
<td>17.1^b</td>
</tr>
<tr>
<td>3</td>
<td>822.7^c</td>
<td>26.9^c</td>
</tr>
</tbody>
</table>

* Means with different letters are significantly different at P < 0.05. Significance was determined using the Ryan-Einot-Welsch Multiple Range Test (SAS).

The values were obtained by averaging the computed measurements of the vessel width and the standard deviation of the measurements for the given background at all flash intensities, model blood vessel fluorescein concentrations, and sizes.

significant effect on computed width (Table 4); however, the standard deviation of the measurements tended to increase with increasing background fluorescence (r = 0.8926, P < 0.0006). This suggests that changing choroidal fluorescence or pigment epithelial differences between subjects should have little or no effect on measured blood vessel width other than a small increase in the standard deviation.

Reproducibility of Measurements on Blood Vessels of Cat Retina

The mean interuser coefficient of variation (CV) in all five of our studies was 0.56% (Figure 4). Variability was greatest on the first day reflecting the user's learning curve; however, the difference between Study A and B, or any other study, never approached statistical significance. The difference between the two users familiar with computers and ophthalmology and the user who was not, did not approach statistical significance. Variability decreased with increasing size and was less than 1% for all vessels greater than 150 μm. The increase in the CV for the smaller vessels reflects the relatively fixed magnitude of the standard deviation of the measurements.

The interuser variability was similar to the interuser variability (Studies A–C, Fig. 5A). The mean coefficient of variation was 0.55%. The mean variability of the measurements made on the same day without recalibration (Studies C–E, Fig. 5B), on the other hand, was 0.24% and did not change appreciably with increasing vessel size. This suggests that the interday and interuser variability is largely a result of system calibration differences. None of these differences approached statistical significance. The overall variability of our technique, including interuser, intrauser on different days, and intrauser on the same day was 0.45%.

Discussion

Reproducibility of the computerized image analysis method (mean CV = 0.45%) for measuring retinal blood vessel diameter was three to four times better than that of any previously reported method.7,9,13,15,16,19,20,24 When measurements were done on the same day by the same user, eliminating the variability induced by calibrating the system, the reproducibility was even better (mean CV = 0.24%). This means changes as small as 1 μm in retinal blood vessel diameter can now be measured.

A direct comparison of the reproducibility of this method with previous methods is difficult because of differences in acquisition of the vessel image and determination of reproducibility. Studies that used fundus or monochromatic photographs to test their measurement technique probably have a higher variability than this technique because the edge resolution on fundus and monochromatic photographs is not as good as on fluorescein angiograms. A higher variability also occurred in studies which report only interuser variability.16 This tends to be higher than the average of interuser and intrauser variability, or in those which used two users instead of three to test their reproducibility.13,19,20 However, these factors are unlikely to explain the substantially lower variability of this technique.

The most reproducible measurement method reported previously is scanning densitometry. Using fluorescein angiograms, Griffiths et al19 reported a CV of 1.8%. However, the use of scanning densitometry is limited because it is time consuming, technically difficult, and results in a higher CV.15,20 The
The image analysis technique presented here combines the power of a computer with the objectivity of densitometric measurements, offering a promising alternative.

Preussner et al. claimed that the variability of their image analysis technique was 0%, reflecting this potential. Unfortunately, data to support this claim were not presented and they used a large computer system that is not practical for general use. Employing an earlier image analysis system, Sandor et al. reported variability of 2 to 10%, depending on fluorescein concentration. Deutsch et al. reported a combined variability of 4% for the photographic and image analysis process.

Visual measurement methods, which are more prone to subjective error, are less reproducible. Grunwald et al. reported a CV of 2% using image projection and measurement with calipers. In another study using the same technique, the interuser variability was so great that there was statistical significance between ten observers with a \( P < 0.002 \). It therefore seems likely that the results of Grunwald et al are artificially low because interuser variability may not have been included in the value they reported. Other visual techniques offered no improvement. Hodge et al. reported that the CV for the Olympic and Nikon screw micrometers were 2.4% and 3.0%, respectively. Skovborg et al. reported a CV of 4.4% using a Zeiss micrometer. Image splitting was equally variable, with an intrauser variability of 4.2%.

Our low CV (0.45%) is most likely due to three factors. First is the use of image analysis itself, which provides objective measurements that are not prone to subjective error. Second is the decreased amount of user input compared to previous methods. With these systems, the user had to mark two points on each negative in order to align them and had to mark the edge of each vessel on its densitometric representation in order to determine its width. Third, averaging 25 or more measurements to determine the vessel width improves the reproducibility of our method.

The linearity of this system (i.e., the ratio of the computed widths to the actual widths) is similar for all cast sizes. This property maintains accuracy of the method under conditions of variable fluorescein concentrations and with the temporal variations seen during a fluorescein angiogram. The effect of dye concentration on computer measurements is the least controllable variable. Table 2 shows that the computed width increased with increasing dye concentration. The apparent width of retinal vessels is known to be widest during the first passage of the dye bolus. This makes it unlikely that the effect of changes in fluorescein dye concentration is due to an artifact of this measurement technique. Therefore, in the clinical and experimental assessment of retinal vessel width, it is essential that dye be administered per unit weight and that measurements be made at identical times after dye injection.

The effect of flash intensity is less important because it can be kept constant. At a fluorescein concentration equivalent to that found in a typical fluo-
rescein angiogram after dye equilibration, the computed versus the actual width were essentially equal at a flash intensity of 150 W/second (Table 4). This intensity can be used clinically.

This work supports the use of fluorescein angiograms to accurately measure the internal diameter of retinal blood vessels including the plasma zone. Bulpitt et al.17 and Hodge et al.20 have shown that the plasma zone accounts for 8–12% of retinal blood vessel diameter. In contrast, Deupree et al.18 reported that the plasma zone was 3% of retinal blood vessel diameter and concluded that demonstration of the plasma zone using fluorescein angiograms was not necessary for accurate retinal blood vessel measurements. The disparity may be due to use of less fluorescein or a lower flash intensity by Deupree et al.18 Furthermore, Lemmingson26 and Bulpitt et al.27 reported that the changes in the retinal blood column, which are relied on in other techniques, are not always proportional to changes in the internal diameter.

In summary, we report a new method for measuring retinal blood vessel width. This technique is rapid, easy to use, and up to four times more reproducible than previous methods. It employs 35 mm fluorescein angiograms and a minicomputer which is practical for clinical use and enables measurement of changes as small as 1 μm in retinal blood vessel diameter. Accuracy of the measurements is dependent on fluorescein dye concentration and flash intensity but not changes in background fluorescence. Standardization of flash intensity and timing as well as strict control of the amount of fluorescein injected per unit weight is, therefore, necessary if measurements from different fluorescein angiograms are to be compared. 

Key words: image analysis, fluorescein angiography, retinal blood vessel diameter, retinal blood flow

Acknowledgments

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References