Noncontact, Two-Dimensional Measurement of Retinal Microcirculation Using Laser Speckle Phenomenon

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Purpose. To report a new apparatus for noncontact, two-dimensional measurement of retinal microcirculation using the laser speckle phenomenon and to demonstrate that this apparatus can document known or expected changes in retinal blood flow.

Methods. The rabbit fundus was illuminated by an argon (blue) laser spot (0.62 × 0.62 mm), and its image speckle was detected with an image sensor. The difference between the average of the speckle intensity (Imean) and the speckle intensity for successive scannings was calculated, and the ratio of Imean to this difference was defined as normalized blur (NB), a quantitative index of blood velocity in the retinal microcirculation. The results were displayed on a color monitor showing the two-dimensional variation of the NB level in the measurement area. Using this apparatus in the rabbit, the NB in the retinal field free of visible surface vessels was determined and compared with the retinal blood flow rate measured using the microsphere technique in the same eye simultaneously. In addition, the effect of the ocular perfusion pressure (OPP) on NB was studied. In the above experiments, a stepwise reduction in OPP was introduced by elevating the intraocular pressure manometrically.

Results. The relative decrease in the average NB (NBav) over the field measured, with the reduction in OPP, showed significant correlation with the relative change in the blood flow rate determined using the microsphere technique (r = 0.59, P < 0.001). Although NBav in the retina was little affected by OPP change when OPP was greater than 50 mm Hg, NB decreased along with OPP at levels less than 50 mm Hg.

Conclusions. The NBav showed significant correlation with the retinal blood flow rate determined with microsphere technique. Retinal microcirculation under various conditions can be studied two dimensionally and noninvasively in the living eye with the present apparatus.


Among the noninvasive methods for studying retinal blood flow, including fluorescein angiography,1 the blue field simulation technique,2 and laser Doppler velocimetry,3,4 laser Doppler velocimetry probably yields the most quantitative and objective results. Laser Doppler velocimetry allows quantitative and noninvasive measurements of the centerline velocity of red blood cells in individual retinal vessels. When combined with vessel diameter measurements, it enables the determination of volumetric flow rate in the major retinal vessels.3,4 However, the status of retinal microcirculation, which is probably a more important index for retinal physiology or pathology, cannot be evaluated with the laser Doppler velocimetry instruments presently available. The speckle phenomenon is one of several interference phenomena associated with coherent light sources such as the laser.5 When the ocular fundus is illuminated by laser, a speckle pattern appears. Depending on the blood flow in the illuminated tissue, the structure of the pattern varies rapidly, and the rate of variation depends on the blood velocity.6 Fercher and Briers7,8 obtained and presented pictures of velocity distribution of red blood cells in the retina by means of laser speckle photography. Although their method had the advantage of giving an overall map of distribution of blood flow velocities in the retina, it allowed only semiquantitative estimation...
of retinal microcirculation and could not follow time-course changes. We have recently described an apparatus for noncontact, two-dimensional measurement of the microcirculation in the optic nerve head (ONH) tissue using the laser speckle phenomenon with a diode laser (wavelength 808 nm). With such an apparatus, it was possible to follow the time change of distribution of peripheral blood flow velocity in a field of 0.42 X 0.42 mm in the ONH tissue every 15 seconds.

We have further improved this apparatus for measurement of the retinal microcirculation by using an argon (blue) laser, which is scattered selectively by the retina, rather than a diode laser. In this article, we discuss the in vitro experimental results and the principle of measurement. Next, we compare the results obtained in vivo to those obtained using the microsphere technique to evaluate the validity of the present methodology. Then we study the effect of change in ocular perfusion pressure on measurement results.

MATERIALS AND METHODS

Apparatus

Figure 1 shows a schematic diagram of the apparatus. A fundus camera (TRC-WT3; Topcon, Tokyo, Japan) was equipped with a blue-component argon laser (wavelength 488 nm, maximum power 3 mW, 2002-3SLL; Uniphase, San Jose, CA) and an image sensor (100 x 100 pixels, BASIS type; Canon, Tokyo, Japan). The ocular fundus is illuminated by a halogen lamp for observation, and the halogen lamp illumination is switched to the argon illumination at the time of measurement. The argon laser beam passes through a dichroic mirror (DM1) followed by a ring mirror (M1), which focuses the beam on the ocular fundus with a field 1.2 mm in diameter in rabbit eyes or 2.1 mm in diameter in human eyes. The scattered laser light passes through the center of M1 and is reflected by a second dichroic mirror (DM2). Thus, the light scattered from a square field of the ocular fundus (0.62 x 0.62 mm in rabbit eyes, 1.06 x 1.06 mm in human eyes) is projected onto the image sensor with 100 x 100 pixels, where a speckle pattern appears. The laser power was adjusted to the minimum level at which the image sensor could obtain a usable signal. The greatest power actually used for the measurement was 0.2 mW. Scanning speed of the image sensor was 540 frames per second. In accordance with the movement of blood cells in the tissue, the structure of the pattern varies rapidly; the greater the blood cell velocity, the greater the rate of variation.

Each successive scan of the image sensor results in a different profile of output signal intensity. The pulse height of the output signal obtained at the horizontally x-th and vertically y-th pixel (x, y; x = 1, 2, 3, . . ., 100; y = 1, 2, 3, . . ., 100) in the k-th scan (k = 1, 2, 3, . . ., 98) is defined as I(x, y, k). Normalized blur (NB(x, y)), a quantitative index of the blurring of a speckle pattern, is calculated for each pixel as follows:

\[
NB(x, y) = \frac{I_{\text{mean}}(x, y)}{\sqrt{\frac{1}{98} \sum_{k=1}^{98} |I_{\text{mean}}(x, y) - I(x, y, k)|}}
\]  

where \(I_{\text{mean}}\) is the mean pulse height at the horizontally x-th and vertically y-th pixel for the 98 scans, and the denominator, \(\sqrt{\frac{1}{98} \sum_{k=1}^{98} |I_{\text{mean}}(x, y) - I(x, y, k)|}\), was used as a substitute for standard deviation to save time in calculation. The NB value is an approximation of the reciprocal of the speckle contrast given by Fercher et al., because the denominator of Equation (1) also represents the temporal variation in the speckle pattern and is thought to serve as an index of blood velocity. The NB values are divided into 25 color-coded levels, in which areas with fast blood flow velocity are displayed in red. The results are displayed as a color map on a color monitor to demonstrate visually the two-dimensional variation of the NB level in the field measured. Figure 2 shows a representative color map of the retina of a Dutch rabbit, where red areas correspond to areas of high flow. The average NB level (NB\(_{\text{avg}}\)) in any square field of interest in a displayed color map can be calculated. To complete 98 scans over the whole area requires 0.18 seconds; 15 seconds are required to display the results of calculation on the color monitor.

To check movement of the animal eye during the 0.18-second measurement period, the difference between output signal intensity for the k-th and (k+1)-th scans is monitored on three horizontal pixel lines.
(25th, 50th and 75th line) in the sensor plane, as follows:

$$TD(k) = \sum_{x=1}^{100} \frac{|I(x, y, k) - I(x, y, k + 1)|}{|I(x, y, k) + I(x, y, k + 1)|}$$

(2)

where TD(k) is defined as the temporal difference (TD). The time course of TD values is displayed continuously throughout the measurement period to check movement of the animal eye on the same monitor displaying the color map of NB values. If an animal eye does not move during measurement, the TD values on the three pixel lines are expected to show little change because the temporal change in the output signal intensity, I(x, y, k), depends only on retinal blood flow. On the other hand, if eye movement occurs, the temporal change in output signal intensity is altered not only by the retinal blood flow itself but also by the movement of the measured retinal field, and the TD values on the three lines should show high peaks by the latter at the same time.

**In Vivo Experiment**

Sixty-six Dutch rabbits, each weighing 1.8 to 2.3 kg, were used in the in vivo experiments and were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. General anesthesia was induced by intravenous injection of 30 mg/kg pentobarbital sodium and was carefully maintained by injecting small doses of pentobarbital sodium when needed. After dilating the pupil with one drop of 0.5% tropicamide (Mydrin M; Santen Pharmaceutical, Osaka, Japan), the image speckles from a retinal field free of visible surface vessels and approximately one papillary diameter from the ONH along the medullary rays (Fig. 3) were recorded to determine the NB value. Examination under red-free light enabled identification of vessels on the retinal surface, which were avoided to confine NB measurement almost exclusively to the retinal microvasculature.

**Check of Eye Movement During Measurement**

After mydriasis, TD values obtained from a retinal field were monitored twice in six eyes of six Dutch rabbits. The first measurements were performed when the animal eye was expected to remain unmoved because of deep general anesthesia. The second measurements were performed when the animal eye movement was possible because the effects of pentobarbital were wearing away. Whether the animal eye moved during measurement was carefully observed through a fundus camera.

**Reproducibility of In Vivo Measurements**

After mydriasis, NB was measured twice in the same retinal field with a 5-minute interval between measure-
FIGURE 3. Measurement field of NB in the retina. The image speckles from the region of the medullary area (0.62 X 0.62 mm) free of surface vessels approximately one papillary diameter away from the optic nerve head (•) were recorded to measure the NB in the retina attributable to retinal microcirculation.

ments in 12 eyes of 6 rabbits. After photographing the fundus to document the measurement site, animals were allowed to recover from anesthesia and were returned to the animal house. The NB of presumably the same retinal site was measured after 24 hours with the aid of the photographic record.

The NB in across the whole measurement field of 0.62 X 0.62 mm, corresponding to 100 X 100 pixels in the sensor plane (NB av[10000]), was calculated, and variation between NB in the first measurement (NB av,1) and that measured at 5-minute (NB av,2) or 24-hour intervals (NB av,3) was calculated to yield a coefficient of reproducibility of measurement in living eyes as follows:

$$\frac{|NB_{av,1} - NB_{av,2(3)}|}{\frac{1}{2} (NB_{av,1} + NB_{av,2(3)})}$$  \hspace{1cm} (3)

The coefficient was also calculated for fields located in the middle of the measurement field corresponding to 10 X 10, 20 X 20, 30 X 30, 40 X 40, 50 X 50, 60 X 60, 70 X 70, 80 X 80, and 90 X 90 pixels in the sensor plane, respectively.

Comparison of NB in Obtained From Medullary and Extramedullary Fields

In rabbits, retinal blood vessels are restricted to only a small horizontal band area along the medullary rays,\(^{12}\) and the NB obtained from fields with retinal microvasculature was compared with that from nonvascularized fields in 12 eyes of 6 rabbits. NB av(10000) was measured three times from a retinal field approximately one papillary diameter away from the ONH where no discrete retinal vessels were visible (medullary field) and from a field approximately one papillary diameter below the ONH (extramedullary field), respectively.

Comparison of NB av and Microsphere Results

NB av(10000) obtained from the medullary field was compared with the retinal blood flow rate determined using the colored microsphere technique.\(^{13}\) General anesthesia was induced as described above. Heparin (500 IU/kg) was given intravenously to prevent clotting. The femoral artery was cannulated unilaterally with a polyethylene catheter for measurements of the arterial blood pressure and pulse rate and for blood sampling during and after injection of microspheres. The ipsilateral internal carotid artery was also cannulated, and the catheter was introduced into the left ventricle. After topical instillations of 0.1% diclofenac sodium (Dilocd; Wakamoto Pharmaceutical, Tokyo, Japan) and Mydrin M, two 25-gauge needle infusion sets filled with artificial aqueous humor (Opeguard MA; Senju Pharmaceutical, Osaka, Japan) were inserted through the peripheral limbus of the cornea into the anterior chamber of the eye on the side opposite carotid cannulation. One set was connected to a pressure transducer (DTX; Spectramed, Oxnard, CA) for monitoring the intraocular pressure (IOP), and the other was connected to a reservoir filled with Opeguard MA, by which the IOP was adjusted. Two-step elevation of the IOP (i.e., from 10 to 50 mm Hg, from 10 to 65 mm Hg, from 10 to 80 mm Hg, from 50 to 65 mm Hg, from 50 to 80 mm Hg, or from 65 to 80 mm Hg) was produced manometrically in separate animals. Five minutes after the adjustment of the IOP, NB av(10000) was measured three times at 1-minute intervals. Immediately after the first consecutive determinations of NB av(10000) at the first IOP level, 0.15 to 0.25 ml of a suspension of nonlabeled, colored (red) microspheres (15 ± 0.3 μm in diameter, 10⁷ spheres per milliliter; E-Z TRAC, Los Angeles, CA) was injected into the left ventricle. A reference blood sample was obtained from the cannulated femoral artery starting at the time of the injection and then continuously until 60 seconds after the injection. The IOP was then elevated to the second level, and the three consecutive NB av(10000) measurements and microsphere injection were carried out in the same manner as above, except that blue microspheres were used. Arterial P0₂, PCO₂, and pH were determined on injection of microspheres at each IOP level using the pH-Blood Gas Analyzer, Model 170 (Corning Glass, Corning, NY), and body temperature was monitored with a rectal thermometer. The rabbit was then killed by intravenous overdose of pentobarbital sodium, and the eye was enucleated and fixed in a 4% formaldehyde solution for 1 hour. The fixed eyeball was dissected, and the retina was excised, blotted, weighed, and dissolved with a 5-M NaOH solution. The dissolved tissues were passed through filter paper (2 μm
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in pore size; RAWG-02500; Millipore, Bedford, MA) and mounted on a glass slide. The number of microspheres in samples on the slide was counted under a light microscope (Nikon, Tokyo, Japan). The spheres at different IOP levels were distinguished by color.\textsuperscript{13} The blood sample was also dissolved with NaOH solution, and the number of spheres was counted.

Effect of Ocular Perfusion Pressure on \( \text{NB}_{av} \)

The effect of changes in ocular perfusion pressure (OPP) on \( \text{NB}_{av}(10000) \) was studied. After induction of general anesthesia, the femoral artery was cannulated for measurements of arterial blood pressure and pulse rate and for blood sampling, and the body temperature was monitored as above. The anterior chamber was cannulated as above, and the IOP was stepwise increased from 10 to 30, 50, 70, and 80 mm Hg. Five minutes after the adjustment of the IOP, \( \text{NB}_{av}(10000) \) was measured three times at 1-minute intervals at each IOP level.

The OPP was calculated according to the formula:

\[
\text{OPP} = \text{FABP}_m - \text{IOP} \quad (4)
\]

where \( \text{FABP}_m \) is the mean femoral arterial blood pressure, which was calculated by:

\[
\text{FABP}_m = \frac{1}{3} (\text{FABP}_d + \text{FABP}_s - \text{FABP}_d) \quad (5)
\]

where \( \text{FABP}_d \) and \( \text{FABP}_s \) are diastolic and systolic femoral arterial blood pressures, respectively. Arterial \( \text{PO}_2 \), \( \text{PCO}_2 \), and pH were determined when NB was measured at each IOP level.

RESULTS

In Vitro Experiment

Figure 4 shows the relationship between the speed of rotation of the ground glass and \( \text{NB}_{av}(10000) \). \( \text{NB}_{av}(10000) \) showed a significant linear correlation with the speed of rotation in the range 5 to 120 mm/second \((r = 0.99, P = 0.00) \).

Check of Eye Movement During Measurement

Figure 5 shows a representative time course of TD values when there was no eye movement during measurement and when an eye showed a small twitch. When there was no eye movement during measurement, the TD values on three pixel lines always showed little change (Fig. 5a). Conversely, when there was a twitch eye movement, the TD values on the three lines always showed a hump at the same time (Fig. 5b). The TD values ranged from 17 to 21 when there was no eye movement and from 39 to 46 when there was a twitch eye movement during measurement.

Reproducibility of In Vivo Measurements

The coefficients of reproducibility of the \( \text{NB}_{av}(10000) \) measurements at the 5-minute and 24-hour intervals were 8.2% ± 1.8%, and 13.0% ± 2.0%, respectively (mean ± SEM, \( n = 12 \)). No significant difference was observed among \( \text{NB}_{av,1} \), \( \text{NB}_{av,2} \), and \( \text{NB}_{av,3} \).

The coefficients of reproducibility of the \( \text{NB}_{av} \) measurements at the 5-minute and 24-hour intervals

\[
\text{TD} \text{ values on three horizontal pixel lines. (a) case without eye movement, (b) case with eye movement. Time-course of TD value on 25th horizontal line (---), 50th line (---), and 75th line (-----) are shown, respectively. For further explanation of TD, see text. This figure was reproduced from a photograph of display in picture tube.}
\]
were correlated with the field of measurement, that is, the number of pixels in the sensor plane (Fig. 6). The coefficient was little affected by the number of pixels employed in measurement when the array was larger than 70 × 70 pixels, which corresponds to a field of approximately 0.42 × 0.42 mm in the rabbit fundus. It still averaged 25% or less when the array was 20 × 20 pixels or larger.

Comparison of NBav Obtained From Medullary and Extramedullary Fields

NBav(10000) obtained from the medullary field was 14.1 ± 0.8 in an arbitrary unit (n = 12). In the extramedullary field, output signal intensity was close to or below the minimum level that could be detected with an image sensor under the same conditions. The speckle pattern from the extramedullary field could be recorded by increasing the laser intensity, which showed almost no blurring.

Comparison of NBav and Microsphere Results

Although the cornea became cloudy at IOP levels of 65 and 80 mm Hg, the speckle pattern from the fundus could still be recorded by increasing the laser intensity. Only rabbits that showed no significant change in the systemic parameters checked during the experiment were accepted. The systemic parameters obtained in these rabbits are summarized in Table 1. All the parameters monitored were within the normal range of healthy rabbits.14,15 The comparison between the average of three consecutive NBav(10000) values, obtained from the retinal field free of visible surface vessels, and the retinal blood flow rate, determined using the microsphere technique in the same eye at the same IOP level, is summarized in Figure 7. There was a significant correlation between the two methods, with a correlation coefficient of 0.59 (P < 0.001, n = 40).

Effect of Ocular Perfusion Pressure on NBav

Only rabbits that showed no significant change in the systemic parameters checked during the experiment were accepted, and all the parameters monitored were within the normal range of healthy rabbits.14,15 The systemic parameters obtained in these rabbits are summarized in Table 2.

A representative color map of the retina at IOP levels of 10, 50, 70, and 80 mm Hg is shown in Figure 8. The color changed from red to blue as the IOP increased to 70 or 80 mm Hg, indicating a pressure-dependent decrease in blood velocity in the field measured. The relationship between the OPP and the average of three NBav values in the retinal field free of visible surface vessels is shown in Figure 9. The average of three NBav(10000) values measured at IOP levels of 30, 50, 70, and 80 mm Hg was expressed as the percentage for that at the IOP level of 10 mm Hg. NBav(10000) showed no significant change either when the IOP was less than 50 mm Hg or when the average OPP was greater than 50 mm Hg. At OPP levels less than 50 mm Hg, however, NBav(10000) decreased with reductions in the OPP. At the average OPP of 27.1 mm Hg and 18.7 mm Hg (IOP of 70 mm Hg and 80 mm Hg), a significant decrease (38.9% and 55.8% on average, respectively) was found (P < 0.01, n = 8, Dunnet’s multiple comparison test).

| TABLE 1. Values of Systemic Condition Parameters Obtained at the Time of Microsphere Injection at Each IOP Level |
|------------------------|--------|--------|--------|--------|
| Systemic Condition Parameter | IOP (mm Hg) |
|                        | 10 (n = 12) | 50 (n = 10) | 65 (n = 8) | 80 (n = 10) |
| FABPm (mm Hg)         | 97.4 ± 3.2 | 98.2 ± 4.8 | 97.1 ± 6.2 | 96.3 ± 7.1 |
| Pulse rate (per minute)| 296 ± 9   | 297 ± 11  | 298 ± 10  | 297 ± 11  |
| Body temperature (°C) | 37.8 ± 0.3| 37.6 ± 0.3| 37.4 ± 0.7| 37.1 ± 0.8|
| pH                    | 7.39 ± 0.02| 7.40 ± 0.02| 7.40 ± 0.03| 7.39 ± 0.03|
| Pco₂ (mm Hg)          | 36.7 ± 1.2| 36.4 ± 5.8| 34.5 ± 2.5| 33.4 ± 1.1|
| Po₂ (mm Hg)           | 82.2 ± 3.9| 86.5 ± 7.4| 84.6 ± 5.2| 85.1 ± 4.4|

IOP = Intraocular pressure; FABPm = mean femoral arterial blood pressure. Figures are mean ± SEM.
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FIGURE 7. Comparison of results obtained from the microsphere technique and NB measurements in the retina. Each plot represents the mean value of the retinal blood flow rate determined by the microsphere technique or the NBw obtained from the medullary area at each intraocular pressure (IOP) level. Error bars represents SEM (n = 12, 10, 8, and 10 for 10-, 50-, 65-, and 80-mm Hg experiment, respectively).

DISCUSSION

Although application of the laser Doppler technique enabled noninvasive measurements of in vivo microcirculation in the ONH16"22 or of the centerline velocity of red blood cells in individual retinal vessels,3'4 in vivo quantitative and consecutive measurements of the retinal microcirculation have not been attempted. This study demonstrated that the in vivo retinal microcirculation could be measured consecutively and quantitatively using the laser speckle phenomenon. The in vitro experiment using our apparatus equipped with an argon laser (blue) revealed a linear correlation between the speed of rotation and the NBav(10000) between 5 and 120 mm/second, indicating that the NB presently adopted as an index of blurring of a speckle pattern parallels the speed of diffusing substance. However, it must be noted that the power spectral distributions of its speckle intensity fluctuations are not exactly the same as those of the rabbit fundus, and, therefore, the values of NB thus obtained should not be directly compared to those obtained from the rabbit fundus. NBw(10000) obtained from the retinal field containing retinal microvasculature averaged 14.1 units. In the field where no retinal microvasculature exists over the choroidal vessels, output signal intensity was close to or below the minimum level that can be detected with an image sensor under the same conditions. Although the speckle pattern that could be recorded by increasing the laser intensity, it showed almost no blurring, that is, NB could not be calculated, because the numerator and denominator of Equation (1) both became close to zero.

This finding indicates either that little of the choroidal scattered light reached the detector, because argon light was strongly scattered in the superficial retina or strongly absorbed in the retinal pigment epithelium and choroid,23 or that the choroidal blood flow rate was too high to be detected with the NB presently adopted. The latter possibility seems unlikely, because incorporation of diode laser (wavelength 808 nm) instead of argon laser (wavelength 488 nm) allowed us to measure the choroidal blood velocity using NB (unpublished data, 1994). Therefore, choroidal blood flow is thought to have little effect on the NB presently obtained.

The colored microsphere method has recently been employed as a substitute for the radioactive microsphere method, and the results of both methods reportedly show a linear correlation in myocardium.13 The retinal blood flow rate presently determined using the colored microsphere method at an IOP of 10 mm Hg, 13.6 mg/minute, agreed with that determined with the radioactive microsphere method in the normal rabbit eye, 9 to 15 mg/minute.24,25 There existed a large variation in the retinal blood flow rate

TABLE 2. Values of Systemic Condition Parameters Obtained When the NB was Measured at Each IOP Level

<table>
<thead>
<tr>
<th>Systemic Condition Parameter</th>
<th>10</th>
<th>30</th>
<th>50</th>
<th>70</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>FABPm (mm Hg)</td>
<td>98.1 ± 5.2</td>
<td>97.2 ± 4.5</td>
<td>98.2 ± 4.1</td>
<td>97.1 ± 5.8</td>
<td>98.7 ± 5.3</td>
</tr>
<tr>
<td>Pulse rate (per minute)</td>
<td>297 ± 12</td>
<td>295 ± 11</td>
<td>298 ± 12</td>
<td>293 ± 11</td>
<td>290 ± 10</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>37.4 ± 0.2</td>
<td>37.7 ± 0.3</td>
<td>37.5 ± 0.2</td>
<td>37.9 ± 0.3</td>
<td>37.8 ± 0.3</td>
</tr>
<tr>
<td>pH</td>
<td>7.39 ± 0.02</td>
<td>7.40 ± 0.01</td>
<td>7.41 ± 0.01</td>
<td>7.39 ± 0.01</td>
<td>7.39 ± 0.01</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
<td>36.2 ± 2.9</td>
<td>37.7 ± 2.1</td>
<td>34.6 ± 3.7</td>
<td>36.5 ± 2.8</td>
<td>35.7 ± 3.0</td>
</tr>
<tr>
<td>PaCO2 (mm Hg)</td>
<td>87.5 ± 5.7</td>
<td>89.7 ± 1.8</td>
<td>89.8 ± 7.8</td>
<td>89.0 ± 4.5</td>
<td>93.4 ± 6.8</td>
</tr>
</tbody>
</table>

IOP = Intraocular pressure; FABPm = mean femoral arterial blood pressure. Figures are mean ± SEM. n = 8.
FIGURE 8. Effect of intraocular pressure (IOP) on NB in the retina. Representative color map of NB in the retina at various IOP levels. Data numbered 1 to 4 were recorded at IOP levels of 10, 50, 70, and 80 mm Hg, respectively.

determined with the microsphere technique for individual eyes, probably due to a small number of microspheres trapped in the retinal tissue. However, the blood flow rate determined with the colored microsphere technique and the average NB across the measurement field, NB_{av}(10000), demonstrated significant correlation with each other, with a correlation coefficient of 0.59 in the IOP range of 16.3 and 87.4 mm Hg. When the average of the blood flow rates determined with the microsphere technique at each IOP level was calculated and correlated with the NB_{av}(10000) in individual eyes, correlation was better, with a correlation coefficient of 0.84. These results suggest that the NB presently obtained in the retina as a quantitative index of tissue blood flow velocity may also quantitatively reflect the blood flow rate through the retinal tissue, if it is averaged across the measurement field.

The NB_{av}(10000) obtained from the retinal field free of surface vessels was little affected by the IOP change when the IOP was above 50 mm Hg, but the NB_{av}(10000) decreased parallel to IOP decreases at levels below 50 mm Hg. This result indicates that the retinal microcirculation in rabbit is autoregulated in the range of IOP above 50 mm Hg (Fig. 9). The results of our study are in agreement with those of previous studies carried out in cats, monkeys, and humans using various methods, which showed that retinal blood flow rate is autoregulated against IOP change when IOP is greater than 50 to 75 mm Hg.

The coefficient of reproducibility of measurements in living eyes should reflect not only instrumental error sources but also physiological fluctuation in the blood flow in the living body. The coefficient of NB_{av} measurements at 5-minute or 24-hour intervals was large when the field measured was smaller than 0.13 X 0.13 mm, or when the number of corresponding pixels of the image sensor was less than 20 X 20. It is possible that the variation in NB_{av} obtained from a smaller field reflects not only instrumental error sources but also spatial fluctuation in the blood flow. Our result suggests that this variation can be considerably reduced by enlarging the measurement field (Fig. 6). When the effects of various treatments are under question, a larger field of measurement is advantageous, because it reduces variation in the result and thus enhances sensitivity of detection. If further advance in technology allows us to use an image sensor with more densely arrayed pixels, it may be possible to obtain a better reproducibility also in the measurement from a smaller field. With laser Doppler flowmetry, temporal fluctuation in the blood flow can be detected by exploiting its high temporal resolution. However, 15 seconds are necessary to display the result of one measurement with the present apparatus, making it difficult to detect rhythmic changes in the blood flow. In the present apparatus, which needs 0.18 seconds for NB measurement, the pulse rate of rabbits (approximately 300 per minute) may be too great for the pulsatile component in the blood flow to affect the results. If animals with lower pulse rates are to be used in future studies, measurements should be synchronized with an electrocardiogram, which can be easily performed.

The main factors that could influence the magni-
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...with those obtained from the site free of these vessels, future use of this technique in humans, we estimated NBav at the IOP level of 10 mm Hg.

The NBav was expressed as the percentage for that measured laser light-scattering properties of the tissue. The for-field of measurement (i.e., eye movement) and spatial variation of NB values may be interpreted to occur during measurement. The latter factor directly affects the laser speckle phenomenon and consequently NB values. Therefore, it is difficult to compare reliably NB values obtained from large surface vessels with those obtained from the site free of these vessels, because a large variation in the light-scattering properties is expected between the two. However, large site-to-site variation of light scattering seems unlikely in the retina free of surface vessels, where the measurement field was placed in the present study. Therefore, spatial variation of NB values may be interpreted to reflect site-to-site variation in the blood velocity. For future use of this technique in humans, we estimated light level safety using the ANSI 1993 standard. In rabbits, retinal exposure is 0.018 W/cm² when the corneal power used is 0.2 mW. This irradiance was sufficient to obtain a usable signal. To obtain a similar signal in humans, one would need a similar retinal irradiance, assuming equal backscattering in humans and pigmented rabbits. Thus, a corneal power of 0.2 mW × (2.1/1.2)² = 0.6 mW may be needed. For this kind of application, it is useful to calculate the maximum permissible exposure time for a given wavelength, area, and corneal power. The maximum permissible exposure time for a 0.6-mW laser beam (wavelength 488 nm) exposure in a 2.1-mm diameter area is 15 seconds. Because the experimental exposure time, including the time for alignment, is typically 5 seconds, it can be concluded that this technique falls within the ANSI safety limits for application to human diagnosis. Further, little functional damage to the retina or choroid should occur during NB measurement in living eyes. In conclusion, the present instrument is promising for studying the physiology and pathology of retinal microcirculation in experimental animals, enabling noncontact, two-dimensional observation of the in vivo blood velocity in the peripheral retina.

**Key Words**
laser speckle phenomenon, retinal microcirculation, two-dimensional color mapping, normalized blur (NB), microsphere technique

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