

## TECHNICAL REPORTS

### Polarized-Light Intravital Microscopy for Study of Cochlear Microcirculation

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#### INTRODUCTION

For many tissues in man and in experimental animals, intravital microscopy is feasible only when incident illumination is used. A simple system uses oblique illumination by bringing the light to the specimen from the side with a light guide. This technique is often applied in microscopy of human nailfold capillaries (Fagrell *et al.*, 1977, Jacobs *et al.*, 1987) and was used in early studies of cochlear microcirculation (Perlman and Kimura, 1955; Seymour, 1954; Weille *et al.*, 1954). This form of illumination, however, has certain shortcomings, the most important of which is the loss of image contrast and resolution caused by reflections and uneven illumination of the specimen. One solution employs the objective lens itself as an incident-light condenser. Such an incident-light microscope (Slaaf *et al.*, 1986) may use a reflector with 50% reflectance and 50% transmittance placed at 45° to the optical axis. However in the cochlea, as in other nonstandard biological preparations, this system is not suitable because of excessive reflection at the air/fluid interface. Fluorescence intravital microscopy (FM) provides an alternative approach, permitting visualization of cochlear microvessels *in vivo* without too much reflection (Nuttall, 1987a,b; Prazma *et al.*, 1989). Combined with telescopic microscopy principles (Slaaf *et al.*, 1982; Wayland *et al.*, 1977) and "dual slit" cross correlation of video signals (Intaglietta *et al.*, 1970; Intaglietta and Tompkins, 1972; Slaaf *et al.*, 1981; Tompkins *et al.*, 1974; Wayland and Johnson, 1967), FM has been used to measure red blood cell (RBC) velocity continuously and to estimate the vessel diameter in the cochlea (Nuttall, 1987a,b). The advantages of FM include (1) enhancement of image contrast for continuous measurement of the flow velocity in the cochlea, and (2) visualization of microvessels, permitting estimation of vessel diameters. The principal disadvantages include possible local phototoxicity and potential disturbances in the cardiovascular system caused by fluorescent dye injection. The intensity of fluorescent signals are also quite low, requiring the use of an image-enhancement technique or other sensitive photo-

detectors. We have explored polarized-light intravital microscopy (PM) in the study of microcirculation of mammalian cochlea as a solution to these disadvantages, combining the principles of the polarizing microscope and spectrophotometry with intravital microscopy (Nuttall, 1987a,b). This report shows that PM significantly improves the ability to measure cochlear blood flow velocity and enhances the visibility of endothelial cells, leukocytes, and red blood cells, allowing rheological studies and improved vessel diameter measurements in the cochlea.

## MATERIALS AND METHODS

### *Surgical Preparation*

Guinea pigs weighing 250–400 g were used in this study. After diazepam (5 mg/kg, ip) tranquilization, analgesia was induced by fentanyl (0.32 mg/kg, im). A surgical level of anesthesia was maintained by a supplementary half-dose of fentanyl every 30 min and half-dose of diazepam every 2 hr. The head of the animal was placed in a heated headholder and a tracheotomy performed to insure free breathing. The contralateral carotid artery and external jugular vein were cannulated in a retrograde fashion for continuous blood pressure measurement and intravenous injections. Rectal temperature of the animal was maintained at  $38 \pm 1^\circ$  with a servoregulated heating blanket.

The auditory bulla was opened by a ventrolateral approach, leaving the tympanic membrane and ossicles intact. In order to observe the blood circulation in vessels of the spiral ligament and stria vascularis, a rectangular fenestra ( $0.2 \times 0.3$  mm) over the third turn at the cochlea was made by gently scoring and elevating the cochlear bony wall with a small knife blade. Usually, this technique resulted in the removal of an intact piece of bone. The approximate size and location of a window opening over the third turn is shown in Fig. 1 (Nuttall, 1987a).

### *Intravital Microscopy*

The microscope used in the current study was developed by Nuttall (1987a,b), and was assembled from components produced by several manufacturers. The main components are a Leitz vertical illuminator unit and a Leitz trinocular head. The microscope components are carried on a sturdy framework, permitting convenient placement of the animal preparation, manipulation of the microscope position in the  $x$ - $y$  plane, and easy interchange of the compound microscope for a dissection microscope in order to view and attend to the specimen preparation at low magnification. A Mitutoyo  $20\times$  M Plan Apo objective having a working distance of 20 mm and numerical aperture (NA) of 0.42 and Olympus ULWDMSPan 50 with 0.56 NA and 9 mm working distance were used in the current experiment. Parallel light rays from the back aperture of the lens are intercepted by a 63-mm diameter (focal length 400 mm) achromat doublet (Melles Griot LAO 333), which acts as a transfer lens and focuses the image on the sensor of a silicon intensified target video camera (Dage MTI model 66). Images were recorded on videotape (Sony Model 5200, 3/4 inch format) for off-line processing. Light is supplied to the vertical illuminator from a 75-W xenon source through a 1.5-m-long, 3-mm-diameter, liquid light guide.

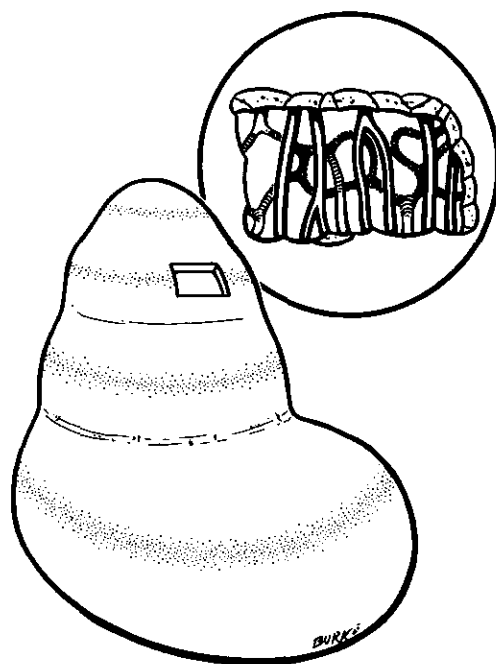


FIG. 1. An illustration of the guinea pig cochlea showing the approximate size and location of a window opening over the membranous labyrinth of the third turn. The inset schematically shows the patterns of stria capillaries (Nuttall, 1987a, reprinted with permission of Elsevier Science Publishers).

### *Configuration for Fluorescence Microscopy*

For fluorescence, the Leitz filter set consisted of an excitation filter (BP 450–490 band pass filter), a dichroic mirror (RKP 510 reflection short pass filter), and the suppression filter set (LP 515 long pass filter) of a typical fluorescence microscope configuration for fluorescein isothiocyanate (FITC). For blood flow velocity determination with FM, FITC conjugated to dextran acts as a contrast medium in the blood (Nuttall, 1987a,b; Olsson *et al.*, 1975) and was administered intravenously to the guinea pig at a dose of 15 mg/100 g (50 mg/ml in physiological saline) infused slowly over a 15-min period.

### *Configuration for Polarizing Microscopy*

For this configuration, a Tu 55.54 filter block in a Leitz Ploemopak illuminator was modified. Exciting filters were replaced with a green band pass filter (wavelength 545 nm, bandwidth 60 nm), and the suppression filter was removed. A plane glass reflector with 50% reflectance and 50% transmittance was placed at 45° to the optical axis. A simple Polaroid filter was mounted in front of the green filter on the illumination side. The direction of polarization was fully crossed with a second Polaroid filter (the analyzer) on top of the cube in the imaging pathway. As a practical matter, the extinction factor (EF) should be as large as possible ( $EF = I_p/I_s$ ; where  $I_p$  is the intensity of light that comes through a polarizing device when the polarizer and analyzer transmission directions are parallel, and  $I_s$  the minimum intensity that can be obtained when the polarizer and analyzer

are crossed). The EF in the current experiment was around  $10^3$ . A diagram of the polarizing intravital microscope is presented in Fig. 2.

For animal experiments, with the lateral wall cochlear vessels in focus, the position for optimal contrast was obtained by simultaneous rotation of polarizer and analyzer. These are the NS and EW directions (Fig. 2). The polarizer was positioned in the illuminating light path, where the light is not focused but spread over as large a surface area as possible to avoid burning the Polaroid filter.

### *Experimental Protocol*

Eight animals were used in the current study. After injection of fluorescent dye, FM and PM with the cross-correlation analyzer were used to determine the blood flow velocity in the same vessel segments. The values of RBC velocity obtained with PM were compared with the values of plasma gap velocity obtained with FM. Off-line analysis was carried out to compare the photometric signals through two signal windows from FM images with those from PM images, based on the same vessel segments. To evaluate the results of cross-correlation analysis for different photometric signals from FM images and PM images, the  $x$  axis signal from the oscilloscope in the RBC velocity-tracking correlator (Instrumentation for Physiology and Medicine, Inc., IPM, Model 102) was used to trigger a PC computer system to average the  $y$  axis signal  $256 \times$ .

## RESULTS

When the cochlear lateral wall soft tissue is exposed, vessels cannot be observed with bright field microscopy because of excessive reflected light. However, after the polarizer and analyzer were in place, images of individual capillaries, arterioles, and even the RBCs can be visualized. Image contrast is further improved when a green filter is used, since RBCs present as dark spots because of the higher absorption of hemoglobin in the green light range. PM and FM images of cochlear microvessels on the lateral wall are shown in Fig. 3. Individual RBCs, white blood cells, and endothelial cells can be seen in the PM images, and even their orientation and shape can be identified. A series of moving RBCs or RBC columns can be seen clearly in the radiating arterioles, branches of the spiral modiolar artery supplying the stria capillaries, or capillaries but their diameters appear much smaller than the vessel diameter measured with the fluorescence technique, due to the existence of the plasma layer between the blood cell column and the vessel wall. With a  $50 \times$  objective, the endothelial cells can be visualized and the vessel diameter can be determined more accurately. Moving leukocytes, RBCs, and their interaction with endothelial cells and vascular geometric variances can be continuously monitored in capillaries. Even relative hematocrit across individual vessels can be estimated based on blood cell density.

The relationship between RBC velocity and plasma gap velocity is shown in Fig. 4. No statistical difference was found between RBC velocity and plasma gap velocity in paired two-tailed  $t$  test ( $t = 3.1021$ ),  $P > 0.05$ ,  $n = 45$ ). Regression analysis indicates a very close positive linear relationship between the two groups ( $y = 3.493 \times 10^{-3} + 0.941x$ ,  $r = 0.9938$ ). Results obtained in the current experiment suggest that velocity measured with PM images is consistent with that

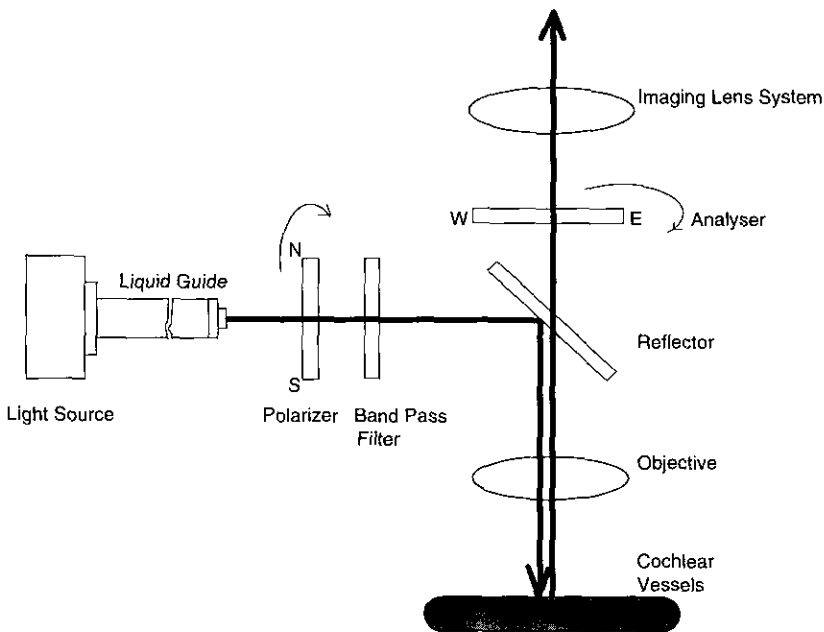


FIG. 2. Diagrammatic representation of polarized-light microscope. The wide-band light is supplied by 75-W high-pressure xenon lamps. A bandpass filter transmits green light from the polarizing filter. The plane glass reflector reflects 50% of the polarized light into the optical pathway of the microscope. The analyzer is crossed with respect to the polarizer, extinguishing directly reflected light.

obtained with FM images and that RBC velocity is essentially equal to plasma gap velocity in the cochlear stria capillaries and radiating arterioles.

Dual-window photometric signals of the same vessel segment obtained from a video photometric analyzer (Model 202A, Instrumentation for Physiology and Medicine, Inc. San Diego, CA) are presented in Fig. 5. Compared with data from FM images, photometric signals of PM images show more and larger oscillations of each signal window light intensity, indicating the higher contrast imaging of individual moving blood cells.

To assess the consequence of these two techniques on cross-correlation analysis, the normalized correlograms from FM photometric signals and PM photometric signals are plotted in Fig. 6. Photometric signals were collected from a stria capillary under the same conditions of window size, time constant, gain, etc. The correlogram from PM images shows higher amplitude and a more symmetrical form than that from FM images, indicating that photometric signals from PM images have a higher signal/noise ratio than those from FM images and that PM is better for cochlear blood flow velocity measurement, using cross-correlation analysis.

## DISCUSSION

### *Principle of Polarization Microscopy*

A light source generates natural, i.e., unpolarized, wide-band light. When passing through the polarizer mounted in front of the mirror, the light is polarized.

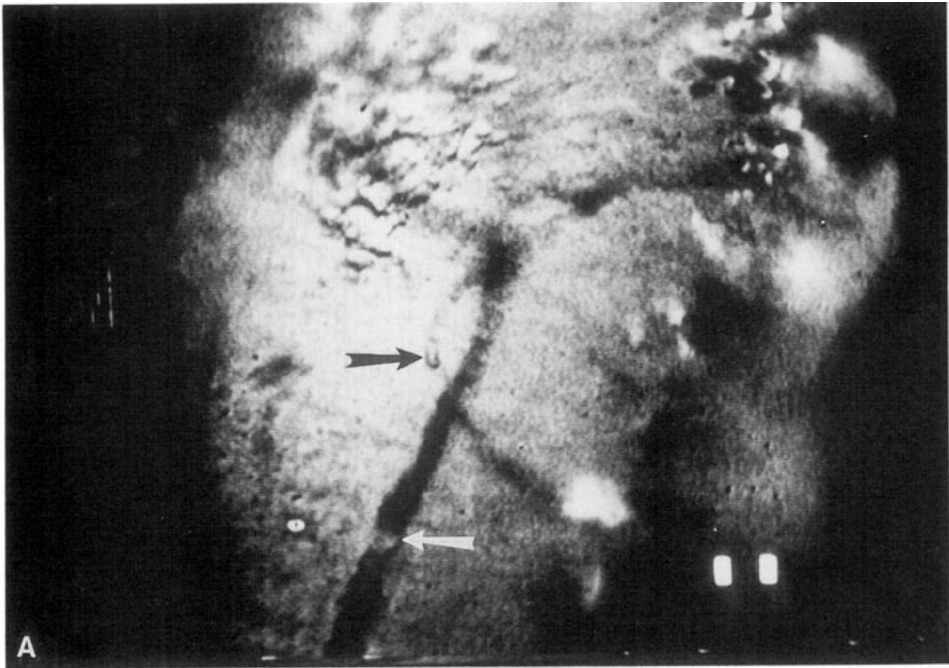


Fig. 3. Microphotographs of the cochlear microvasculature taken from the monitor. (A) A single RBC (black arrow) and white blood cell (white arrow) are indicated in a PM image. (B) FM image has no information on the blood cells. (C) Endothelial cells (arrow) can be identified in PM image of a capillary. The distance between the two white markers in each micrograph is 10  $\mu\text{m}$ .

The oscillating linearly polarized waves emerging from the polarizer are directed to the specimen surface by the reflector. An isotropic phase will reflect the wave front without affecting polarization. These wave fronts will not pass the second polarizing filter, the analyzer, if its transmission direction is perpendicular to the polarizer. Isotropic specimens remain dark between two crossed polarizing filters, while anisotropic objects will be bright and dark alternately as the direction of polarizing filters is rotated through  $360^\circ$ . Those parts of the illuminating light that are reflected by the lens surface or by the fluid covering the tissue are extinguished at the crossed-analyzer. The light which is reflected from structures within the tissue is depolarized as a result of multiple scattering. As a consequence, part of this light will pass the crossed analyzer and form an image (Slaaf *et al.*, 1987).

The concentration of a substance is often determined by spectrophotometry from the transmittance through a sample of known path length. The ability of a substance to absorb light is directly related to its extinction coefficient. To achieve the minimum photometric error or maximum signal, it is clear that a wavelength on the extinction coefficient peak must be chosen based on the absorption spectra of the material to be studied. It has been shown that there are two extinction coefficient peaks in absorption spectra for oxy- and deoxyhemoglobin in the visible wavelength range, 410–440 nm peak and 540–570 nm peak (Pittman, 1986). We proposed that the image contrast of RBCs in the cochlea is determined by the extinction coefficient difference between hemoglobin in RBCs and substances in

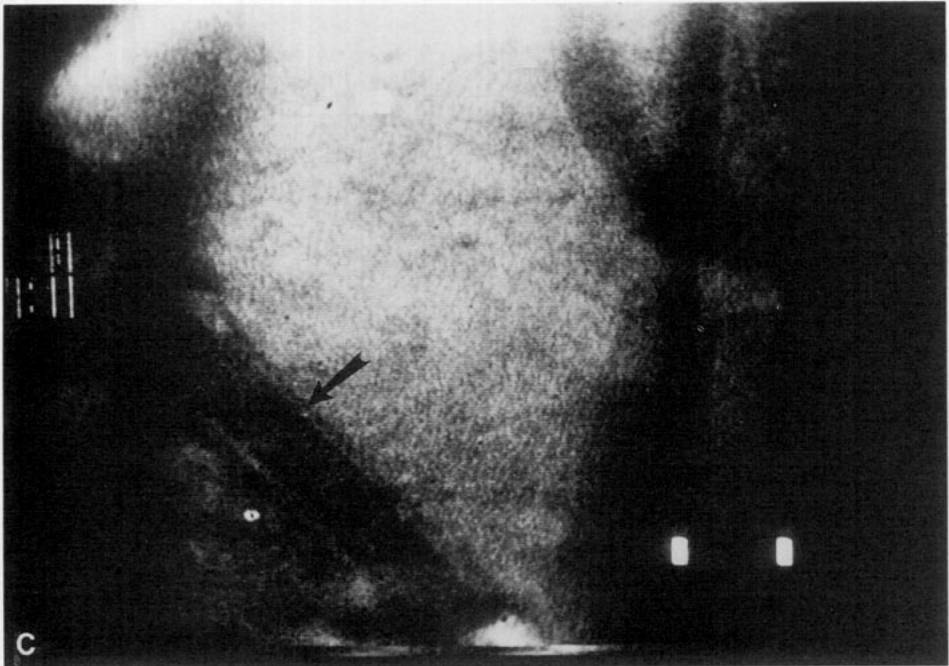
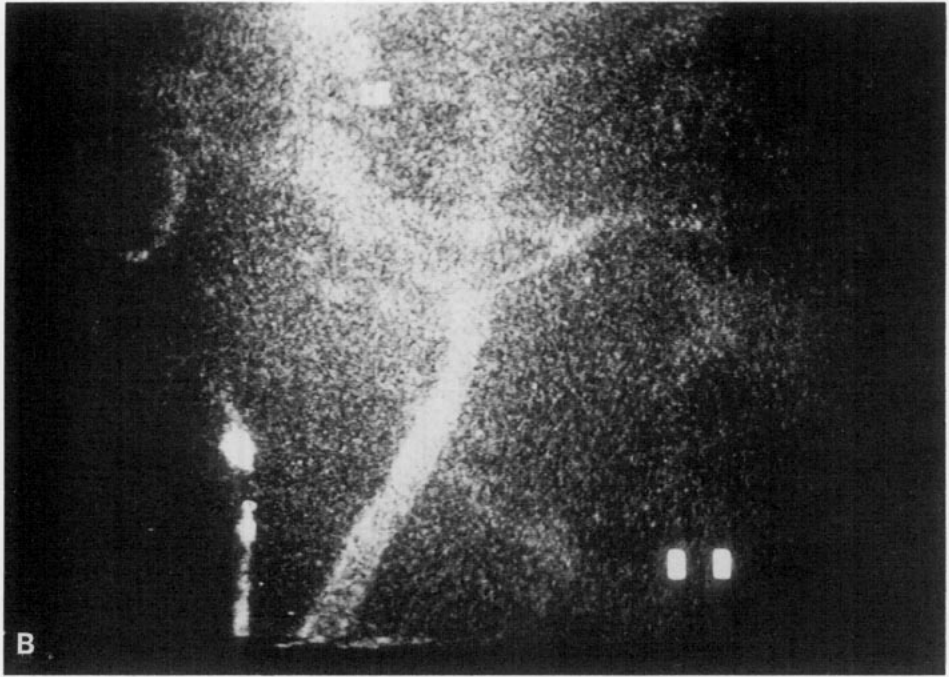


FIG. 3—Continued

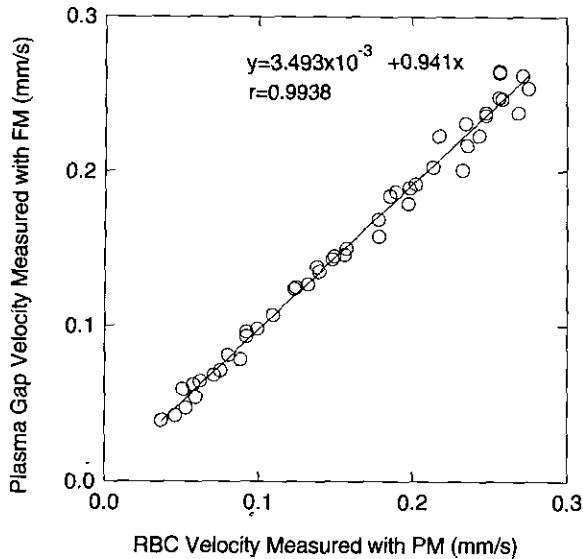


Fig. 4. Correlation between two methods for the blood flow velocity measurement in guinea pig cochlea. Results indicate no significant difference in the values obtained with FM and PM, and RBC velocity is almost equal to plasma gap velocity in cochlear capillaries and arterioles.

surrounding tissues. Thus, the green band pass filter (wavelength 545 nm, bandwidth 60 nm) was used to improve the image contrast in the current study.

As for other parenchymal tissues, such as brain and kidney, the cochlea is studied using epi-illumination (Costa and Branemark, 1970; Lawrence, 1970; Perlman and Kimura, 1955; Seymour, 1954; Weille *et al.*, 1954). To improve image quality, fluorescent labeling of plasma and RBCs has been used to enhance the optical signal for cross-correlation analysis (Nuttall, 1987a,b). The principles of polarization microscopy and spectrophotometry were incorporated into the intravital microscope developed by Nuttall (1987a) to develop polarized-light intravital microscopy. To the best of our knowledge, this configuration has not been used with cross-correlation analysis to study cochlear microcirculation. The purpose of the present study was to evaluate the usefulness of PM to measure erythrocyte velocity by analog cross-correlation analysis. We found that the method could be used successfully to measure RBC velocity in guinea pig cochlea.

PM was found to be convenient with the use of an incident illuminator (Ploemopak), equipped with a cube, consisting of a reflector with 50% reflectance and 50% transmittance placed at 45° to the optical axis of the microscope, in combination with a polarizer and analyzer. No disturbing reflections were present in the images obtained, and the image contrast of RBC and surrounding tissue was improved. Individual erythrocytes, leukocytes, and endothelial cells as well as the RBC column can be studied in the guinea pig cochlea using this microscopy. For RBC velocity measurement, PM can produce a better signal/noise ratio of photometric signals. The correlogram of PM images has a bigger amplitude and more symmetrical shape than that for FM images, indicating credible velocity measurement.

The main advantages of PM include: (1) higher image contrast and higher



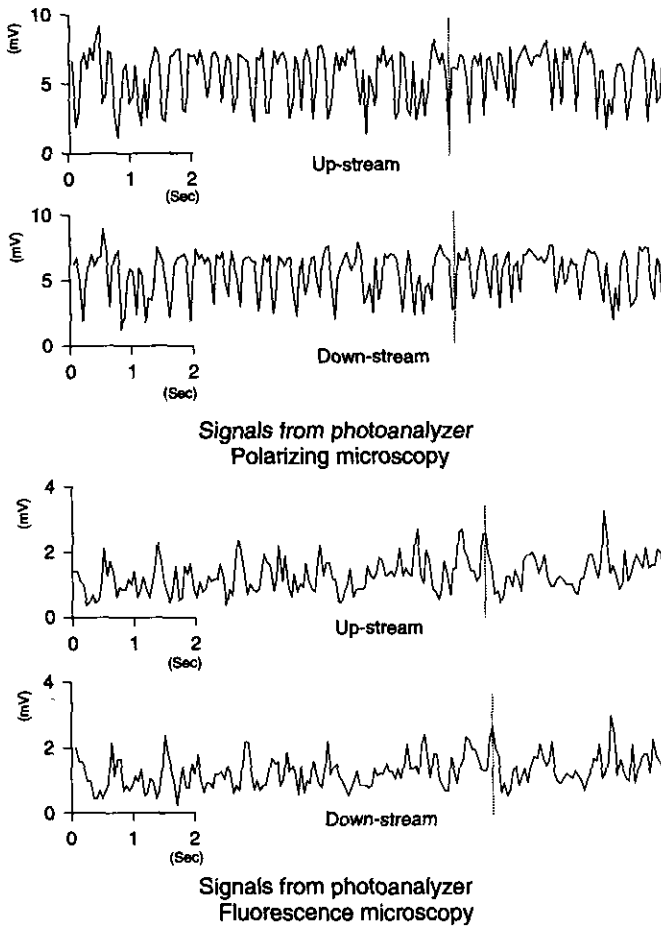


FIG. 5. Comparison of dual-window photometric signals obtained using FM and PM at the same location on a strial capillary. PM photometric signals show more oscillation and negative peaks, reflecting each moving RBC. The peaks of photometric curves are sharper and the turns of curves are swifter, suggesting better image contrast.

resolution which probably improve RBC velocity measurement in the cochlea; (2) the capacity to distinguish individual RBCs, leukocytes, endothelial cells, and the RBC columns, which can provide information about the blood flow pattern and the interaction of blood cells and microvessels; (3) higher spatial resolution, permitting more accurate measurement of the vessel diameter; and (4) the ability to avoid probable hemodilution and phototoxicity caused by fluorescence dye injection.

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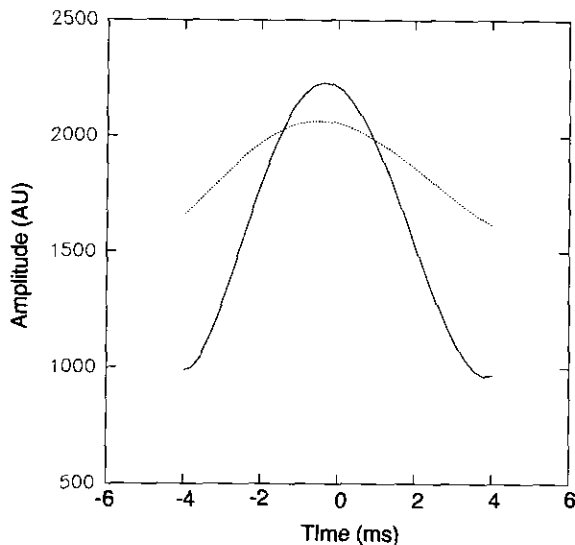


FIG. 6. Amplitude of correlogram is indicated with arbitrary units (AU) and the width with milliseconds (ms). PM correlogram has a much sharper shape and greater amplitude, indicating better crosscorrelation than that for FM (broken line) photometric signals.

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