STEPHEN D. WAJER, D. SCOTT McLEOD, and GERARD A. LUTTY

CONFOCAL MICROSCOPIC IMAGING OF FLUORESCENTLY LABELED SICKLE ERYTHROCYTES IN THE RETINAL VASCULATURE

Erythrocytes, or red blood cells (RBCs), from individuals with sickle cell disease are less pliable and deformable than normal RBCs and, therefore, can be trapped within the lumen of blood vessels supplying various organs. Trapping or adhesion of sickle RBCs (SS-RBCs) has been suggested as the initiating event in the characteristic vaso-occlusions that occur in sickle cell retinopathy, but this hypothesis has never been directly demonstrated. In this study, fluorescent dye-labeled SS-RBCs were injected intravascularly into rats, and the retinas were analyzed by both traditional light microscopy and laser scanning confocal microscopy (LSCM) to determine vascular sites of adhesion and obstruction. The LSCM system optically sectioned the specimen through vertical focal planes, and the sections were overlaid to produce three-dimensional views not directly observable with a conventional microscope. Preliminary data suggest that the initial site for SS-RBC retention is within the precapillary arterioles of the peripheral retina.

INTRODUCTION

Laser scanning confocal microscopy (LSCM) is a relatively new technique that offers optical sectioning of transparent or semitransparent tissue specimens, improved resolution, reduced scattered light, digital imaging, and electronic signal enhancement. Since a conventional light microscope is part of the LSCM system, all of the standard imaging modes are also available. In the confocal mode, the optical system does not collect the entire image at once. Rather, the LSCM system focuses an intense laser beam of collimated light to a diffraction-limited point at one focal plane in the specimen (Fig. 1). The image returning from this point is focused through the confocal optics onto the detector photomultiplier tube (PMT) and is the conjugate (confocal) of the image from the specimen plane. A pinhole diaphragm in front of the PMT suppresses light from nonfocal planes, and the image from the focal plane is passed without attenuation and captured digitally. Only a limited area is viewed at any time, which greatly reduces the degrading effect of scattered light on image quality. As noted by Wilson and Sheppard,[1] a PMT detector registers a greatly reduced signal if the light originates from out-of-focus planes. Thus, reducing the out-of-focus signal improves contrast and enhances resolution beyond the limits of conventional fluorescence microscopy. The resultant increase in lateral and depth resolution makes the LSCM system ideal for optical sectioning.[2]

SYSTEM DESCRIPTION

The LSCM system comprises two optical systems focused to the same point in the specimen (Fig. 2). An illuminating system focuses the laser to a point in the specimen. A second optical system, conjugate to the first, focuses the image on the pinhole of the PMT detector.

After passing the *X/Y* scanners and two beamsplitter systems for video output and observation, the laser beam is focused onto the specimen plane. The coherent laser light is scanned across the specimen surface by galvanometer scanners driven by a digitally controlled scan generator. The laser beam is scanned in two orthogonal
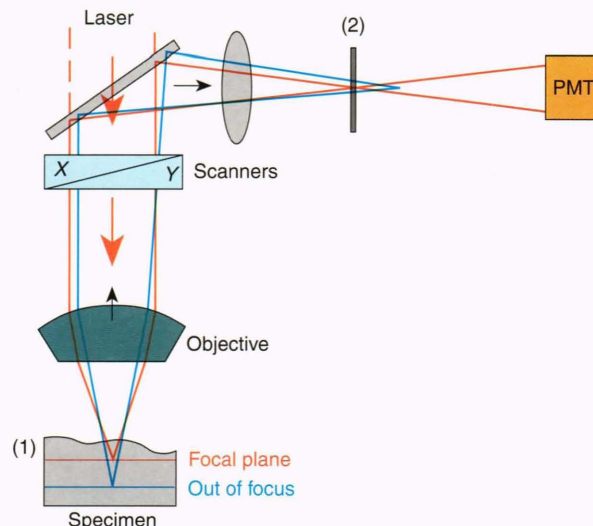

Figure 1. Schematic representation of the confocal optical microscope. A laser light source that has been expanded to fill the optical elements passes a beamsplitter and is scanned point by point as a diffraction-limited spot onto the focal plane at (1). The objective lens then reflects the image from this focal plane to the beamsplitter and re-forms the image onto the pinhole diaphragm at (2) in front of the photomultiplier tube (PMT). The image at (1) is confocal with the image at (2). Out-of-focus data are attenuated by the pinhole diaphragm, and this signal falls off sharply.
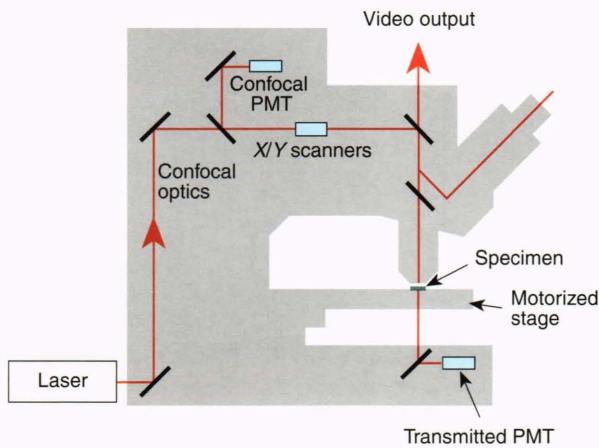
Figure 2. Schematic overview of the laser scanning optical microscope system. A conventional research microscope is equipped with the laser light source. The beam is expanded to fill the optics and is subsequently translated by digitally controlled X/Y scanners. After passing the two beamsplitters, the beam is focused on the specimen by the objective lens. A motorized stage translates the specimen along the optical or z axis for optical sectioning. Reflected or fluorescence images are detected by the photomultiplier tube (PMT) in the confocal optics. Transmitted images are collected by a PMT in the base of the microscope.

axes: one mirror scans the lines and the other the frame. A sophisticated optical system is placed between the scan mirrors to use the full aperture of the objective regardless of the scan angle. Reflected or fluorescent light is captured by one detector via the confocal optical path, and transmitted light is imaged by the condenser onto a second detector (not confocal). This dual-detector configuration allows simultaneous acquisition of a confocal fluorescence image and a conventional dark-field, transmitted-light image.

An advantage of the confocal optical scanning method is that the brightness and contrast of the image can be varied electronically using signal enhancement. For a weak signal, the PMT detector output will show only a small modulation, but electronic amplification of the signal can improve contrast of the image.[2] Therefore, less excitation energy is needed to record the image, prolonging the life of fluorescent markers in tissue specimens. This is a major advantage over conventional fluorescence microscopy, where samples require large amounts of excitation energy to be seen.

The optical sectioning feature of the LSCM system allows three-dimensional (3D) volume visualizations to be constructed from serial two-dimensional (2D) slices of a specimen. As the specimen is translated vertically along the optical axis, the 2D images retain contrast and resolution wherever the specimen comes into focus. When serial 2D optical slices of the object are overlaid, a 3D volume representation with extended focus results. The confocal optical sectioning technique is free of the monumental problems of alignment and registration associated with classical sectioning methods. The conventional optical microscope is restricted in performing 3D sectioning by fundamental limitations from low signal-to-noise ratio; the light from a plane of focus deep in the sample

is scattered and degraded as it passes through the sample matrix.

Images from the LSCM system contain digital information in the x, y, and z planes and intensity data for each volume pixel, known as a "voxel." Volume renderings (made with Vital Images, Inc., Voxel View software) represent the 3D object as a collection of these cubelike voxels. A user-specified, measurable property from the original data set is used to reveal the regions of interest. Because the images are captured digitally, quantitative measurements can be obtained anywhere in the 3D data set, i.e., in any plane or direction or along some irregular path throughout the volume. This flexibility provides an extra dimension of data for (volume) analysis that is not possible with traditional 2D images. Thus, the researcher is free to browse through the volume from any orthogonal or oblique plane to obtain views not directly observable with the microscope alone. Using Vital Images Voxel Animator software, these views can be set into motion by creating animations to reveal otherwise obscure features.

STUDY OBJECTIVES

In this study we investigated how red blood cells (erythrocytes) from subjects with sickle cell disease interact with blood vessels in the sensory retina—the semi-transparent vision tissue on which light is focused in the eye. Sickle cell disease is caused by single-point mutations in the hemoglobin gene. The presence of mutant hemoglobin in the red blood cell (RBC) causes hemoglobin tetramers to polymerize under hypoxic or acidic conditions, which gives the RBC the characteristic sickle shape (Fig. 3).[3] Accelerated destruction of sickle RBCs (SS-RBCs), called hemolysis, results in the characteristic sickle cell anemia. The SS-RBCs can initiate blockage of blood vessels (vaso-occlusions) in most major organ systems of the body. Experimental models suggest that they either adhere to endothelial cells lining the blood vessels or become physically entrapped within the microvasculature, or both.[4] In the retina, vaso-occlusions result in sickle cell retinopathy, which is characterized by the growth of new blood vessels—neovascularization—adjacent to areas of the retina made ischemic (blood deficient and therefore deprived of oxygen and nutrients) by vaso-occlusive processes.[5] The site of the initial vaso-occlusions is thought to be at the level of the precapillary arterioles,[6,7] arterial blood vessels immediately preceding the capillaries, but this theory has never been directly demonstrated. In this study we attempted to elucidate the sites of SS-RBC retention within the retinal vasculature.

EXPERIMENTAL METHODS

We injected either fluorescently labeled human sickle erythrocytes or normal human erythrocytes into anesthetized adult rats. The retinas were prepared by the adenosine diphosphatase (ADPase) histochemical technique[8] for visualization of the retinal vasculature. The enzyme ADPase is almost exclusively localized to the vasculature in the retina and can be visualized by the lead reaction product that results from incubation for ADPase activity. Flat-mount preparations of ADPase-incubated retinas
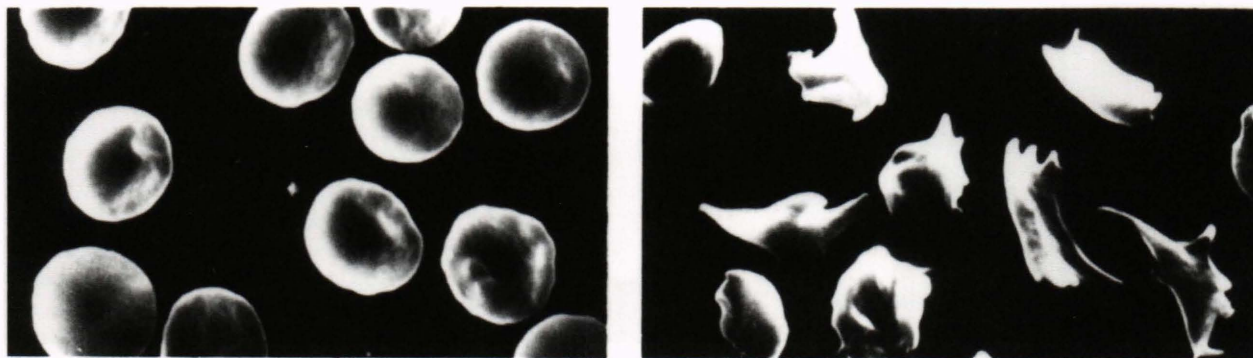
Figure 3. Scanning electron micrographs of human sickle red blood cells. These cells change from a normal biconcave disk shape (left) to the typical sickle shape (right) under deoxygenation. (Reproduced from *The Journal of Clinical Investigation*, 1983, vol. 72, pp. 22–31, Fig. 1, by copyright permission of The Society for Clinical Investigation.)

were examined by both conventional microscopy and LSCM. Dark-field illumination was used to visualize the ADPase reaction product in blood vessels; fluorescence illumination was used to visualize labeled SS-RBCs.

Rat Model of Human Sickle Cell Adhesion within the Retinal Vasculature

Erythrocytes from human sickle cell patients (Fig. 3) were isolated[9] and labeled with either fluorescein isothiocyanate or rhodamine isothiocyanate[10] and then injected into the left ventricle of anesthetized rats. Cells were allowed to circulate for 5 min, the animals were sacrificed, and the retinas were prepared by the ADPase flat-mount technique.[8] Normal human fluorescein isothiocyanate-labeled RBCs were injected into other rats as a control. The specimens were viewed under conventional dark-field and fluorescence microscopy using a Zeiss inverted microscope. Identical specimens were examined with a Zeiss LSCM.

Laser Scanning Confocal Microscopy

We collected LSCM optical sections through the inner (near the retinal surface) and outer retinal vascular networks using a Zeiss LSCM. A 63X/1.4NA oil-immersion Zeiss objective was used with a multiline argon laser illuminating source. Optical sections were taken with 0.45-μm separation through a depth range of 30.6 μm of retinal tissue. A dark-field image and fluorescence image were produced in one scanning series using independent detectors, and the two images were displayed simultaneously as overlays. Two-dimensional slices from the optical series were superimposed to produce a single, extended-focus image. Stored optical slices also were used to construct 3D volume renderings and stereo images, and the 3D representations were set into motion using a Silicon Graphics workstation and Vital Images animation software.

RESULTS

The entire retinal vasculature could be visualized by incubating the retinas for ADPase activity and then viewing the retina in flat mount under dark-field illumination. The lead ADPase reaction product was confined almost

exclusively to the vasculature, as can be seen in the left panel of Fig. 4. Arteries and arterioles show the greatest ADPase activity and reaction product when compared with veins and venules; capillaries show the least activity.[8] This differential staining and position in the vascular hierarchy permitted exact blood vessel type to be identified. Combining the ADPase method with injection of fluorescently labeled target cells allowed us to determine the location of the SS-RBCs in the vasculature with traditional fluorescent microscopy by simply changing the light source (Fig. 4, right panel). In the rat, the primary vasculature near the surface of the retina has arteries, arterioles, veins, and venules, whereas the secondary vasculature in the inner nuclear layer contains almost all of the capillaries. This spatial segregation of microvasculature and large vessels necessitated a mechanical sampling of each plane in the retina by step-focusing each area to determine where in the vascular hierarchy the fluorescently labeled cells were retained.

We also combined fluorescent images with ADPase images to create a composite of SS-RBCs and the associated retinal vasculature by manually creating double exposures using photographic processes (Fig. 5). The SS-RBCs were most often observed at precapillary arteriolar bifurcations, suggesting that this site within the vascular hierarchy was the most vulnerable for retention of SS-RBCs. The normal human RBCs injected into control rats were not observed in the retinal vasculature (data not shown). Trapped SS-RBCs were also observed in clusters, apparently adhering to the walls of large veins in a few examples, and obviously not obstructing those vessels (Fig. 6).

The LSCM analysis of the retinal flat mounts enhanced our analytical capabilities primarily by improving the collection and visual presentation of the data. The spatial segregation of microvasculature and large vessels, which required a mechanical step-focus scan of each area with the traditional fluorescent microscope, was done automatically by the LSCM. All components of the bilayered vasculature could be presented in one stereo image (Fig. 7). The LSCM system permitted the lead ADPase reaction product and the fluorescently labeled cells to be scanned simultaneously. The stacks of images from both scans could be easily aligned and presented
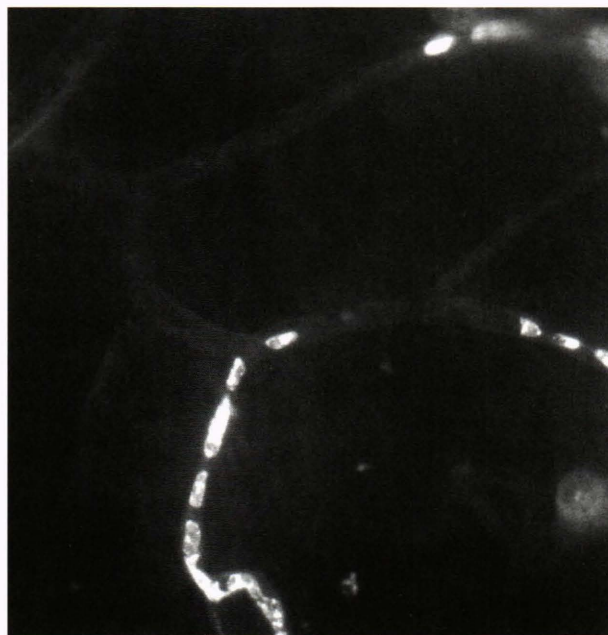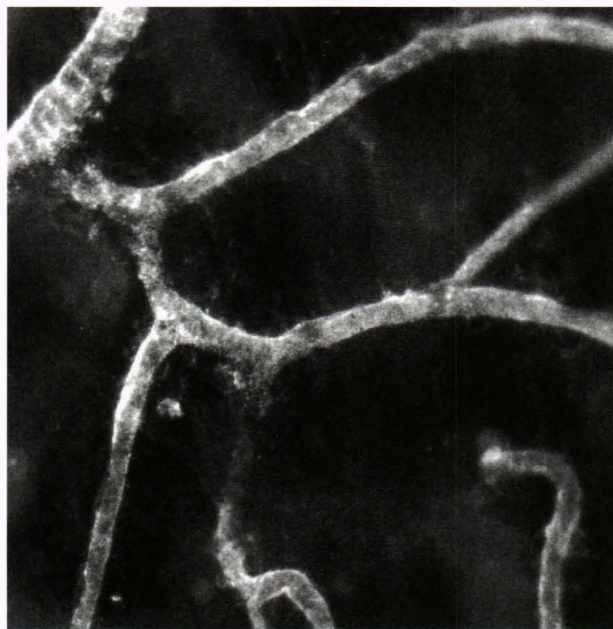
Figure 4. (Left) Flat-mounted rat retina incubated for the histochemical demonstration of adenosine diphosphatase activity and viewed using dark-field illumination. (Right) The same area showing distribution of retained fluorescein-labeled sickle red blood cells in precapillary arterioles.

as a single data set using the Vital Images Voxel View software. A single LSCM 2D optical section through a precapillary arteriole of a test rat indicated less than total obstruction of blood flow (Fig. 8). When views were set in motion with animation, several perspectives demonstrated that the lumen was not totally obstructed (Figs. 9 and 10). The superior resolution of the LSCM system permitted the intimate relationship of SS-RBC and vascular endothelial cells to be observed. At the bifurcation shown in Fig. 10, the SS-RBCs appear to conform to the shape of the vessel wall, suggesting adhesion of the cells to the vascular endothelium.

DISCUSSION

Because the retinal vasculature is bilayered, the ADPase flat-mount retina method lends itself to the optical sectioning and 3D reconstruction permitted by LSCM. Combining fluorescent-dye labeling of SS-RBCs with the ADPase flat-mount technique permitted us to identify sites of adhesion and obstruction by SS-RBCs within the retinal vasculature. Using LSCM, we could collect the fluorescence and dark-field images in one scanning series and display them together as an overlay.

The LSCM optical series of 2D sections provides views not directly observable with the microscope alone. As a result, the extent of lumenal obstruction by SS-RBCs can be examined without the monumental problems of alignment and registration associated with conventional histological sectioning techniques, the only other method for observing the tissue in cross section.

Clinical fluorescein angiography of human subjects[7] and ADPase flat-embedded analysis of retinas from young sickle cell subjects[6] suggest that vaso-occlusions in sickle cell disease occur initially at the level of precapillary arterioles. Our animal model suggests that this echelon
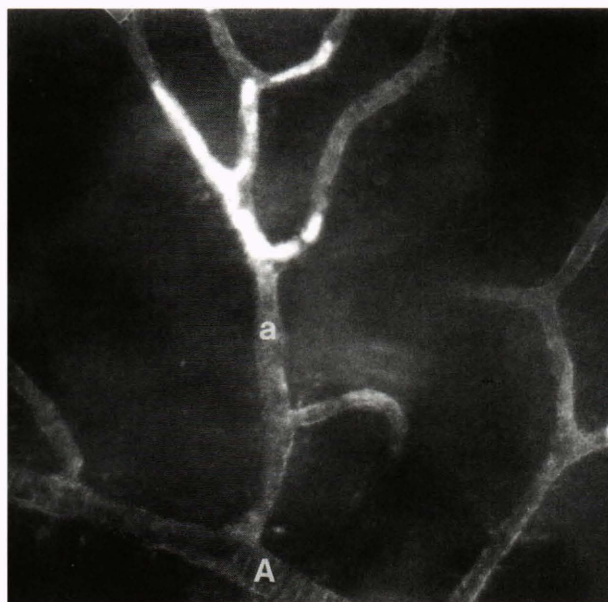

Figure 5. Multiple-exposure micrograph of a flat-mounted rat retina made by combining the fluorescent image of sickle red blood cells with the dark-field image of the vascular adenosine diphosphatase reaction product. This method allows visualization of the retinal vascular hierarchy and demonstrates the retention of these cells at three successive dichotomous arteriolar branches within the peripheral vasculature. (A = artery; a = arteriole.)

of retinal vascular hierarchy is particularly susceptible to the deposition of SS-RBCs. Therefore, our study confirms the precapillary arteriole as the initial site for SS-RBC retention and suggests that in some cases a single cell may not totally obstruct the blood vessel lumen. Kurantsin-Mills, Klug, and Lessin[11] suggested that vortex

Figure 6. Multiple-exposure micrograph of adenosine diphosphatase reaction product in retinal vessels and fluorescein-labeled sickle red blood cells adherent within a vein. (A = artery; V = vein.)
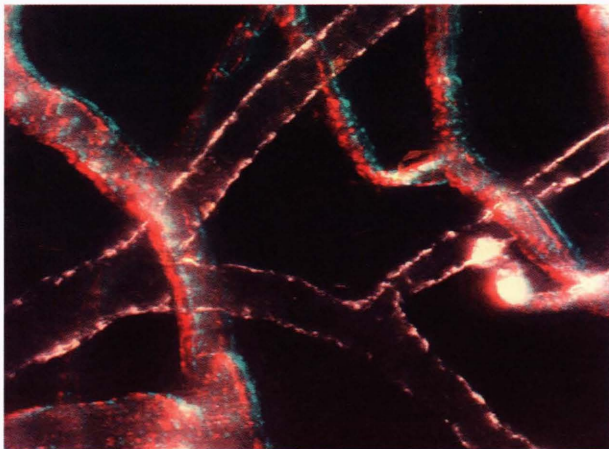

Figure 7. Red/blue stereo three-dimensional image demonstrating the bilevel vasculature of the rat retina. The retained sickle red blood cells were found in the primary vascular network.

Figure 8. The same field of view presented in Fig. 7 showing sickle red blood cells retained within the bifurcation of a precapillary arteriole. In this view, it appears that the obstruction of blood flow is less than total.

Figure 9. Single frame from a video animation of the human sickle red blood cells within the bifurcation of a rat precapillary arteriole. Several video animations were produced using the same series of optical sections from the laser scanning optical microscope.

Figure 10. Single frame made from a second animation. The entire volume was rotated counterclockwise so observers could look into the lumen of the arteriole. From this viewpoint, it appears that the obstruction of blood flow is less than total.

currents at the arteriolar bifurcation contribute to the deposition of SS-RBCs at this site. In nonocular tissue such as mesoappendix[3] and mesocecum,[4] SS-RBCs have been trapped at the level of postcapillary venules. Different sites of adhesion may occur in different tissues due to the surface molecules on the endothelial cells. Normal RBCs were not retained in the retinal vasculature, demonstrating the validity of our observations using SS-RBCs.

Subsequent work with this model will focus on demonstrating which class of SS-RBC initiates vaso-occlusions in the retinal vasculature. We also hope to show that the cells are truly adherent by flushing the vasculature before enucleation. This model provides a
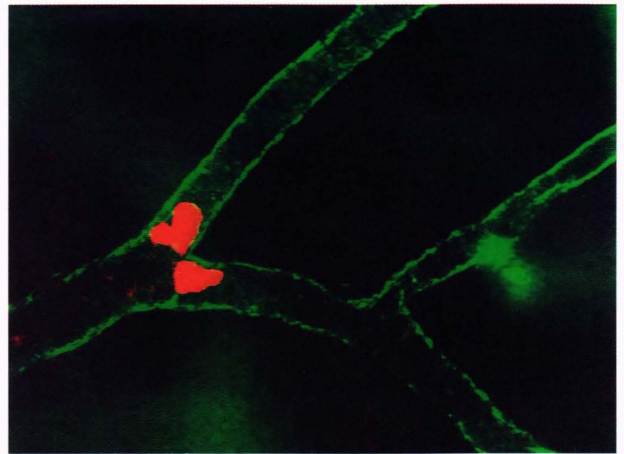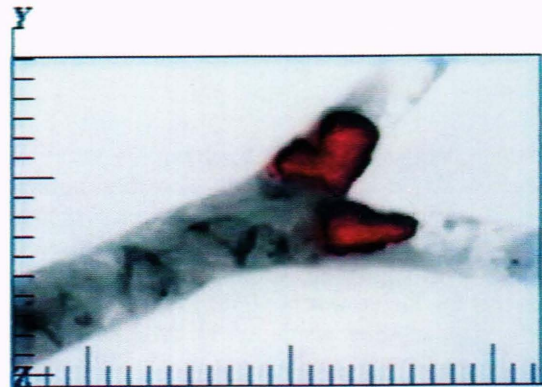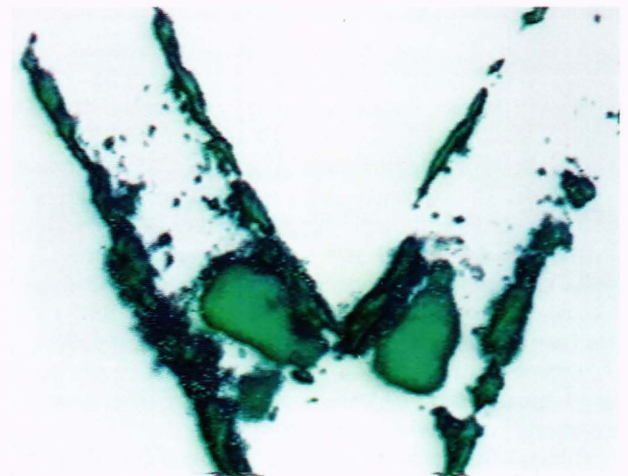
system for determining the mechanism of adhesion and retention of SS-RBCs and for evaluating therapeutic modalities for preventing SS-RBC-initiated vaso-occlusions in the sickle cell patient.
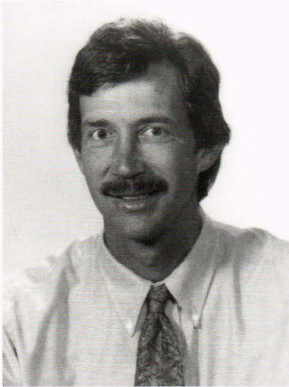
REFERENCES

[1] Wilson, T., and Sheppard, C., *Theory and Practice of Scanning Optical Microscopy*, Academic Press, New York (1984).

[2] Inoué, S., "Foundations of Confocal Scanned Imaging in Light Microscopy," in *Handbook of Biological Confocal Microscopy*, J. Pawley (ed.), Plenum Press, New York, pp. 1–14 (1990).

[3] Kaul, D., Fabry, M., Windisch, P., Baez, S., and Nagel, R., "Erythrocytes in Sickle Cell Anemia are Heterogeneous in Their Rheological and Hemodynamic Characteristics," *J. Clin. Invest.* **72**, 22–31 (1983).

[4] Kaul, D., Fabry, M., and Nagel, R., "Microvascular Sites and Characteristics of Sickle Cell Adhesion to Vascular Endothelium in Shear Flow Conditions: Pathological Implications," *Proc. Nat. Acad. Sci. USA* **86**, 3356–3360 (1989).

[5] Goldberg, M. F., "Sickle Cell Retinopathy," in *Clinical Ophthalmology, Vol. 3*, T. D. Dwane (ed.), Harper and Row, Hagerstown, MD, pp. 1–44 (1976).

[6] McLeod, D., Goldberg, M., and Lutty, G., "Dual Perspective Analysis of Vascular Formations in Sickle Cell Retinopathy," *Arch. Ophthalmol.* **111**, 1234–1245 (1993).

[7] Raichand, M., Goldberg, M. F., Nagpal, K. C., Goldbaum, M. H., and Asdourian, G. K., "Evolution of Neovascularization in Sickle Cell Retinopathy," *Arch. Ophthalmol.* **95**, 1543–1552 (1977).

[8] Lutty, G. A., and McLeod, D. S., "A New Technique for Visualization of the Human Retinal Vasculature," *Arch. Ophthalmol.* **110**, 267–276 (1992).

[9] Fabry, M. E., Mears, J. G., Patel, P., Schaefer Rego, K., Carmichael, L. D., et al. "Dense Cells in Sickle Cell Anemia: The Effects of a Gene Interaction," *Blood* **64**, 1042–1046 (1984).

[10] Butcher, E., and Weissman, I., "Direct Fluorescent Labeling of Cells with Fluorescein or Rhodamine Isothiocyanate: I, Technical Aspects," *J. Immunol. Meth.* **37**, 97–108 (1980).

[11] Kurantsin-Mills, J., Klug, P. P., and Lessin, L. S., "Vaso-Occlusion in Sickle Cell Disease: Pathophysiology of the Microvascular Circulation," *Am. J. Ped. Hematol./Oncol.* **10**, 357–372 (1988).

THE AUTHORS

STEPHEN D. WAJER is currently a member of the Materials Laboratory of the APL Technical Services Department. He is actively involved in the field of micro-analysis with special applications involving scanning electron microscopy and laser scanning confocal microscopy. Current technical interests include high-resolution imaging of nonconductive and semiconductive materials using low-voltage scanning electron microscopy. Mr. Wajer received a B.S. in mathematics from The Johns Hopkins University Whiting School of Engineering in 1982 and joined APL in 1985. Before that, he worked at The Johns Hopkins University School of Medicine, where he studied ocular diseases related to premature birth. He is a member of the Microscopy Society of America and the Microbeam Analysis Society.
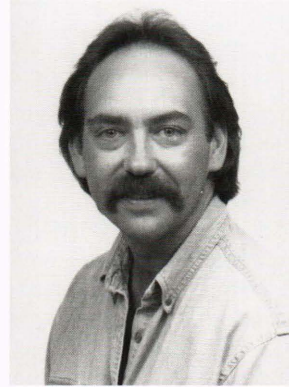
D. SCOTT McLEOD is a member of the Associate Staff of APL's Biomedical Research and Engineering Group. Mr. McLeod specializes in the development of techniques to study the blood vessels of the eye and the application of these techniques in improving analysis of the vascular complications that occur in various diseases. He worked for The Johns Hopkins University School of Medicine before joining APL in 1988. He is currently collaborating with scientists at the Wilmer Eye Institute Department of Ophthalmology on a blinding disorder that affects infants born prematurely and on the causes and effects of vascular obstruction in diabetes and sickle cell anemia.
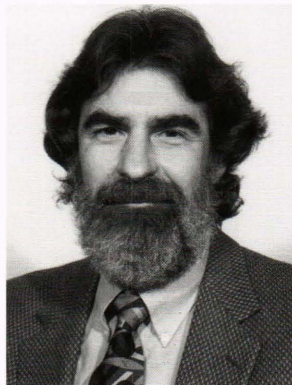
GERARD A. LUTTY is an Assistant Professor of Ophthalmology at the Wilmer Eye Institute, The Johns Hopkins University School of Medicine. He received a B.S. in zoology in 1970 and an M.S. in microbiology in 1980 from The Catholic University of America, Washington, DC. He received a Ph.D. in cell biology from The Johns Hopkins University School of Medicine in 1992. Dr. Lutty has worked at the Wilmer Eye Institute for 20 years and has participated in many collaborative research projects with APL staff. Currently, his major interest is the processes that cause vaso-occlusions in both sickle cell and diabetic retinopathy. He is also interested in factors controlling the neovascularization that occurs in these retinopathies and in the retinopathy of prematurity.