Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 6527–6531, October 1985
Biophysics

Visualization of oriented hemoglobin S in individual erythrocytes by differential extinction of polarized light

(linear dichroism/sickle cells)

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ABSTRACT The distribution of oriented, polymerized sickle cell hemoglobin (hemoglobin S) in erythrocytes is visualized with a microscope that produces an image proportional to linear dichroism. Monochromatic light alternately polarized along two perpendicular directions is incident on the sample. The image is focused on a diode array, and the digital output is used to form two images. One is the usual image proportional to the average transmitted light intensity of the two incident polarizations of light; the other is a linear differential image proportional to the linear dichroism of the sample. This quantitative image can specifically reveal oriented hemoglobin molecules with a sensitivity of about 4000 oriented molecules per picture element of the image.

Sickle cell anemia is a severely debilitating, inherited disease characterized by painful blockage of the circulation and by hemolytic anemia. The symptoms of the disease are due to the polymerization of mutant hemoglobin S (Hb S) when it is deoxygenated. This causes decreased deformability and decreased survival of erythrocytes containing polymerized Hb S (1–5). Most sickle cells appear as morphologically normal, biconcave discs when well-oxygenated but appear as sickle-shaped or holly-leaf-shaped cells with sharp spicules when deoxygenated. These reversibly sickled cells revert to normal shape upon reoxygenation. Subpopulations of cells remain elongated even when reoxygenated (irreversibly sickled cells) or remain discoid when deoxygenated. Fundamental to these cellular changes and to the understanding of sickle cell disease are the amount, distribution, and alignment of polymerized Hb S within sickle erythrocytes (5). Thus, a microscopic method for quantitative measurement of oriented, polymerized hemoglobin throughout an erythrocyte will be very useful in studies of sickle cell anemia.

We have developed a microscope that can determine the quantitative distribution of oriented Hb S polymer throughout an individual cell. The microscope measures the linear dichroism (the difference in extinction for incident light polarized along two directions perpendicular to each other) at each point in the object. Thus, a computer-reconstructed image from these determinations is a measure of the linear dichroism ($A_{\parallel} - A_{\perp}$) of the object rather than its absorbance. Linear dichroism is produced only by oriented matter (such as aligned, polymerized Hb S) in the object; unoriented chromophores do not have linear dichroism.[§] This microscopic technique detects macromolecular organization within cells; it will be useful for analysis of basic aspects of Hb S polymerization and alignment and its importance in sickle cell disease.

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MATERIALS AND METHODS

Fresh blood cells were washed three times with 0.15 M NaCl; the erythrocytes were then suspended to a hematocrit of about 10% in phosphate-buffered saline (0.145 M NaCl/5 mM phosphate, pH 7.4). Oxygenated suspensions of sickle cells equilibrated with room air and deoxygenated suspensions equilibrated under an atmosphere of 100% N_2 for 1 hr were fixed by mixing with an equal volume of 3% glutaraldehyde/50 mM phosphate, pH 7.4, that had been similarly oxygenated or deoxygenated.

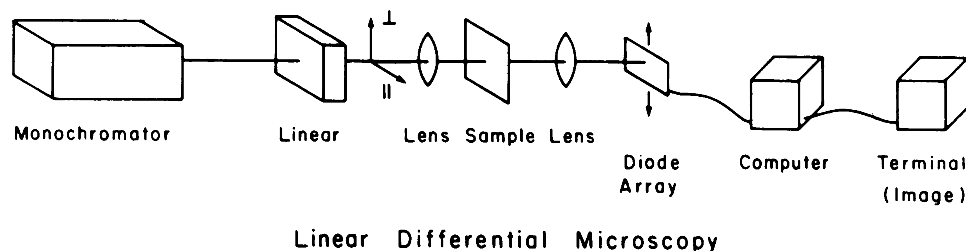
The microscope (Fig. 1 *Upper*) consists of a monochromatic light source that is modulated to produce alternately horizontal (parallel) and vertical (perpendicular) linearly polarized light. A Zeiss microscope focuses the modulated light beam on a target erythrocyte; the resultant image is focused on a linear diode array. The diode array is mechanically scanned over the image and its output is digitized to 12-bit accuracy and stored on a computer. Transmission images and linear dichroism images can be reconstructed from the digitized data. The transmission image is a measure of the extinction of the object; it is the sum of the transmitted intensities for incident parallel and perpendicular polarized light. The linear dichroism image is a measure of the amount and orientation of aligned polymeric Hb S; it is proportional to the linear dichroism ($A_{\parallel} - A_{\perp}$) and is the quotient of the difference of transmitted intensities ($I_{\perp} - I_{\parallel}$) divided by the sum ($I_{\perp} + I_{\parallel}$).

$$\frac{I_{\perp} - I_{\parallel}}{I_{\perp} + I_{\parallel}} = \frac{10^{-A_{\perp}} - 10^{-A_{\parallel}}}{10^{-A_{\perp}} + 10^{-A_{\parallel}}} = \tanh\left[\frac{2.303}{2}(A_{\parallel} - A_{\perp})\right] \simeq \frac{2.303}{2}(A_{\parallel} - A_{\perp}). \quad [1]$$

Absorbance is defined in the usual way as $A = \log(I/I_0)$ and the linear dichroism is usually small enough so that the hyperbolic tangent can be replaced by its argument. In Eq. **1**, $A_{\parallel}$ and $A_{\perp}$ are phenomenological. They refer to two arbitrary orthogonal directions of the incident linearly polarized light. To obtain information about the direction of orientation and the amount of oriented chromophore, it is necessary to measure the ratio of intensities in Eq. **1** for two different orientations of incident linearly polarized light.

The orientation of chromophores in a plane can be characterized by the angle (θ) of a principal absorption axis of the chromophore relative to a fixed direction (see Fig. 1 *Lower*).

§There can be a (usually) small contribution to linear differential extinction (linear dichroism) from depolarization of scattered light even from randomly oriented molecules. We will use absorbance (*A*) to represent the decrease in the transmitted light, although the quantity actually measured is the extinction. The extinction includes absorbance and scattering. The effects of instrumental stray light and of apparent linear dichroism from refraction and reflection can be evaluated for each experimental arrangement.



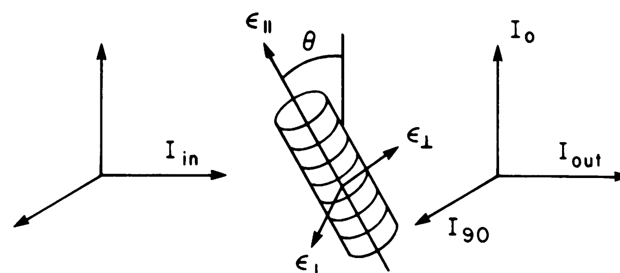


FIG. 1. (*Upper*) Schematic diagram of the linear differential microscope. Two orthogonal states of polarization of light are alternately incident on the sample. Each transmitted intensity is measured by the linear diode array that is scanned across the image plane. The images are stored digitally in the computer. (*Lower*) Definition of the angle θ between the incident direction of polarization designated as zero and the axis of the hemoglobin polymer.

For the hemoglobin polymer we choose $\epsilon_{\|}$ and $\epsilon_{\perp}$ as the molar extinction coefficients parallel and perpendicular to the axis of the cylindrically symmetric Hb S polymer, and the angle θ specifies the orientation of $\epsilon_{\|}$. Then from Eq. **1** (6)

$$\frac{I_{90} - I_0}{I_{90} + I_0} = \frac{2.303}{2}(\epsilon_{\|} - \epsilon_{\perp})lc\cos 2\theta. \quad [2]$$

Here absorbances have been replaced by $A = \epsilon lc$, with l, the path length, and c, the molar concentration of the oriented chromophore. From a second set of differential intensities measured with a 45° rotation of the orthogonal beams of incident polarized light, we obtain

$$\frac{I_{135} - I_{45}}{I_{135} + I_{45}} = \frac{2.303}{2}(\epsilon_{\|} - \epsilon_{\perp})lc\sin 2\theta. \quad [3]$$

We use these two linear differential images to construct two further images—one that is dependent on θ (the orientation of the hemoglobin polymer) and one that is dependent on the amount of hemoglobin polymer $(\epsilon_{\|} - \epsilon_{\perp})lc$. This latter quantity is obtained from the square root of the sum of the squares of the two differential images. The sign and magnitude of the angle θ is determined from either the image proportional to $\sin 2\theta$ or $\cos 2\theta$; the largest absolute value of $\sin 2\theta$ or $\cos 2\theta$ at each pixel is used.

RESULTS

The digitized intensity and linear dichroism images can be processed in several ways. Here we present the intensity images in gray scale and the linear dichroism images as color-coded images photographed from a color display terminal, with positive linear dichroism in green and negative linear dichroism in red.

Fig. 2 shows images of six erythrocytes that were deoxygenated under 100% N_2 for 1 hr (from patients homozygous for sickle cell disease), fixed with glutaraldehyde, and measured at a wavelength of 415 nm, a strong absorption band for hemoglobin (Soret band). There is a wide variety of morphologies and a wide range of linear dichroisms. The linear dichroism images provide information about the amount and orientation of polymerized hemoglobin. Qualitatively, in Fig. 2, green represents hemoglobin polymer oriented vertically, whereas red indicates parallel orientation. The top two images (Fig. 2*A* and *B*) show that the sickle shapes of the cells follow the orientation of the hemoglobin polymer. The middle left image (Fig. 2*C*) shows a cell in which hemoglobin polymer spirals around in a circular pattern. The bottom left image (Fig. 2*E*) shows a normal-appearing discoid cell with negligible linear dichroism. These images clearly demonstrate the power of linear dichroism. In particular, they show that the 415-nm absorption band for hemoglobin (Soret band) is useful for identifying aligned hemoglobin polymer in cells that were reoxygenated after having been fixed while deoxygenated.

The amount of aligned hemoglobin can be measured quantitatively, as shown in Fig. 3. Two linear dichroism images are obtained with the orthogonal planes of polarization for the two images rotated by 45°. From these two images the gray-scale image shown on the right is calculated (Eqs. **2** and **3**). This image is a pixel-by-pixel representation of the number of aligned hemoglobin molecules at each point in the erythrocyte. The orientation of the aligned hemoglobin at each point can also be obtained (image not shown). For these cells the aligned hemoglobin polymer follows the shapes of the cells.

Fig. 4 illustrates the advantage of a variable-wavelength light source. The intensity image (gray-scale) and the linear dichroism image next to it were obtained at 450 nm, where hemoglobin absorbs very little. Here the edges of the cells are emphasized; a quantitative measure of the shape of each cell is obtained. The intensity image is similar to what would be

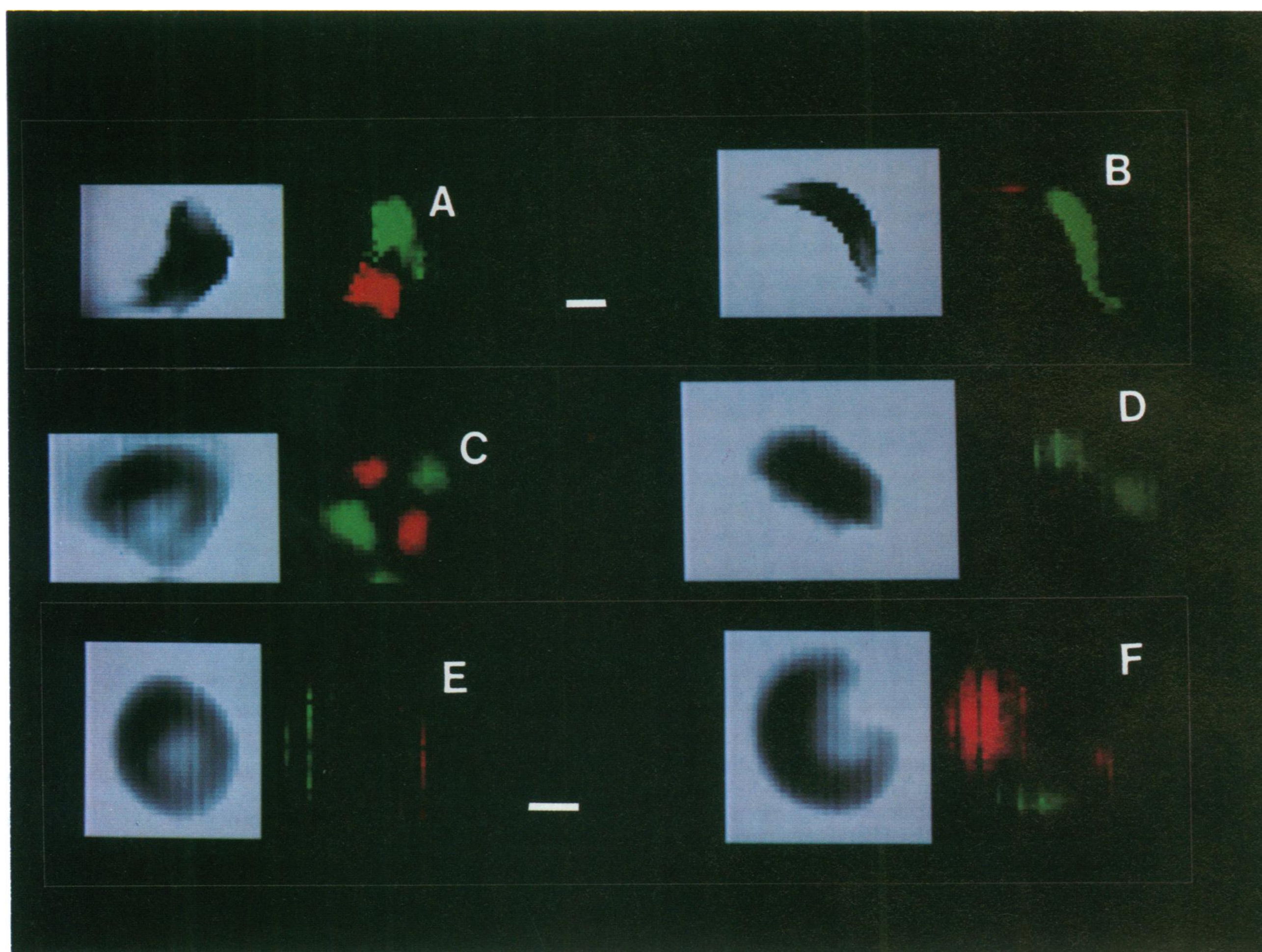


FIG. 2. Images of deoxygenated and glutaraldehyde-fixed erythrocytes from patients homozygous for sickle cell disease. The gray-scale images are the transmitted intensity ($I_\perp + I_\parallel$); the color-coded images are the linear dichroism ($(I_\perp - I_\parallel)/(I_\perp + I_\parallel)$). A wavelength of 415 nm with a bandwidth of 10 nm is used to correspond to the Soret absorption band of hemoglobin. The linear dichroism ranges from approximately +0.01 (green) to −0.01 (red). For images in *A–C*, each pixel represents a 0.28 × 0.28 μm square; for images in *D–F*, each pixel represents a 0.20 × 0.20 μm square. The white bars are 2 μm long.

seen by eye using white light. The linear dichroism is mainly caused by polarization-dependent refraction and reflection at refractive index gradients. The right-hand image at 415 nm (strong hemoglobin absorption) shows no oriented polymer for the discoid cell but a large amount of orientation for the elongated cell.

DISCUSSION

Monochromatic linear dichroism imaging adds more versatility to optical microscopy. Chromophores can be studied that are intrinsic to the sample, as in erythrocytes, or they can be specific dyes added to the sample. Oriented material can be selectively detected in the presence of random absorbers. Thus, ordered structures can be identified and quantified, and their orientation can be determined in complex objects. The sensitivity depends on the polarization dependence of the extinction coefficients of the chromophore; heme is thus especially suitable. Chromophores with a differential extinction only one-tenth as great as that of heme (such as DNA bases) should still be measurable in amounts present in cells.

Our results with other oxygenated and deoxygenated sickle erythrocytes are consistent with the pictures shown here. At 415 nm reversibly sickled cells show large amounts of oriented, polymerized Hb S. Distorted discs with high axial ratios show little or no aligned polymer; they are presumably irreversibly sickled cells with high concentrations of unaligned hemoglobin polymer (7). Discoid erythrocytes generally show no linear dichroism whether fixed at room oxygen tension or deoxygenated; however, a small percentage of deoxygenated discs contained large amounts of aligned polymer that seemed to be wound concentrically around the cell (Fig. 2*C*). Oxygenated erythrocytes containing normal hemoglobin (A) show little or no linear dichroism in the Soret band. Their linear dichroism is comparable to that of the sickled erythrocytes at 450 nm (see Fig. 4).

Hofrichter and co-workers (8, 9) in a pioneering study used a microspectrophotometer to measure the spectrum of the polarization ratio ($P = A_\perp/A_\parallel$) within single sickled cells; here $\parallel$ is parallel to the axis of the hemoglobin polymer. They observed 1-μm^2 regions of deoxygenated single cells in the Soret band and found a maximum value of $P = 3.0 \pm 0.1$. They assumed that this maximum value of P corresponds to completely aligned deoxyhemoglobin molecules within the polymer. From the known average extinction coefficient at the absorption maximum of the Soret band and this polarization ratio, a value of $(\epsilon_\parallel - \epsilon_\perp) = -1.1 \times 10^5$ M^{-1}·cm^{-1} is obtained. This corresponds to a difference in photon extinction cross sections of $(\epsilon_\parallel - \epsilon_\perp) = -1.9$ Å^2 per hemoglobin molecule = -1.9×10^{-8} μm^2 per hemoglobin molecule.

The linear dichroism images shown on the right in Fig. 3 give $(\epsilon_\parallel - \epsilon_\perp)lc$ at each location in the erythrocyte. Using

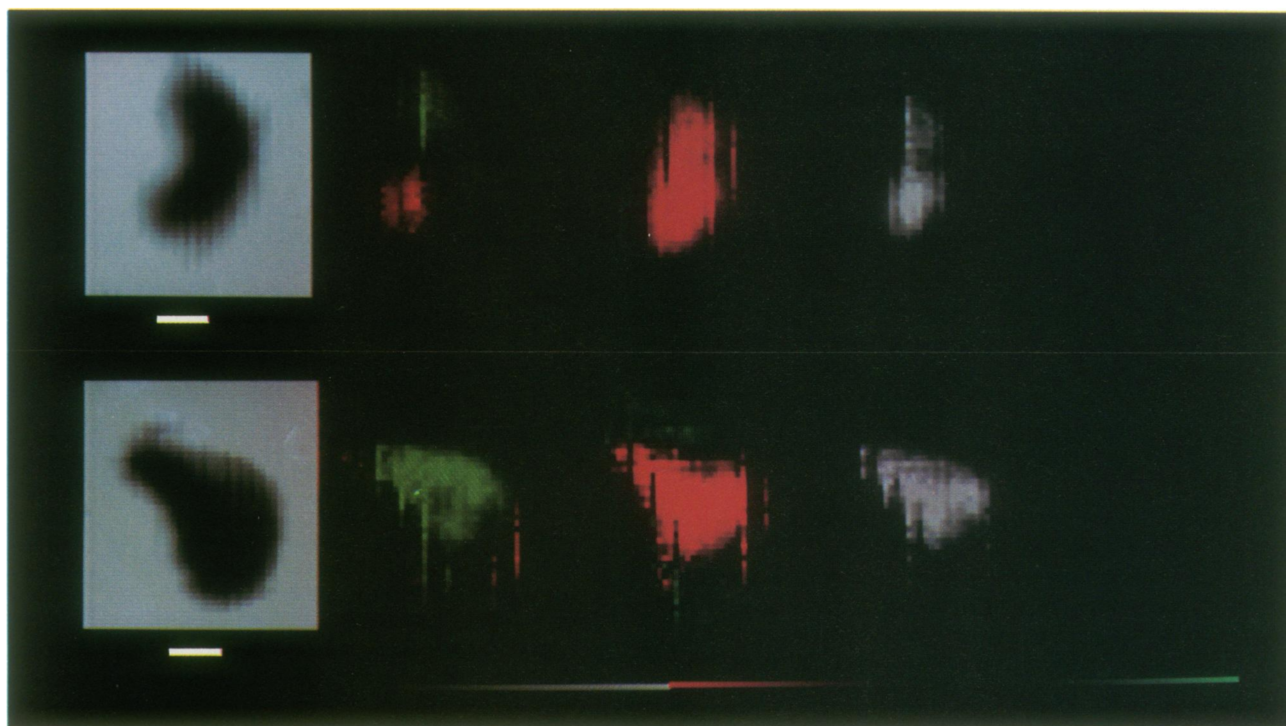


FIG. 3. Images that show the transmitted intensity (left), linear dichroism (middle two images), and amount of aligned hemoglobin polymer (right). The conditions are the same as in Fig. 2. The two linear dichroism images have the two orthogonal planes of polarization rotated by 45°. The image of the amount of aligned hemoglobin polymer is obtained as the square root of the sum of the squares of the two linear dichroism images. This gives $(2.303/2)(\epsilon_{\parallel} - \epsilon_{\perp})lc$; the difference in molar extinction coefficient parallel and perpendicular to the axis of the polymer $(\epsilon_{\parallel} - \epsilon_{\perp})$ must be known to calculate the amount (path length, l, times concentration, c) of aligned polymer.

the known (8, 9) value of $(\epsilon_{\parallel} - \epsilon_{\perp})$ we obtain the amount (hemoglobin molecules per μm^2) and distribution of oriented hemoglobin throughout the erythrocyte. We can detect and color code a linear dichroism $(A_{\parallel} - A_{\perp}) = 0.002$; therefore, the image can display $\approx 10^5$ oriented hemoglobin molecules per μm^2. Each pixel in Fig. 3 corresponds to a square in the object about 0.2 μm on a side; this allows visualization of a minimum of about 4000 oriented hemoglobin molecules. The resolution limit for the microscope is >0.2 μm, so this will decrease the sensitivity. To be able to calculate the concentration of aligned hemoglobin (c) at each location we would also need to measure the erythrocyte thickness (l) at each location.

The total amount of *aligned* hemoglobin in an erythrocyte can be obtained by summing the oriented amount measured at each pixel; a background amount is subtracted using the 450-nm image. The total hemoglobin in the erythrocyte is obtained by a similar process applied to the transmission image. For the deoxygenated sickle cells that we have studied there is a range of values of percentage of aligned hemoglobin from 0% to 4%. Noguchi *et al.* (10), using a NMR method, found up to 70% polymerization of hemoglobin in sickle erythrocytes. Thus, the aligned hemoglobin polymer is a small percentage of the total polymer, but it is apparently the portion responsible for the morphological distortions of sickle cells (11) that influence the rheologic abnormalities associated with sickle cell disease (12).

The origins of the large variation in the amount and distribution of aligned hemoglobin are unknown. The total concentration of hemoglobin in each cell is important (13). If this is a kinetically controlled system (14), we may be viewing sites of early onset of polymerization or multiple domains. If the percentage of aligned material is thermodynamically controlled, the small values of the thermodynamic parameters estimated for this system (15) may lead to large fluctuations in percentage of aligned hemoglobin. These measurements of individual cells may offer new insights into the molecular mechanism of sickling at a higher resolution than those obtainable by measurement of bulk properties.

The apparatus described here can also be used to produce circular dichroism images (16). The only difference is that left and right circularly polarized light is alternately incident on the sample. Instead of ordered structures, the circular dichroism image is sensitive to chiral structures, whether oriented or not. Linear dichroism images and circular dichroism images at specific wavelengths can reveal different classes of structures in the object. Digital images can be processed in many ways to improve image quality and to quantify observations. Furthermore, a phase- and polarization-sensitive detector can be added to measure the state of polarization of the transmitted light. Phase-contrast and polarizing microscopes are, of course, not new, but quantitative images based on separation and measurement of linear and circular birefringence and dichroism are. We think that digital, differential imaging with either linearly or circularly polarized, monochromatic light will broaden the applications of optical microscopy.

Note Added in Proof. Measurements on deoxygenated, *unfixed* cells give values of percentage of aligned hemoglobin approximately twice that of fixed cells.

We thank Mr. Joseph Katz and Mr. G. Kropp for excellent technical assistance. This work was supported in part by National Institutes of Health Grants GM 10840 (I.T.), AI 08247 (M.F.M.), and HL 20985 (S.E.), by National Science Foundation Grant RR 01612 to the San Francisco Laser Center, and by the U.S. Department of Energy, Office of Energy Research Contract 03-82ER60090.

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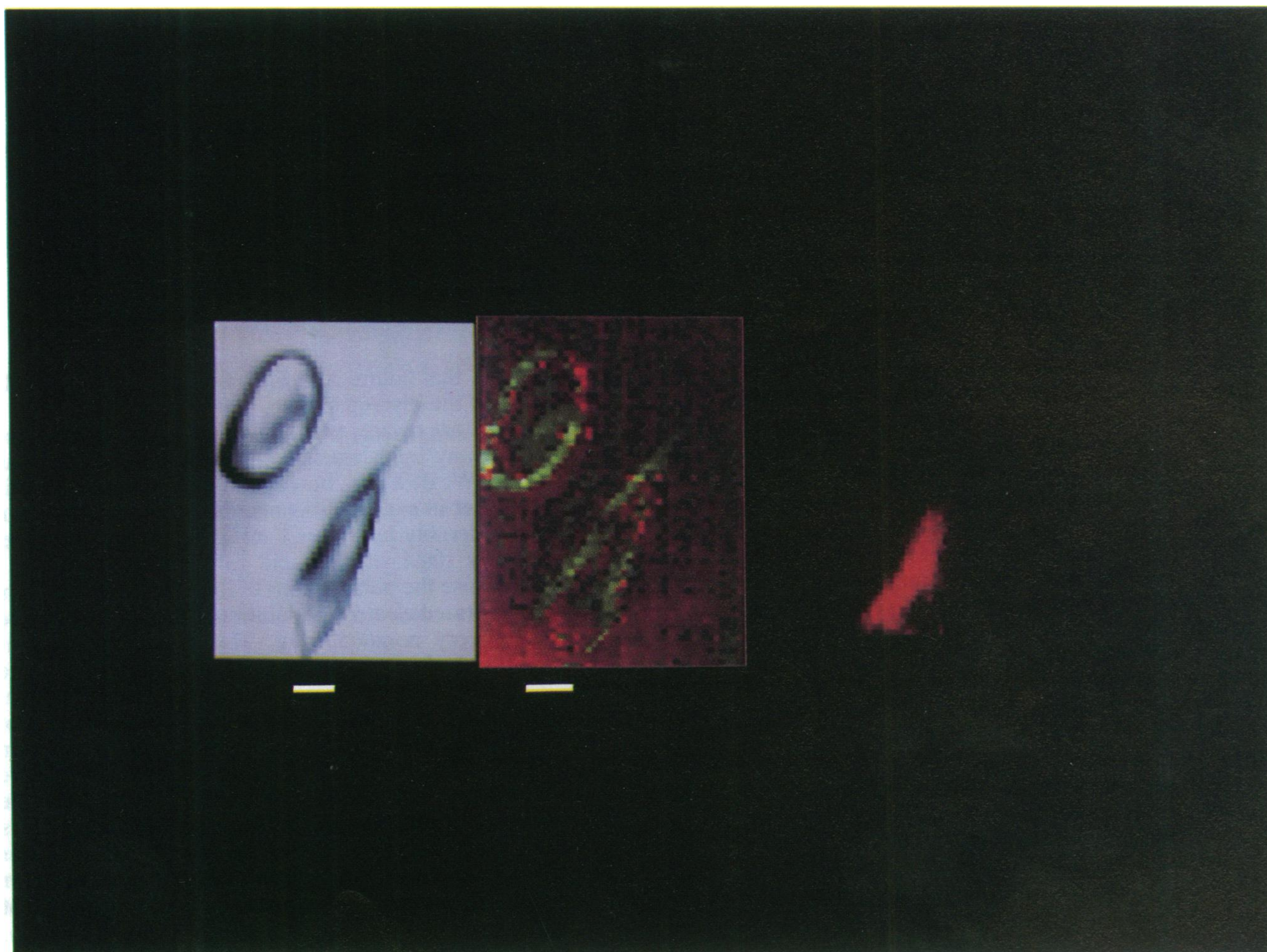

FIG. 4. Images of two erythrocytes visualized with a wavelength of 450 nm (outside the Soret absorption band of hemoglobin) are shown as the left two images. Both the intensity image (gray-scale) and the linear dichroism image emphasize the edges of the cells. The right image is a linear dichroism image at 415 nm (strong absorption of hemoglobin); note that the discoid cell is invisible in this image, whereas the sickle cell is very bright. The cells were treated the same as in Fig. 2. The white bars are 2 μm long.

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