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Cell Differentiation Based on Absorption and Scattering

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Improvements of the flow system allow calibrated cell length measurements down to less than 2 μm at a very high rate. An optical index match to plane viewing windows perpendicular to the optical axis in the flow system keeps the axial symmetry for forward scattered light. Cell size, axial light loss and scattering intensity within different angles were found to be powerful tools to differentiate cell populations. Red cells were analyzed according to various cell surface structures. Lymphocyte populations isolated from different parts of the lymphatic system in rats have been distinguished. Experimental tumor cells showed typical data pattern after different chemical treatments.

During the last years several investigators in flow cytometry have described the need of detecting more than one parameter for propriate cell or particle characterization. Various kinds of fluorescence stainings, multiwavelength fluorescence detection with single or multiwavelength excitation, as well as the Coulter volume are most popular in multiparameter analysis (1, 8, 10, 18, 21, 23-28). Since all staining procedures are time consuming and may give rise to artifacts due to improper handling, light scattering was studied as additional parameter. In a number of earlier experiments, small angle forward scattering has been used for particle size analysis, instead of detection of the Coulter volume (14, 24). A more detailed study of light scattering phenomena on cells has been introduced by using multiangle detector configurations (6, 9, 12, 13, 19, 20) allowing up to 64 forward rings or segments to be monitored. More recent theoretical studies on cells with different internal refractive indexes showed no clear relationship of the intensity of scattered light to the size of cells (3, 4, 11, 15-17). Other theoretical studies demonstrated the influence of the orientation of nonspherical particles with respect to the optical axis on the intensity of scattered light (3).

The following describes an approach in correlating light scattering information and cell size measurements suppressing influences of particles being not sufficiently spatially aligned and oriented, and the formation of cylindrical lenses in the fluid—air transition by proper design of the flow system. Biological cells have been investigated showing different surface and/or different internal structures.

MATERIALS AND METHODS

Optical and Electronic Arrangement

The flow system was designed for high spatial reproducibility of the particle stream and orientation of ellipsoidal particles in the direction of flow. A long distance was chosen for the hydrodynamic focussing to keep the acceleration rate below 1000 m/sec^{-2} and therefore the velocity difference seen by a 10 μm particle (front to end) below 2,5 mm/sec^{-1} avoided artificial stretching of the cells. The flow velocity is typically about 5 m/sec^{-1} and can be changed between less than 2 m/sec^{-1} and 12 m/sec^{-1} to meet specific experimental requirements while maintaining laminar flow conditions. The diameter of the sample stream is hydrodynamically reduced to 5 μm, but may be further reduced to 2 μm if necessary. The advantageous introduction of a second sheath fluid for optical index matching of the cells to plane viewing windows has already been described (5, 7). The flow chamber is designed for small distances between the viewing windows and the particle stream. A working distance of less than 1 mm allows the use of optics with apertures up to 0.8. Presently a ×40 microscope objective (n.a.:0.61) is used. Because only plane windows are placed perpendicular to the optical axis, the entire optical system maintains axial symmetry.

Particle length is derived from the pulse width t_i of the intensity change generated from specific and "unspecific" (7, 10, 14, 22) absorption while passing the laser beam. The diameter ($1/e^2$-points of the laser focus) has been detected as 0.9 μm and therefore it is smaller than most biological cells. Because of the spatial alignment of the cells, with their longest axis in direction of flow, an elliptically shaped particle will show its smallest radius of curvature as leading and trailing parts while intersecting the focus. This results in a short rise and decaytime of the absorption signal, and the errors in size measurement due to the beam divergence can be reduced by proper spatial alignment of the flow. The error of the analyzing electronics has been found as less than 3% and the total error of the size detection in flow as about 5% (6). For latex microspheres of a mean diameter of 2.01 μm, a coefficient of variation of the diameter of 6.2% was detected in accordance with other measurements (6). For a calibration to absolute size values, the velocity of the flow needs to be monitored continuously allowing internal electronic correction of the size measurement (Fig. 1). This technique has been described elsewhere (6).

Because the optical system maintains axial symmetry, a specially designed fiberoptic system with five bundles forming rings around a sixth center bundle has been used (Fig. 1b). Every individual bundle is fluid matched to a single photomultiplier resulting in a high sensitivity for light detection. Light scattering is monitored in five different angles in parallel. Intensity ratios of different angles are easily accessible. The angular range can be changed by moving the detector or changing the optical components. In most of the cases under investigation, the cells are not stained and do not show specific absorption. Therefore the change of the transmitted intensity is due to losses by scattering and deflection out of the forward cone, known

as "unspecific" absorption. The change in light intensity transmitted into the small forward detection cone of 5° solid angle is therefore a measure of the total intensity of scattered light integrated over all remaining solid angles.

Particle length information as well as the peak heights of scattered intensities are converted into square wave pulses of heights proportional to the measured value. The correlated values of both parameters are analyzed and stored on a two parameter multichannel pulse-height analyzer of sufficiently high analyzing rate. This allows a direct correlation of the size of a cell with its light scattering intensity into various forward angles or with its total loss for transmitted light.

Biological Material

A) human blood: Human blood was taken by venipuncture from healthy young volunteers and stored in 10 cc syringes at 4°C. Na-citrate was added as anti-coagulant.

B) lymphocytes: Lymphocytes were purified by Ficoll-gradient centrifugation from various parts of the lymphatic system of rats five days after transplantation of an additional kidney into the abdomen. Cells were a generous gift from Dr. Wagner, Department of Surgery, Medical School, Hannover F. R. G.

C) L 1210: L 1210 lymphocytes (Flow Labs., Inc., Rockville, Md.) were grown under standard culture conditions.

D) buffy coat: Buffy coat was separated from banked human blood after 24 hr of storage after mild centrifugation and drawn in a separate plastic bag under sterile conditions.

RESULTS

During the course of aging, human red blood cells undergo a dramatic change in their shape. Figure 2 shows six different stages of the transformation of the normal disc-shaped erythrocyte, known as discocytes, to the spherical shape with rough

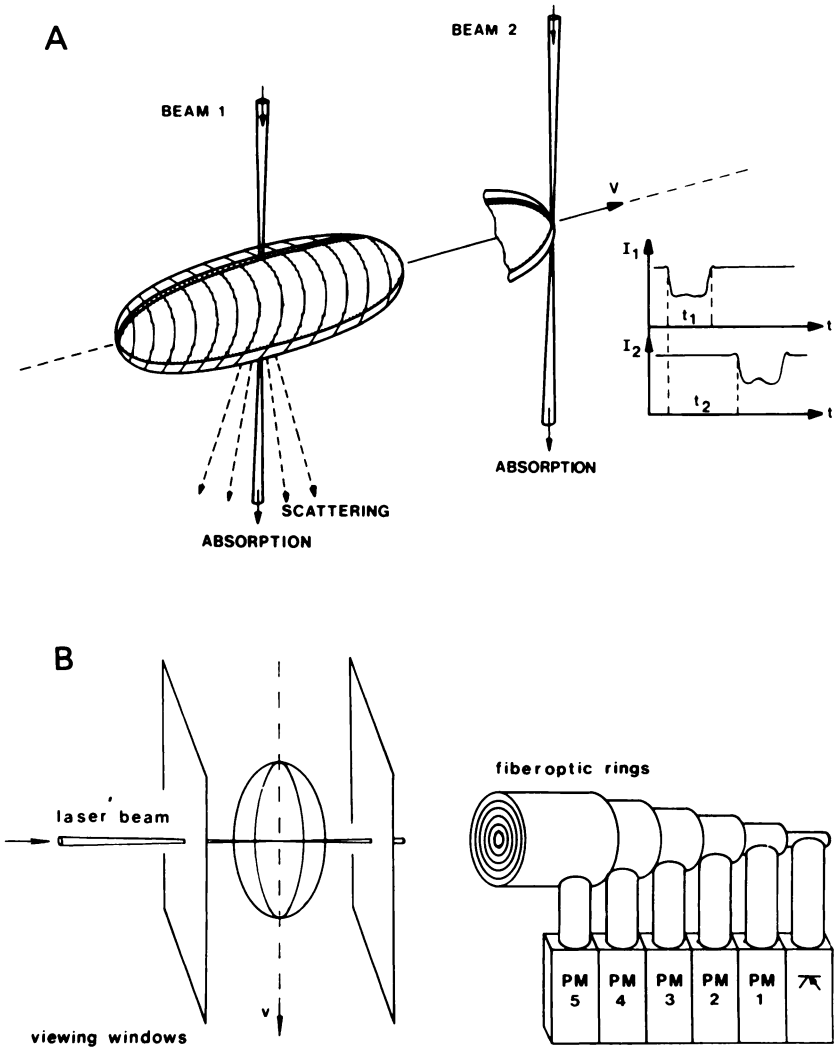

FIG. 1. A) Principle of calibrated size and scattering measurement. Cells are aligned in a single file and oriented in the direction of flow. Passing beam 1, always smaller in diameter than the cells, an absorption pulse is detected in forward direction, the width t_1 being proportional to the particle length. Passing beam 2, being parallel to beam 1 and displaced a known distance generates an equivalent absorption pulse. The time interval t_2 between the two leading edges is taken for velocity measurement and internal calibration of the particle size measurement. Light scattering of beam 1 is detected in various forward angles. B) Concentric fiberoptic light guide for the detection of scattered light in various forward directions. The fibers forming a single ring are connected to an individual photomultiplier. The center bundle is used for the detection of the transmitted light and is attached to a photodiode.

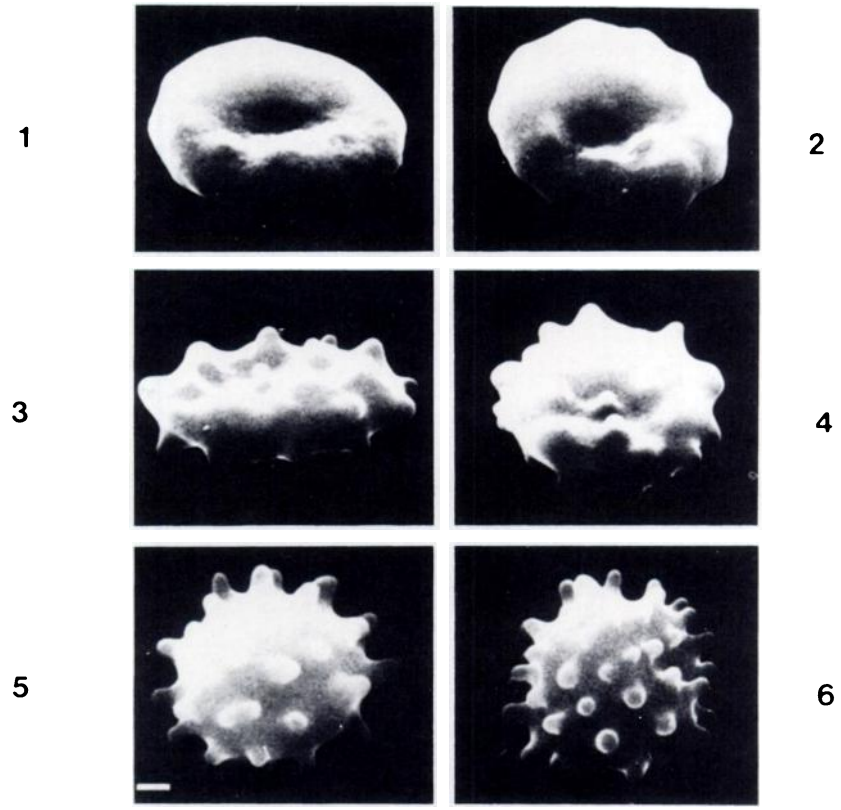

FIG. 2. Various stages of shape transformations of a human red blood cell under experimental storage conditions. The cells change from the normal shape 1 known as discocyte, to the spherical shape 6, known as echinocyte. (Taken from (2)). (Photo is from Nouvelle Revue Francaise d'Hematologie.)

surface structures, known as echinocyte. Only a very few of the latter are found in circulation in man, but they can be generated by experimental storage conditions. No chemical treatment is necessary and severe changes of the optical properties of the membrane itself are not expected. The change in surface structure, i.e., different stages of "spicule" formation, should result in increasing light scattering intensities. Since the volume of the erythrocyte remains constant during the cell transformation from the discocyte to the ball shaped echinocyte, the largest diameter of the cell being measured by this technique is reduced.

The contour plots of Figure 3 show the correlation of the cell diameter and total loss of the transmitted light for human blood after four different storage intervals. Three different clusters can be distinguished after 2 hr of storage. Group one has a mean size of about 8 μm and shows low light scattering intensities representing discocytes. Group two shows a mean size of about 6 μm and higher light scattering intensities (echinocytes), whereas group 3 covers a wide size range with nearly equal light scattering behavior. This subclass is identical with nucleated white cells. The different subclasses have been identified in previous experiments after purification by gradient centrifugation. During the course of storage, more and more cells from group one shift over to the characteristics of group two, the echinocyte cell type. After 8 days most of the cells are found in group two. There is an obvious decrease in the number of cells in group three over the storage period.

A kidney transplant induces a very complex response of the immunologic system. Lymphocytes are part of this response. Purified rat lymphocytes from different parts of the lymphatic system, obtained 5 days after kidney transplantation, have been analyzed. Figure 4 shows the relation of the size to the total scattering intensity, measured for unstained cells as light loss out of the 5° foreward cone, for lymphocytes purified from the peripheral blood, the thymus, the inguinal lymph nodule and the spleen. Although no significant differences in morphology were obvious in light microscopy, the contour plots of the flow examination give some distinctions. Lymphocytes from the peripheral blood show a reasonable linear correlation of total scattering intensity and cell diameter. This correlation is not linear for lymphocytes taken from a lymph nodule. The medium sized cells give raise to the highest scattering intensities. Most of the lymphocytes taken from the spleen are found in a smaller size interval than those from the peripheral blood and the lymph nodule, but with equivalent light scattering intensities. However, a smaller number of lymphocytes have been detected, being larger in diameter than the majority and showing a high light-scattering intensity. In the case of thymus lymphocytes, cells with two different light scattering/size characteristics seem to overlap giving raise to this "one arm" contour plot. Correlations of these contour patterns to various responses of the lymphocytes within the immunological reaction are presently under investigation.

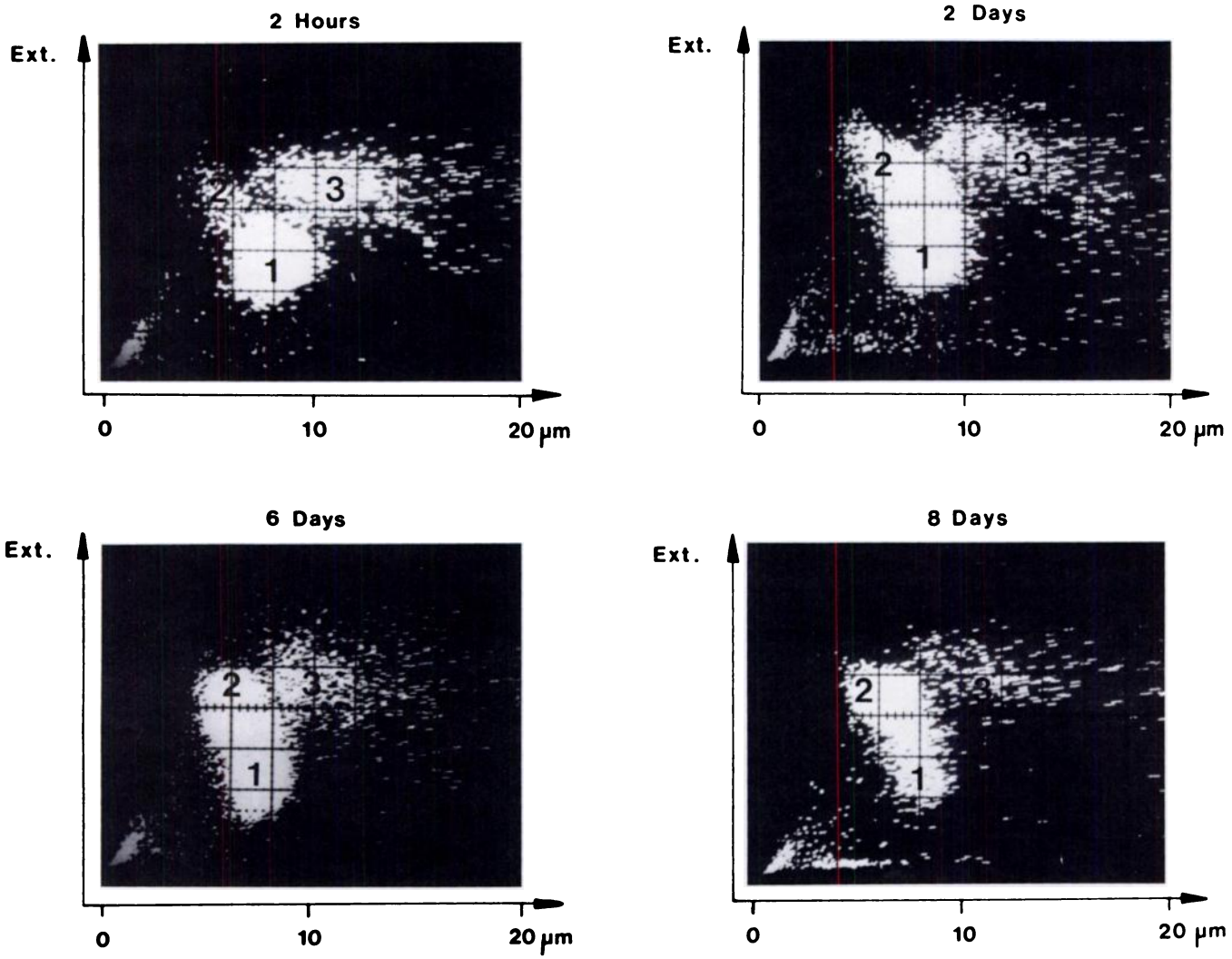

FIG. 3. Contour plots of cell size vs light loss in forward direction from unstained human blood after different storage intervals. Cells in group 1 are identified as discocytes, cells in group 2 as echinocytes and cells in group 3 as white blood cells.

The fraction of human blood that consists mainly of platelets, white blood cells and plasma, known as buffy coat, has been mixed with L 1210 lymphocyte tumor cells (Fig. 5, left column). The administration of a persantin derivative at the time of mixing the two different cell types changes the optical behavior of a number of cells (Fig. 5, right column), showing remarkable total unspecific extinction within a size range covered by other cell types also (marked: X). Persantin and some of its derivatives are known to interact with the cell membrane, generating a more rigid type of membrane. From the present knowledge it is believed that the remarkable difference between the treated and untreated sample is due to changes in the cell surface induced by that persantin derivative.

DISCUSSION

The information from light scattering from a cell is rather complex. Scattering intensities into several solid angles are needed as individual parameters for cell classification if no other independent parameter such as cell size or fluorescence intensities is available. Biological cell systems have been used in this study for the evaluation of cell classification from light scattering and absorption. The information on particle length, internally calibrated and displayed in absolute values, has been most helpful as an additional parameter. Red blood cells are known to change their shape when stored under experimental conditions. Although the cell volume remains nearly constant, the cell diameter decreases. Because red blood cells show only a limited number of internal structures compared with other classes of blood cells, the change in the characteristic light scattering can be related to the differences of the surface structures. No external manipulation with dyes, antibodies or microspheres are necessary for these surface changes. Therefore this system seems to be of more general interest for probing light scattering.

The results obtained from lymphocytes show different scattering characteristics for cells obtained from different parts of the lymphatic system. This gives rise to the hope that with better knowledge of the scattering properties of the lymphocyte subclasses, more immunological information may be

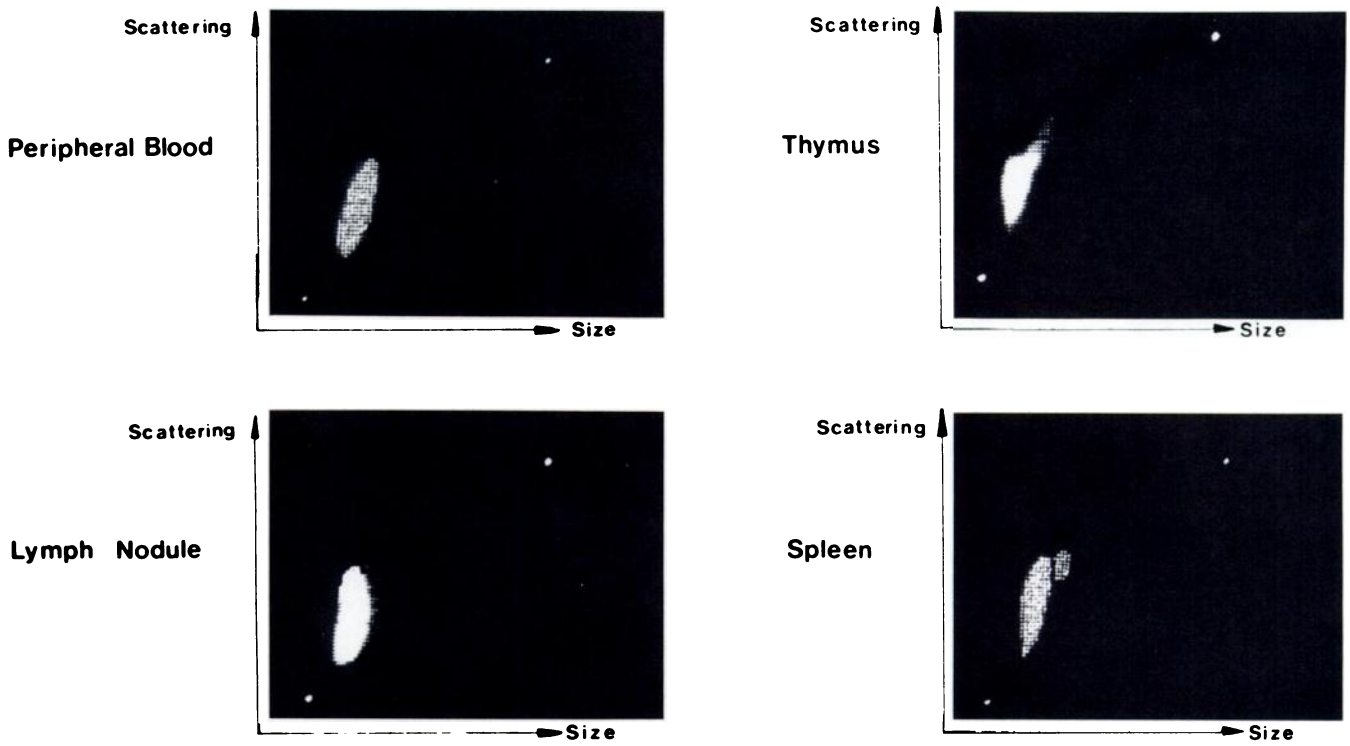

FIG. 4. Contour plots of cell size *vs* total light scattering (taken from O.D. measurements) of unstained lymphocytes. Cells were purified from different parts of the lymphatic system of a rat 5 days after transplantation of a kidney into the abdomen.

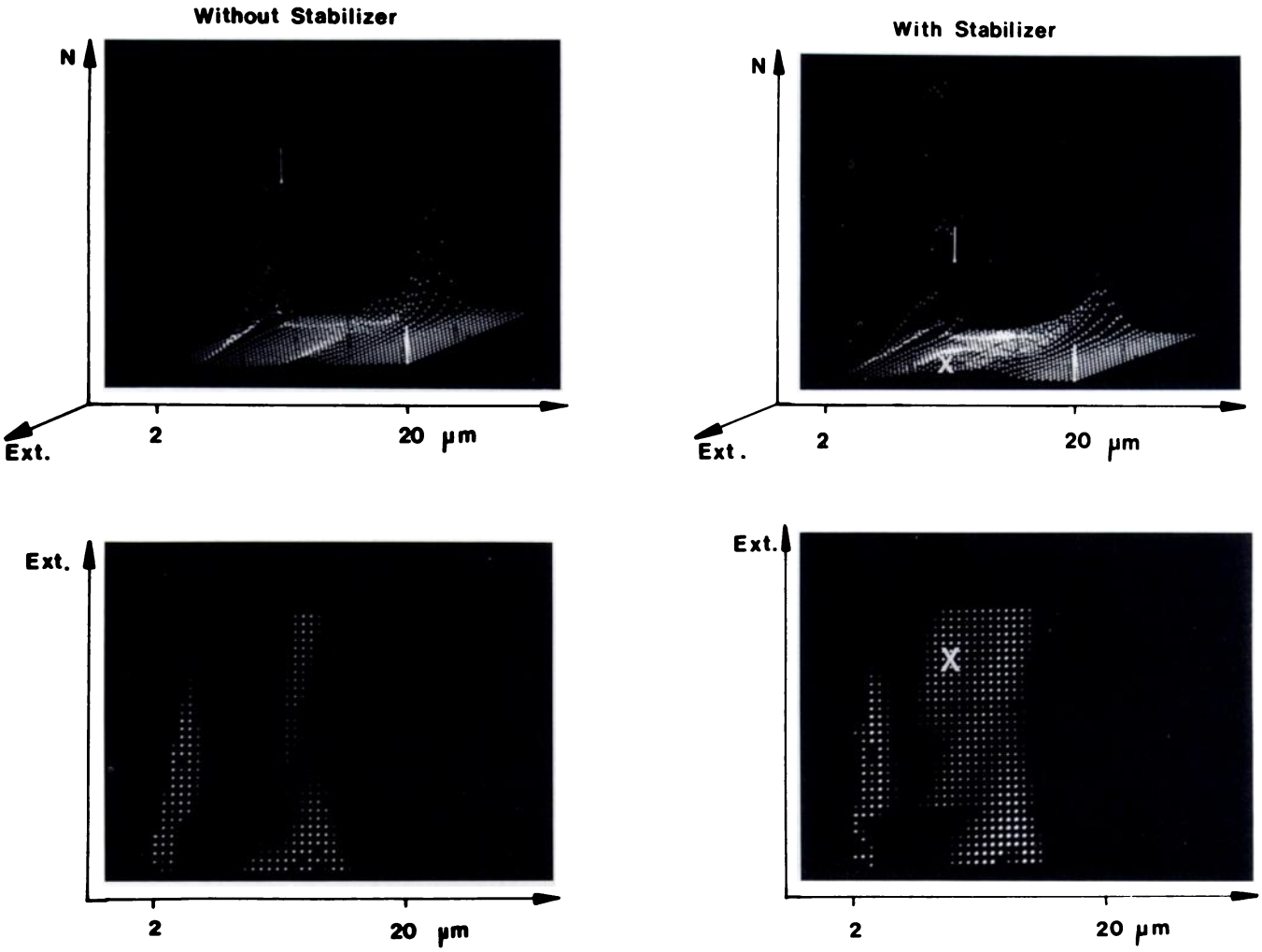

FIG. 5. Contour and profile plot of cell size *vs* light loss in forward direction from unstained L 1210 cells mixed with human buffy coat. With (left column) and without (right column) the addition of a persantin derivative.

drawn from light scattering. All results demonstrate that in biological material showing a wide variety of internal structures of different optical indexes and a broad spectrum of different surface structures, in addition to other parameters, light scattering can be of help for further classification; but deriving cell size information from light scattering peak intensities only may be erroneous. However, further experimental and theoretical investigations of model systems are necessary to optimize the use of light scattering techniques for cell classification.

LITERATURE CITED

1. Arndt-Jovin DJ, Jovin TM: Analysis and sorting of living cells according to deoxyribonucleic acid content. J Histochem Cytochem 25:585, 1977
2. Bessis M, Weed RJ: The structure of normal and pathologic erythrocytes. Adv Biol Med Phys 14:35, 1973
3. Brunsting A, Latimer P: Effects of asphericity on single particle scattering. Presented at the Sixth Engineering Foundation Conference on Automated Cytology, Schloss Elmau, April 1978
4. Cooke DD, Kerker M: Response calculations for light-scattering aerosol particle counters. Appl Opt 14:737, 1975
5. Eisert WG: Fast cell size distribution analysis by laser flow microphotometry—Applications to ciliate populations. Microsc Acta 78:228, 1976
6. Eisert WG, Nezel M: Internal calibration to absolute values in flow-through particle size analysis. Rev Sci Instrum; 49:0084, 1978
7. Eisert WG, Ostertag R, Niemann E-G: Simple flow microphotometer for rapid cell population analysis. Rev Sci Instrum 46:1021, 1975
8. Goehde W, Dittrich W: Simultane Impulsfluorimetrie des DNS- und Proteingehaltes von Tumorzellen. Z Anal Chem 252:328, 1970
9. Jovin TM, Morris SJ, Striker G, Schultens HA, Digweed M, Arndt-Jovin DJ: Automatic sizing and separation of particles by ratios of light scattering intensities. J Histochem Cytochem 24: 269, 1976
10. Kamentsky LA: Cytology automation. Adv Biol Med Phys 14:93, 1973
11. Kerker M: Internal structure by light scattering and fluorescence. Presented at the Sixth Engineering Foundation Conference on Automatic Cytology, Schloss Elmau, April 1978
12. Loken MR, Parks DR, Herzenberg LA: Identification of cell asymmetry and orientation by light scattering. J Histochem Cytochem 25:790, 1977
13. Loken MR, Sweet RG, Herzenberg LA: Cell discrimination by multiangle light scattering. J Histochem Cytochem 24:284, 1976
14. Mansberg HP, Saunders AM, Groner W: The hemalog d white cell differential system. J Histochen Cytochem 22:711, 1974
15. Meyer RA, Brunsting A: Light scattering from nucleated biological cells. Biophys J 15:191, 1975
16. Morris VJ, Jennings BR: Anomalous diffraction approximation to the low-angle scattering from coated spheres. Biophys J 17:95, 1977
17. Mullaney PF, Fiel RJ: Cellular structure as revealed by visible light scattering: studies on suspensions of red blood cell ghosts. Appl Opt 15:310, 1976
18. Mullaney PF, Steinkamp JA, Crissman HA, Cram LS, Crowell JM, Salzman GC, Martin JC: Laser flow microphotometry for rapid analysis and sorting of mammalian cells. Ann NY Acad Sci 267:176, 1976
19. Salzman GC, Crowell JM, Goad CA, Hansen KM, Hiebert RD, La Bauve PM, Martin JC, Ingram ML, Mullaney PF: A flow-system multiangle light-scattering instrument for cell characterization. Clin Chem 21:1297, 1975
20. Salzman GC, Crowell JM, Hansen KM, Ingram M, Mullaney PF: Gynecological specimen analysis by multiangle light scattering in a flow system. J Histochem Cytochem 24:308, 1976
21. Shapiro HM, Schildkraut ER, Curbelo R, Turner RB, Webb RH, Brown DC, Block MJ: Cytomat - R: a computer-controlled multiple laser source multiparameter flow cytophotometer system. J Histochem Cytochem 25:836, 1977
22. Sharpless TK, Melamed MR: Estimation of cell size from pulse shape in flow cytofluorometry. J Histochem Cytochem 24:257, 1976
23. Steinkamp JA, Fulwyler MJ, Coulter JR, Hiebert RD, Horney JL, Mullaney PF: A new multiparameter separator for microscopic particles and biological cells. Rev Sci Instrum 44:1301, 1973
24. Steinkamp JA, Hansen KM, Crissman HA: Flow microfluorometric and light-scatter measurement of nuclear and cytoplasmic size in mammalian cells. J Histochem Cytochem 24:292, 1976
25. Steinkamp JA, Romero A, Horan PK, Crissman HA: Multiparameter analysis and sorting of mammalian cells. Exp Cell Res 84:15, 1974
26. Stöhr M: Double beam application in flow techniques and recent results. Pulse—Cytophotometry II:39, 1976
27. Stöhr M, Eipel H, Goerttler K, Vogt-Schaden M: Extended application of flow microfluorometry by means of dual laser excitation. Histochemistry 51:305, 1977
28. Wheeless LL, Patten SF: Slit-scan cytofluorometry. Acta Cytol 17:333, 1973