FLOW CYTOMETRIC MEASUREMENT OF THE POLARIZATION OF FLUORESCENCE FROM INTRACELLULAR FLUORESCEIN IN MAMMALIAN CELLS

T. LINDMO AND H. B. STEEN, *Department of Biophysics, Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo 3, Norway*

ABSTRACT Based on the description of a laboratory-built flow cytometer, the necessary modifications of this instrument for the measurement of fluorescence polarization are described. At a maximum rate exceeding 1,000 cells/s, the instrument is capable of measuring simultaneously the horizontally and vertically polarized component of the fluorescence emitted from stained cells excited with vertically polarized light. By mathematical analysis of the accumulated data, the distribution of polarization values in the population is obtained. Various sources of instrumental error have been investigated. The large aperture of the detector optics leads to systematic underestimation of the polarization values. Other errors are negligible, and the instrument is shown to to give results consistent with the theory of fluorescence polarization. Application of the instrument is illustrated by experiments with mammalian cells exposed to the fluorogenic substrate fluorescein diacetate (FDA). The polarization of the fluorescence from intracellular fluorescein produced by hydrolysis of FDA is measured, giving information on the cytoplasmic microviscosity. It appears that this microviscosity is constant over the cell cycle. On the other hand, it is significantly affected by the osmolarity of the medium.

INTRODUCTION

Measurements on fluorescence polarization have been utilized to gain information on various physical properties of cells. With suitable fluorescent probes, such parameters as intracellular microviscosity (1–5), or the viscosity of the cellular membrane lipids (6, 7), as well as the rotational relaxation time of lectins bound to the cellular surface membrane (8), have been measured.

In contrast to the conventional technique of measuring fluorescence polarization on a sample of cell suspension in the cuvette of a spectrofluorometer, Arndt-Jovin et al. (9) introduced the technique of measuring fluorescence polarization on a single-cell basis in a flow cytometer. Their instrument is also capable of sorting cells according to their polarization properties.

The present work shows how a general-purpose laboratory-built flow cytometer (FCM) may be modified for the measurement of fluorescence polarization. Various possibilities for instrumental artifacts are investigated. Application of the instrument

is illustrated by the measurement of the polarization of fluorescence from intracellular fluorescein produced by hydrolysis of fluorescein diacetate (FDA) (10) in cells of the established human cell line NHIK 3025.

INSTRUMENTATION

The Flow Cytometer

The instrument is built as a general-purpose flow cytometer with two fluorescence detectors and one light-scatter detector, and is basically similar to already published designs (11–13).

Some special optical components must be used for the measurement of fluorescence polarization. The principal parts of the instrument, as equipped for this purpose, are shown in Fig. 1. The sample stream emerges as a free jet from the nozzle of a flow chamber and intersects the exciting light in air, thus defining the optical focus for the detection optics. The sample, in general a monodisperse suspension of fluorescently stained cells, is kept in a pressurized sample container. The sample stream is introduced along the axis of the flow chamber, which is built around a glass capillary (Specialty Glass Products, Inc., Willow Grove, Penn.) with an orifice diameter of 100 μm. A flow of sheath fluid, usually distilled water, from another pressurized reservoir is also introduced into the flow chamber, thus creating a coaxial, laminar flow of sample and sheath fluid. The sample stream is confined to the central 15 μm of the jet. The relative amount of sample to sheath flow is adjustable by means of a needle valve in the sheath fluid flow path. The diameter of the sample stream within

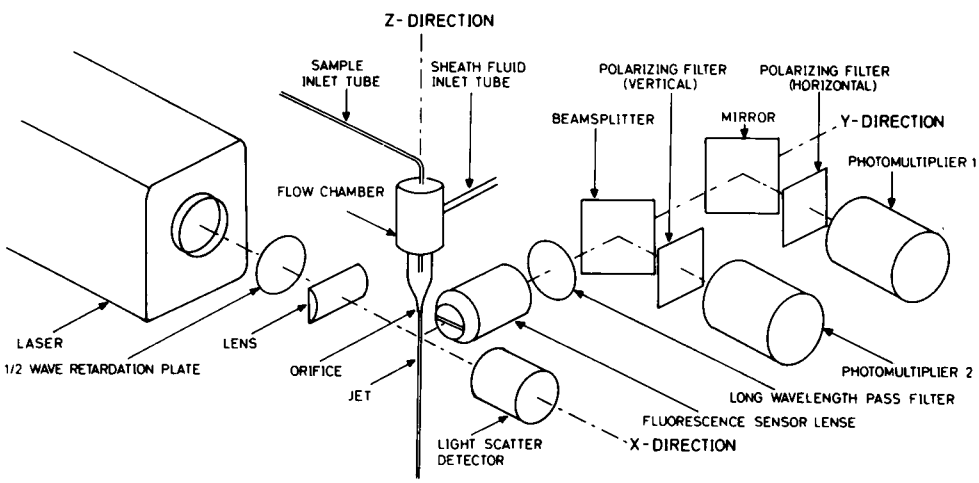

FIGURE 1 Principal components of the flow cytometer as equipped for the measurement of fluorescence polarization. The laser light is vertically polarized, and the direction of observation is at right angles to both the direction of propagation and the direction of polarization of the exciting light. The half-wave retardation plate is used to rotate the polarization vector from vertical to horizontal for calibration. The pressurized containers for the sample and for the sheath fluid are not shown, nor is any of the electronic equipment.

the cylindrical liquid jet may easily be monitored by observing the jet with a stereomicroscope when running a fluorescent sample solution. Usually the total flow rate is about 9 ml/min and the sample flow rate about 0.2 ml/min.

The exciting light comes from an argon laser (4W, Spectra Physics, Mountain View, Calif.) with a beam diameter of 1.4 mm. By means of a cylindrical lens (focal length 25 mm) the beam is focused to a 15-μm-thick horizontal band of light that intersects the liquid jet about 1 mm below the nozzle tip. Thus, in the central part of the jet the intensity of the illumination is constant in the plane perpendicular to the jet.

Nonfluorescent particles or cells may be detected by light scatter (14). For this purpose a silicone photodiode (PIN 10, United Detector Technology, Inc., Santa Monica, Calif.) is placed in the laser beam, collecting light scattered at small angles around the forward direction. The detector is protected by a small beam stop at the center of the detector surface.

The fluorescence light is collected by a microscope objective (32x, E. Leitz, Inc., Rockleigh, N.J.) of large numerical aperture (0.6) and long working distance (5.7 mm) focused to the spot where the sample stream intersects the laser beam. An external obscuration in the form of a 0.5-mm-thick strip of metal is placed in front of the objective to stop laser light spread in the horizontal plane by the cylindrical surface of the water jet. To prevent scattered light at the laser wavelength from entering the fluorescence detection optics, a long-wavelength pass interference filter (LWP 525 nm, Optisk Laboratorium, Copenhagen, Denmark) is placed behind the fluorescence collecting objective. By means of a beam-splitter and a front surface mirror, the fluorescent light is directed onto two photomultipliers (9558 and 9658, EMI Gencom, Plainview, N.Y.). By a proper selection of filters in front of each photomultiplier, two different properties of the emitted fluorescence may be isolated and independently measured; e.g. the measurement of two-color fluorescence (14) or the detection of two polarization components.

The pulses of fluorescence emitted from stained cells or particles as they pass through the laser beam generate electrical pulses from the photomultipliers with pulse-length of about 1 μs, and height proportional to the intensity of the emitted fluorescence. The pulses are amplified, integrated, and fed into a multichannel analyzer (MCA) (BA-163, Intertechnique, Dover, N.J.) that classifies the pulses according to pulse height and accumulates the results so that channel number in the storage matrix is proportional to pulse height. The MCA has the capability of two-parameter analysis. For that purpose, the 4,096-channel memory is organized in a two-dimensional array of 64 $\times$ 64 channels.

The special components needed for polarization measurements are polarizers (Polacoat, Ltd., Cincinnati, Ohio) in front of each photomultiplier oriented so as to transmit only the vertically or the horizontally polarized component of fluorescence; an achromatic beam-splitter (TF-MT-45, Balzers High Vacuum Corp., Santa Ana, Calif.); and a $\frac{1}{2}$-wave retardation plate (zero-order quartz 488 nm, Oriel Corp. of America, Stamford, Conn.), which by 45° rotation changes the direction of polarization of the laser beam from vertical to horizontal.

The polarization of the emitted fluorescence is expressed as (15, 16):

$$P = (I_v - I_h)/(I_v + I_h). \quad (1)$$

I_v and I_h are the intensities of the vertically and horizontally polarized components of fluorescence. A calibration procedure is required to obtain the same gain factor for the detector channels measuring these two components.

Calibration is based on the fact that with horizontally (y-direction, Fig. 1) polarized light, i.e. with the electric vector of the exciting light parallel to the direction of observation, the fluorescence emitted in this direction is completely unpolarized (17, 18). In this case, therefore, the vertically and horizontally polarized components of the emitted light are equally intense, and the detector system may be adjusted to give signals of equal magnitude for the two components. For the calibration procedure it is convenient to use standard particles such as fluorescent microspheres (Particle Technology, Inc., Los Alamos, N.M.) or ethanol-fixed chick erythrocytes stained with ethidium bromide, as illustrated in Fig. 2. When a series of biological samples is analyzed, calibration is usually performed for each sample. After the measurement with vertically (z) polarized exciting light is finished and data are stored on tape, the polarization of the exciting light is changed to horizontal (y), and a calibration recording is made while the same sample is still running. No instrument adjustments are made, but the calibration recording is used in the subsequent data analysis to correct for possible instrumental drift.

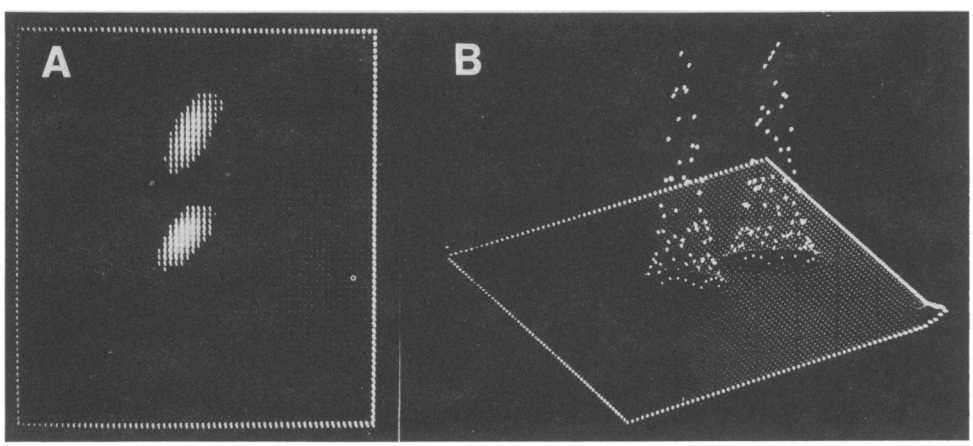

FIGURE 2 Primary data of polarized fluorescence recorded from ethanol-fixed chick erythrocytes stained with ethidium-bromide (10 mg/liter in Tris buffer). Measurements were first performed with horizontally (y) polarized exciting light and the detector gain was adjusted to give counts on the diagonal of the display. Afterwards vertically (z) polarized exciting light was used, resulting in the cluster above the diagonal. A: Contour view. The intensity of the horizontally polarized component of fluorescence is measured on the horizontal axis, the vertically polarized component on the vertical axis. B: Isometric view of the same data as shown in A. The origin is to the left.

Data Analysis

Data are transferred in digital form from the MCA to a computer for mathematical analysis. The first step in the analysis is to correct the channel numbers (V and H) of the two-dimensional data-matrix for zero offset in the detector electronics measuring I_v and I_h. The corrected channel numbers, being proportional to the fluorescence intensities I_v and I_h, are:

$$V' = V + a_v \quad \text{and} \tag{2}$$

$$H' = H + a_h, \tag{3}$$

where a_v and a_h are the independently determined corrections for zero offset.

The measurements of polarized fluorescence may be calibrated if a recording with horizontally (y) polarized exciting light is available. A calibration factor is computed from the calibration recording according to the expression:

$$f = (1/N)\Sigma[n(V'_y/H'_y)]. \tag{4}$$

In this expression, the sum is taken over all memory positions, n indicates the number stored in a certain memory position, and N is the sum of all counts stored in the data matrix. With accurate adjustments of the photomultiplier voltages, the calibration recording gives counts on the diagonal of the display, and the value of f is 1.00.

The calibrated mean value of the polarization of fluorescence measured with vertically (z) polarized exciting light, is obtained as:

$$\bar{P} = (1/N)\Sigma[n(V'_z - fH'_z)/(V'_z + fH'_z)]. \tag{5}$$

The transformation of the two-parameter primary data into the one-parameter frequency distribution of polarization values is accomplished by calculating for each channel position the corresponding value of P:

$$P = (V'_z - fH'_z)/(V'_z + fH'_z). \tag{6}$$

The number of counts, n, associated with this channel position is then added to the polarization frequency distribution in the appropriate interval on the P-axis as determined by Eq. 6. This procedure is repeated for all channels in the primary data matrix, thus giving the frequency distribution showing how the polarization P varies within the sample.

With known corrections for zero offset as input to the computer program, the mathematical analysis of the data will yield histograms, mean values, and standard deviations for the distribution of polarization values as well as for the projection of data onto the vertical and horizontal axes.

ANALYSIS OF INSTRUMENTAL ERRORS

Aperture of Detector Optics

Analysis of polarization of fluorescence is usually based on the same geometry as shown in Fig. 1. The exciting light is vertically polarized (electric vector parallel to Z-axis) and propagates in the X-direction. Fluorescence emitted along the Y-axis is observed.

The polarization of light emitted along the Y-axis from a sample of randomly oriented molecules for which the transition moments for absorption coincide with those for emission, is $P_{max} = \frac{1}{2}$ (15). This is however, under the assumption of vanishingly small solid angle of detection. In practice a certain solid angle is required to obtain adequate sensitivity, and the light will have different degrees of polarization depending on the direction in which it is emitted. A mathematical analysis of the problem is included in the Appendix.

For our detector optics of numerical aperture 0.6, the value found for P_{max} in this theoretical situation is 0.47. Table I shows how P_{max} varies with numerical aperture of the detector optics. Taking into account the obscuration protecting the lens from deflected laser light leads to a small additional reduction in the mean value of P. With horizontally polarized exciting light, the average polarization detected over the entire solid angle defined by the detector lens should be zero, but the obscuration in front of the lens leads to an average P-value of 0.008. Hence, with the high numerical aperture of our detector lens, polarization values will systematically be found too small. The reduction is about 10% when the obscuration in front of the lens is also taken into account.

TABLE I

THE EFFECT OF NUMERICAL APERTURE OF THE DETECTOR OPTICS ON POLARIZATION VALUES.

NA = sin α_{max}	α_{max}	P_{max}
0.0	0.0°	0.500
0.1	5.7°	0.499
0.2	11.5°	0.497
0.4	23.6°	0.489
0.6	36.9°	0.472

The direction of observation is at right angles to the direction of propagation and the direction of polarization of the plane-polarized exciting light. The sample is assumed to consist of randomly oriented fluorescent molecules with coinciding transition moments for absorption and emission. The values have been found by numerical integration of Eq. 12 over the detector aperture.

Scattered Light

Whereas error due to any constant level of laser light scattered from the liquid jet is largely eliminated in measurement of pulses of emitted fluorescence, particles passing the laser beam will give rise to pulses of scattered light that are highly polarized and could give a false contribution to the polarization of the fluorescence signal. By running nonfluorescent polystyrene particles (14-μm diameter, Coulter Electronics, Inc., Hialeah, Fla.) through the instrument and using the light scatter detector in the forward direction as a monitor, it was verified that the long wavelength pass interference filter behind the fluorescence collecting objective efficiently eliminates scattered light at the laser wavelength.

Polarizing Power of Sheet Polarizers

The degree to which the polarizers were able to transmit only one component selectively was checked by putting crossed polarizers in front of one photomultiplier. The signal amplitude decreased to about 1% of the value obtained with parallel polarizers, indicating that the fluorescence signal is resolved with sufficient discrimination into its two polarization components.

Polarizing Effects in Optical Components

Since in the present instrument no components are optically active, i.e. able to rotate the plane of polarization, effects due to different transmission properties for the two polarization components, notably in the beam splitter, are fully compensated for when the gain of the detectors is adjusted so as to yield $P = 0$ with horizontally (y) polarized excitation. Possible variation in the photomultiplier response to vertically and horizontally polarized light is also compensated for by this procedure, as well as difference in gain between the photomultipliers.

According to the theory (see Appendix), the horizontally polarized component of fluorescence should be of equal intensity for vertically (z) and horizontally (y) polarized exciting light. By analysis of data shown in Fig. 2, it is found that the intensity of the horizontally polarized component does indeed remain constant (within 3%) when changing direction of polarization of the exciting light. This result confirms that the intensities of the two components of polarized fluorescence are independently measured.

Chromatic Errors

In most components, the optical properties are not invariant to a change in wavelength. This may cause error if calibration is performed with a sample having fluorescence in a different wavelength region than the sample studied for polarized fluorescence. In the present instrument, the calibration factor was found to change by about 5% when running samples with emission maxima at 550 and 590 nm. By performing the measurements also with cross-exchanged photomultipliers, the effect was shown to be mostly due to differences in spectral response between the two detectors. With the

present calibration procedure using horizontally polarized exciting light, chromatic errors are excluded since the sample of interest for polarization measurements is also used for calibration.

Linearity and Zero Offset of Pulse-processing Electronics

Linearity of the detector system was found to be satisfactory, but zero offset was present in both detector channels, so that only the part of the signal pulse rising above a certain threshold would be analyzed. Measurement of fluorescence polarization is very sensitive to nonlinearities and zero offset in the detectors measuring the two polarization components, since the expression for P (Eq. 5) involves the difference between the two signals divided by their sum.

Fig. 3 A shows how the zero offset was determined for each of the two detectors. With voltage divider circuits, the input to the pulse amplifiers and processing electronics could be chosen in discrete steps from 0.01 to 1.00. The response of the detector system could thus be measured for a series of well-defined levels of input amplitudes. The fraction of the input signal applied was varied in discrete steps, and for each step a recording was made from about 10^4 particles. In Fig. 3 A the channel position of the cluster of counts is plotted against the fraction of input signal applied. The amount

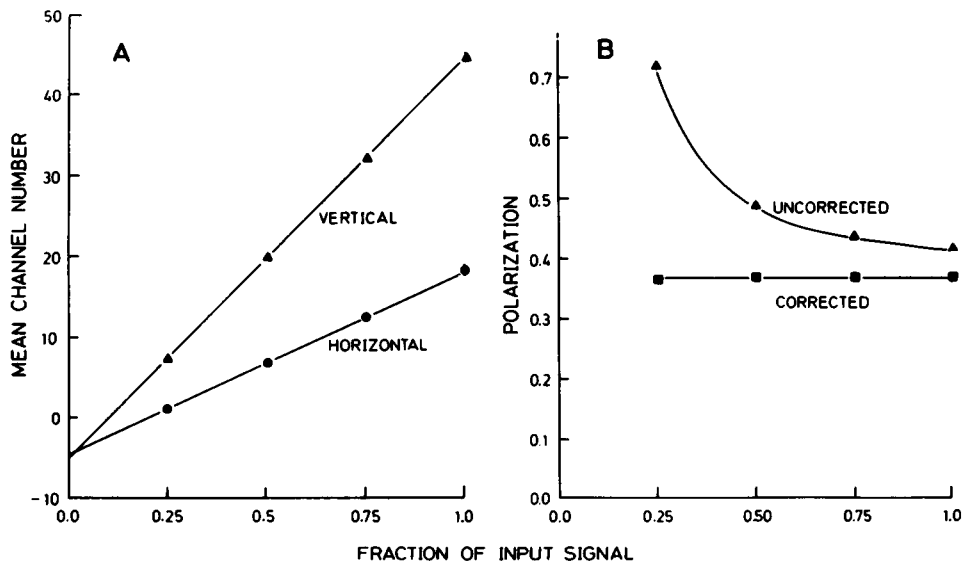

FIGURE 3 A: Determination of zero offset for pulse-processing electronics. A series of recordings were made using fluorescent microspheres and vertically (z) polarized exciting light. By mathematical analysis the position of each cluster of counts was determined as the mean channel number on the vertical (▲) and horizontal (●) axes. These values are plotted as a function of the fraction of input signal applied to the pulse processing electronics. The zero offset was found to be for the vertical axis a_v = 5.0 channels, for the horizontal axis: a_h = 4.5 channels. B: The polarization value as function of input signal magnitude with (■) and without (▲) correction for zero offset. Same primary data as used in A.

of zero offset was determined for each axis by regression analysis, and the lines drawn in Fig. 3 A represent the least squares fit to the data.

Fig. 3 B shows how the apparent values of P would vary with the signal level if the correction for zero offset was omitted, clearly demonstrating the necessity of this correction. It is also shown in Fig. 3 B that with proper correction, the polarization values found are independent of signal magnitude. It was verified that this correction also gave constant polarization values for a series of different signal amplitudes obtained by varying the laser output power.

BIOLOGICAL APPLICATIONS

Material and Methods

The cells used were of the established human cell line NHIK 3025 (19–21), which originates from a cervix carcinoma. The cells were cultivated as monolayers in plastic flasks (75 cm^2, Nunclon, A/S Nunc, Roskilde, Denmark, or Falcon, Div. of BioQuest, Oxnard, Calif.) in medium E2a (22). Synchronous populations of these cells were obtained by the method of mitotic selection (23, 24). Single cell suspensions were obtained by trypsinization and resuspension in phosphate-buffered saline (PBS) (Grand Island Biological Co., Grand Island, N.Y.).

1 μM FDA was prepared by vigorously shaking 5 μl of 20 mM FDA (Koch-Light Laboratories, Ltd., Colnbrook, Bucks, England) in spectroscopic grade acetone (Eastman Kodak Co., Rochester, N.Y.) into 100 ml of lukewarm PBS or distilled water, depending on the application.

0.4 ml of cell suspension (usually about $2.5 \cdot 10^5$ cells/ml) was added to 0.8 ml of 1 μM FDA. FDA is readily taken up by the cells because of its low polarity (10) and hydrolyzed into fluorescein, which does not easily leak out through the cell membrane. The intracellular

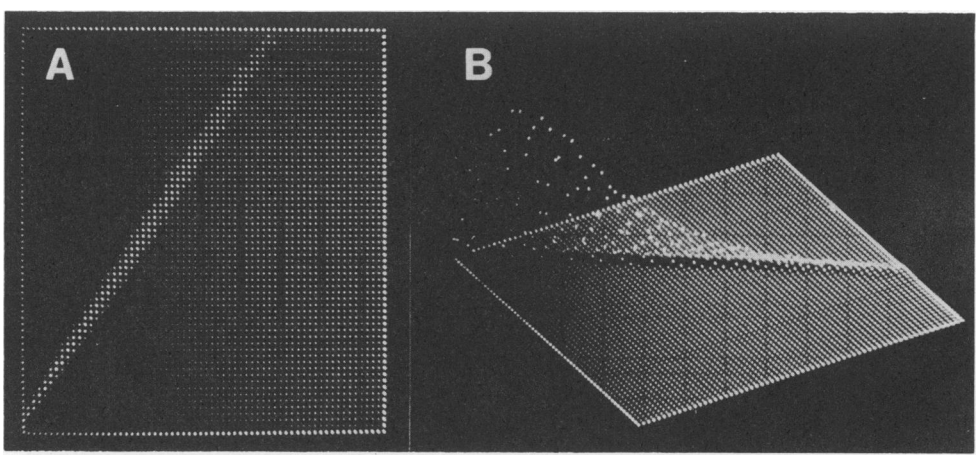

FIGURE 4 Primary data of polarized fluorescence from an exponentially growing population of NHIK 3025 cells, recorded for 3 min, starting 3 min after mixing of cell suspension and FDA solution. Final concentration was 0.67 μM FDA in PBS. The diagram shows data from 53,000 cells. The (calibrated) mean value of polarization found after mathematical analysis is $P = 0.174$, and the standard deviation of the distribution is $\sigma = 0.025$. A: Contour view. The intensity of the horizontally polarized component is measured on the horizontal axis, the vertically polarized component on the vertical axis. B: Isometric view of the same data as shown in part A. The origin is to the left.

concentration of fluorescein will therefore increase with time during the first 10–30 min after mixing. The recording of fluorescence polarization was started after 3 min and lasted for 3 min. After data were stored on tape, a calibration recording with horizontally polarized exciting light was performed on the same sample. The spread in calibration values for a series of samples was 2–3%. The experiments were done at a temperature of 26 ± 1°C, with exciting light of 800 mW at 488 nm.

Results

Fig. 4 shows primary data for a population of exponentially growing NHIK 3025 cells. In Fig. 5 A the polarization distribution of this sample is combined with data from two other samples obtained under the same experimental conditions. The mean value of the resulting polarization distribution if $P = 0.177$ and the standard deviation is $\sigma = 0.025$, resulting in a coefficient of variance of 14%. Taking into account the systematic

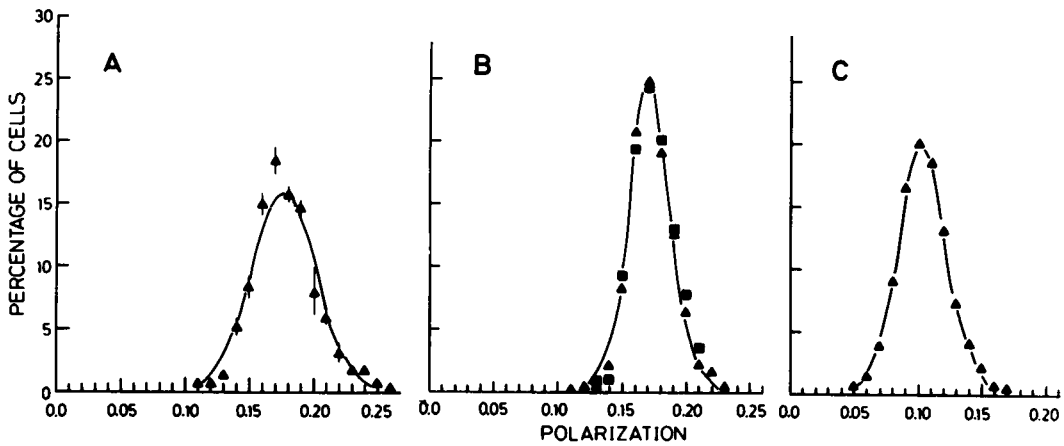

FIGURE 5 Various distributions of polarization values found after mathematical analysis of data of polarized fluorescence from populations of NHIK 3025 cells. Measurements were taken in the time interval 3–6 min after addition of FDA to a final concentration of 0.67 μM. A: Exponentially growing cell population. Equivalent data to those shown in Fig. 4 were obtained from three different samples, normalized to the same total number of registered cells, and combined to yield the distribution shown. Each diagram contained data from about $5 \cdot 10^4$ cells. The (calibrated) mean value of polarization is $P = 0.177$. The standard deviation of the distribution is $\sigma = 0.025$. The variance of the results obtained is illustrated by the vertical bars indicating ±SEM value obtained from the three different samples. The fully drawn line is the Gaussian distribution with the same mean value and standard deviation as the distribution of polarization values. B: Synchronous cell populations harvested at 4 (▲) and 10 (■) h after mitotic selection, corresponding to the middle of the G1 and S phases of the cell cycle. The (calibrated) mean value of polarization of G1 cells is $P = 0.174$ and the SD of the distribution is $\sigma = 0.017$. The corresponding values for cells in the middle of S phase are $P = 0.175$, and $\sigma = 0.016$. For both G1 and S cells normalized data from two different samples, each about 4,000 cells, were combined. C: Exponentially growing cell population in hypotonic PBS of osmolarity 0.10. The (calibrated) mean value of polarization is $P = 0.105$, and the SD of the distribution is $\sigma = 0.021$. Normalized data from two different samples were combined. For each sample about $5 \cdot 10^4$ cells were analyzed.

underestimation of P, this result is in good agreement with the value $P = 0.186$ obtained by conventional fluorometry.[1]

When a calibration recording was made in the same time interval as for polarization measurement (3–6 min after mixing), the P-values of the calibration recording showed the same standard deviation as those for polarization measurement. Thus, the variance in P-values is the same for polarized and unpolarized fluorescence when recorded under conditions of equal fluorescence intensity. When the calibration recording was performed after the measurement of polarized fluorescence, i.e. at higher fluorescence intensity, the distribution of P-values for the calibration recording showed smaller standard deviation than the distribution of polarized fluorescence. The conclusion may be drawn that the width of the distribution of polarization values is not due to differences in polarization between individual cells, but only due to instrumental factors of which photon statistics is an important contribution. If it is assumed that variances due to different factors are additive, the true coefficient of variance of the distribution of polarization values must be less than 5% to make no significant contribution to the total variance of the experimentally measured distribution.

The cells used to obtain the data shown in Fig. 5 A were in exponential growth. The distribution of cells among the various phases of the cell cycle was determined by FCM measurement of DNA content (25) and subsequent mathematical analysis of the DNA histogram (26). The result for this particular population was 46% cells in G1, 39% in S, and 15% cells in G2 and mitosis, in good agreement with previous data for this cell line (24). Since the distribution of polarization values obtained from this population (Fig. 5 A) has a rather low coefficient of variance (14%), mostly due to experimental factors, it is therefore concluded that no significant fraction of the cell population can have polarization values that differ markedly from the value found for the majority of the cells.

This result is at variance with that reported by Cercek et al. (5), who found a significant change in polarization through the cell cycle for synchronous populations of Chinese hamster ovary cells. They found the value $P \simeq 0.16$ for G1 cells, compared to the value for S-cells of $P \simeq 0.10$.

Our conclusion that for NHIK 3025 cells the polarization does not vary through the cell cycle was verified by similar measurements on synchronous populations taken 4 and 10 h after mitotic selection, i.e. in the middle of the G1 and S phases of the cell cycle as illustrated by the DNA-histograms shown in Fig. 6. The distribution of P-values for the two cases are shown in Fig. 5 B. The mean values of polarization were 0.174 and 0.175 for G1 and S-cells, respectively. The distributions obtained for cells in G1 and for cells in S are identical, and they correspond well with the distribution obtained for the asynchronous population (Fig. 5 A).

The degree of fluorescence polarization measured in NHIK 3025 cells suspended in FDA solution was found to change with the osmolarity of the solution. Fig. 5 C shows

[1] Steen, H. B., T. Lindmo, and E. O. Pettersen. Intracellular viscosity during the cell cycle of NHIK 3025 cells and upon stimulation of lymphocytes by mitogens. Submitted for publication.

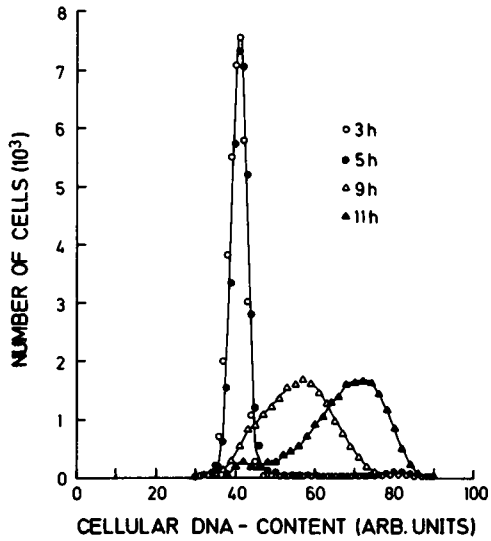

FIGURE 6 DNA-histograms of synchronous populations of NHIK 3025 cells at various times after mitotic selection. The channel numbers on the abscissa are proportional to cellular DNA content. Unfixed cells were stained with mithramycin and analyzed in the flow cytometer by using the laser wavelength of 457.9 nm. 40,000 cells were recorded for each histogram. Populations of NHIK 3025 cells synchronized by mitotic selection enter S-phase about 7 hr after selection, and DNA synthesis then lasts for approximately 8 h (24). Cells in G2 and mitosis (observed after 15 h) exhibit a peak centered at channel 82 with the same coefficient of variance as for the G1 peak (4.5%) centered at channel 41.

the polarization distribution of an asynchronous population of NHIK 3025 cells in PBS diluted to a final osmolarity of 0.1. The mean value of the distribution is P = 0.10 and the standard deviation is 0.02. The result was obtained for duplicate samples, and was confirmed in another independent experiment. The decrease in fluorescence polarization from P = 0.177 (Fig. 5 A) in a solution of 0.31 osM (PBS) to P = 0.105 in a solution of 0.1 osM is in accordance with the decrease from P = 0.185 to P = 0.11 reported by Cercek and Cercek (1) for lymphocytes at the same osmolarities.

CONCLUSION

The method of flow cytometry (FCM) has several advantages compared to conventional fluorometry of cell suspensions: (*a*) The fluorescence characteristics of each single cell are measured. This implies that in addition to the average values determined by conventional fluorometry on suspensions, one may also obtain the distribution of the various parameters, e.g. intensity and polarization, within a population of cells. Thus, subpopulations with properties different from those of the majority of cells may readily be detected. (*b*) A large number of cells can be measured in a short time, i.e. more than 10^3 cells/s. Thus, time resolution and statistics are greatly improved compared to the time-consuming measurement of cells using microscope fluorometers (3). (*c*) By FCM only fluorescence from the cells themselves is recorded, whereas any background arising from the medium goes undetected, so that corrections and possible er-

rors due to this background are eliminated. (*d*) FCM may readily be extended to include cell sorting (9), so that viable cell populations may be separated on the basis of their fluorescence properties.

However, in dynamic studies FCM has certain limitations. To reduce statistical variability to a reasonable level, each FCM recording takes a certain time, e.g. 30,000 cells in 30 s, whereas the measurement in conventional fluorometry is virtually instantaneous in comparison.

The fact that any background fluorescence is eliminated by FCM may also be a disadvantage. Hence, conventional fluorometry is needed to obtain information on extracellular fluorescence, e.g. if the leakage of fluorescein in experiments with fluorochromasia is to be studied.

Obviously, a combination of FCM and conventional fluorometry provides the most powerful experimental approach.[1]

APPENDIX

The Polarization Observed at an Angle β From the Direction of Polarization of the Exciting Light

Because of the rotational symmetry around the direction of polarization of the exciting light, it is sufficient to consider light emitted in the plane normal to the direction of propagation of the exciting light (the plane *OZY*). The polarization of the ray of light reaching the detector lens at an angle α with the detector axis is sought. This ray is at an angle β with the direction of polarization of the exciting light ($\alpha + \beta = 90°$). Fig. 7 shows the coordinate system used for analysis of the problem. Compared to Fig. 1, the coordinate system has been rotated around the *X*-axis so that the instantaneous direction of observation is along the *Y*-axis, the angle between the *Z*-axis and the electric vector **e** of the exciting light thus

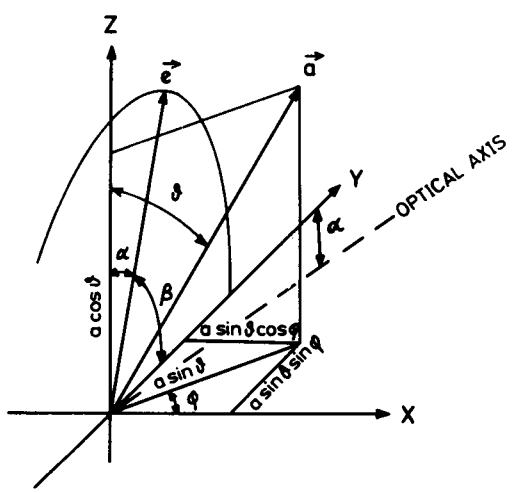

FIGURE 7 Coordinate system for the theoretical analysis of polarization of fluorescence emitted in the *Y*-direction, at an angle β to the direction of polarization of the exciting light and at an angle α to the axis of the detector optics ($\alpha + \beta = 90°$).

being α. The transition moments for absorption and emission of the molecule considered are assumed to be parallel and fall along the unit vector **a**.

The intensity of the vertically and horizontally polarized light emitted along OY is (15):

$$I_v \propto |p|^2 \cdot \cos^2 \vartheta, \tag{7}$$

$$I_h \propto |p|^2 \cdot (\sin \vartheta \cdot \cos \varphi)^2. \tag{8}$$

p is the projection of the vector **e** along the direction of **a**, and $|p|^2$ is proportional to the probability of absorption. By vector analysis from Fig. 7, it is seen that:

$$|p|^2 = (\cos \beta \cdot \sin \vartheta \cdot \sin \varphi)^2 + (\sin \beta \cdot \cos \vartheta)^2. \tag{9}$$

By taking all molecular orientations into consideration with equal probability by integrating ϑ and φ over the spherical surface, it is found that

$$I_v(\beta) = 2 \sin^2 \beta + 1, \tag{10}$$

$$I_h(\beta) = 1, \tag{11}$$

$$P(\beta) = \sin^2 \beta / (\sin^2 \beta + 1). \tag{12}$$

Common constants in the expressions for $I_v(\beta)$ and $I_h(\beta)$ have been omitted in the above equations. The maximum value of polarization is $P = \frac{1}{2}$ for $\beta = 90°$, corresponding to emission along the optical axis of the detector system. The minimum value of polarization is $P = 0$ for $\beta = 0°$, i.e. fluorescence emitted in the direction of polarization of the exciting light is completely depolarized. Numerical integration of Eq. 12 over the detector aperture yields the results given in Table I.

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