Fluorescence Resonance Energy Transfers Measurements on Cell Surfaces via Fluorescence Polarization

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ABSTRACT A method has been developed for the determination of the efficiency of fluorescence resonance energy transfer efficiency between moieties located on cell surfaces by performing individual cell fluorescence polarization (FP) measurements. The absolute value of energy transfer efficiency (*E*) is calculated on an individual cell basis. The examination of this methodology was carried out using model experiments on human T lymphocyte cells. The cells were labeled with fluorescein-conjugated Concanavalin A (ConA) as donor, or rhodamine-conjugated ConA as acceptor. The experiments and results clearly indicate that determination of *E* via FP measurements is possible, efficient, and more convenient than other methods.

INTRODUCTION

Measurements of Fluorescence Resonance Energy Transfer have been applied to a wide range of problems in molecular biology (Stryer, 1978). This technique has been used to obtain both static and dynamic information about intramolecular (Hahn and Hammes, 1978; Zukin et al., 1977; Luedtke et al., 1981) and intermolecular (Epe et al., 1982; Damjanovich et al., 1977; Stryer et al., 1982; Lobb and Auld, 1980; Thomas and Stryer, 1982) distance relationships.

In systems with donor and acceptor fluorophores located in well-defined sites, the interpretation of the energy transfer efficiency (*E*) is straightforward. In molecular systems with a random distribution of donors and acceptors, the measured *E* represents an averaged value over the different individual donor–acceptor orientations and stoichiometry (Gennis and Cantor, 1972; Gennis et al., 1972).

Resonance energy transfer between specific sites on the cytoplasmic membrane of mammalian cells has been investigated experimentally on bulk cell suspension using steady-state macrofluorimeter (Dale et al., 1981), on a single-cell basis using static (Fernandez and Berlin, 1976), and flow cytometry (Chan et al., 1979; Jovin and Arndt-Jovin, 1982; Jovin, 1979). The measurements of energy transfer using static and flow cytometry have been further improved and advanced by the Jovin couple (Jovin and Arndt-Jovin, 1989), who applied photobleaching techniques on microscope, and by Tron et al. (1984), who used the flow cytometry energy transfer (FCET) method.

Unfortunately, as concluded by others (Tron et al., 1987), the above-discussed methods have some shortcomings despite their broad use. In short, the basic problem in using microscope fluorimetery is its inherently poor statistics. Because of the time- and labor-consuming features of fluorescent microscopy, reliable transfer efficiency data are hard to obtain for cell populations with frequently occurring wide cell-to-cell variation.

Using spectrofluorimeters, it is impossible to obtain information about individual cells. Furthermore, very careful experimentation is required and care must be taken in carrying out all the necessary corrections to obtain undistorted data. The concentration of all the samples must be checked with great accuracy. Thorough rinsing of the labeled cells is critical because incomplete elimination of the fluorophor reconjugated unbound ligands may result in false fluorescence intensity (FI) readings. Cell debris-bound fluorophore emission also contributes to the detected intensities. Because binding of ligands to cell debris cannot be controlled and there is no way to determine the exact amount of this contribution, cell debris-free preparation of the sample is fairly critical.

The inherent limitation of the FCET technique is as follows. The application of the method is limited by the number of available sites for the donor and, what is more critical, acceptor-labeled ligands. The absolute limit of the sensitivity cannot be given because it is dependent on the autofluorescence of the cells. Furthermore, the necessity of FCET method to calculate several correction factors complicates this method (Damjanovich et al., 1997).

The current problem of the photobleaching energy transfer method is that its data acquisition is not rapid enough to obtain data from a significantly large cell population. Furthermore, data from individual cells must be averaged to eliminate errors due to biological variations. Other sources of error can be introduced by particular experimental conditions (Damjanovich et al., 1997).

In the present study, a method for the direct determination of *E* via FP measurement occurring on an individual trapped cell is described, which uses the in-house designed and built *Individual Cell Scanner (ICS)*.

This methodology 1) requires a smaller correction factor compared to the FCET method; 2) its statistical aspect is far more representative than that obtained by the static cytometry method, because it enables repeated testing of minute

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number of cells, or alternatively many cells; 3) background, scatter signals, and autofluorescence, can easily be deduced from the fluorescence signal on individual cell basis, because their location is predetermined; and 4) Scanning of cells is rapid and enables data acquisition from a significant number of cells.

THEORY

Determination of *E* by polarization measurement

The relation between FP (P), the fluorescence lifetime (τ_F), and the rotational correlation time of a globular fluorescent probe suspended in a homogeneous solution is given by the Perrin equation (Perrin, 1929),

$$1/P - \tfrac{1}{3} = (1/P_0 - \tfrac{1}{3})(1 + \tau_F RT/\eta V). \quad (1)$$

Where V is the molar volume of the rotating fluorophore, R is the gas constant, T the absolute temperature, and η the viscosity of the embedding medium. $(RT/\eta V)^{-1}$ is defined as τ_R, the rotational correlation time of the probe. P_0 is the intrinsic polarization as measured in cases where $T/\eta \rightarrow 0$.

From the Perrin equation, one can write the fluorescence lifetime of donor in the absence (τ_D) and in the presence of the acceptor (τ_D^A), as a function of the degree of polarization as follows.

$$\tau_D = \tau_R\left(\frac{1/P_D - 1/P_0}{1/P_0 - \frac{1}{3}}\right), \quad (2)$$

$$\tau_D^A = \tau_R\left(\frac{1/P_D^A - 1/P_0}{1/P_0 - \frac{1}{3}}\right). \quad (3)$$

Where P_D^A and P_D are the degrees of the donor polarization in the presence and absence of the acceptor, respectively.

In contrast, E as a function of fluorescence lifetime is given by (Tron, 1994)

$$E = 1 - \tau_D^A/\tau_D. \quad (4)$$

By introducing Eqs. 2 and 3 into Eq. 4, it is easy to show that E as a function of FP degree is given by the formula,

$$E = \frac{P_0(P_D^A - P_D)}{P_D^A(P_0 - P_D)}. \quad (5)$$

Eq. 5 clarifies that, for positive values of E, P_D^A must increase relative to P_D. The physical interpretation of this fact is as follows. When energy transfer occurs, an additional path for evacuation of the donor-excited level is available. As a consequence, the donor fluorescent lifetime decreases, and, consequently, its FP increases, according to Perrin's equation. Indeed, under biological conditions, one cannot assume rotation of spheres in an isotropic environment as requested from Perrin equation. However, the actually determined apparent steady-state (or time-resolved) anisotropy values may carry a large amount of valuable biological information. In a number of cases, not only the sometimes determinable absolute values of particular parameters are of biological interest and importance, but their much more easily determined changes, which can inform us about active or passive participation of certain molecular or other structural elements in given biological functions (Damjanovich, 1994).

It should be emphasized that, other than donor–acceptor energy transfer, there are four main factors that can affect FP: homotransfer between donors, the fluorophore's fluorescence lifetime, and the viscosity, and temperature of the hosting medium. However, during the staining procedure, we paid special attention to keep constant the latter two and, as a consequence, they do not contribute to the observed change in FP. A similar case is that of homotransfer. Because the density of fluorescein-conjugated Concanavalin A (ConA-F) molecules attached to the single and double-stained cells is the same, homotransfer will probably not contribute to the change in the FP. As far as contributions to the changes in FP due to alterations in lifetime, these are relevant because they are the dominant mechanism, which plays a role in altering FP due to energy transfer.

MATERIALS AND METHODS

Cells

Human peripheral blood lymphocytes were obtained from healthy donor whole-heparinized blood by Ficol paque gradient solution (1.077 g/cm^3, Pharmacia, Uppsala, Sweden). After centrifugation, cells accumulated at the Ficol–sera interphase were collected by gentle pipeting, washed three times with phosphate buffered saline (PBS), following resuspension in PBS at a final working concentration of 6×10^6 cells/ml and kept at 4°C.

Concanavalin A (ConA) and Succinylated ConA (SConA)

Nonconjugated ConA, (ConA-F), and tetramethylrhodamine-conjugated ConA (ConA-R) were purchased from Molecular Probes (Eugene, Oregon). Nonconjugated succinylated ConA (SConA), fluorescein-conjugated SConA (SConA-F), and tetramethylrhodamine-conjugated (SConA-R) were purchased from Vector (Burlingein, CA). The average labeling ratios (fluorophore/ConA tetramer) for ConA-F, ConA-R, SConA-F, and SConA-R were 2.9, 4.5, 4.2, and 6.7, respectively.

Instrumentation

The ICS apparatus

The multiparametric, computerized ICS used in performing this work was designed, built, and upgraded at our laboratory (Sunray et al., 1999). Its central feature is a cell tray incorporating a 100×100 dimensional array of 7-μm-diameter holes, 20-μm pitched, in which individual cells are trapped. The cell tray is mounted on a computer-controlled stage, which enables repeated multi-scanning of the same individual cells.

Cells were individually irradiated with a 442-nm He-Cd laser at an intensity of 30 μW on the cell plane. The emission intensity from each cell

was simultaneously measured by four photomultipliers at 530 and 580 nm at two directions of polarization, parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the excitation-beam polarization. The polarization degree of fluorescence from each cell was calculated by

$$P = (I_\parallel - MI_\perp)/(I_\parallel + MI_\perp). \quad (6)$$

Where M, the microscope correction factor, equals the ratio $I_\parallel/I_\perp$, when measuring unpolarized light. It compensates for the distortion of FP measurement due to the microscope optical arrangement, especially the numerical aperture, and the electronic intensifiers of the different detection channels. Under the staining and excitation power-density conditions used here, the sampling time for obtaining 10,000 counts from a single dye-labeled cell varied from 0.001 to ~0.5 s.

The ICS uses a preset count mode instead of the preset time (velocity) mode, used in laser scanning microscopy and flow-through systems. Moreover, down-counting mode is used to terminate counting. The detector, which reaches the preset count first, stops the counting of the other three. Nevertheless, because the counting time is measured as well, the intensity per channel can be calculated. In this way, the user can define the photonic statistic error, and the weaker and brighter stained cell show eqi-photonic error, which might be crucial in the determination of E.

The acquired data, including cell position, measurement duration for each cell, absolute sampling time, intensity at two different wavelengths, computed polarization values, and test set-up information are shown on-line, graphically and numerically displayed on the screen and stored in the memory. Software enables the determination of range and other statistical characteristics of all parameters for either the entire cell population, or an operator-selected subpopulation before, or during the scan.

The FACS apparatus

A fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, San Jose, CA) was used to measure the fluorescence intensity of stained cells. The 488-nm argon ion laser line was used for excitation. The fluorescence intensity emitted from the cells was measured in two wavelengths: 530 and 580 nm.

Labeling of the cells

ConA-F and ConA-R reacted simultaneously with the cells at a total concentration of 200 μg/ml at 4°C for 30 min. Control samples were cells labeled with ConA-F plus the equivalent amount of unlabeled ConA in place of the other fluorophore. Cells were washed once free of ConA by centrifugation for 5 min at 4°C through 5 ml of 5% fetal calf serum in PBS, the supernatant was removed, and cells were resuspended in cold PBS (5% fetal calf serum). The labeling of cells with SConA was performed similarly.

Cell loading onto the ICS cell tray

An aliquot of 80 μl unstained cell suspension (6×10^6 cells/ml) was loaded onto the cell tray. Initial scanning was then performed to detect background scattering and autofluorescence. This undesired signal was recorded for each cell location and subtracted from the emission intensity signal (after staining) recorded from the same location to obtain the net fluorescence signal.

FCET measurement

Three samples of cells were prepared: one labeled with ConA-F only, the second with only ConA-R, and the third sample of cells, which were double stained simultaneously with both ConA-F and ConA-R. The final concentration of ConA in the cell supernatant, the temperature and duration of incubation, and cell washing conditions were all identical to those described above.

Determination of *E* and error estimations

Polarization measurement

Stained cells were loaded onto the cell carrier and individually measured for their FP using the ICS. Two FP measurements were needed for the determination of E: FP measurement of ConA-F-labeled cells, and of ConA-F–ConA-R double-labeled cells. In each of these two measurements, at least three different fields of cells were scanned, and the mean FP value of each field was calculated. The average FP values (P_F and P_F^R for fluorescein and fluorescein–rhodamine, respectively) and their coefficients of variation were then obtained from three measurements and inserted into Eq. 5, yielding E. In our calculation, we assumed that $P_0 = 0.4922$, according to Szalay et al. (1962). The error of E (ΔE) was calculated according to

$$\Delta E = \left[\left(\frac{\partial E}{\partial P_D^A}\Delta P_D^A\right)^2 + \left(\frac{\partial E}{\partial P_D}\Delta P_D\right)^2\right]^{1/2}. \quad (7)$$

The partial derivatives of E, $\partial E/\partial P_D^A$ and $\partial E/\partial P_D$ were calculated from Eq. 5 and then introduced in Eq. 7, yielding,

$$\Delta E = P_0\{[P_D^A(P_0 - P_D)]^2 \times [(P_D^A)^2(P_D^A - P_0)^2(\Delta P_D)^2 + (P_D)^2(P_D - P_0)^2(\Delta P_D^A)^2]^{1/2}\}, \quad (8)$$

where ΔP_D^A and ΔP_D are the standard deviations (SD) of P_F^R and P_F, respectively.

FCET measurement

Determination of E using FCET measurement was carried out via Eq. 9 following Szöllösi et al. (1987):

$$E = \frac{I_2/I_1\left(1 + S_4\frac{C_R\varepsilon_R}{C_F\varepsilon_F}\alpha\right) - S_1 - \frac{C_R\varepsilon_R}{C_F\varepsilon_F}\alpha}{I_2/I_1 - S_1 + \alpha}, \quad (9)$$

where I_2 and I_1 were the individual double-stained cell, which is measured at 580 and 530 nm, respectively. S_1 and S_4 are the correction factors that aimed to compensate for the spectral overlap associated with direct excitation of the respective fluorophores. The ratio I_2/I_1 determined S_1 when measuring cells labeled with only ConA-F, whereas I_1/I_2 determined S_4 when measuring cells labeled with only ConA-R. Excitation with 488 nm yields null values of S_4. The parameter α compensates for the poor quantum yield at long wavelengths of commonly used photomultipliers in comparison to that of short wavelengths. Practically, α computed as

$$\alpha = M_R L_F \varepsilon_F / M_F L_R \varepsilon_R, \quad (10)$$

where M_R and M_F are the mean values of fluorescence-density distributions of I_2 and I_1, determined using cell suspensions saturated either with ConA-R or with ConA-F, L_R and L_F are the labeling ratios for ConA-R and ConA-F and ε_R and ε_F are their molar extinction coefficients, respectively.

TABLE 1 Comparison of FP values of fluorescein in glycerin solution measured by ICS and SLM

Glycerin Solution (%)	FP (ICS)	FP (SLM)
62	0.158 ± 0.004	0.153 ± 0.006
64	0.169 ± 0.005	0.176 ± 0.008
65	0.173 ± 0.0047	0.169 ± 0.009
66	0.186 ± 0.005	0.184 ± 0.012
68	0.208 ± 0.006	0.205 ± 0.01
69	0.214 ± 0.0045	0.209 ± 0.007
73	0.246 ± 0.0055	0.242 ± 0.014
80	0.316 ± 0.007	0.320 ± 0.016

The ratio C_R/C_F, stand for the actual rhodamine-to-fluorescein molar ratio on the cell surface. The SD of E (σ_E) was calculated from

$$\sigma_E^2 = \frac{(1 + C^2)}{(r - S_1 + \alpha)^2} \times [\alpha^2(\sigma_r^2 + \sigma_{S_1}^2) + (S_1 - r)^2\sigma_\alpha^2 + \frac{\alpha^2(r - S_1 + \alpha)^2}{(1 + C)^2}\sigma_C^2, \quad (11)$$

where, $r = I_2/I_1$, $C = \varepsilon_R C_R/\varepsilon_F C_F$, and σ_r is the SD of r, which is defined as

$$\sigma_r^2 = \left[\frac{\sigma_{I_2}}{I_1}\right]^2 + \left[\frac{I_2\sigma_{I_1}}{I_1^2}\right]^2. \quad (12)$$

RESULTS AND DISCUSSION

Control measurements

The real value of FP of a microscopic fluorescent sample, when measured via microscope, might be distorted due to the high numerical aperture of an objective (Lindmo and Steen, 1977; Axelrod, 1989; Deutsch et al., 2002). To ensure correct measurement of FP with the ICS, which is based on microscope optical arrangement, a comparison between bulk versus microscopic measurements of FP of 1 μM fluorescein-glycerin in PBS solutions were performed. For bulk measurements, the spectrofluorimeter (Aminco-Bowman, Series 2, SLM-Aminco Spectronic Instruments, New York) was used. The excitation wavelength was 442 nm and the emission and excitation slit widths were 2 mm each. The results are shown in Table 1. The SD in the table was calculated from results of 10 and 100 repeated measurements performed by the spectrofluorimeter and the ICS, respectively. The degree of similarity of the two measurement setups is best shown in Fig. 1.

Fluorescence resonance energy transfer measurements

In the experiments discussed below, cells labeled with ConA-F or ConA-R were used to demonstrate the applicability of the measuring methodology as well as the calculation method for determination of energy transfer efficiency via polarization measurement. Instrumental factors were kept constant for these experiments.

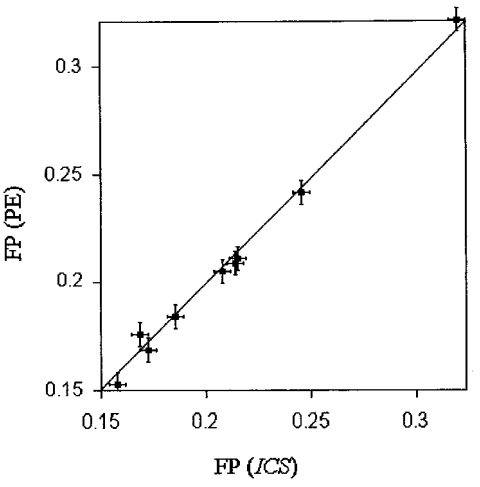


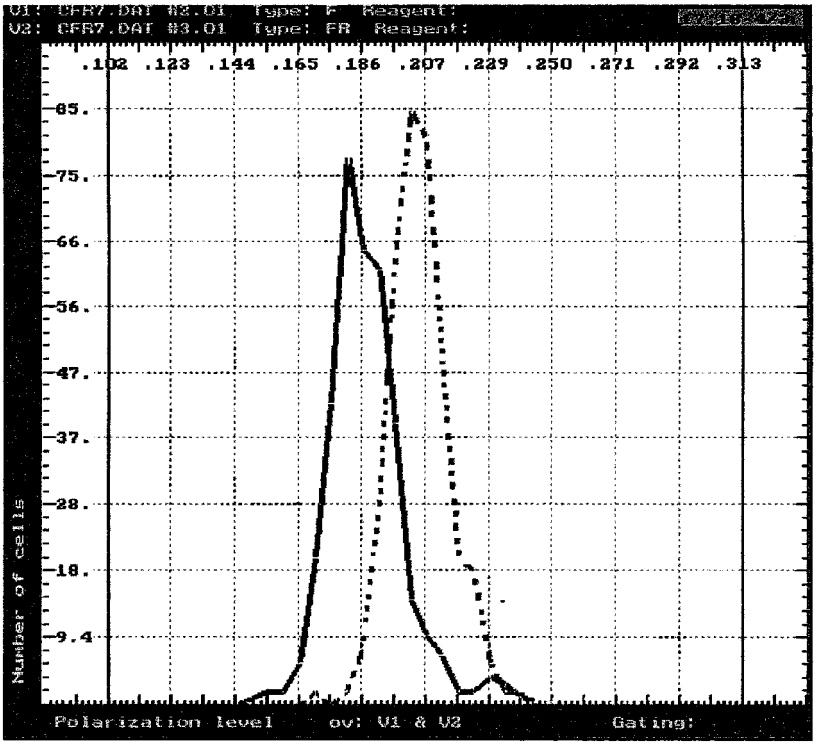
FIGURE 1 Macroscopic versus microscopic FP measurements of fluorescein in glycerin–water solution as measured by spectrofluorimeter and ICS, respectively.

Determination of *E* between ConA-F and ConA-R bound to human lymphocytes

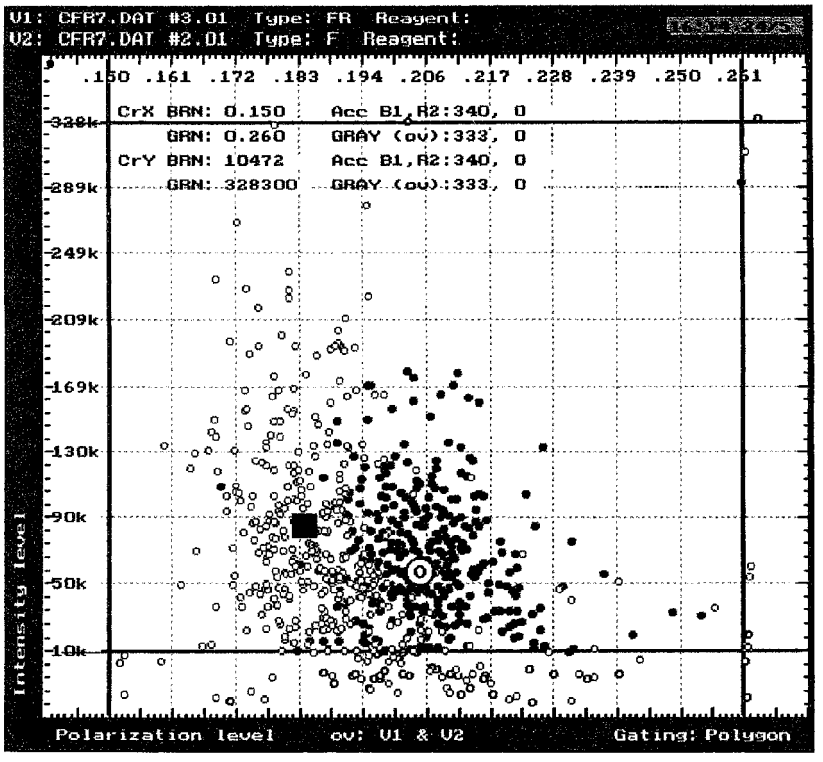
Cells were prepared and measured as described in Materials and Methods. A typical individual cell FP histogram and scatter diagram of FI versus FP are shown in Fig. 2 *A* and *B*, respectively. The data in Fig. 2 *A* show an increase in mean FP level (measured at 530 nm), from 0.185 (*solid line*) for F-labeled cells, to 0.206 (*broken line*) for double (F-R) labeled cells. Repeated measurements of two other fields on the same cell tray, for each of the two types of labeled cells yielded $P_F = 0.188 \pm 0.002$, $P_F^R = 0.211 \pm 0.003$. Inserting these values into Eqs. 5 and 8, gives $E = (17.6 \pm 2)\%$. Similar behavior was observed for at least 10 different healthy donors.

The increase in the donor FP values shown above is the outcome of shortening donor lifetime due to a more rapid evacuation of its excited energy level via energy transfer in the presence of the acceptor, in accordance with the Perrin equation (Eq. 1). It should be emphasized here that practically, the measured FP at 530 nm in double-labeled cells reflects the FP of F rather than that of R. This is because both absorption power of ConA-R at 442 nm excitation and its emission intensity at 530 nm are very small compared to those of ConA-F (Fig. 3).

The FI versus FP scattergram shown in Fig. 2 *B*, indicates two major clusters: the open circles, which presents the ConA-F stained cells, and the full circles, which presents the ConA-F and ConA-R double-stained cells. As can be



(a)



(b)

FIGURE 2 (*A*) Individual cell frequency histogram of fluorescence polarization (measured at 530 nm) of human lymphocyte labeled only with ConA-F (——) and with both ConA-F and ConA-R (- - - -). Abscissa, FP values; Ordinate, cell frequency. (*B*) Scatter diagram of FI (ordinate) versus FP (abscissa) of human lymphocytes labeled with ConA-F only (○) and with both ConA-F and ConA-R (●). The FI of the CM of first cluster is marked by ■ and that of the latter by ◎.

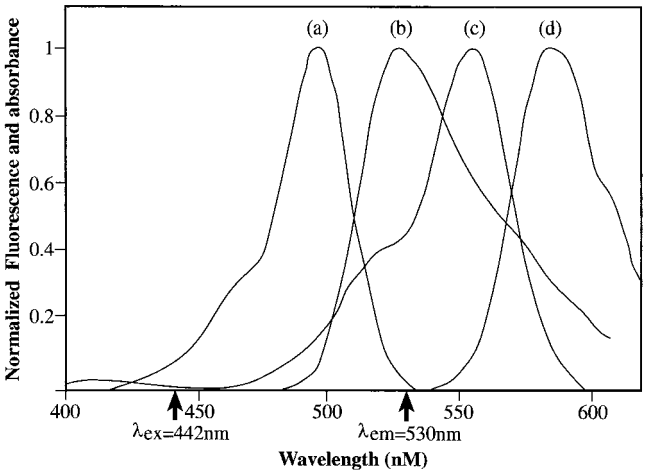


FIGURE 3 Normalized absorption and emission spectra for ConA-F (*a*, *b*) and for ConA-R (*c*, *d*) in PBS, respectively. λ_{ex} is the wavelength of the excitation beam, and λ_{em} is the wavelength in which the emission was measured.

seen in this figure, the FI of the center of mass of the first cluster is higher than that of the latter, 84,733 and 69,239 FI arbitrary units, correspondingly. This decrease of FI, following the addition of the acceptor, indicates quenching of donor due to energy transfer.

Sensitivity

The sensitivity of E to changes in donor–acceptor proximity, measured via FP utilizing the ISC, was evaluated as follows.

Change in E as a function of ConA-R to ConA-F ratio

In these experiments, the proximity of cell membrane ConA-R to ConA-F was investigated. The proximity was varied by controlling ConA-R versus ConA-F concentration (C_R/C_F) during the process of cell labeling. The measured E values obtained for different C_R/C_F values are listed in Table 2. The total ConA concentration was kept constant (0.2 mg/ml). The results indeed indicate that E increases as the relative portion of the acceptor increases and the proximity between acceptors and donors decreases.

TABLE 2 E as a function of C_R/C_F ratio

C_R/C_F	E (%)
0.64	13.1
1.07	16.5
1.93	21.7
4.5	25.4

C_R/C_F is the ConA-R to ConA-F cell labeling ratio.

Use of Succinylated ConA for no-capping situation

Capping is a physical phenomenon occurring in lymphocyte membranes, in which surface protein macromolecules polarize from their normal diffuse distribution to form a dense cluster at one pole of the cell (Chahn and Alderete, 1990). ConA is one of the most widely used and well-characterized lectins used to induce patch and cap formation. However, SConA fails to induce these phenomena (Yahara and Edelman, 1973). It was thus expected that measured E for the cells treated with SConA would be smaller than that obtained from cells treated with ConA.

For these experiments, cells were stained as described in Materials and Methods. As can be seen in Fig. 4, FP measurement of SConA-F-labeled lymphocytes, before and after the addition of SConA-R, did not yield significant change in FP, i.e., $P_F \approx P_F^R = 0.258$.

As can be seen, the FP of SConA-F is significantly higher than that of ConA-F (0.258 versus 0.188). This may be explained by assuming two competing causes of homotransfer, which both, by and large, lower FP (Badley, 1976): the first, between fluorescein molecules settling on different ConA molecule, the second, between fluorescein molecules settling on the same ConA molecule. The first cause might be dominant in the case of capping, which increase the proximity among fluorescein molecules, such as in the case of ConA-F treated cells in comparison with SConA-F treated cells. The second cause is dominant when capping is avoided as in the case of dissolving SConA-F and ConA-F in PBS, to measure their FP. The FP of the former was found to be lower than the latter, 0.100 and 0.120, correspondingly, in agreement with the fact that the average labeling ratios of SConA-F is higher (4.2) than that of ConA-F (2.9).

FLUORESCENT POLARIZATION VERSUS FCET MEASUREMENTS

The calculation of E through FCET measurements was carried out using Eq. 9, where S_1 (the slope of the scattergram of Fig. 5) = 0.276, S_4 (the slope of the scattergram of Fig. 6) $\approx$ 0 as expected, $M_R = 127$, $M_F = 717$, $L_R = 4.5$, $L_F = 2.9$, and $\varepsilon_F/\varepsilon_R = 10.3$ (the last ratio is taken from Szöllösi et al., 1987). Therefore, $\alpha = 1.17$. According to our measurement, $C_R/C_F = 0.97$, therefore $C_R\varepsilon_R/C_F\varepsilon_F = 0.094$. Substituting these values in Eq. 9, one gets

$$E = \frac{I_2/I_1 - 0.385}{I_2/I_1 + 0.894}. \tag{13}$$

Eq. 13 enables the calculation of E values of single cells double stained with both ConA-F and ConA-R. For the discussed FCET measurements, ~5000 cells were measured. The results are shown in Fig. 7. The mean value of the E was found to be 17.9% with SD of 3%. This result is

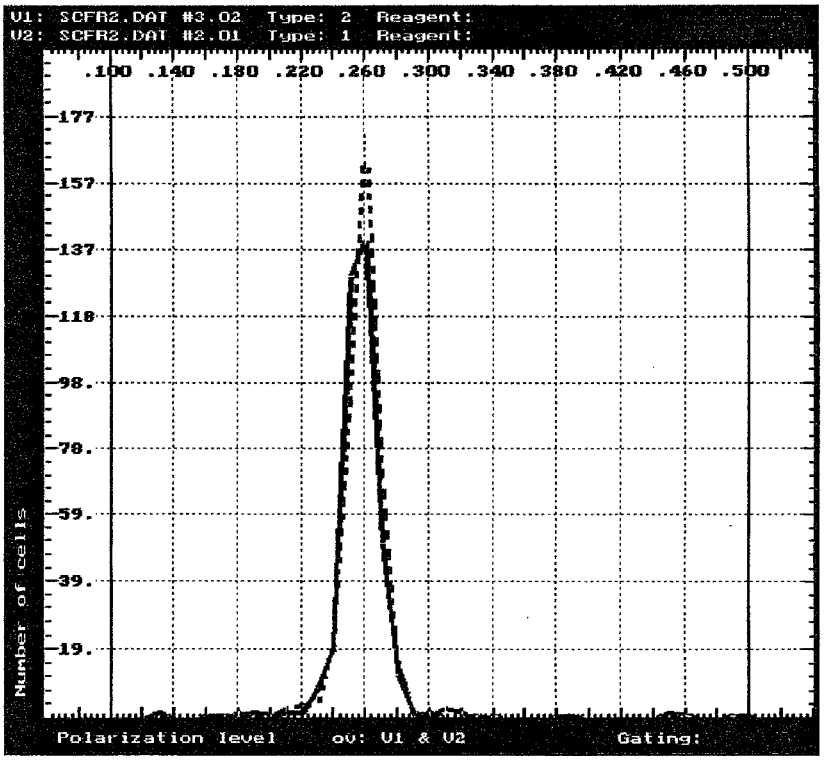


FIGURE 4 Individual cell frequency histogram of FP (measured at 530 nm) of human lymphocytes labeled only with SConA-F (——) and with both SConA-F and SConA-R (- - - -). Abscissa, FP values; Ordinate, cell frequency.

in good agreement with the E value (about 17.6%) obtained through FP measurements.

CONCLUSIONS

A novel cytometric technique is reported, which combines characteristics from ICS and FP measurements to provide a new method for the determination of E. The goals of this combination were: improvement of FCET measurement by shifting the transfer efficiency determination to the donor side, simplification of FCET by reducing the number of correction factors, improvement of poor microscope fluorimetry statistics, and giving a solution to the background problem of the spectrofluorimeter and autofluorescence problem of flow cytometry.

The use of ICS enables the calculation of E for each cell in the following ways: first by determining the donor FP from each cell in the sample, and thereafter, acceptor molecules are added to the cells trapped on the cell tray in order

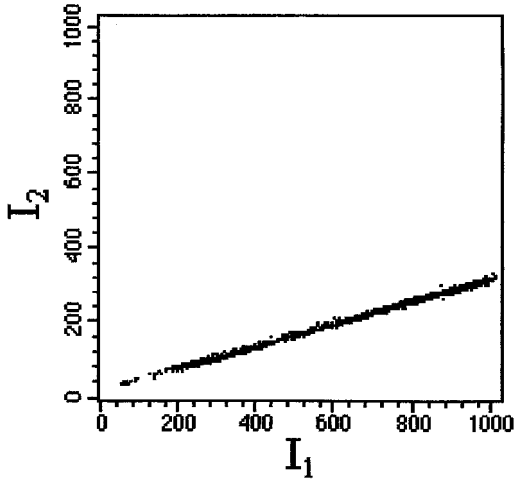


FIGURE 5 Scattergram of FI of ConA-F-labeled cells measured at 580 nm (I_2, ordinate) and at 530 nm (I_1, abscissa). The slope defines S_1.

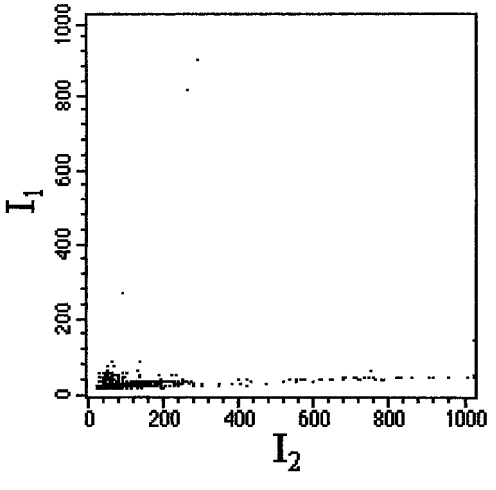


FIGURE 6 Scattergram of FI of ConA-R-labeled cells measured at 580 nm (I_1, ordinate) and at 530 nm (I_2, abscissa). The slope defines S_4 and equals approximately zero.

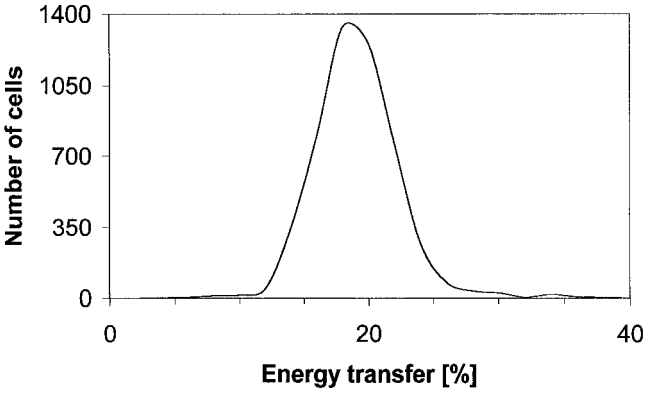


FIGURE 7 Frequency distribution histograms of E values (calculated via Eq. 14) of ConA-F and ConA-R double-stained cells. Mean value is 17.9% ± 3%.

to determine the donor in the presence of acceptor FP from each cell. The measurements of FP versus FCET showed good correlation.

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