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ARTICLE *in* CYTOMETRY · MARCH 1987

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Cytometry 8:120–128 (1987)

Flow Cytometric Measurements of Fluorescence Energy Transfer Using Single Laser Excitation[1]

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Received for publication February 28, 1986; accepted October 23, 1986

Flow cytometric energy transfer (FCET) measurements between labeled specific sites of cell surface elements (Szöllosi et al., Cytometry, 5:210–216, 1984) have been extended in a simplified form using a flow cytometer equipped with single excitation beam. This versatile and easily applicable method has several advantages over any nonflow cytometric (i.e., spectrofluorimetric) energy transfer measurements on cell surfaces: 1) The labeled ligands can be applied in excess, without washing, thereby enabling the investigation of relatively labile receptor–ligand complexes. 2) Contributions of signals from cell debris, from cell aggregates, or from nonviable cells can be avoided by gating the data collection on the light scatter signal. 3) The heterogeneity of the cell population with respect to the proximity of the labeled binding sites can be studied. 4) In the cases of homologous ligands or of ligands binding to the same molecule but at different epitopes, the determination of fluorescence resonance energy transfer efficiency values can be carried out on a cell-by-cell basis, offering data on intramolecular conformational changes.

This modified FCET method enabled us to demonstrate the uniform density of glycoproteins, specific for Con A binding, in the plasma membrane of normal and Gross virus leukemic mouse cells of different sizes. The utility of this procedure has also been demonstrated by using the mean fluorescence intensities of the distribution curves in the calculation of the fluorescence energy transfer efficiency.

Key terms: Resonance energy transfer, concanavalin A, Gross virus leukemia, flow cytometry, flow microfluorimetry, receptor density

The cytoplasmic cell surface membrane plays a major role in communication between the environment and the cell interior. The cell senses specific external stimuli through surface receptors. The information necessary for cell function can be communicated by the aggregation state of these receptors or by changes in the distribution state of receptors on the membrane.

The proximity relationship of membrane receptors can be studied by measuring the efficiency of fluorescence resonance energy transfer (FRET) between donor and acceptor fluorophores conjugated to components in question (21). FRET measurements have been performed on cell surfaces using spectrofluorimetry (4,18–20), microscopy (9), and flow cytometry (3,5,10,13–15,24,25). The FRET efficiencies determined using spectrofluorimeters are values averaged over the entire homogeneous or heterogeneous population. The data obtained with spectrofluorimeters are highly perturbed by the amount of debris, dead cells, aggregates, etc. Furthermore the procedures used to eliminate excess labeled ligands also remove cells from the samples and present an almost insurmountable problem when comparing fluorescence intensities of single-labeled samples with those of double-labeled samples. We have developed and recently published (5,24,25) a new method using a flow cytometer capable of dual-wavelength excitation to determine FRET efficiency between labeled binding sites on a single cell basis, thus revealing the heterogeneity of cell populations. This method, designated flow cytometric energy transfer (FCET) requires dual-wavelength excitation, correlated data acquisition, and analysis.

[1]This work was partially sponsored by the National Science Foundation (USA), grant INT-8405545.

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In the present study we describe a modification of the FCET method permitting, in case of homologous ligands, determination of FRET efficiency values on a cell-by-cell basis employing flow cytometers equipped with single-wavelength excitations. In the study of proximity relationships of heterologous ligands the method is far superior to spectrofluorimetric measurements because it minimizes sources of possible errors listed above. This modified FCET method has been employed with normal and Gross virus leukemic mouse cells labeled with fluorescein-conjugated concanavalin A (F-Con A) and tetramethylrhodamine-conjugated Con A (R-Con A) in order to study the proximity of Con A-binding glycoproteins on the cell surface. Our results suggest that although the total number of Con A-binding sites per cell was different for normal and leukemic mouse cells, the effective surface density of Con A receptors, determined from FRET efficiency values, was the same for both normal and leukemic cells in spite of significantly different sizes.

MATERIALS AND METHODS

Cells

HK22 ((C3H×DBA/2)F_1) murine lymphoma cells (formerly designated as T41 cells (12)) were maintained in Dulbecco's minimum essential medium containing 10% fetal calf serum (FCS) and antibiotics.

Normal lymphocyte suspensions were prepared from 8-week-old female (C3H/He-mg/Lati×AKR/Lati)F_1 hybrid mice as follows: the mesenteric lymph node was cut into small pieces, suspended in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.7 mM Na_2HPO_4, 1.45 mM KH_2PO_4; pH 7.4) and filtered through a piece of gauze.

Gross virus leukemia cells were originally obtained from an AKR mouse, and maintained in (AKR/Lati × C3H/He-mg/Lati)F_1 hybrid mice by serial transplantation. One-tenth milliliter of 1×10^7 cell/ml suspension, taken from the spleen of moribund, leukemic F_1 hybrid mice was injected intraperitoneally into F_1 hybrid mice. Leukemic cell suspensions for analysis were prepared from the mice, as described above, on the 9th day after inoculation (8,22).

Proteins and Dyes

Fluorescein-5′-isothiocyanate (FITC) and tetramethylrhodamine-5′-isothiocyanate (TRITC) were obtained from Molecular Probes, Inc. (Junction City, OR) and PolySciences Inc. (Warrington, PA), respectively. Concanavalin A (Con A) was purchased from Serva Feinbiochemica GmbH and Co. (Heidelberg, West Germany).

Con A (10 mg/ml) was labeled with fluorophore in 0.1 M $NaHCO_3$ buffered at pH 9 containing 0.5 M NaCl, 2 mM Ca^{++} and 1mM Mg^{++} in the presence of 1 mM α-D-methylmannopyranoside by 1-h incubation at room temperature (3). Unreacted dye was removed by extensive dialysis and the labeled Con A was further purified by affinity chromatography on Sephadex G-75 (fine) column (1). After elution by 1 mM α-D-methylmannopyranoside, the sugar was removed by dialysis. The extent of labeling was determined spectrophometrically for F-Con A (6). Because the ratio of optical densities of TRITC chromophore at 280 nm and 553 nm was different before and after conjugation and changed from batch to batch, the protein concentration of R-Con A was determined using competition reactions involving labeled and unlabeled Con A. The extinction coefficients used were: for Con A at 280 nm, 1.22×10^5 $M^{-1}cm^{-1}$ (1), for FITC at 495 nm, 6.3×10^4 $M^{-1}cm^{-1}$ (6), and for TRITC at 553 nm, 3.4×10^4 M^{-1} (3). The average labeling ratios (chromophore/Con A) were 2.0 for fluorescein and 1.4 for rhodamine. The ratio of the molar extinction coefficient of F-Con A to that of R-Con A, as determined from excitation spectra, was 10.3 and 1.09 at 488 nm and 514 nm, respectively (our determination).

Unlabeled Con A was also affinity purified on a Sephadex G-75 column as described above.

Labeling of the Cells With F-Con A and R-Con A

Cells were collected from media or prepared from mice, washed twice with PBS and incubated (1×10^7 cells/ml in PBS) in the presence of 0.2 mg/ml total Con A concentration at 4°C for 60 min with gentle agitation every 10 min. The cells were washed by centrifugation through a layer of 3 ml 50% FCS onto a cushion of Lymphoprep (Flow Laboratories GmbH, Meckenheim, West Germany) to avoid aggregation, fixed in 1% formaldehyde, and analysed in the flow cytometer. Centrifugation and fixation were carried out at 4°C.

For determination of the number of Con A binding sites per cell, the concentration of F-Con A was changed from 5 μg/ml to 200 μg/ml. Scatchard analyses of binding curves were performed as described by Kavai et al. (17).

Instrumentation

A fluorescence activated cell sorter (Becton Dickinson, FACS-III) was used to measure the scattered light and fluorescence intensities of cells. The 488 nm or the 514 nm line of an 5-watt Argon ion laser was used for excitation. The fluorescence of cells was detected with two photomultiplier (PM) tubes using a single condenser lens, beam splitter, and appropriate optical filters. PM1 detected the emitted fluorescence through a 520 nm longpass and a 540 nm bandpass interference filters (Ditric Optics, Inc., Marlboro, MA); this signal is designated I_1. PM2 detected the fluorescence beyond 580 nm using a 520 nm longpass interference and a 580 nm longpass colored glass filter (Ditric Optics); this signal is designated I_2. Data were collected using a multichannel, pulse-height analyzer and displayed as frequency distribution histograms. For data processing and mathematical analysis, the histograms were transferred to a PDP-11/34 computer.

Determination of the Efficiency of Fluorescence Resonance Energy Transfer (FRET)

Quenching of the fluorescence emission of the donor and the sensitization of the emission of the acceptor molecules can be observed when FRET occurs between

donor and acceptor pairs. Experimental measurements of these phenomena may be used for the calculation of the numerical value of FRET efficiency (E):

From the quenching of donor fluorescence, E can be determined as:

$$E = 1 - \frac{Q_D^A}{Q_D} = 1 - \frac{F_D^A}{F_D} \quad (1)$$

where Q_D^A and Q_D are fluorescence quantum yields of the donor in the presence and absence of acceptor, F_D^A and F_D are the fluorescence intensities of the donor in the presence and absence of acceptor.

The sensitization of acceptor emission is described by:

$$\frac{F_A^D}{F_A} = 1 - \frac{\epsilon_D C_D}{\epsilon_A C_A} E \quad (2)$$

where F_A^D and F_A are the fluorescence intensities of the acceptor in the presence and the absence of donor. C_D and C_A are the molar concentrations and ϵ_D and ϵ_A the molar extinction coefficients of the donor and acceptor at the excitation wavelength.

The data obtained in a spectrofluorimeter are very sensitive to the number of cells in the measuring cuvettes as well as to the free-labeled ligand concentration and a number of other parameters already mentioned. However, equations 1 and 2 can be applied to flow cytometric measurements and population averaged values for fluorescence intensities can be used to yield mean FRET efficiency values.

Using the modified version of our new method (24,25) the flow cytometric measurements of E are carried out as follows:

Separate cell samples are labeled either with fluorescein-conjugated Con A (F-Con A) serving as donor or tetramethylrhodamine-conjugated Con A (R-Con A) serving as acceptor alone (single-labeled samples), or with both F-Con A and R-Con A (double-labeled samples). Two fluorescence signals, I_1 and I_2, were measured for each cell of the three differently stained samples as described above. The excitation wavelength could be either 488 nm of 514 nm as both fluorescein and rhodamine are excited by these wavelengths. The measured intensities are described analytically by equations 3 and 4. (For simplicity we introduce the notation $I(\lambda_1; \lambda_2)$ which is defined as the fluorescence intensity measured at λ_2 nm with excitation at λ_1 nm.)

$$I_1(488 \text{ or } 514; 540) = I_F(1-E)+I_R S_4 \quad (3)$$

$$I_2(488 \text{ or } 514; 580) = I_F(1-E)S_1+I_R+I_F E\alpha \quad (4)$$

I_F and I_R are the theoretical, directly excited fluorescence intensities of the fluorescein and of the rhodamine when no energy transfer occurs. S_1 and S_4 are corrections for the spectral overlap associated with direct excitation of the respective fluorophores. S_1 is I_2/I_1 determined using cells labeled only with F-Con A, S_4 is I_1/I_2 determined using cells labeled only with R-Con A. S_4 is negligible with 488 nm excitation. The energy transfer process has been neglected in equation 3 because the contribution of the process to the I_1 signal through rhodamine sensitization is less than 2%. The parameter α in equation 4 takes care of the fact that the detectability of an excited flourescein molecule differs from that of an excited rhodamine molecule. In our case α can be determined according to ref 25 as:

$$\alpha = M_R L_F \epsilon_F / M_F L_R \epsilon_R \quad (5)$$

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 R-Con A or with F-Con A. L_R and L_F are the labeling ratios for R-Con A and F-Con A.

I_F and I_R intensities of an individual double-labeled cell can be estimated by equations 6 and 7:

$$I_F = q\frac{F-[\text{Con A}]}{[\text{Con A}]_{sat}} M_F \quad (6)$$

$$I_R = q\frac{R-[\text{Con A}]}{[\text{Con A}]_{sat}} M_R \quad (7)$$

Table 1
Median Flourescence Intensities of HK22 Murine Lymphoma Cells Labeled With Flourescent Con A

Cells labeled with	F-[Con A] (%)[a]	C_R/C_F	Exc. 488 nm I_{540}	Exc. 488 nm I_{580}	Exc. 514 nm I_{540}	Exc. 514 nm I_{580}
Con A	0	—	2.9	2.3	4.2	3.5
F-Con A	100	—	533.3	41.0	214.2	25.3
F-Con A + Con A	52	—	287.7	22.5	113.4	16.7
R-Con A	100[b]	—	3.9	15.3	11.2	63.5
R-Con A + Con A	60[b]	—	3.6	10.1	8.5	39.5
F-Con A + R-Con A	62.8	0.41	294.9	38.1	123.5	42.5
F-Con A + R-Con A	42.0	0.96	182.8	33.7	79.0	52.0
F-Con A + R-Con A	19.4	2.88	78.5	26.3	39.2	59.7

[a]Percentage of Con A-binding sites occupied by F-Con A.
[b]Percentage of Con A-binding sites occupied by R-Con A.

where q is the ratio of the actual number of Con-A-binding sites of a given cell to the average number of Con-A-binding sites for the whole cell population. The F-[Con A]/[Con A]$_{sat}$ and R-[Con A]/[Con A]$_{sat}$ ratios are the proportions of Con A binding sites occupied by F-Con A and R-Con A. From equations 5–7, I_R:

$$I_R = I_F \frac{R - [\text{Con A}]L_R\epsilon_R}{F - [\text{Con A}]\,L_F\epsilon_F}\alpha \tag{8}$$

Since R-[Con A]L_R = C_R and F−[Con A]L_F = C_F, the equation 8 can be written as:

$$I_R = I_F \frac{C_R\epsilon_R}{C_F\epsilon_F}\alpha \tag{9}$$

By the aid of equation 9 equations 3 and 4 can be solved for E:

$$E = \frac{I_2/I_1\left(1 + S_4 \frac{C_R\epsilon_R}{C_F\epsilon_F}\alpha\right) - S_1 - \frac{C_R\epsilon_R}{C_F\epsilon_F}\alpha}{I_2/I_1 - S_1 + \alpha} \tag{10}$$

This term is suitable for the determination of FRET efficiency values on a cell-by-cell basis since all the parameters at the right side can be measured separately or determined experimentally.

The C_R/C_F, which is the actual rhodamine to fluorescein molar ratio on the cell surface, can be determined by dissolving the labeled cell samples using sodium-dodecyl-sulphate (23). Accurate C_R/C_F values can also be calculated from the initial composition of the incubation mixture (F-Con A and R-Con A) knowing the eventual different binding affinities of the donor and acceptor conjugated ligands. The later can be determined from competition experiments in the presence of unlabled Con A. In our experiments the competition method was used for the determination of C_R/C_F ratios.

Error Estimation

The standard deviation of the E values can be determined from the individual standard deviations for parameters used in equation 10 according to the standard formula in equation 2 for the propagation of error. To demonstrate the propagation of errors in our calculations, the equation 10 was rewritten into a simplified form. Since the S_4 parameter is negligible if the samples are excited at 488 nm, equation 10 becomes:

$$E = \frac{r - S_1 - C\alpha}{r - S_1 + \alpha} \tag{11}$$

where $r = I_2/I_1$ and $C = (\epsilon_R C_R)/(\epsilon_F C_F)$. The standard deviation of r (σ_r) can be determined as:

$$\sigma_r^2 = \left[\frac{\sigma_{I_2}}{I_1}\right] + \left[\frac{I_2\sigma_{I_1}}{I_1^2}\right]^2 \tag{12}$$

The standard deviation of E (σ_E) was calculated from equation 13:

$$\sigma_E^2 = \frac{(1+C)^2}{(1-S_1+\alpha)^2} \cdot \left[\alpha^2(\sigma_r^2 + \sigma_{S_1}^2) + (S_1 - r)^2\sigma_\alpha^2 + \frac{\alpha^2(r - S_1 + \alpha)^2}{(1+C)^2}\sigma_C^2\right] \tag{13}$$

Equation 13 describes the way errors resulting from the error propogation in individual parameters affect the accuracy and precision of the energy transfer efficiency.

RESULTS

The FRET efficiency calculation procedure described above makes possible determination of the energy transfer efficiency on a single cell basis. In a flow cytometer lacking the capability of storing data in a correlated manner, FRET efficiency values were calculated from mean fluorescence intensities of distribution curves. The accuracy of these values is far superior compared to the accuracy of similar data obtained in a spectrofluorimeter for reasons listed in the first part of this article.

In order to demonstrate the versatility of the method described, the mean values of fluorescence intensity distribution curves of HK22 murine lymphoma cells labeled with fluorescent Con A derivatives are given in Table 1. The raw data of individual cells, giving the fluorescence distribution curves, were gated by cell size (light scatter) to exclude cell debris and cell aggregates. Because the distribution curves were slightly skewed, only 90% of the distribution curves was taken into account in the calculation of the mean fluorescence intensity to avoid the effect of the up-scale tail on the results. The third and the fifth line of the table demonstrate the competition between the fluorescent and the nonfluorescent Con A. Except for cells labeled with R-Con A (especially using 488 nm excitation and 580 nm detection), the autofluorescence of cells labeled with nonfluorescent Con A was usually low compared to the fluorescence of cells labeled with fluorescent lectin.

Using the mean fluorescence intensities of double-labeled samples, the FRET efficiency can be calculated by different methods (mean efficiency values of three sets of samples are summarized in Table 2). The calculation methods based upon the quenching of donors (equation 1) and the sensitization of acceptors (equation 2) can be applied only using mean fluorescence intensities. In these cases the F_D and F_A values were determined knowing the percentage of Con A binding sites occupied by F-Con A. Using correlated data acquisition and storage, the flow cytometric method offers the possibility to determine FRET efficiency on a cell-by-cell basis. Without this capability, the mean values of fluorescence intensities (as shown in Table 1) can be used for calculation. The efficiency of FRET increased with the increase of the rhodamine-to-fluorescein molar ratio on the cell surface. FRET efficiency data showed good agreement independent of the calculation method used.

Table 2
Energy Transfer Efficiency (E) Between F-Con A and R-Con A Bound to HK22 Cells

	Calculation methods						
					Flow cytometric		
	Quenching		Sensitization		Single beam		Double beam[a]
C_R/C_F	488[b]	514	488	514	488	514	488/514
0.41	12 ± 2[c]	12 ± 2	8 ± 2	8 ± 2	10 ± 2	11 ± 2	10 ± 2
0.96	19 ± 2	20 ± 2	13 ± 2	18 ± 2	16 ± 2	18 ± 2	18 ± 2
2.88	27 ± 3	28 ± 3	22 ± 3	26 ± 3	25 ± 3	26 ± 3	26 ± 3

[a]Calculation method is described in (24).
[b]Wavelength of excitation in nm.
[c]$E \pm \sigma_E$ values in percent.

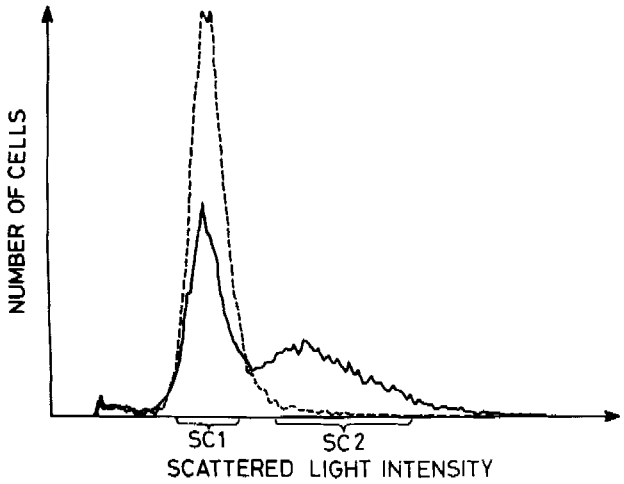

Fig. 1. The scattered-light intensity histogram of normal (dashed line) and gross virus leukemic mouse cells (solid line). Data collection of scattered-light intensity curves was triggered by the fluorescence intensities of cells stained with fluorescein diacetate (FDA); this method eliminates the contributions of the red blood cells and the dead cells to the distribution curves of scattered light intensity. (FDA staining procedure is described in details in ref 22.)

However, the sensitization method with 488 nm excitation gave systematically somewhat lower FRET efficiencies compared to the other values.

Using 488 nm excitation we have determined the standard deviation of E values which were calculated by the flow cytometric single-beam method, using the error propagation function described by equation 13. Estimated from the experimental data, the following relative errors for individual parameters were used in the calculation: 2% and 6% for I_1 and I_2 intensities, respectively, 6% for S_1, 6% for α, and 10% for C parameters. (The errors caused by the autofluorescence of cells are included in the errors of the fluorescence intensities.) The resulting absolute values of standard deviations for energy transfer efficiencies were 2%, 2.5%, and 3% at values CR/CF = 0.41, 0.96, and 2.88, respectively. The standard deviations calculated from the error propogation function showed good agreement with the standard deviations determined from repeated experiments.

The FRET procedure was employed with heterogeneous populations of cell samples prepared from leukemic mice. Figure 1 shows the scattered-light distribution curves of live lymphocytes prepared from normal and leukemic mice. Two peaks can be observed on the distribution curve representing cells of leukemic mice (Fig. 1, solid line). In this case the normal lymphocytes (SC1 region) can easily be distinguished from the leukemic cells (SC2 region) by size, as revealed by scattered-light intensity.

Correlations between the scattered-light and the fluorescence intensities of cells of leukemic mice, labeled with both F-Con A and R-Con A, are shown on Figure 2. An interesting finding was the close correlation between red and green fluorescence in contrast to the fact that light scatter intensity correlate well with none of the fluorescence intensities (Fig. 2). Since I_2/I_1 is the only parameter yielding a change in the numerical value of E for individual cells of the sample (all other parameters are constant for each cell), the heterogeneity of the cell suspension in FRET efficiency can be estimated from this dot-plot distribution (Fig. 2, bottom). The location of data around a straight line in this plot indicates that the FRET efficiencies are the same for normal and leukemic cells. The width of the line indicates the CV of the FRET efficiency. Having performed the calculation using the mean fluorescence intensities of distribution curves triggered either by SC1 (small cells) or SC2 (large cells), no significant differences were found between FRET efficiency values of normal and leukemic cells (Table 3). Average values of FRET efficiency of three independent sets of samples measured at both 488 nm and 514 nm excitation are summarized in Table 3. Calculation of FRET efficiencies was performed using equation 10. The relative errors of the average FRET efficiencies were 10–20%. The data of Table 3 demonstrate that FRET efficiency values at a given rhodamine-to-flourescein molar ratio are the same regardless the cell type on which the measurements were carried out.

While the binding constants of Con A receptors were very similar on the different cell types there were great differences in the average number of Con A binding sites per cell, as shown in Figure 3. Surface densities of Con A receptors were calculated from the number of Con A binding sites per cell, for the different cell types

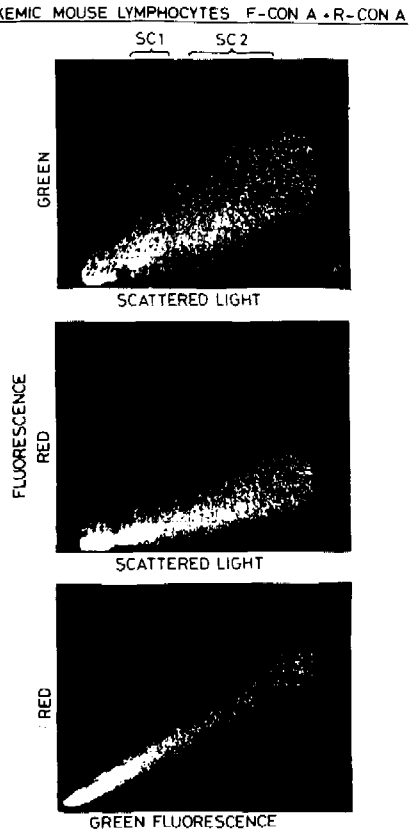

Fig. 2. Scattergrams of cells prepared from leukemic $AKCF_1$ mice. Cells were labeled with both F-Con A and R-Con A and analyzed using 488 nm excitation as described in Materials and Methods. Green fluorescence was measured at 540 ± 10 nm, the red above 580 nm. *Top:* Correlation of green emission on the ordinate to the scattered-light on the abscissa. *Middle:* Correlation of red emission on the ordinate to the scattered-light on the abscissa. SC1 and SC2 regions correspond to the normal and leukemic cells, respectively. *Bottom:* Correlation of red emission versus the green emission. In each of the three dot-plots the clusters of dots near the origin represents red blood cells which were not considered in calculations of fluorescence mean values.

Table 3
Energy Transfer Efficiency (E) Between F-Con A and R-Con A Bound to Different Cells

F-[Con A][a]/ F-[Con A]$_{sat}$ (%)	HK22 cells	Normal mouse lymphocytes	Leukemic mouse lymphocytes	
			SC1[b]	SC2[c]
72.0	—	7	6	7
62.8	10	—	8	9
52.5	—	14	13	13
42.0	17	—	18	19
27.0	—	21	20	20
19.4	26	—	25	27

[a]Percentage of Con A binding sites occupied by F-Con A.
[b,c]Data analysis was triggered by SC1 (small cells) or SC2 (large cells) regions.

assuming a smooth spherical shape for the cell surface. The average diameters of the cell types were estimated from the scattered light intensity distribution curves (as compared to those of cells with known average diameters), and these data are summarized in Table 4. Another estimate of Con A surface density was obtained by fitting the FRET efficiency data to theoretical curves describing the energy transfer processes in two dimensions (7,26). The critical closest distance used in these calculations was assumed to be 6 nm, which is in good agreement with the crystallographic data of the Con A molecule (11,16). The FRET efficiency values were the same regardless of the cell type, suggesting the same effective surface density of Con A receptors for the dif-

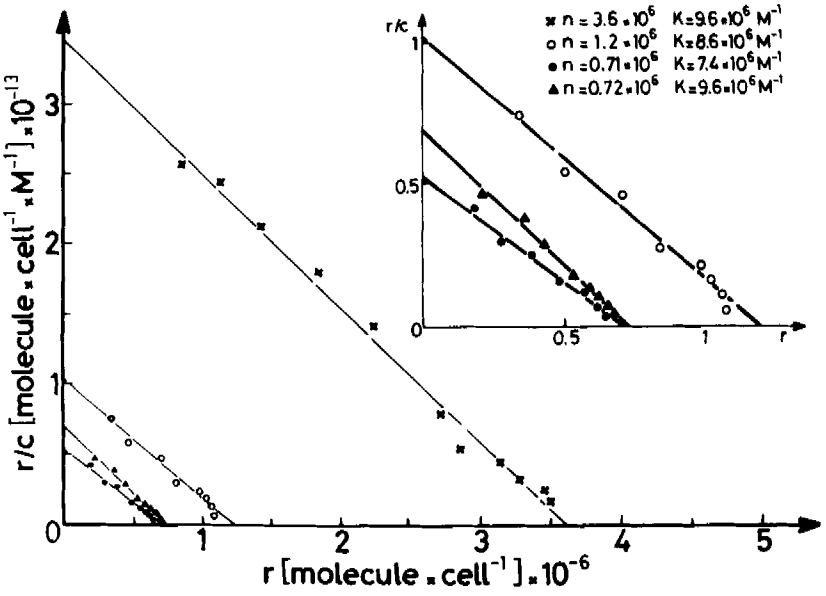

Fig. 3. Scatchard plots demonstrating the variation in number (n) of Con A binding sites for different cell types all having the same average association constant (K). HK22 cells (x); leukemic cells (o) and "normal" lymphocytes (●) prepared from leukemic $AKCF_1$ mice; normal lymphocytes (▲) prepared from normal $AKCF_1$ mice.

Table 4
Cell Surface Density (D_{surf}) of Con A Binding Sites (BS) on Different Cells

Parameters	HK22 cells	normal mouse lymphocytes	Leukemic mouse lymphocytes SC1[a]	Leukemic mouse lymphocytes SC2[b]
Diameters [μm]	15	8	8	11.5
Con A [BS/cell]	3.6×10^6	7.1×10^5	7.2×10^5	1.2×10^6
D_{surf} [BS/μm^2]				
Assuming smooth sphere surface	5.1×10^3	3.6×10^3	3.6×10^3	2.9×10^3
Calculated from ET efficiency values[c]	1.0×10^4	1.0×10^4	1.0×10^4	1.0×10^4

[a,b]Regions in light scatter histograms (SC1 = small cells, SC2 = large cells).
[c]Energy transfer (ET) efficiency data were fitted to theoretical curves (12) describing ET processes in two dimensions.

ferent cells (Table 4). This value was two to three times higher than that of a smooth sphere with a uniform Con A distribution.

DISCUSSION

Previously, the flow cytometric energy transfer method was the only available technique for studying heterogeneous cell suspensions with respect to FRET efficiency values between ligand binding sites on cell surfaces (5, 24, 25). Here we have shown that, with modifications of the FCET method, FRET efficiency can be measured on a single cell basis using a single beam flow cytometer. Experiments carried out on HK22 cells demonstrated the applicability of the method. Since the mean fluorescence intensities of distribution curves were used for the determination of FRET efficiency values, the results of the different calculation procedures could be compared as shown in the Table 2. Except for the results gained with the sensitization method using 488 nm excitation, which were less than the other corresponding values, the different calculation methods yielded almost identical FRET efficiency data. This is explained by the rather low excitability of rhodamine at 488 nm, which results in a fluorescence signal of low intensity, consequently the autofluorescence of cells contribute significantly to the signal diminishing the accuracy of measurement using the sensitization method. Cell autofluorescence may contribute to the experimental error of the other fluorescence intensities as well. Correlation for the autofluorescence can be easily accomplished using mean fluorescence intensities. When the determination of FRET efficiency is performed on a single-cell basis, the correction for autofluorescence can also be achieved by subtracting the averaged value of autofluorescence from

the fluorescence intensities; however, such corrections give rise to some broadening of the distribution curve of FRET efficiency. Making this correction on a cell-by-cell basis would result in much better data but, unfortunately, there is no simple way to do it because of the weak correlation between cell size and cell autofluorescence.

Using equation 13 for the determination of the propogation of errors demonstrated that the main source of error in E calculation was the standard deviation of fluorescence intensity measured above 580 nm (I_2). This error can be decreased by improving the red sensitivity of the detector system and by increasing the "red" signal, i.e., the concentration of the rhodamine in the system. Lowering the autofluorescence of cells above 580 nm would also reduce of the standard deviation of energy transfer efficiency.

Because there are fewer sources of error, our calculation method is superior to the quenching and the sensitization methods even in the case when mean fluorescence intensities as well. Correction for the autofluorescence can be easily accomplished using mean fluorescence intensities. When the determination of FRET efficiency is performed on a single-cell basis, the correction for autofluorescence can also be achieved by subtracting the averaged value of autofluorescence from the fluorescence intensities; however, such corrections give rise to some broadening of the distribution curve of FRET efficiency values. The stability of laser power is critical only in the determination of factor α.

Adaptation of the FCET method (5,24,25) to a single-beam flow cytometer has not reduced the information obtained, compared to a double-beam flow cytometer, for homologous ligands and for ligands that bind to the same membrane protein albeit to different epitopes. The calculation of FRET between heterologous ligands can also be carried out by this procedure using mean fluorescence intensities of the distribution curves, when investigation of the heterogeneity of FRET efficiency is not necessary. This method is still free of errors coming from unbound ligands, cell debris, and nonviable cells with altered or perturbed ligand binding capacity. Thus the extended and simplified FCET method may become more widespread than the more sophisticated double-beam method, because it requires only the simplest single-beam flow cytometer.

In this work the method of calculating FRET effiicieincy has been applied to heterogeneous cell populations, i.e., to cell suspensions prepared from Gross virus leukemic mice. Previously, it has been demonstrated that normal and leukemic cells present in the sample can easily be distinguished according to their size (8,22,23). This finding has been used to follow the development and the spontaneous regression of leukemia in different mouse strains (8,22). Our results show that the values of FRET efficiency were the same for both the normal lymphocytes and the leukemic cells, strongly supporting a uniform distribution pattern of Con A receptors on the cell surface of the respective cells. This finding was also supported by the fact that there was only a single straight line in the dot-plot showing the correlation between the red and the green emission of cells labeled with both F-Con A and R-Con A (Fig. 2, bottom). If a cell suspension is heterogeneous with regard to FRET efficiency, several lines of differing slope would be observed in this dot plot. This follows because the ratio of red to green fluorescence intensities is the only independent variable determining the numerical value of E. In fitting the FRET efficiency data to theoretical curves describing FRET processes in two dimensions (6,25), the same values of effective surface densities of Con A binding sites were found for HK22 cells as well as for normal and leukemic mouse cells. Their surface density was significantly higher for these cell types than that determined using the assumption of a smooth spherical cell surface and uniform Con A distribution. This finding supports the hypothesis raised earlier (3,24,25) that the Con A receptors are not evenly distributed but form microclusters at submicroscopic level. This microclustering can explain the results of Dale et al. (4), who found no differences in FRET efficiencies between cells labeled evenly with F-Con A and R-Con A and cells capping the same labels.

The use of the mean fluorescence intensities of distributions to calculate energy transfer by the flow cytometric method has important advantages compared to the spectrofluorimetric measurements carried out on cell suspensions. Although both procedures yield average values for FRET efficiency, free ligands, cell debris, and cell aggregates present in the sample may distort the results of the spectrofluorimetric measurements while the gated data analysis in the flow cytometric measurements minimizes the effects of these factors. Moreover the heterogeneity of the cell population can be studied by the flow cytometer using gated data analysis and/or appropriate dot plot distributions.

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