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Cell-by-Cell Autofluorescence Correction for Low Signal-to-Noise Systems: Application to Epidermal Growth Factor Endocytosis by 3T3 Fibroblasts

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Autofluorescence of cells can be a major portion of the fluorescence signal in many systems, especially when fluorescent conjugates are used to study receptor-ligand systems for which there are less than 70,000 receptors per cell. We have devised a method for the cell-by-cell correction of autofluorescence for flow cytometric data by using an additional parameter to measure and correct for autofluorescence in the fluorescence channel. The principle has been extended to allow simultaneous correction for autofluorescence and dual-fluorescence spillover compensation in samples labeled with two different fluorochromes; all corrections were done in software, making them applicable to any flow cytometer. The autofluorescence correction method was used to analyze the acidification of epidermal growth factor (EGF) by Swiss 3T3 cells. EGF is acidified to pH 6.2 starting two min after labeling, with a half-time for acidification of 45 s.

Key terms: Ligand acidification, multiparameter flow cytometry, receptor-mediated endocytosis, endosome pH

Flow cytometric analysis of mammalian cells is often complicated by high levels of autofluorescence. This autofluorescence has been attributed mostly to pyridine and flavin nucleotides (1,2). The autofluorescence spectrum of most cell types is typically very broad; the emission resulting from excitation at 488 nm ranges from 500 to 700 nm, with a peak emission at 550 nm (2). Thus the background caused by autofluorescence is a particular problem with fluorescein-conjugated probes.

By comparison of unlabeled cells with calibration microspheres having known numbers of fluorescein equivalents, we have estimated the magnitude of the autofluorescence signal to be equivalent to, on the average, 33,600 molecules of fluorescein per Swiss 3T3 cell. Thus, for samples with less than 70,000 fluorescein-equivalents of antibody or ligand bound per cell the signal due to autofluorescence is more than one-third of the total signal. The degree to which small numbers of bound molecules can be measured can be expressed as a "signal-to-noise" ratio (S/N) in which the sole contributor to "noise" is the autofluorescence background.

Low S/N are common for ligand-receptor systems studied by flow cytometry. For example, neutrophils have 60,000 to 75,000 receptors for fluoresceinated formylated hexapeptide, yielding a S/N of 3 to 5 (7). To study the processing of epidermal growth factor (EGF) by Swiss 3T3 cells we have prepared a fluorescein isothiocyanate (FITC) conjugate that retains full binding activity. Using this conjugate under saturating conditions, we typically observe a S/N of 1.35 (i.e., 19,500 receptors per cell). Of course, the S/N decreases dramatically as the FITC-EGF is internalized and quenched by acidification. In combination with the large coefficient of variation typically observed for autofluorescence in cultured cells (e.g., 45% for 3T3 cells), such a low S/N makes accurate measurements extremely difficult. The variation in cell size, which can affect both receptor number and autofluorescence, is large enough that in a low S/N system, large unlabeled cells may be more fluorescent than small labeled cells.

To overcome some of these problems, we describe here a method for using either light scattering or a second wavelength "autofluorescence" emission to correct for autofluorescence on a cell-by-cell basis. This method is generally applicable to systems in which autofluorescence is a major part of the measured fluorescent signal, and can be extended to correct simultaneously more than one fluorescence parameter. We have implemented

the correction with software; it can also be implemented in hardware directly on the flow cytometer.

MATERIALS AND METHODS

Cells and Reagents

FITC-dextran (MW 70,000) was purchased from Sigma. TRITC-dextran (tetramethylrhodamine-conjugate) was prepared similarly (dibutyltin-dilaurate method). FITC-EGF was prepared by the reaction of fluorescein isothiocyanate (FITC) with the N-terminus of EGF in 100 mM sodium carbonate buffer (pH 9) for 2 h at room temperature. The reaction mixture was dialyzed against two changes of distilled water overnight at 4°C. Aliquots were frozen until the day of use.

Subconfluent Swiss 3T3 cells were grown on 60-mm culture dishes in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 1% Penicillin-Streptomycin. For EGF labeling (Figs. 2,4) cells were washed twice with phosphate-buffered saline (PBS) (without Ca^{2+} or Mg^{2+}) and 0.5 ml of medium (without serum) was added. After 10 min, FITC-EGF was added to a concentration of 10 nM. To determine the specificity of endocytosis (Table 2), some plates received, in addition, unlabeled EGF to 325 nM. After various times at 37°C the cells were washed twice with PBS and suspended either by scraping in PBS with a rubber policeman or by incubation for 5 min at 37°C in 0.5% trypsin or 10 min at 0°C in 10% trypsin (after trypsinization an equal volume of PBS containing 1% bovine serum albumin was added to inhibit further enzyme action). Unless otherwise indicated, trypsinization was done at 37°C. To determine the spillover between F(488,530) and other fluorescence parameters, strongly fluorescent samples were prepared by incubating cells in 1 mg/ml FITC-dextran for up to 2 h at 37°C followed by washing ten times with PBS and scraping in PBS. For the three fluorescence spillover correction (Fig. 3, Table 3) cells were labeled and washed identically with the incubation time being 30 min; TRITC-dextran (1 mg/ml) was used in addition to the FITC-dextran.

Flow Cytometry

All flow cytometric analyses were performed using a FACS 440 and a Consort 40 Data Management and Control System (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Forward light scatter, right-angle (side) scatter, and two fluorescence signals were measured simultaneously in list-mode (pulse height was measured in all cases unless otherwise indicated). The 488-nm line of an argon ion laser (Spectra-Physics, Mountain View, CA) was used for excitation (laser power was 600 mW). Fluorescence was measured using appropriate photomultipliers and optical filters: fluorescein emission (designated F[488,530]) with a 9924A PMT (EMI Gencom, Inc., Plainview, NY) (high voltage 800 V) and a 530-nm bandpass filter with a 30-nm bandwith, and autofluorescence (designated F[488,625]) with a 9798A PMT (high voltage 900 V) using a 625-nm bandpass filter with a 35-nm bandwidth. For three fluorescence spillover correction, F(488,575) was measured instead of side scatter, using a 9924A PMT (high voltage 950 V) and a 575-nm bandpass filter with a 15-nm bandwidth; F(488,600) was collected instead of F(488,625) using the 9798A PMT (700 V) and a 600-nm longpass filter; F(488,535) was collected using a 9924A PMT at 700 V. For each sample, 10,000 to 20,000 live cells (selected with a threshold on forward scatter) were acquired.

For calculating fluorescein-equivalents of autofluorescence, samples of unlabeled cells were compared with calibration microspheres obtained from Becton Dickinson (BDRC beads) and Ortho Diagnostics (Fluorotrol). Under our conditions, the calculated number of channels per fluorescein equivalent differed by a factor of 1.7 to 2.1 between these two sets of standards; this may be attributed to differences in the definition of fluorescein-equivalent. The values obtained with the Fluorotrol were used.

Autofluorescence correction was performed using CALC4, a utility for performing user-specified calculations, including dual-fluorescence compensation, on four-parameter list mode data.

RESULTS

When excited at 488 nm, cultured fibroblasts show an emission spectrum ranging from 500 nm to beyond 600 nm (1,2). However, the autofluorescence emission spectrum is wider than that of fluorescein. Therefore, measuring emissions at 520 nm and 620 nm, for instance, should yield partially independent values from which the relative amounts of autofluorescence and fluorescein emission can be determined.

Selection of Autofluorescence Parameter

In order to use a parameter to correct for autofluorescence, two criteria must be met: first, the parameter must be highly correlated with the autofluorescence exhibited by an unlabeled control under all conditions of the experiment, and second, the parameter must not be significantly affected by fluorescence from the probe being used. We have chosen F(488, 625) because it is well suited for autofluorescence correction as measured by these criteria. Figure 1 shows dual-parameter histograms of F(488,530) in combination with four other parameters: forward scatter, side scatter, side scatter pulse width, and F(488,625) for unlabeled 3T3 cells. The correlation coefficients for these combinations of parameters for 3T3 cells are given in the legend to Figure 1. The correlations typically displayed little variance between different cell preparation methods. The highest correlation with F(488,530) was observed for F(488,625), followed by side scatter, side scatter pulse width, and forward scatter. Some correlation of autofluorescence with side scatter is to be expected, since side scatter is to at least some degree a measure of cell size and autofluorescence would be expected to increase with increasing cell volume (assuming a relatively constant

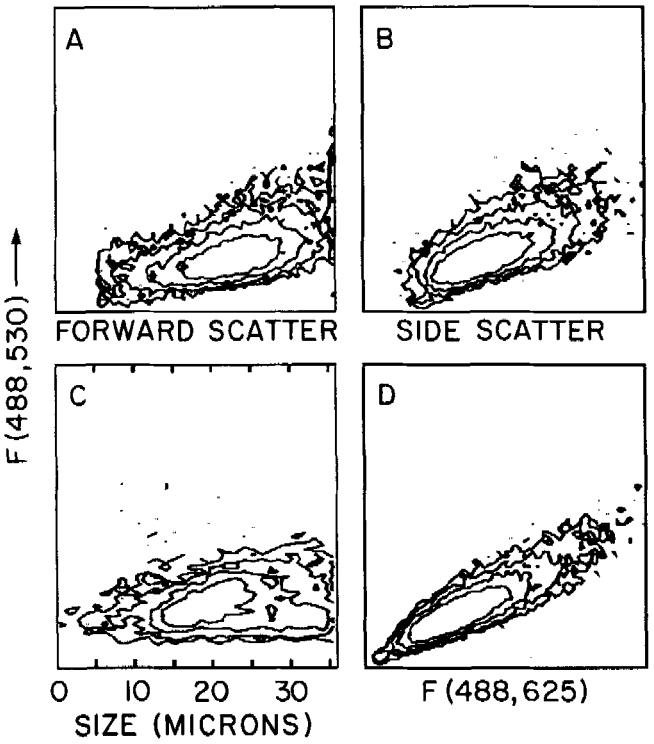

FIG. 1. Correlations of autofluorescence at 530 nm with various parameters measured by flow cytometry. Dual-parameter histograms of $F(488,530)$ vs. forward scatter (A), side scatter (B), side scatter pulse width (C), and autofluorescence at 625 nm (D) are shown. Side scatter pulse width was calibrated with microspheres of known size to obtain an estimation of cell size (ordinate axis in C). Samples are unlabeled, scraped 3T3 cells. Contours are drawn at 2, 6, 18, and 48 cells per bin. Correlation coefficients for these samples are (A) 0.416, (B) 0.457, (C) 0.487, and (D) 0.824. Correlation coefficients for samples of cells that were trypsinized instead of scraped and cells that were treated with methylamine (a lysosomotropic amine that induces vacuolization) varied by less than 10% from these values.

cytoplasmic concentration of autofluorescent cell constituents). Since these concentrations may vary with the cell cycle and other factors influencing cell metabolism, the correlation is not expected to be perfect. In contrast, if the *relative* concentrations of all autofluorescent molecules are the same from cell to cell (or only one species dominates the autofluorescence under all conditions), a very high degree of correlation between $F(488,530)$ and $F(488,625)$ might be expected. The observed correlation coefficients above 0.8 are consistent with this assumption; squaring these correlation coefficients reveals that 67–76% of the variation in $F(488,530)$ is accounted for by variation in $F(488,625)$. This parameter thus fulfills the first criteria for use in autofluorescence correction.

To determine whether $F(488,625)$ would be affected by the presence of a fluorescein-conjugated probe in a given sample, cells were incubated with FITC-dextran under conditions that produced a S/N (ratio of $F[488,530]$ for labeled and unlabled samples) of 70. The spillover was 12% for this sample (Fig. 2A).

Table 1 lists correlation coefficients and spillover constants for other optical filters. While it showed the highest degree of correlation with $F(488,530)$, $F(488,575)$ was eliminated as a potential autofluorescence correction parameter because of the high degree of spillover from fluorescein fluorescence.

Table 1
Autofluorescence Correlations and Spillover[a]

Wavelength (nm)	Bandwidth (nm)	Correlation with $F(488,530)$	FITC spillover (%)
575	25	0.843	16.1
625	35	0.824	12.0
600	Long pass	0.814	3.5
585	15	0.727	23.4

[a]Cells were labeled with FITC-dextran at a concentration of 1 mg/ml for 100 min, then scraped and run. The signal-to-noise ratio in the fluorescein channel (530 nm) was greater than 70.

Illustration of Autofluorescence Correction: One-Way Method

To perform simple autofluorescence correction the spillover constants were calculated by fitting a straight line to data for unlabeled cells:

$$F(488,530) = c + S_{530,625} * F(488,625). \quad (1)$$

$S_{530,625}$ is a constant defining the spillover from the 625-nm channel into the 530-nm channel; it is the slope of the line fit to the unlabeled cell data. Figure 2A shows data and the fitted line for a typical sample. The calculated value for the spillover constant varied less than 2.5% among samples within an experiment. The "background" in the 625-nm channel was then calculated as:

$$B_{625} = -c/S_{530,625}. \quad (2)$$

This background, which was always less than 4.8% of the full range (n = 18), was most likely due to noise in the photomultiplier or imperfect calibration of the offset circuit of the analog-digital converter. The values obtained from the control were used to correct experimental samples as follows:

$$F_{530} = F(488,530) - [F(488,625) - B_{625}] * S_{530,625} \quad (3)$$

where F_{530} is the amount of fluorescein fluorescence that would have been observed in the absence of any autofluorescence. This calculation was done on a cell-by-cell basis; Figures 2D,E show dual-parameter histograms for the corrected labeled and unlabeled samples. The overlap between the single-parameter histograms is reduced (Fig. 2F) compared with the uncorrected samples (Fig. 2C). The number of positive cells (those with fluorescence greater than the 99th percentile for the unlabeled control) increased from 17% before correction to 65% after correction (Table 2). Note that the control level is, by definition, 1%. To demonstrate the specificity of internalization of FITC-EGF, cells were incubated

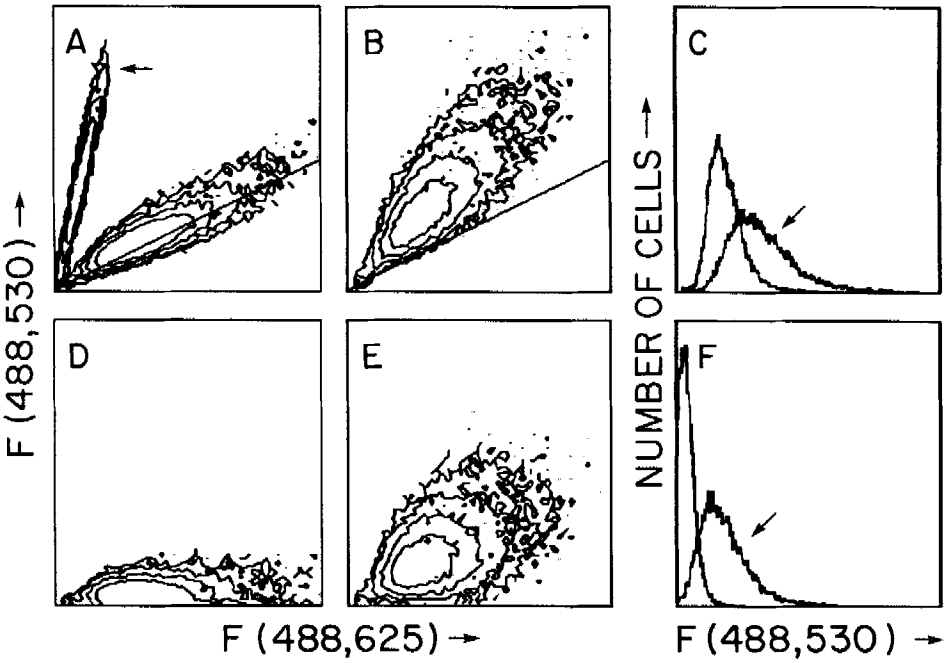

FIG. 2. Illustration of dual-parameter autofluorescence correction method. A,D. Dual-parameter histograms of control cells scraped from tissue-culture dishes. B,E. Dual-parameter histograms of cells labeled with FITC-EGF at 10 nM for 30 min. C,F. Histograms of $F(488,530)$ for the contol and the labeled samples are overlayed. A–C. Uncorrected data. D–F. Autofluorescence-corrected data. Note the decrease in overlap between the control and FITC-EGF histograms from the uncorrected to the corrected histograms. The second set of contours shown in A (arrow) show the amount of spillover of fluorescein fluorescence into the autofluorescence channel. Cells were labeled with FITC-dextran as described in the text. The line drawn through panel A represents the least-squares best fit as described in the text (Eq. 1) (it is reproduced in panel B for reference).

Table 2
Specificity of Internationalization of FITC-EGF[a]

	Correction	Control	Labeled	Blocked	Percent specific[b]
Percent positive[c]	No	0.96	17.09	1.72	
	Yes	0.99	64.96	3.27	
Mean $F(488,530)$	No	49.11	84.41	50.76	95.33
	Yes	9.45	46.64	15.03	85.00

[a]Samples were labeled with 10 nM FITC-EGF and blocked with a 33-fold excess of unlabeled EGF. Cells were trypsinized from tissue culture plates before running on the cytometer.
[b]Calculated by subtracting the mean of the controls from the labeled and blocked samples.
[c]Percentage of cells having a measured fluorescence greater than that of 99% of the control cells.

with FITC-EGF with or without an additional 33-fold excess of unlabeled EGF for 30 min at 37°C and trypsinized. At a concentration of 1×10^6 cells/ml and a concentration of FITC-EGF close to the K_d, this excess of unlabeled EGF should reduce the amount of signal due to specifically bound EGF by 94.2%. Thus, the values in Table 2 reflect signal from FITC-EGF that is specific based on competition by unlabeled EGF.

Two-Way Spillover Correction

When spillover from the fluorochrome into the autofluorescence channel is sufficient to provide a significant portion of the signal in that channel, then a more exact, two-way correction must be done. This two-way correction is identical to dual-fluorescence compensation, using the autofluorescence parameter as of the two fluorochromes.

The error introduced by correcting for autofluorescence by one-way spillover only as opposed to correction by two-way spillover is as follows:

$$E = \frac{S_{625,530} * S_{530,625}}{1 - S_{625,530} * S_{530,625}} \quad (4)$$

where $S_{625,530}$ is the spillover from the 530-nm channel into the 625-nm channel and $S_{530,625}$ is the spillover from the 625-nm channel into the 530-nm channel. For the FITC-EGF acidification data in this paper, this error was approximately 9%. Thus, this data was analyzed using one-way spillover correction as opposed to the more complicated two-way correction.

Correction for Several Fluorochromes

To test whether autofluorescence correction could be done simultaneously with dual fluorescence compensa-

tion, cells were labeled with FITC- or TRITC-dextran, and analyzed using 488-nm excitation. Three emission bands were measured to provide sufficient information to estimate FITC, TRITC, and autofluorescence concentrations on a cell-by-cell basis. The FITC emission was predominant in the 530-nm band, while all three fluorochromes contributed to the 575- and 600-nm emissions. Because the TRITC was excited with 488-nm light, it showed a considerably weaker signal than the FITC. Thus, though the *S/N* for FITC was over 10, the *S/N* for TRITC was only about 2.

The method described below was used to correct simultaneously for autofluorescence in emission from two different fluorochromes; however, it is a general method for the correction of the spillover of any number of fluorochromes when an equal number of independent measurements are available.

We wish to obtain from a spectrum, $M(w)$ (a vector containing the fluorescences measured at each wavelength w), the relative concentrations of fluorochromes, $C(f)$ (a vector where f is the fluorochrome number). In our case both vectors are of length three and the fluorochromes are FITC, TRITC, and autofluorescence. The two vectors are related by a matrix of coefficients $A(w,f)$ (with the first index being the rows and the second the columns of the matrix):

$$M(w) = A(w,f) * C(f). \quad (5)$$

We may solve for C(f) by premultipying by the inverse of the coefficient matrix. To calculate C(f), we must determine the matrix of coefficients A(w,f). This calibration is simply done by measuring n samples, which are labeled only (or predominantly) with each of the n fluorochromes. In our example, the three samples were cells only, cells plus FITC-dextran, and cells plus XRITC-dextran: thus, all samples contained autofluorescence. If the measurements of fluorescence for each sample are denoted by the matrix A'(w,s), where s is the calibration sample number, then

$$A(w,f) = A'(w,s) * B^{-1}(s,f) \quad (6)$$

where the matrix $B(f,s)$ represents the relative concentration of each fluorochrome in the samples. If each sample consists of pure fluorochrome, then $B(f,s)$ is simply the identity matrix and $A(w,f) = A'(w,s)$. However, in our example, there is autofluorescence in all three samples and the matrix $B(f,s)$ is:

$$B(f,s) = \begin{matrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 1 & 1 & 1 \end{matrix}$$

In this matrix, the columns are cells only, cells plus FITC-dextran, and cells plus TRITC-dextran, respectively, and the values represent the relative concentrations of FITC, TRITC, and autofluorescence, respectively, in the three samples. This operation just represents subtraction of autofluorescence from the FITC and TRITC signals. For the samples we used, the following coefficient matrix was obtained:

$$A(w,f) = \begin{matrix} 86.13 & 1.18 & 3.68 \\ 47.12 & 11.50 & 6.82 \\ 25.96 & 12.30 & 31.77 \end{matrix}$$

In this matrix, each column represents the *relative* contribution of FITC-dextran, TRITC-dextran, and autofluorescence, respectively, to the 530-emission band (first row), 575-emission band (second row), and 600-emission band (third row).

Figure 3 demonstrates the results of this three-fluorescence spillover correction. Histograms of four samples are shown: cells only, cells labeled with each fluorochrome, and cells labeled with both fluorochromes simultaneously. Figure 3A shows the histograms of the uncorrected data; Figure 3B shows the histograms of the spillover-corrected data. The first three samples were used to calculate the correction matrix. Table 3 lists the standard deviations for the distributions and the percent positive cells before and after correction. There are several noteworthy features: (1) There is a considerable decline in the spillover between parameters in the controls after correction. (2) The amount of each fluorochrome in the coincubation sample is much closer to the amounts in the singly labeled controls after correction. This is expected since the incubation conditions were identical, and is reflected in the percent positives and the overlayed histograms in Figure 3(3). The amount of autofluorescence (F_{600}) after correction is constant, whereas before correction it varied because of spillover from the fluorochromes (4). The standard deviations of the distributions decreased after correction. Overall, these indicate the success of the correction in reducing spill-over and simultaneously correcting for autofluorescence.

Application to FITC-EGF Acidification

As discussed above, accurate measurements of acidification of ligands with low numbers of receptors are made difficult by high levels of autofluorescence. We have used the autofluorescence correction method described above to determine whether the kinetics of acidification of EGF were consistent with those obtained for other ligands. Biphasic acidification of internalized ligands was first observed for insulin (4,5), and then for internalized antibodies directed against H-2K (6). In these cases, ligand was acidified to pH 6 within 5 min, followed by a slower subsequent acidification to pH 5.

Cells were incubated with FITC-EGF for various times between 0 and 10 min. After trypsinization on ice, the cells were analyzed to obtain the fluorescence of the internalized FITC-EGF. Methylamine was then added to raise the pH of acidic vesicles, allowing an accurate

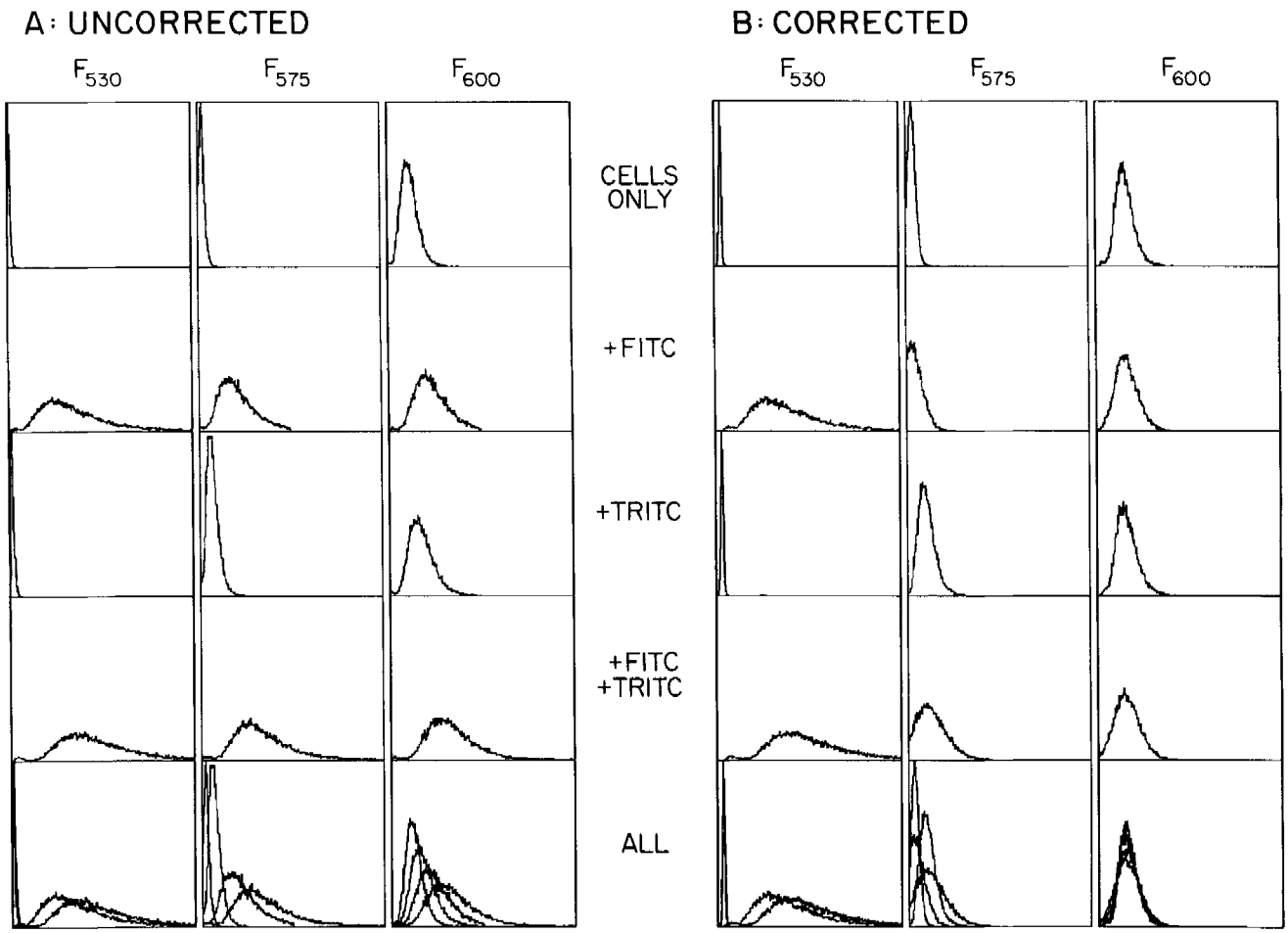

FIG. 3. Three-fluorescence spillover correction. 3T3 cells were incubated in the absence or presence of 1 mg/ml FITC-dextran, 1mg/ml TRITC-dextran, or both for 30 min. After incubation, the samples were washed ten times with PBS and scraped. Histograms of FITC signal (530 nm), TRITC signal (575 nm), and autofluorescence (625 nm) are shown for the four samples. A. Uncorrected data. B. Data corrected by three fluorescence spillover correction using the cells-only, FITC, and TRITC samples for calibration. An offset of ten channels was added to each corrected histogram after the calculations according to equation 6 but before histogramming. This was done to move the peak away from the axis.

Table 3
Three-Fluorescence Spillover Correction

Sample[a]	Corrected	FITC SD[b]	FITC % Pos[c]	TRITC SD	TRITC % Pos	Autofluorescence SD	Autofluorescence % Pos
Blank	No	21.21	0.88	21.77	0.99	22.76	0.99
	Yes	5.23	0.99	14.67	0.98	21.52	1.00
FITC	No	57.34	98.91	57.34	90.34	51.62	16.92
	Yes	55.60	99.43	49.87	12.89	40.13	5.77
TRITC	No	21.42	1.33	23.08	17.36	23.98	3.62
	Yes	13.92	1.12	5.71	40.64	25.41	1.10
FITC+ TRITC	No	57.29	98.99	57.34	97.08	54.63	43.33
	Yes	46.23	99.47	56.67	55.29	38.32	6.78

[a]Indicates with which dextran conjugates cells were labeled. See Figure 3 for labeling conditions.
[b]Standard deviation of the distribution for each sample.
[c]The percentage of cells that have a fluorescence greater than that of 99% of the control sample (blank).

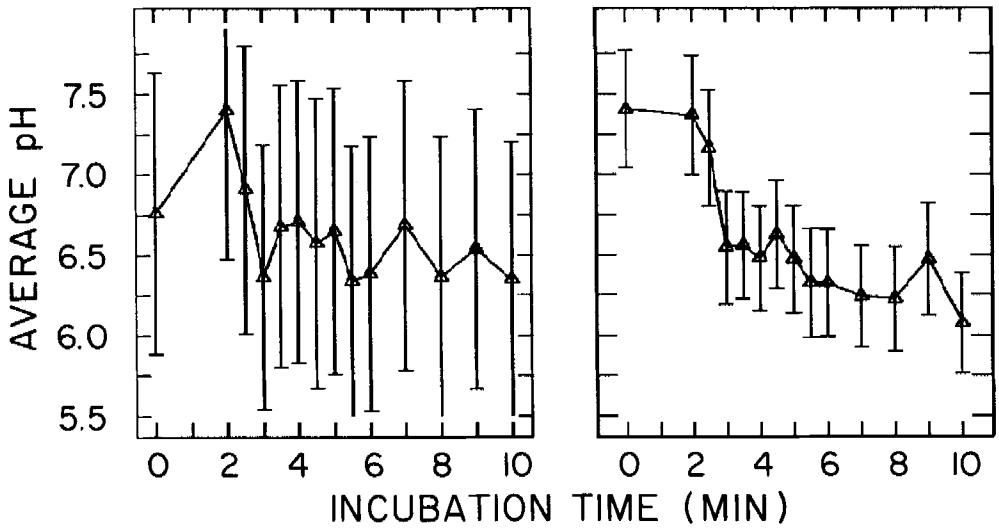

FIG. 4. Acidification of internalized FITC-EGF. 3T3 cells were incubated with 10 nM FITC-EGF for the indicated times (the 0-min sample was washed immediately after adding FITC-EGF). After the incubation, the plates were put on ice, washed, and trypsinized for 10 min. Samples were analyzed on the FACS, and methylamine was added to 200 mM. After 5 min at room temperature, samples were analyzed again to obtain the unquenched FITC-EGF fluorescence. The pH at which FITC-EGF is found is plotted as a function of time for uncorrected (A) and corrected (B) data. Error bars represent ± 1 standard deviation of the mean of each sample. The acidification is very rapid, beginning 2 min after the addition of the conjugate, and proceeding to pH 6.2 with a halftime of 45 s.

measurement of the total cell-associated FITC-EGF. The ratio of fluorescence before and after amine addition is thus a measure of pH (3) and was compared against a standard curve.

Figure 4 shows the pH of the internalized EGF as a function of time for the uncorrected and corrected samples. The noise in the measured pH is significantly reduced by the correction (note the 0-min and 3-min points). The autofluorescence-corrected data clearly demonstrate a very rapid acidification of 1 pH unit starting 2 min after labeling, with a halftime of about 45 s. Acidification then proceeds further at a slower rate, eventually reaching pH 5.2 by 45 min (data not shown).

The correction for autofluorescence on a cell-by-cell basis in these samples improved the coefficient of variation of the individual fluorescence intensity histograms considerably; the coefficient of variation was reduced an average of 59 ± 7% (n = 47). This is reflected in the error bars in Figure 4.

DISCUSSION

Quantitative estimation of (and correction for) autofluorescence is an important prerequisite for the accurate analysis of fluorescence data. Of course, the gain in accuracy by correcting for autofluorescence is dependent on the signal-to-noise over autofluorescence. In systems in which this ratio is several orders of magnitude above 1, correcting for autofluorescence is unnecessary. Once the magnitude of the autofluorescence is on the order of the experimental variance, however, appreciable improvement can be obtained by correction.

The only previous method used to correct for autofluorescence is the subtraction of the mean of the unlabeled population from that of the labeled. This, in general, is appropriate for samples in which the deviation *within* a population is less than that *between* the labeled and unlabeled population. However, this is frequently not the case for ligand binding measurements and it becomes desirable to use a cell-by-cell correction for autofluorescence.

We have described a method in which the value of a separately measured parameter is used for this correction. There are two main criteria to consider when choosing this parameter: the cell-by-cell correlation between it and the autofluorescence in the parameter to be corrected, and the amount of spillover of the fluorochrome's fluorescence into the autofluorescence parameter. The higher the correlation between the parameter and the autofluorescence in the fluorescence channel the better the correction. We find a fair correlation between side scatter and autofluorescence, presumably because of the correlation between autofluorescence and cell size. However, the best correlation is obtained by choosing a longer wavelength emission as a measure of autofluorescence in the fluorescein channel. Of course, different correlations may be observed for other cell types.

Spillover into the autofluorescence channel is an important consideration. Although not a problem when using scatter channels, it may become significant when using other wavelength emissions. Spillover into this channel introduces a dependence of the "autofluorescence" value on the level of labeling in the sample and simple correction is invalid. For S/N of less than 5, we chose $F(488,625)$ because the spillover into this channel was small compared to the actual autofluorescence at this wavelength. The error arising from ignoring the

spillover into the autofluorescence channel is given by equation 4 and was roughly 9% for our conditions.

The method we have described is generally applicable to any fluorescence measurements that can be made on individual cells or particles. We have extended the method to correct for spillover between more than two parameters and have used this derivation to corrrect simultaneously for autofluorescence in measured FITC and TRITC fluorescences. Although we have implemented the correction with software, it is possible to carry out the correction with hardware. Many flow cytometers are already equipped for dual flourescence spillover compensation. The disadvantage of doing the correction with hardware is that all the settings must be made manually. The advantage, however, is that sorting can then be done on corrected fluorescences.

We have also used this method to measure the acidification of internalized FITC-EGF. The correction resulted in much smaller variations within samples and gave a smoother curve of pH as a function of time. From this data, we can conclude that FITC-EGF is acidified to pH 6.5 from pH 7.4 between 2 and 3 min after binding to the cell surface. This rapid acidification indicates either that proton pumping begins very soon after the primary vesicle has formed from the plasma membrane or that the primary vesicles fuse within 3 min with a pre-acidified compartment. Further acidification is much slower and continuous to pH 5.2. This indicates either a slow delivery of ligand to lysosomes (or other more acidic compartments), or a maturation of the early compartment into a lysosome through slow acidification concomitant with delivery of lysosomal enzymes.

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