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A Single Laser Method for Subtraction of Cell Autofluorescence in Flow Cytometry[1]

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In flow cytometry cell autofluorescence often interferes with efforts to measure low levels of bound fluorescent antibody. We have developed a way to correct for autofluorescence on a cell-by-cell basis. This results in improved estimates of real staining and better separation of the fluorescence histograms of stained and non-stained cells.

Using a single laser, two-color fluorescence measurement system and two-color compensation electronics, autofluorescence and one fluorescent reagent are measured (rather than two fluorescent reagents). With fluorescein-conjugated antibodies the signal in the 515 to 555 nm range (green fluorescence) includes both fluorescein emission and part of the cellular autofluorescence. In the cases we have investigated, autofluorescence collected at wavelengths above 580 nm ("red") is well correlated with the green autofluorescence of the cells. A fraction of this red fluorescence is subtracted from the green fluorescence to produce an adjusted fluorescein output on which unstained cells have zero average signal.

Use of this method facilitates the selection of rare cells transfected with surface antigen genes. Culture conditions affect the level of autofluorescence and the balance between red and green autofluorescence. When applied with fluorescein-conjugated reagents, the technique is compatible with the use of propidium iodide for live/dead cell discrimination.

Key terms: Antibody detection, fluorescence compensation

Cell autofluorescence, and particularly variation in autofluorescence from one cell to another, is often a problem in cytofluorometry because it lowers discrimination between stained and unstained cells. If the autofluorescence of stained cells can be estimated on a cell-by-cell basis, it can be subtracted from the original measurement, leaving a residual signal proportional to the amount of fluorescent reagent bound. In this article we demonstrate that such an autofluorescence estimate can be made and that it provides a useful correction for measurements on cells stained with immunofluorescent reagents. The method is simple, and the instrumentation needed is already available on most commercial instruments. The reagents used in this work were fluorescein conjugated, but the method should be applicable to other systems in which there is sufficient difference between the reagent dye spectrum and the cell autofluorescence spectrum.

The correction principle is the same as for dual fluorescence compensation (4). In that system two dyes are used, which can be excited by a single source but which emit fluorescence in different wavelength regions. Fluorescence signals are measured by two detectors, one matched to the emission of each dye. Generally there is some overlap between the two emission spectra so that each fluorescence measurement includes contributions from both dyes. Compensation for this overlap is accomplished by making (subtractive) linear combinations of the two signals to yield independent measurements of the two dyes. The more difference there is between the emission spectra, the better the quality of the correction.

MATERIALS AND METHODS

Cell Lines

Mouse thymidine kinase deficient (TK^-) L cells (2) and human 143 TK^- cells (5) were transfected with high molecular weight DNA or plasmid DNA using the calcium phosphate precipitation technique (2,5). Cultures

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were analyzed and sorted in exponential growth conditions except as indicated.

When the influence of culture conditions on cell autofluorescence was studied, various cultures were seeded simultaneously at different densities in 100-mm-diameter Petri dishes. In a typical experiment, 3 days before the analysis 2×10^6, $.5 \times 10^6$, and $.12 \times 10^6$ cells were seeded in different dishes. The day before the analysis all cultures were fed. The final cell density in each dish was recorded for comparison with the cytofluorometric data. Cultures that were confluent at the time of harvest are referred to as confluent, and those that were distinctly non-confluent are referred to as sparse.

Antibodies

The fluorescein-isothiocyanate conjugated (FITC) anti-Leu-1 and anti-Leu-2 antibodies were provided by Becton Dickinson (Becton Dickinson Immunocytometry, Mountain View, CA). The anti-Trop-1 and anti-Trop-2 antibodies (3) were coupled to FITC by conventional methods (1). The fluorescein to protein molar ratio of the anti-Trop-1 antibody was 1.9. Cell staining was performed essentially as described previously (6). Unstained cell samples were incubated and washed like the stained samples. The influence of antibody binding on the autofluorescence of unstained cells was tested by reacting unconjugated antibody to Leu-1, Leu-2, Trop-1 or Trop-2 with cells transfected for the appropriate cell surface antigen. The amount of unconjugated antibody used was matched to the amount of fluorescein conjugated antibody used for staining. Dead cells were identified by staining with propidium iodide (.5 μg/ml) (6).

Flow Cytometry

Fluorescence analyses were made on a fluorescence-activated cell sorter (Becton Dickinson FACS-II) with dual compensation preamplifier and logarithmic amplifiers designed in our laboratory. Cellular autofluorescence was subtracted by adjusting two-color compensation (4). In effect, the autofluorescence is treated as a second dye in a two-color system. We adjust the compensation while running an unstained sample of the cells to be analyzed. The compensation electronics includes two potentiometers, one for adjusting the fraction of the green signal to be subtracted on the red channel and the other for adjusting the red signal to be subtracted on the green channel. Monitoring the oscilloscope traces from the green channel linear amplifier output, we adjust the second potentiometer to minimize the output for unstained cells. Since the correction is not perfect, some cells are slightly under-corrected or over-corrected, leaving a pattern of residual signals that is symmetric around the baseline. Unstained cells thus average zero net signal on the green channel, and when stained cells are run the net positive signals are proportional to the real fluorescein staining. The signal evaluation system assigns to the lowest channel any cell whose estimated fluorescence is below zero after compensation. Using a logarithmic amplifier, the proper adjustment gives 50% of the cell events in the lowest channels. Any compensation electronics similar to that described should work for this application.

Laser excitation was normally 400 mW at 488 nm. The optical filters used in the fluorescence channels were 1) a 515-nm longpass filter (Kodak Wratten 12) to

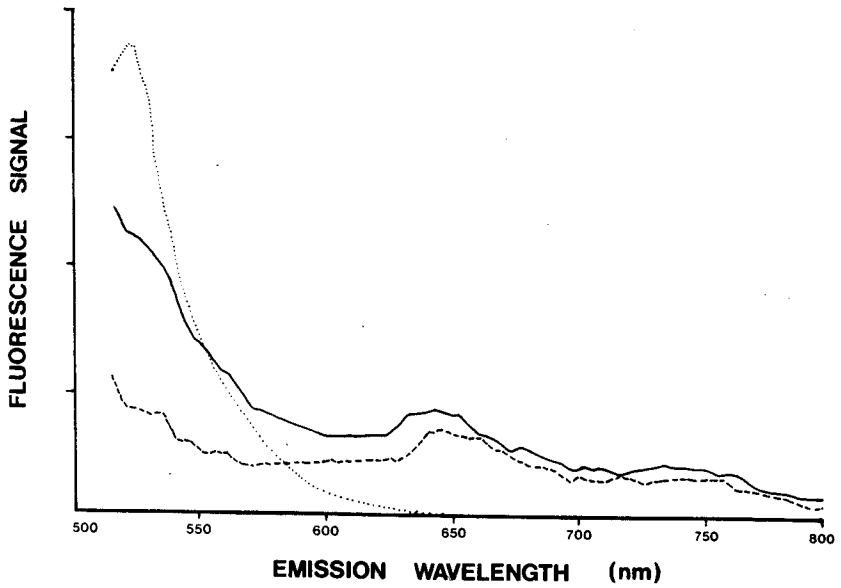

FIG. 1. Fluorescence emission spectra of L cells (solid line), 143 cells (broken line), and FITC-conjugated antibody to Trop-1 (dotted line) excited at 488 nm. Excitation window = 8 nm. Measurements were taken in 5-nm steps. The Raman scatter peak (570–600 nm) has been removed from the spectra. The spectra are uncorrected for photomultiplier spectral sensitivity to approximate what would be expected with the flow cytometer photomultipliers.

block the 488-nm scattered light; 2) a 560-nm dichroic reflector to divide light between the green and the red channels; 3) a 515–555-nm bandpass filter in the green channel; and 4) various longpass filters, principally a 580 nm in the red channel.

Spectrofluorometry

Spectra were obtained using a SLM 8000 spectrofluorometer (SLM instruments, Urbana, IL) equipped with a Hamamatsu R928 photomultiplier (PMT). The light source was a 450 W xenon arc lamp.

RESULTS

Figure 1 shows fluorescence emission spectra of L cells and 143 cells in suspension and FITC-anti-Trop-1 antibody in solution, all excited at 488 nm. The spectra are intended to illustrate the signals available to the PMTs in the cell sorter, so they are not corrected for differences in PMT sensitivity as a function of wavelength. The details of such spectra will vary somewhat between different samples of a particular cell type or between different PMTs. In the L cell spectrum 38% of the signal above 515 nm is between 515 and 555 nm (the fluorescein channel pass band), 50% is above 580 nm, and 38% is above 620 nm. The 143 cell spectrum shows a similar profile but with a larger proportion of red emission (25% from 515–555 nm, 66% above 580 nm, and 53% above 620 nm). The signal from FITC antibody above 515 nm is mostly in the 515–555-nm band (77%), while 7% of the detected signal is above 580 nm, and 1% is above 620 nm. Using the signal above 580 nm to correct cell autofluorescence results in some subtraction from the fluorescein signal due to the small amount of fluorescein emission above 580 nm. For the cell spectra illustrated the correction would decrease the observed fluorescein signal by 7% (L cells) or 3% (143 cells), but, since the fraction is uniform for a given compensator setting, it has very little effect on the data.

Figure 2 shows typical contour plots of red vs green autofluorescence for L cells in different culture conditions. Very similar results were obtained for 143 cells. The usual observation was that, compared to cells from sparse cultures, cells from confluent cultures had more red component in their autofluorescence and more variable autofluorescence brightness from cell to cell.

The correction technique relies upon there being a good correlation between the red and green autofluorescence. In log-log plots perfectly correlated data would fall on a line at 45°. As can be judged from the narrow width of the distributions in Figure 2, the correlation is good but not perfect in both culture conditions. This could be due to inadequacies in the measurements (counting statistics variation in the number of photoelectrons collected) or to small but real differences in the cell emission spectra. If counting statistics were important, we would expect the width of autofluorescence plots like those in Figure 2 to be a function of signal level. This was tested by varying the laser power from 200 mW to 1200 mW. No significant differences were found, indicating that most of the deviation from perfect correlation was due to real differences among the cells.

Multiple observations on L cells and 143 cells showed that the relative proportion of red and green autofluorescence is generally consistent but not identical for multiple samples of a particular cell type grown under standard conditions. Thus, although the amount of correction required is approximately reproducible, it is still necessary to adjust the autofluorescence correction using an unstained sample of the actual cell population being stained.

To control for any effect of the staining procedure on cell autofluorescence, the autofluorescence control cells

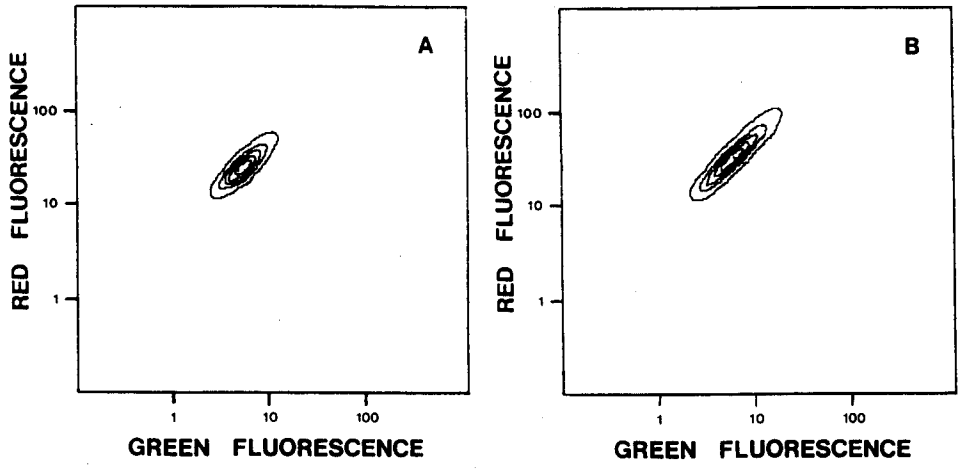

FIG. 2. Contour plots of green (x axis) vs red (y axis) fluorescence of unstained L cells from sparse (A) and confluent (B) cultures. The cells from the confluent culture averaged 16% higher in green fluorescence and 33% higher in red fluorescence than the cells from the sparse culture. Each area between the contours contains 18% of the analyzed cells.

should be processed with nonfluorescent versions of the reagents used in the actual staining. In our work the cells are kept on ice for as much as possible of the several hour period between harvest and analysis on the flow cytometer, so we expect the cells to have little ability to respond to the reagents. This expectation was confirmed by "staining" appropriate L cell transfectants with unconjugated antibodies to Leu-1, Leu-2, Trop-1, and Trop-2. Their autofluorescences were compared to those of the same transfectants totally unstained but handled in the same way, and no differences were detected. This confirms that unstained cells were adequate controls in our work. In systems where the cells might respond to the reagents, the proper controls must be tested before using unstained cells to adjust compensation.

The technique was tested by analyzing mixtures of untransfected L cells and transfected L cells expressing different levels of specific surface markers. The fluorescence intensity distributions of positive and negative cells were measured with and without compensation. Two examples are shown in Figures 3 and 4. The transfected cells used in Figure 3 expressed very low amounts of Leu-1 (Fig. 3C). Panels 3A and 3B show the fluorescence profiles of a mixture of L cells and Leu-1 transfectants without (3A) and with (3B) compensation. Much better separation of the peaks was achieved after compensation. In a selection for rare positive cells we would typically sort the brightest 0.5% of negative cells, recovering whatever fraction of rare positive cells is above the cutoff. From the data in panels 3C and 3D we would predict recovery of 79% of the positive cells with compensation but only 19% without it. This is an important difference. In this case we would expect about 4 times better selection efficiency with the correction than without it.

The transfected cells used for the experiment shown in Figure 4 expressed the Leu-2 antigen. Their average staining brightness was greater than that of the Leu-1 transfectants (panel 4C versus panel 3C). When mixed with untransfected L cells (panels 4A and 4B), better separation of the peaks was achieved with compensation. Estimating positive cell recoveries above the top 0.5% of the unstained cells, we get 98% with compensation (4D) and 62% without it (4C). Although these Leu-2 transfectants could be selected fairly efficiently without compensation, the expected selection efficiency is still increased significantly by autofluorescence correction, and the estimates of real staining are improved.

When the test procedure was applied to mixtures of L cells and transfectants from either sparse or confluent cultures, compensation improved the peak separation in all cases, but more improvement was usually observed with confluent cultures. This would be predicted from the greater range of autofluorescence typically found in confluent cultures (Fig. 2). The cells used for Figure 3

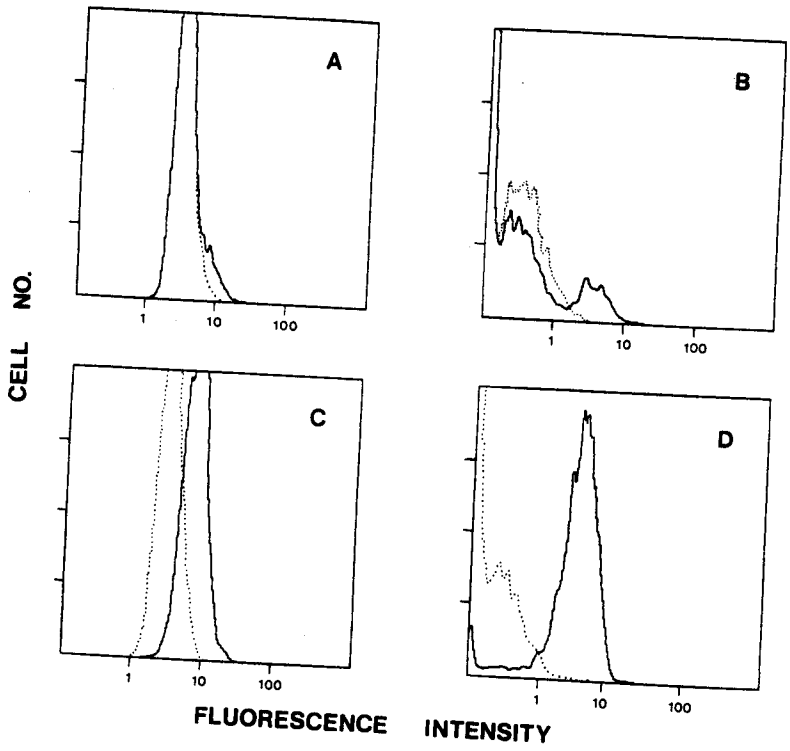

FIG. 3. Fluorescence profiles of stained (solid lines) and unstained (dotted lines) cell populations. A) and B): mixture of Leu-1 transfectants (15%) and L cells (85%), uncompensated (A) and compensated (B). C) and D): Leu-1 transfectants uncompensated (C) and compensated (D).

were from confluent cultures, while those shown in Figure 4 were from sparse cultures.

Different filters were tested on the red channel. The correlation of green/red autofluorescence was best with a 580-nm longpass filter and became progressively worse using longpass filters from 590 to 620 nm (data not shown). Using no filters on the red channel (essentially collecting all emission above 560 nm), very poor peak separations were obtained, possibly because of the high proportion (about 23%) of FITC signal collected in the red channel.

The addition of propidium iodide to the medium for live/dead discrimination (6) did not influence the pattern of compensation, since propidium-iodide stained dead cells were excluded from the analysis.

DISCUSSION

Compared to uncorrected measurements, the simple method described here for subtracting autofluorescence on a cell-by-cell basis results in better estimates of real staining, better peak separations in mixed populations, and higher selection efficiencies for rare positive cells. The amount of improvement and its value in any particular application depend on how much of a problem autofluorescence was in the first place. The improvements are particularly apparent with very dim stains on highly autofluorescent cells, as in the case of the Leu-1 transfectants used in this work. We are now using this method for sorting rare transfected cells, and we can routinely isolate dimly staining transfectants, showing that the method has clear practical value. The correction works well on both confluent and sparse cell cultures. When compared directly, confluent cell populations show greater improvement in peak separation than sparse cultures. However, the cloning efficiency of confluent L cells is about one half that of sparse L cells, so we routinely sort transfected cells from sparse cultures.

Among brightly autofluorescent cell types we have observed that the autofluorescence spectrum is always sufficiently different from that of fluorescein to allow effective compensation. We have seen good correlation between green and red autofluorescence in L cells, 143 cells, and a number of other cell lines, including HeLa, A 875, and SK-Hep 1 (unpublished observations), implying that the emission spectra of the individual cells in a population are similar. The correlation could be due either to autofluorescence emission dominated by a molecular species that emits in both wavelength ranges or to correlated amounts of different molecules emitting green or red fluorescence. The observation that the correlation is good but not exact indicates that the green and red fluorescences are not entirely due to a single fluorescent species. Interestingly, the change from a 580-nm to a 620-nm longpass filter on the red channel results in a significantly poorer correlation, indicating that much of the reddest light is from molecules whose concentration in the cells is not well correlated with

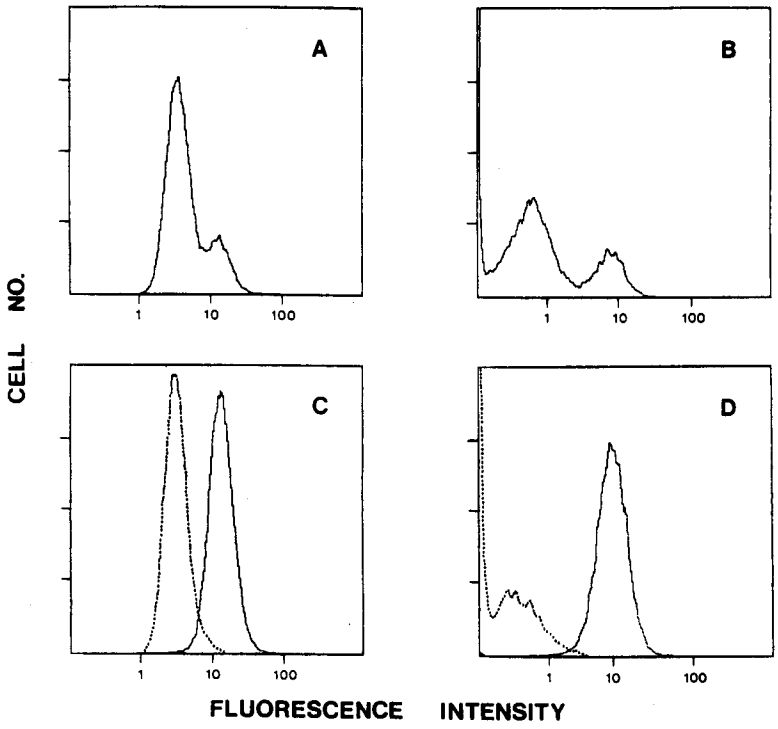

FIG. 4. Fluoresence profiles of stained (solid lines) and unstained (dotted lines) cell populations. A), B): mixture of Leu-2 transfectants (15%) and L cells (85%), uncompensated (A) and compensated (B). C), D): Leu-2 transfectants uncompensated (C) and compensated (D).

that of the species responsible for the green autofluorescence.

The binding of nonfluorescent antibody to transfectants expressing the appropriate cell surface antigens did not change cell autofluorescence. This was expected, since a change in autofluorescence due to metabolic activation by a ligand is unlikely under our staining conditions (4°C in the presence of 0.1% sodium azide). Moreover, cell surface molecules transfected into L cells may not have the functional relevance they had in their cells of origin. However, in other experimental conditions the possible influence of antibody binding on cell autofluorescence must be evaluated before using totally unstained cells for setting the autofluorescence correction.

Steinkamp and Stewart (7) have developed a similar autofluorescence correction technique, which relies on the difference between the excitation spectra of cellular autofluorescence and the immunofluorescent dye. They excite the cells at a wavelength matched to the immunofluorescent dye and collect an immunofluorescence plus autofluorescence signal in a wavelength range matched to the dye. A second laser at a wavelength that excites the dye poorly is used to generate an autofluorescence correction signal detected in the same wavelength range as the first signal. Both techniques work in practice, and, in the absence of detailed information on the molecules contributing to autofluorescence, there is no good reason to expect one technique to yield better results than the other. The dual excitation approach requires two lasers operating at appropriate wavelengths and custom electronics to subtract signals occurring at different times. Our technique is more suitable for widespread application since it uses a single laser and commonly available analog compensation electronics.

This method is in principle applicable to any experimental situation where a range of wavelengths is available for the measurement of autofluorescence. The autofluorescence measurement must be little influenced by fluorescence emission of the bound fluorochrome and must show good correlation with the autofluorescence in the range of interest. Since autofluorescence acts as the second dye in a two-color system, the channel used for defining the autofluorescence correction cannot be used simultaneously for the detection of a second antibody. The system should work at least as well for phycoerythrin reagents as for fluorescein. In that case one detector with an appropriate phycoerythrin bandpass filter would give the primary signal, and a second detector with the normal fluorescein bandpass filter would provide the signal for autofluorescence correction.

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