THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY
Vol. 25, No. 7, pp. 899-907, 1977
Printed in U.S.A.

TWO-COLOR IMMUNOFLUORESCENCE USING A FLUORESCENCE-ACTIVATED CELL SORTER[1]

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Received for publication January 11, 1977

A technique for the analysis of fluorescein and rhodamine in a flow system using a single wavelength of excitation is described. Both optics and electronics are used to discriminate the fluorescein and rhodamine signals. This technique has been used to study the relationship between immunoglobulin M and immunoglobulin D on mouse splenic lymphocytes.

Quantitative immunofluorescence has been used in flow systems to study the distribution of antigens on various populations of cells (9, 10, 14, 17, 18, 20). The correlation between two antigenic determinants, as assessed by staining the cells with reagents bearing different fluorochromes, has usually been performed by fluorescence microscopy. Studies using multiple immunofluorescent labels on cells by a fluorescence-activated cell sorter (FACS) have required a dual-pass protocol (10, 11). Cells were first separated according to one antigenic determinant, then were restained and analyzed for the second determinant.

The separation and re-analysis is required in a flow system since the fluorochromes most frequently used in immunofluorescence, fluroescein and tetramethyl rhodamine, have emission spectra which overlap. In a fluorescence microscope the light emitted by these two dyes is separated by changing both the excitation and emission filters. In a flow system using one wavelength for the excitation of the two dyes, the emission cannot be completely separated by optical filters.

This article describes a technique for the analysis of double-labeled cells in a flow system without prior separation of the cells. Using a combination of optics and electronics, cells labeled with both fluorescein and rhodamine can be analyzed so that the emission from either chromophore is detected independently even though the emission spectra of the dyes overlap. Two antigenic determinants on a population of cells can now be rapidly and quantitatively analyzed and the number of cells which express either one or both of the antigens can be determined. In addition, the relation between the relative amounts of the two antigens on the different cells can be assessed. The electronically corrected signals can be fed directly to the sorting logic of the FACS so that sorting decisions can be made based on the relationship of the two dyes.

[1] Supported in part by Grant GM17367 from the National Institute of General Medical Sciences.

MATERIALS AND METHODS

The instrument used in these studies was a fluorescence-activated cell sorter (FACS-II, Becton Dickinson Electronics Laboratory, Mountain View, Calif.). A detailed description of this instrument has been published previously (2, 12). The FACS-II system has two photo tubes which receive light from the same light collecting objective as illustrated in Figure 1. This schematic diagram is drawn from a top view, looking in the direction of cell flow. The 400 mW, 514.5 nm line from an argon ion laser (Model 164, Spectra-Physics Inc., Mountain View, Calif.) intersects the stream just below the nozzle so that the cells are illuminated while they are in the fluid jet. The 535 nm long-pass filter, a colored glass filter (No. 3-68, Corning Glass Works, Corning, N.Y.), removes the 514.5 nm light which is scattered by a cell or by the stream. A dichroic mirror (LP 580, #466305, Carl Zeiss, Inc., New York, N.Y.) is used to split the light emitted by a passing cell so that the green light is reflected into one detector while red light passes to the other detector. Further separation of green emission is provided by a 540 nm short-pass filter (Dictric Optics Inc., Marlboro, Mass.). The 590 nm long-pass filter, (No. 2-73, Corning Glass Works) improves the red color separation.

The photo tubes used in this instrument were Model 9798B (EMI-Gencom Inc., Plainview, N.Y.). The signal subtracting network uses two differential amplifiers (Model AM502, Tektronix, Inc., Beaverton, Ore.). Each photo tube output goes directly to the negative input of one amplifier (the amplifiers are used to invert the signals in addition to performing signal subtraction). A selected fraction of each

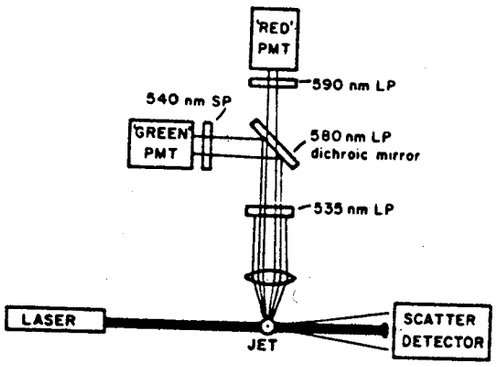

FIG. 1. Schematic diagram of the two-color fluorescence detection system. In this view from the top, the cells flow down the center of the jet and through the laser beam. Fluorescent light is collected by a lens and split by optical filters into red and green components which are detected by separate photo tubes.

photo tube output taken from a 5000 ohm potentiometer goes to the positive input of the opposite amplifier. The two amplifier outputs are then proportional to fluorescein and rhodamine fluorescence.

As the stained cells pass the laser beam three signals are generated, two corrected fluorescence signals and a forward-angle light scattering signal. These signals are digitized and directed into a PDP-11 computer (Digital Equipment Corp., Maynard, Mass.) and processed by a three-parameter software program (16). The light scattering signal discriminates live lymphocytes from red cells (15) and nonviable lymphocytes (12, 13). In addition, a cell size parameter can be generated using a light scattering (5). All data presented in this report were gated by the light scattering signal so that the fluorescence data was obtained only for viable, small lymphocytes.

Antisera: The rabbit anti-mouse immunoglobulin (R anti-MIg) was prepared by injecting rabbits with a mixture of myeloma proteins. The goat anti-rabbit Ig (G anti-RIg) was made by injecting the Ig fraction of normal rabbit serum into a goat. The goat anti-mouse IgM (G anti-MIgM) was made against the myeloma protein MOPC-104E and was specifically eluted from an immunoadsorbant column. This serum was kindly provided by Dr. John Kearney, University of Alabama. The rabbit anti-mouse IgD was prepared by Drs. Erika Abney and Michael Parkhouse (National Institute for Medical Research, London, England) (1) and was kindly provided by them. The coupling of fluorescein and rhodamine isothiocyanate to these antibodies followed the protocol of Cebra and Goldstein (3). Sera that bear fluorescein or rhodamine are designated by an F or an R, respectively, in the abbreviation, *e.g.*, ^{F}G anti-MIgM. Spleen cells were obtained from 2-month old BALB/cN mice and were stained as previously described (13).

RESULTS

Independent detection of fluorescein and rhodamine: In fluorescence microscopy the emission of fluorescein can be separated from the emission of rhodamine primarily as a result of the difference in their absorption spectra. Using a mercury-arc lamp as light source, fluorescein is usually excited by the 435.8 nm line which is a portion of the spectrum where the extinction coefficient of rhodamine is very low. Similarly, rhodamine is usually excited by the 546 nm line where its extinction coefficient is high while that of fluorescein is low. In fluorescence microscopy both the excitation and emission filters are changed to discriminate between the two dyes.

In a flow system the simplest technique to achieve dual-color analysis is to use a single wavelength for excitation and two detectors having different emission filter combinations to analyze a cell as it passes the laser beam (19). Sequential illumination of a cell by multiple wavelengths of exciting light has also been described (4).[2] In a single wavelength excitation the 514.5 nm line from an argon-ion laser can be used for the simultaneous excitation of both fluorescein and rhodamine. Even though this wavelength is above the absorption maximum for fluorescein and below the maximum for rhodamine, enough light is absorbed by both dyes to obtain a strong fluorescence signal from each of them.

The greatest difficulty encountered in dual-color analysis using single wavelength excitation is the separation of emission of the two dyes. Since the emission spectra for fluorescein and rhodamine overlap, it is not possible to optically separate the light signals from the two fluorochromes so that one detector system observes only fluorescein while the other system detects only rhodamine. A detector observing red emission cannot distinguish a cell with a large amount of fluorescein from one which has a small amount of rhodamine. Both cells may give the same red signal as observed by the detector.

The filter combinations which gave the best spectral separation for fluorescein and rhoda-

[2] Stohr M: Personal communication.

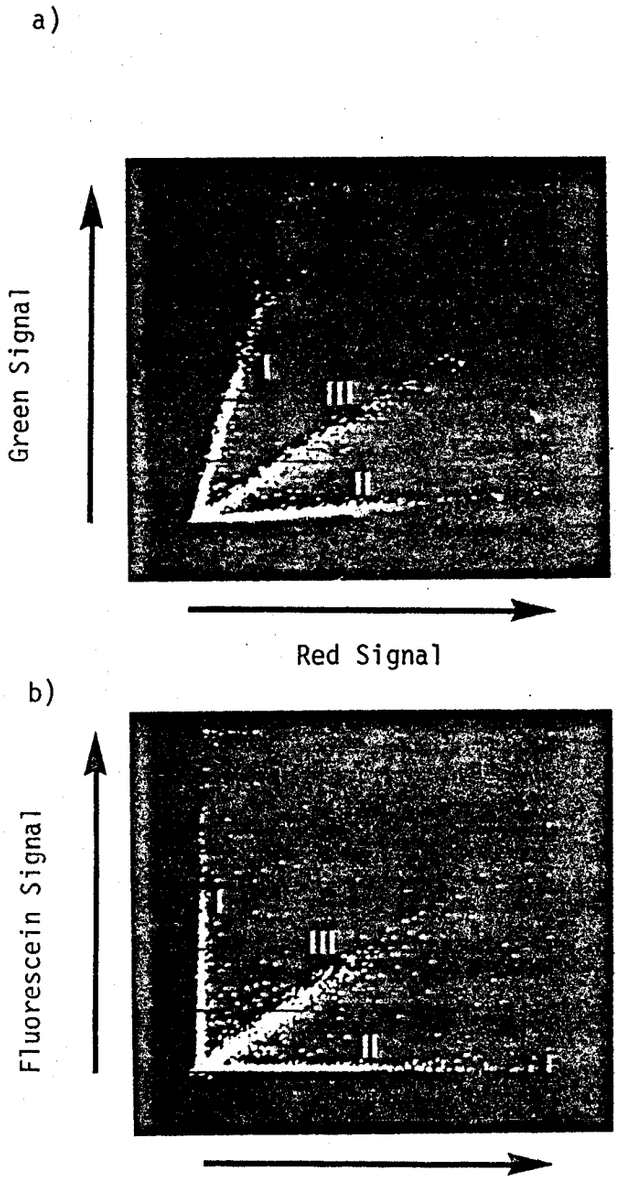

FIG. 2. Mouse spleen cells stained with anti-immunoglobulin. Group I cells were stained with fluorescein; Group II cells were stained with rhodamine; Group III cells were labeled with both fluorescein and rhodamine. An artificial mixture of these cells was analyzed (a) without, and (b) with the electronic correction for the spectral overlap of the two dyes.

mine are listed in Materials and Methods. The degree of separation of the fluorescein from the rhodamine signals is demonstrated in Figure 2. Mouse spleen cells were stained with R anti-MIg followed by either (I) ^{F}G anti-RIg, (II) ^{R}G anti-RIg, or (III) a mixture of ^{F}G anti-RIg and ^{R}G anti-RIg. Cells in group I had only fluorescein attached while cells in group II had only

rhodamine attached. Cells in group III were double-stained with both fluorescein and rhodamine. These three groups of cells were mixed together and then analyzed by means of a two-color detection system. The correlation of the signals from one detector with those generated in the other detector were observed by displaying the signals on an X-Y storage oscilloscope. Each cell is represented by a dot whose position is determined by the relative amplitude of the signals generated by that cell in the two detectors.

The two-parameter display of these three cell groups is shown in Figure 2a. Since mouse spleen cells were used, only half of the cells were stained with these anti-Ig reagents and the distribution of amount of Ig per cell was rather broad (13). In this artificial mixture, cells in group I produced large signals in the green channel and small signals in the red channel. Likewise, the cells in group II yielded strong signals in the red channel and small but significant signals in the green channel. These signal groupings are not orthogonal in the figure because of the spectral overlap of the two dyes. Although the doubly-stained cells, group III, are distinct from the other two populations, a quantitative assessment of the expression of either or both of the chromophores is difficult using this technique.

Compensation for this spectral overlap is made by an electronic cross-coupling system. A portion of each signal from the green and red channels is cross-coupled to the other channel through a differential amplifier system. In this way the component of unwanted fluorescence signal which passes each filter system is electronically removed.

The algebraic basis for this technique is simple. The signal in the green channel, $F1$, is a combination of light coming from fluorescein and rhodamine on a cell:

$$F1 = \alpha \cdot F + \beta \cdot R \qquad (1)$$

where F and R are the number of photons emitted by fluorescein and rhodamine, α and β are the efficiencies for the detection of the two colors in this channel.

A similar equation can be written for the signal in the channel, $F2$:

$$F2 = \gamma \cdot F + \delta \cdot R \qquad (2)$$

where F and R are defined as before, γ and δ are the efficiencies for the detection of fluorescein and rhodamine, respectively, in this channel. Subtracting an appropriate fraction of each signal from the other yields signals F' and $F2'$:

$$F1' = F1 - (\beta/\delta)F2 = \alpha\left(1 - \frac{\beta\gamma}{\alpha\delta}\right) \cdot F \qquad (3)$$

and

$$F2' = F2 - (\gamma/\alpha)F1 = \delta\left(1 - \frac{\beta\gamma}{\alpha\delta}\right) \cdot R \qquad (4)$$

By selecting the proper amount to subtract, the output signal $F1'$ is made proportional to F (fluorescein) and is unaffected by the presence of rhodamine emission. Likewise, $F2'$ can be made proportional only to rhodamine emission and will not be affected by fluorescein. After this correction the green channel can be referred to as the fluorescein channel while the end channel becomes the rhodamine channel.

The constants β/δ and γ/α are empirically determined. Their magnitude depends upon the spectral properties of the dyes, on the filter combinations used, and the spectral response of the photo tubes. This signal subtraction occurs prior to amplification so that a change in gain on the amplifier does not affect the fraction of the signal subtracted.

The constants are set on the feedback system by analyzing cells that carry a single-color stain. For example, if fluorescein labeled cells are run first, the potentiometer controlling subtraction of $F1$ and $F2$ is set so that the rhodamine differential amplifier output ($F2'$) is zeroed. Similarly, rhodamine labeled cells are run and the potentiometer for the fraction of $F2$ to be subtracted from $F1$ is set to zero the fluorescein amplifer output ($F1'$). Once the circuit is adjusted, the signals in the $F1$ channel are porportional to the amount of fluorescein on the cells while the signals in the F' channel are proportional to the amount of rhodamine. Because of the spread in subtracted signal values, some cells carrying little or no fluorescein will yield a slightly negative fluorescein amplifier output signal. The same is true for rhodamine. The signal processing electronics places such cells in the lowest channels in the data histograms (along with real zero signals).

Re-analyzing the three groups of cells from Figure 2a with the cross-coupling system yields a scatter plot shown in Figure 2b. The cell groups which are single-stained now lie orthogonal to each other; the amount of signal due to spectral overlap has been electronically re-

moved. The cells which are double-stained, group III, can now be analyzed for their relative amounts of fluorescein and rhodamine.

Relationship between IgM and IgD: This technique of analyzing double-labeled cells has been applied to study the relationship between the two major cell surface immunoglobulin (Ig) heavy chain classes on lymphocytes, IgM and IgD (23). These two classes of Ig appear on the same cells and express the same antigenic combining sites (6, 7). Flow analysis was used to determine how many cells express either or both classes of Ig on their surface.

Spleen cells from BALB/cN mice were stained first with a directly labeled ^{F}G anti-MIgM followed by R anti-MIgD and ^{R}G anti-RIg. In this way the IgM determinants were colored with fluorescein while IgD determinants were stained with rhodamine. These reagents were used at concentrations which gave maximum fluorescence, *i.e.*, in antibody excess. These double-labeled cells were then analyzed using the two-color detection system just described.

The scatter plot of spleen cells double-stained for IgM and IgD is shown in Figure 3. Many cells are not stained with either reagent and appear at the origin. This group of cells comprises approximately half of the spleen and includes the T and the null cells. Most of the remaining cells express both IgM and IgD on their surfaces. A few cells, which lie along the vertical axis, have only IgM while essentially none express only IgD.

The proportion of cells which express either or both antigens can be better quantitated by analysis of the histograms of these two stains. These histograms are presented in Figure 4 where the relative number of cells is plotted as a function of increasing fluorescence. (The peak occurring at the far right side of these histograms is a result of those signals which were off-scale; these were counted in the last few channels.) The upper histogram, Figure 4a, shows the distribution of IgM on these spleen cells. A clear distinction between positive and negative cells is not evident in the IgM staining distribution. This shape is typical of spleen cells labeled with anti-IgM (17). The IgD profile is truly bi-modal so that a reasonable threshold for distinguishing positive from negative can be set at the minimum between the two peaks.

Since both fluroescein and rhodamine signals are generated simultaneously, it is possible to

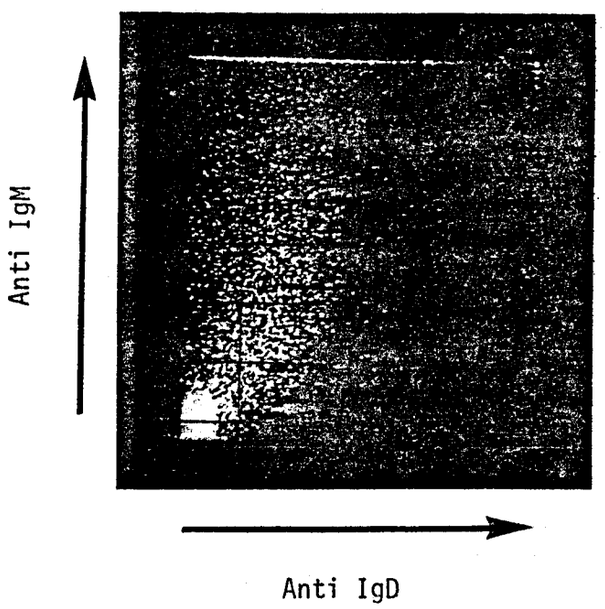

FIG. 3. Correlation of anti-IgM and anti-IgD stains on adult mouse spleen cells. Cells were first labeled with ^{F}G anti-Igm followed by R anti-IgD and ^{R}G anti-RIg. The cluster of dots in the lower left corner is a result of cells unstained by either reagent, the T and null cells. The line along the top is a result of signals which are off-scale.

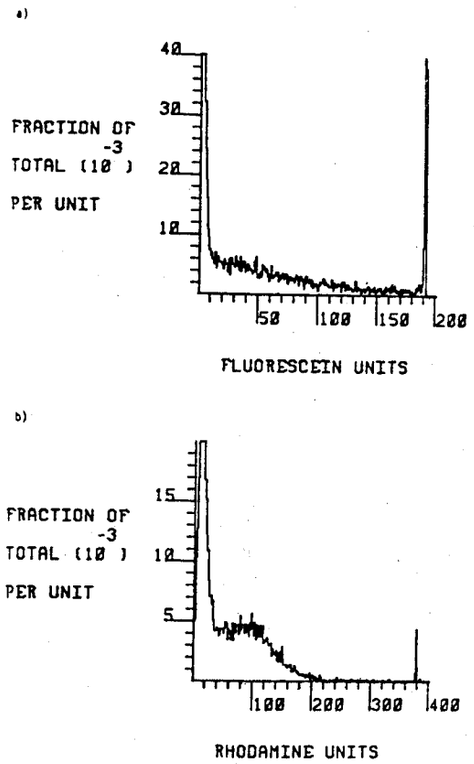

FIG. 4. Histograms of staining of the same double-labeled cell sample shown in Figure 3; (a) anti-IgM and (b) anti-IgD. The peaks on the right side of these curves are a result of cells that would appear off-scale being counted in the final channels.

collect the signals from one channel only for cells where the other signal meets certain criteria. In this manner the IgM distribution of IgD-positive cells can be obtained.

Figure 5a shows the IgM distribution of cells with greater than 60 units of rhodamine fluorescence (Fig. 4b). It is clear that those cells which express IgD also express IgM. Because the IgM distribution is continuous there is no distinct population of these IgD-positive cells which do not express IgM.

The reciprocol data on the correspondence of IgD cells with IgM-positive cells can be obtained by gating on cells bearing more than 20 units of fluorescein (Fig. 4a) and collecting the IgD fluorescence profile. This gated histogram is shown in Fig. 5b. It is clear that a population of cells expresses IgM but does not express IgD. This population comprises approximately 5% of the total splenic lymphocyte population. These data suggest that all of the cells which express IgD have IgM on their surface; however, some cells which express IgM do not bear IgD. These surface markers do not delineate exactly the same populations.

In addition to enumerating the cells that express either or both of the antigens, a correlation between the relative amounts of the two antigens can be made using this technique. The distributions of IgD can be collected by gating on cells bearing different amounts of IgM. Referring to Figure 4a, cells having 25 to 50 units of fluorescein fluorescence were termed dull IgM-positive while cells bearing 100 to 150 units were called bright IgM cells. The IgD fluores-

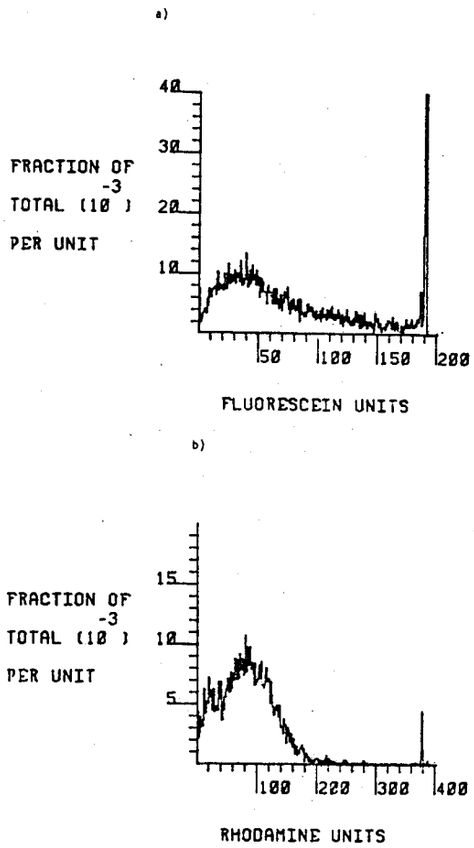

FIG. 5. Flourescence histograms of anti-IgD staining. (a) histogram of anti-IgM for cells which are IgD positive. The fluorescein signals were collected only for those cells which showed >60 units of anti-IgD stain in Figure 4b. (b), anti-IgD histogram for cells exhibiting >20 units of anti-IgM stain in Figure 5a.

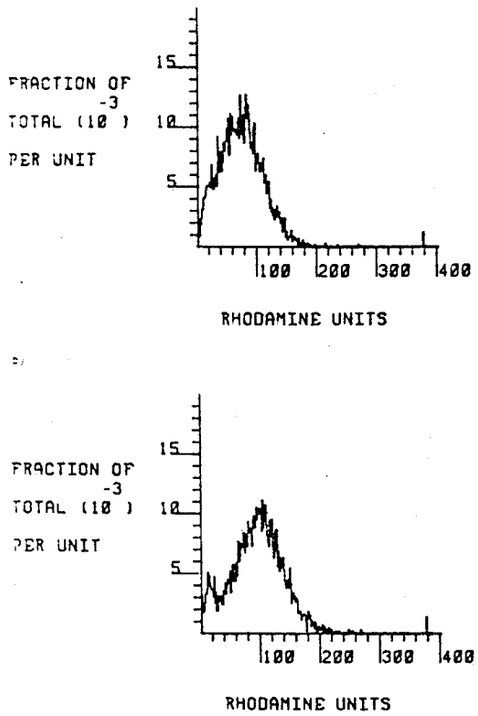

FIG. 6. Anti-IgD distributions for cells bearing different amounts of anti-IgM. (a), anti-IgD histogram of IgM dull cells, *i.e.*, those cells which exhibit between 20 and 50 units of anti-IgM stain in Figure 5a. (b), anti-IgD histogram of IgM bright cells, exhibiting between 100 and 150 units of anti-IgM stain in Figure 5a.

cence profiles of IgM dull and bright cells are compared in Figure 6. It should be noted that only small, viable lymphocytes as assessed by forward-angle light scattering were studied, minimizing variations due to cell size.

The IgM dull cells give a fluorescence distribution for IgD which has very few IgD-negative cells. The population of IgM-positive-IgD-negative cells is much more pronounced in the IgM bright group. This suggests that the IgM only cells are proportionately more frequent in the IgM bright population than in the IgM dull population.

A second conclusion can be made about the relative amounts of the two antigens on the cells which are double-labeled. The IgM dull cells express less IgD than do the IgM bright cells (Figure 6). Although there is a slight positive correlation between these antigens, the two IgD distributions overlap considerably. The conclusion that can be drawn is that IgM bright cells tend to have slightly more IgD than do the IgM dull cells. A statistical comparison of the difference between these two curves is presented elsewhere in this volume (22).

DISCUSSION

This technique of separating the emission of two dyes in a flow system by a combination of optics and electronics is applicable to any dye pair which can be excited at a single wavelength. Optical filters provide the optimum color separation while the electronics network removes the remainder of the signal coming from the opposite chromophore. Using the optics and electronics together, new signals are generated which are proportional to the amount of emission of each dye. The only requirement for using this subtraction network is that the two detector systems discriminate at least partially between the emissions of the two dyes. This means that in equations 3) and 4) ("Results") $\beta\gamma/\alpha\delta$ must not equal 1. Fluorescence data quality is optimized by selecting optical filters for best discrimination between the two dyes without excessive attenuation of the desired signals. As the ratio of efficiencies, β/δ and γ/α, becomes smaller, through the use of better chosen filters, the amount of noise introduced by this subtraction system decreases.

Even though this signal separation could be accomplished by computer software, the advantage to separating the emission by hardware is that the corrected signals can be used directly in the sorting logic of the FACS. In this way, cells can be sorted according to the two antigenic determinants without further hardware manipulation. The two new signals, F1′ and F2′, are used as though they came directly from the photo tubes.

In using this technique it is assumed that the two dyes emit independently so that adding one dye does not affect the emission of the other dye. Chromophore interactions, such as reabsorption, non-radiative energy transfer, and exiplex formation, may change the linear relation between the amount of emission and the amount of each dye. This problem is not limited to this technique for color separation but applies to any system using two or more dyes to study cells. It is hoped that identifying and

measuring these dye-dye interactions will provide more information about the cell membrane and its constituents.

Dye-dye interactions can be reduced by utilizing certain properties of the cells. If the first step of immunofluorescent staining is done under capping conditions (21), the dye is localized into one area on the cell. The second step can then be done under non-capping conditions so that the two dyes are physically isolated from each other on the cell surface. It is possible that just sequential staining is sufficient to separate the dyes since many immunofluorescent stains undergo patching even when not exposed to capping conditions (21), thereby isolating each dye into separate patches.

Using two markers to identify different cell populations, then, allows investigation of the relation between two antigenic determinants. Besides enumerating the cells bearing either or both determinants, quantitative correlations between the amounts of antigens can be made. The technique for signal separation described here permits such studies to be done with fluorescein and rhodamine as the fluorophors and allows the quantitative analysis to be done in a flow system.

ACKNOWLEDGMENTS

The authors would like to thank Mr. Richard Sweet for his helpful discussions and for his critical reading of this manuscript. The work of Mr. Tom Nozaki and Mr. Charles Brumm, who designed and assembled the cross-coupling system, is greatly appreciated. A special thank you is expressed to Ms. Jean Anderson for help in preparation of this manuscript.

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