# Dual immunofluorescence – new frontiers in cell analysis and sorting

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*Investigations of the nature and functions of the immune system and its cell populations have been revolutionized by the techniques of fluorescence-activated cell sorting (FACS) and flow cytometry. Over the last few years, however, it has become increasingly clear that making only a single immunofluorescence measurement on each cell is not adequate for many investigations of lymphoid cell subpopulations. In this review the authors discuss why adequate resolution of functional subsets will increasingly require multiparameter definition including measurements on two or more fluorescent labels.*

The value of dual immunofluorescence observations using fluorescein and rhodamine conjugated reagents has been demonstrated in many microscope-based studies[1]. Multiple fluorescence measurements have been implemented in FACS and other systems with a number of dye combinations and fluorescence excitation schemes, but dual immunofluorescence has not been widely used. This has been due to limitations of the dyes routinely used for immunofluorescent labelling and of the machines used for the measurements.

This situation is being changed by the development of new dyes for immunofluorescence and by the availability of both laboratory models and commercial cell sorters designed to accommodate two lasers. Thus, it is now feasible to make optimized, independent measurements on at least two fluorescent markers on each cell.

FACS dual immunofluorescence is already proving to be very valuable for defining and characterizing lymphoid cell subpopulations and for tracing developmental sequences which are not specified by a single characteristic cell-surface marker and must therefore be defined on a multiparameter basis. For our studies, we use a modified Becton Dickinson FACS system which incorporates an argon-ion laser to excite fluorescein, and a tunable dye laser to excite the recently developed dye Texas Red (cat. no. T-353, Molecular Probes, Inc., Junction City, Oregon). The dye laser is tunable from 570 nm to 650 nm and can thus be adjusted to match new dyes excited in this region as they become available.

## Background for two-laser-system developments

In usual cell-sorter systems, cells pass through a focused laser beam at a rate of several hundred to several thousand per second. As each cell passes, fluorescence excited by the laser and light scattered from the laser beam are measured by appropriate detectors. The fluorescence and/or scatter signals from a large number of cells are stored for analysis. In addition, any subpopulation of cells defined by the light-scatter and fluorescence measurements can be sorted for further observation or functional studies.

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In practice, light-scatter measurements are quite useful for indicating approximate cell size or for distinguishing live and dead cells of similar original size[2]. However, since light-scatter signals depend both on cell size and internal structural properties, they do not assay specific properties such as DNA content or amounts of particular cell-surface antigens. These are the province of fluorescent labels. Multiple fluorescent markers and corresponding fluorescence measurements on each cell can be used to study the correlated expression of two or more antigens on cells in a mixed population.

Since staining brightness is often the limiting factor in immunofluorescence measurements, it is important for a labelling dye to have good fluorescence efficiency and to be well matched to an available strong laser line. In addition, a pair of dyes to be used for dual fluorescence must be different enough to allow independent measurements. These conditions have been difficult to fulfill with single-laser excitation, although a variety of FACS investigations have been carried out in this way[3-8]. For dual immunofluorescence we have used an argon-ion laser in all lines mode (emitting several lines from 458 nm to 514.5 nm in a single beam) to excite fluorescein and rhodamine[6,7,8].

In this fluorescein/rhodamine system, fluorescein was excited efficiently but much of its fluorescence emission was lost in output filtering required to reject scattered laser light on one side and rhodamine fluorescence on the other. Rhodamine fluorescence could be collected fairly efficiently, but the dye is not excited very well by the argon-ion laser. Even with optimized filtering of the light going to the two detectors, each detector had some sensitivity to both dyes necessitating electronic subtraction of the signals to obtain true fluorescein and rhodamine outputs. The result was a workable system, but the diminished sensitivity compared to single immunofluorescence restricted its use to bright stains, and even with these the data quality was not as good as for the corresponding single stains.

## Two-laser-system design and description

Use of laser beams matched to two immunofluorescent labelling dyes to make independent measurements on each dye has proved to be a much more successful approach to two-color FACS analysis. The use of a tun-

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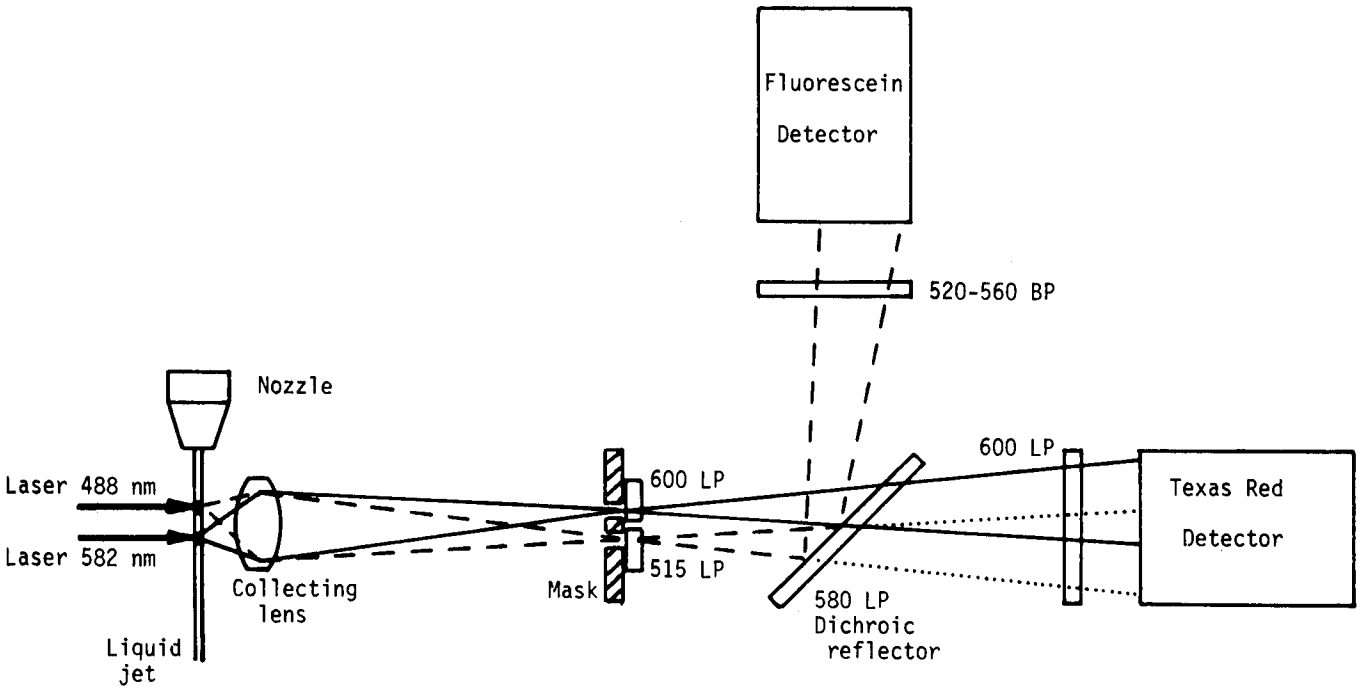


**Fig. 1 Diagram of the optical system of the two-laser fluorescein/Texas Red system**

Fluorescein emission is indicated by the dashed line, Texas Red emission by the solid line, and possible 488 nm-excited red fluorescence by the dotted line. Optical filters are designated by their transition wavelength(s) and type (LP = long wavelength pass, BP = band pass). The functions of the components are described in the text. Some parts have been rotated from their actual orientations so that all elements could be drawn in one plane.

The instrument we use employs a modified Becton Dickinson FACS IV two-laser optical bench. One side carries an argon-ion laser providing excitation for fluorescein at 488 nm (power 400 mW) while the other side has been modified to accept a Spectra Physics model 375 tunable dye laser. The dye laser is pumped by a second argon-ion laser operated at 2.5 W in all lines mode. Circulating Rhodamine 6G in the dye laser yields an output of over 200 mW at any wavelength between 577 nm and 615 nm. Currently we use it at 582 nm (250 mW) to excite Texas Red. Processing of the fluorescence signals from cells is accomplished by Becton Dickinson FACS II electronics including circuitry which takes into account the time delay (about 10 $\mu$s) between the two fluorescence signals. The digitized light-scatter, fluorescein and Texas Red signals are accumulated in list mode on a Digital Equipment Co. VAX 11-780 computer and stored for subsequent analysis.

able dye laser as a second excitation source in a cell-sorter system was pioneered by Arndt-Jovin *et al.*[9], and their system has been used to select cells on the basis of DNA content and fluorescein diacetate viability[10]. Our experience using a similar dye laser has been very positive. Its output stability is good, and it runs for many months between dye changes.

A diagram of the optical system is shown in Fig. 1. The two laser beams are focused on the jet about 0.15 mm apart. The light collected from each laser spot is imaged onto a mask and filtered to block scattered laser light while passing fluorescence. Fluorescences emitted by laser-illuminated cells are separated by a dichroic reflector, filtered further and converted to electronic signals.

As illustrated in Fig. 2, fluorescein is excited ideally by the 488 nm laser light from an argon-ion laser, while Texas Red is excited well by the dye laser at 582 nm. The 520–560 nm bandpass filter allows efficient collection of fluorescein fluorescence, and 600 nm longpass filtering is good for Texas Red fluorescence. The combination of position/time separation of the laser signals with the minimal overlap on the excitation and the emission spectra of two dyes allows us to make fully independent and optimized measurements on each dye. Each measurement is as good as that made on individually stained cells.

So far our dual immunofluorescence work has usually been with fluorescein directly conjugated to one antibody and biotin coupled to the other. Texas Red–avidin (dye to protein ratio about 1.5) is used to reveal the biotin reagent[11]. This reagent works quite well, and it has shown no deterioration in over 18 months of use. Our limited experience with Texas Red-labelled antibodies and that of Thomas Chused (personal communication) has been fairly good with goat or rabbit antibodies, but most monoclonal antibodies have been inactivated by the conjugation process.

In a comparison of Texas Red–avidin and fluorescein–avidin (dye to protein ratio about 5.2) as second steps on separate samples with the same first-step biotin-coupled antibody, the fluorescein reagent gave about 6 times as much fluorescence signal as the Texas Red in our system. However, under the same conditions cell autofluorescence in the fluorescein channel was 12 times as great as in the Texas Red channel. This explains why we have sometimes observed better separation of stained from unstained cells with Texas Red than with fluorescein.

## Illustrations/applications

### *IgM vs. IgD: two-dimensional logarithmic analysis of subpopulations*

During the last year, we have employed two-color immunofluorescence methods to measure the correlated amounts of surface IgM and IgD on a broad array of mouse lymphoid cell populations from different strains, ages and organs[12]. For these studies, we stained cells with a directly fluorescein-labelled rat monoclonal antibody to mouse IgM[13] together with a biotin-labelled monoclonal

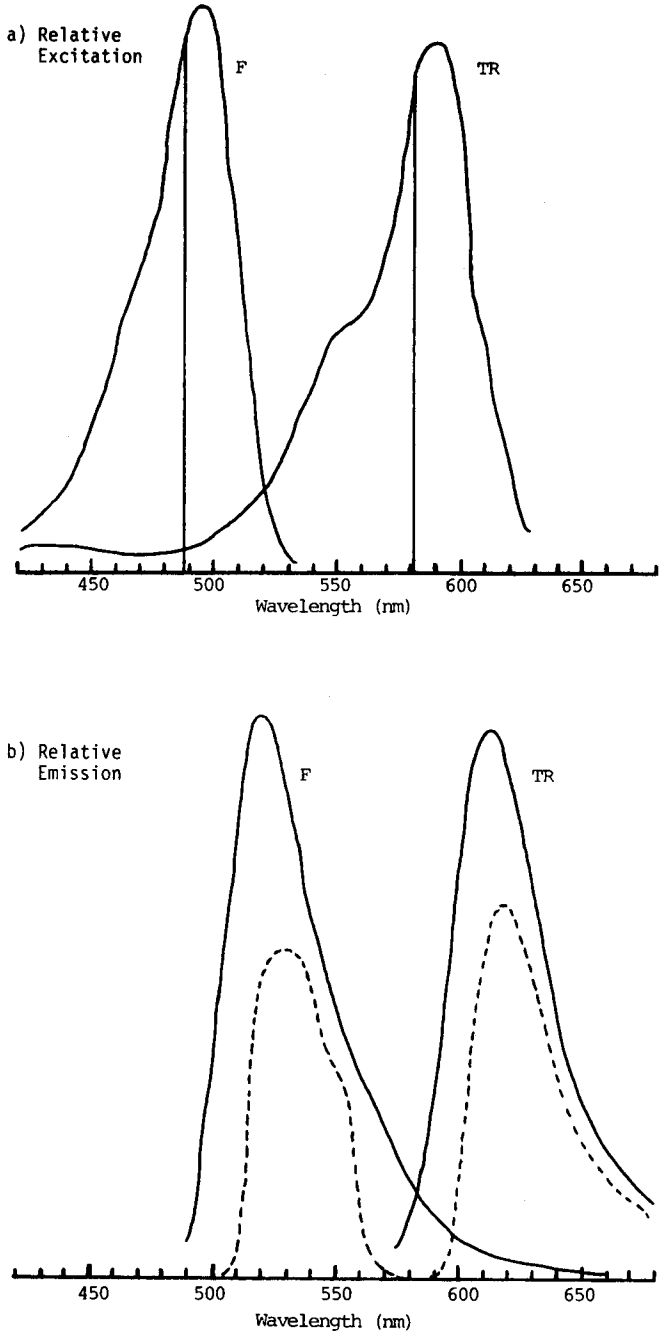


**Fig. 2. Fluorescence excitation and emission spectra for the two-laser fluorescein/Texas Red system.**

(a) Excitation spectra for fluorescein (F) and Texas Red (TR) with vertical lines marking the 488 nm and 582 nm laser wavelengths. (b) Emission spectra of the two dyes (solid lines) and fluorescence detected through the two-laser system emission filters (dashed lines).

anti-IgD specific for the Ig-5a allotype[14] and analysed the cells on our dual-laser FACS.

Previous work suggested the existence of more than one population of IgM + cells in spleen and other organs[15]; however, two-dimensional analysis provides a much clearer indication of the presence of such B-cell subpopulations and allows the different subpopulations to be sorted and analysed for function. We can clearly distinguish IgM dull and bright populations; furthermore, the proportion of cells in these two phenotypes, while very consistent within a particular mouse strain, varies considerably among different mouse strains. Specifically, some strains (BALB/cN) have many more IgM-dull cells (85–90%) while other strains (CBA/J) have fewer dull cells (60%).

Early in these studies, we found that logarithmic amplification of immunofluorescence signals usually results in data displays that are easier to analyse and interpret correctly than the corresponding linear displays. This is especially true in dual immunofluorescence, as the data contrasted in Fig. 3 show. Several displays of the IgM vs. IgD staining pattern for spleen cells from a two-month-old NZB mouse are shown in this figure. Panels (a) and (b) show log–log displays of the data while (c) and (d) show linear–linear displays of the same analyses.

The log–log displays (Fig. 3, panels a and b) demonstrate clearly that the brightest $IgM^+$ cells in the sample are mostly $IgD^+$, that the bright $IgD^+$ are virtually all $IgM^+$, and that there is roughly 50 times as much IgM or IgD on the brightest cells as on the dullest. All cells are on scale, even 'double negative' cells that do not carry detectable levels of either IgM or IgD.

The smaller dynamic range of the linear displays prevents all cells from being displayed on scale when the range of surface antigen levels approximates that of the immunoglobulins examined here. In the linear displays shown in Fig. 3, panels (c) and (d), we chose a gain for Texas Red low enough to keep the brighter $IgD^+$ cells on scale. This results in a display in which there appears to be a major $IgM^+$, $IgD^-$ population; however, at a higher linear gain (not illustrated) or in the log–log display, most of the cells in this population can be seen to carry at least some IgD. The fluorescein-IgM gain, in contrast, was chosen to be just high enough to show that most $IgD^+$ cells also have at least some surface IgM; however, this gain is already too high to accurately reveal the brighter $IgM^+$ cells which therefore form an off-scale pile-up at the right edge of the displays. Thus, careful analysis of data taken at several pairs of linear gains would be required to reach conclusions that are obvious from the log–log display.

*Murine B-cell subpopulations and the CBA/N defect*

CBA/N mice carry an X-linked immunodeficiency[16] which renders them unable to respond to Type-II thymus-independent antigens, for example TNP-Ficol. These mice also have a number of other abnormalities such as low levels of serum IgM, a high IgM/IgD ratio on their splenic B cells, the lack of a B-cell surface marker called LyB3 and an unusually small number of B cells in their spleens[17].

Fig. 4 shows anti-IgM vs. anti-IgD staining of splenocytes from two-month-old CBA/J (normal) and CBA/N (defective) mice. Comparing panels (a) and (b), it is immediately obvious that a particular subpopulation of cells found in the CBA/J is missing in the CBA/N. This low IgM, relatively high IgD population may well be responsible for functions in which the CBA/N is defective. This hypothesis is currently being tested by sorting the various B-cell subpopulations and studying them using both *in-vivo* and *in-vitro* assays. Dual immunofluorescence is necessary for isolating these populations since anti-IgD alone does not separate the high and low IgM groups, while anti-IgM alone does not fully separate the low IgM cells from the unstained population seen in the lower-left corner of the contour plots.

A comparison of the IgM/IgD staining pattern for spleen and lymph nodes from normal mice (not illustrated) shows that besides distinguishing lower IgM and higher IgM populations we also distinguish two subpopulations among the higher IgM cells. Spleen contains bright IgM staining cells with both high and low IgD densities while lymph node has only the high-IgM, high-IgD cells. This leads us to divide the B-cell population into three groups which are separated but not fully resolved by the IgM vs. IgD stain.

*ThB vs. IgM and ThB strain differences*

The cell-surface antigen ThB is found on mouse thymocytes and peripheral B cells but not on mature T cells[18]. Mouse strains fall into high and low ThB categories on the basis of staining brightness of splenic B cells with anti-ThB (Ref. 19). We have employed a biotin-labelled rat monoclonal antibody specific for ThB[19] to study the variation of this marker on IgM bright and dull cells as defined by the rat monoclonal anti-IgM mentioned above. Fig. 5 shows data from an analysis of anti-IgM vs.

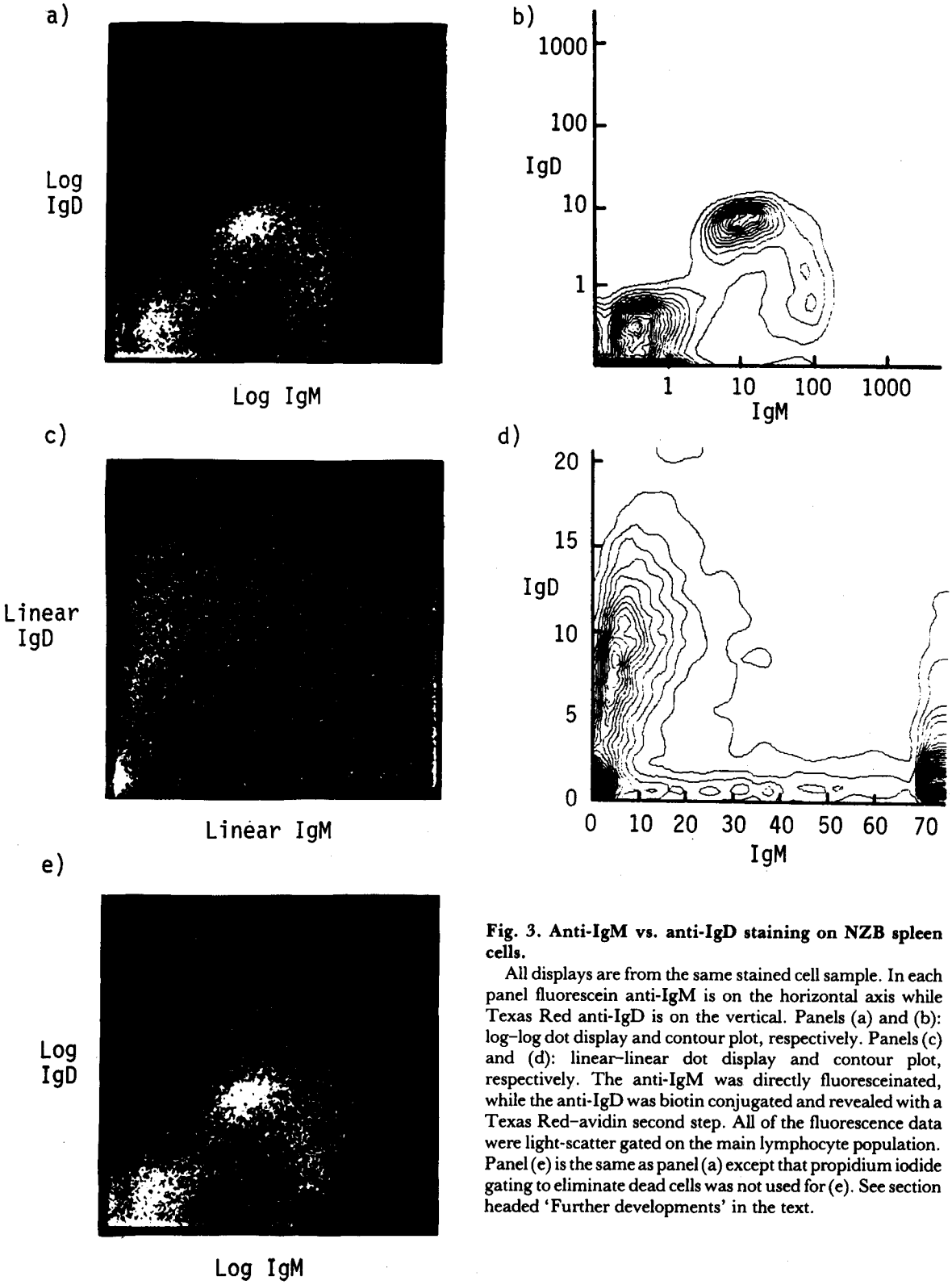


**Fig. 3. Anti-IgM vs. anti-IgD staining on NZB spleen cells.**

All displays are from the same stained cell sample. In each panel fluorescein anti-IgM is on the horizontal axis while Texas Red anti-IgD is on the vertical. Panels (a) and (b): log–log dot display and contour plot, respectively. Panels (c) and (d): linear–linear dot display and contour plot, respectively. The anti-IgM was directly fluoresceinated, while the anti-IgD was biotin conjugated and revealed with a Texas Red–avidin second step. All of the fluorescence data were light-scatter gated on the main lymphocyte population. Panel (e) is the same as panel (a) except that propidium iodide gating to eliminate dead cells was not used for (e). See section headed 'Further developments' in the text.

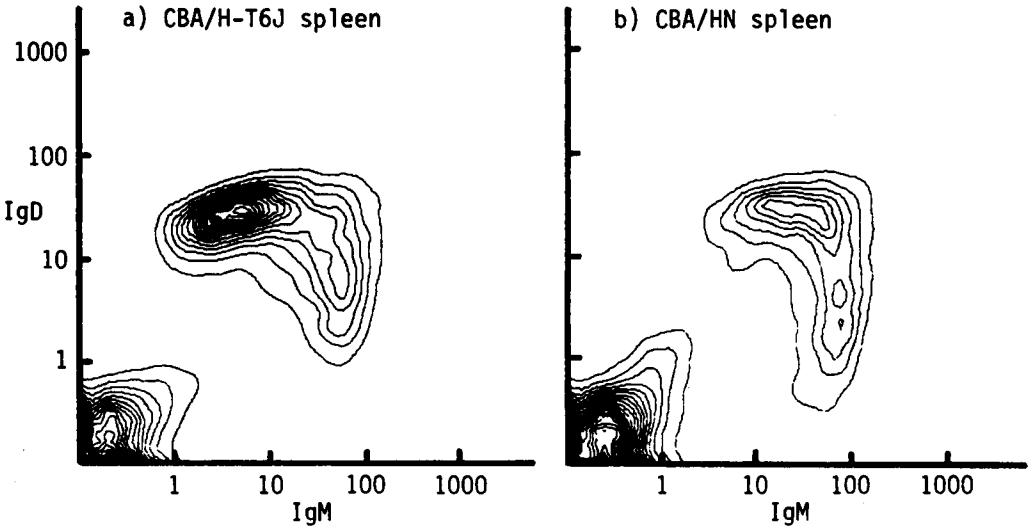


**Fig. 4. Anti-IgM vs. anti-IgD staining of CBA/J and CBA/N spleen cells.**
Log–log contour displays of the staining of (a) CBA/J and (b) CBA/N cells. The staining and analysis conditions are the same as those in Fig. 3.

anti-ThB staining of BALB/cN (low ThB) and SJL/J (high ThB) spleen cells.

The dual immunofluorescence measurements help to clarify the difference between ThB high and low strains by demonstrating that most of the difference in ThB staining profiles is due to the duller IgM-staining cells. The ThB staining of these cells is duller and much less uniform in BALB/cN than in SJL/J, while the bright IgM cells stain similarly for ThB in the two strains. Thus, the genetic regulation of ThB levels on B cells is different for the IgM bright and IgM dull populations.

*Lyt-1 vs. IgM defines a novel B-cell subpopulation*

Recent work in our laboratory[20] and elsewhere[21] has demonstrated that a small number of IgM-bearing cells express the Lyt-1 antigen, which until recently was thought to be found exclusively on T cells[22]. About 2–5% of B cells are found to stain with an anti-Lyt-1 rat monoclonal antibody. Interestingly, in the autoimmune strain NZB this population is considerably elevated (to 15% or more of B cells) and these cells are responsible for the high spontaneous secretion of IgM when NZB spleen cells are cultured[20].

Fig. 6 presents contour plots of staining for IgM vs. Lyt-1, Lyt-2 and Thy-1. A significant double-staining population is found only with Lyt-1. Further work has shown that the Lyt-1 B-cell population is typically bright for IgM and dull for IgD and so coincides with one of the three populations defined by IgM/IgD staining. It appears that most (if not all) of the B cells in this IgM/IgD population express Lyt-1. This population, called Ly-1 B, appears very distinct from the rest of the B cells and may, in fact, represent a separate lineage of B cells[20].

## Further developments

*Fluorescein and Texas Red with propidium iodide exclusion of dead cells*

An initial problem with the dual immunofluorescence system was that its potential for identifying very small cell populations could be limited by the presence of non-specifically stained dead cells in the preparations which could not be completely gated out by light scatter. We have incorporated a third fluorescence measurement to exclude dead cells using propidium iodide, which does not enter live cells, to mark dead cells for exclusion from fluorescein analyses.

Propidium iodide excites well at 488 nm, but its fluorescence emission is largely above 600 nm so that, as shown by the dotted lines in Fig. 1, a cell stained with propidium iodide will generate a signal on the Texas Red detector timed with the passage of the cell through the 488 nm laser beam. Cells with a significant propidium iodide signal are dead and are excluded from further analysis. We have modified the FACS electronics to allow this signal to be processed along with the fluorescein and Texas

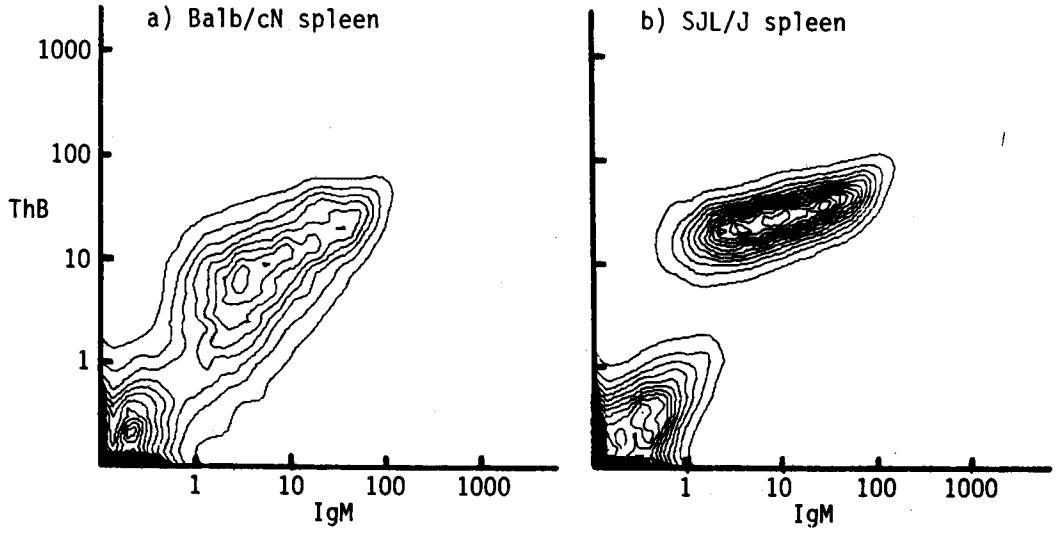


**Fig. 5. Anti-IgM vs. anti-ThB staining of BALB/cN and SJL/J spleen cells.**
Log–log contour displays of the staining of (a) BALB/cN and (b) SJL/J cells. The anti-IgM was directly fluoresceinated, while the anti-ThB was biotin conjugated and revealed with a Texas Red–avidin second step. All of the fluorescence data were light-scatter gated on the main lymphocyte population.

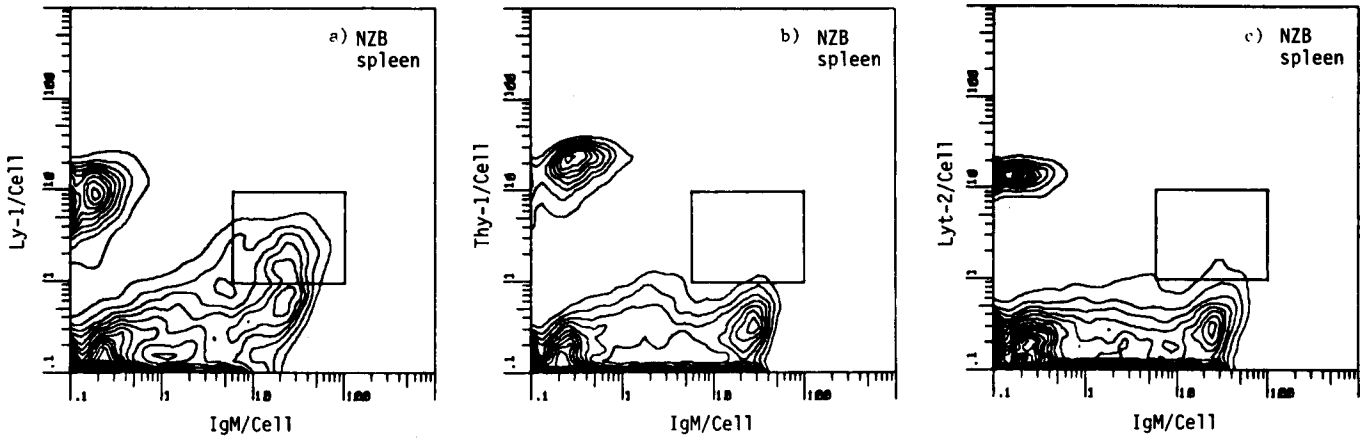


**Fig. 6. Anti-IgM vs. anti-Lyt-1, anti-Lyt-2 and anti-Thy-1 staining of NZB spleen cells.**

Log–log contour displays of IgM staining vs. Lyt-1 (a), Lyt-2 (b) and Thy-1 (c). The IgM reagent was the same as in Fig. 5 and the other reagents were all biotin-conjugated (revealed by Texas Red–avidin). The anti-Lyt-2 and anti Thy-1 rat monoclonals were included to control for non-specific binding as they are the same rat isotype as the anti-Lyt-1 reagent and were labelled in the same manner. The fluorescence data were light-scatter and propidium iodide gated to eliminate dead cells and cell doublets.

Red signals. In appropriate cases exclusion of such dead cells can result in clearer and more accurate results in dual immunofluorescence analyses. This is illustrated in Fig. 3 by panels (e) and (a). In this case some cells, including most of the brightest Texas Red 'IgD' cells shown in (e), are found to be dead and are excluded from the real analysis shown in (a).

*New dyes*

Recently we have been associated with a project which is upgrading single-laser dual immunofluorescence to give data quality more like the two-laser system than like the fluorescein/rhodamine single-laser system described above. Oi, Glazer and Stryer[23] demonstrated the usefulness of photosynthetic pigments from red algae and cyanobacteria (blue-green algae) as immunofluorescent labels. Phycoerythrins, one family of these pigments, excite well at 488 nm, have good fluorescence quantum efficiencies and have emission spectra peaking at 570 nm. Avidin and immunoglobulin conjugates have been prepared by various chemical cross-linking agents.

In the fluorescein/phycoerythrin system both dyes are excited at 488 nm, and fluorescein fluorescence is collected between 520 nm and 560 nm as usual, while phycoerythrin fluorescence is collected above 570 nm. Each detector has some sensitivity to both dyes, so that electronic adjustment is necessary to give true fluorescein and phycoerythrin signals, but the resulting signal levels are similar to those obtained in single-stain measurements. Perhaps soon the single-laser system will serve for most two-color studies and we will be able to reserve the two-laser system for three fluorescence work.

We have come a long way since 1969, when only one fluorescence parameter was available[24]. In the future we expect that multiple fluorescence, new light-scatter measurements, fluorescence polarization and possibly other parameters will come into widespread use.

**Acknowledgements**

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