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Flow cytometry

Flow cytometry has become a standard laboratory tool in the evaluation of hematopoietic cells including the identification of leukocyte populations and subpopulations, a method referred to as immunophenotyping. The clinical application of this technology has been facilitated by the development of instruments and data analysis systems suitable for routine use in diagnostic laboratories. In addition, the expanded range of monoclonal antibodies specific for lymphocyte (and other hematopoietic cell) surface antigens directly conjugated to a number of different fluorescent indicators (fluorochromes) provide an extensive panel of reagents that facilitate multicolor (polychromatic) studies.

The clinical needs that pushed this technology relate to the emergence of absolute CD4 T-cell counts as a critical measure for disease assessment and follow-up in managing patients infected with the human immunodeficiency virus (HIV). Flow cytometry applied in the monitoring of HIV infection was followed by the routine application of cell characterization by flow cytometry in the evaluation of lymphoproliferative disorders, and more recently in the study of immunodeficiency disorders and other immune-mediated diseases.

Recent advances in instrumentation and fluorochrome chemistry now allow for routine polychromatic flow cytometry studies, with concomitant assessment of cell surface markers and intracellular parameters, including intracellular proteins, phosphoproteins and cytokines, as well as identification of changes linked to cellular activation and apoptosis. Intracellular flow cytometry also can be applied to evaluate cell cycle status (i.e., G_0-G_1, S, G_2-M) based on DNA staining, useful in evaluating tumor cells and assessing the *in vitro* lymphocyte response to various stimuli. Additonally, evaluation of lymphocyte proliferation can be performed with cell tracking dyes that allow quantitation of the rounds of cell division associated with cell activation. Finally, characterization of antigen-specific T cells following immunization or associated with normal and/or abnormal immune responses in association with disease states can be accomplished using multimer technology as well as intracellular cytokine detection following antigen exposure.

This chapter focuses on basic concepts of flow cytometry including instrument characteristics, data management, lymphocyte gating and directed use of test reagents. In addition, a brief overview of intracellular protein detection, cell activation studies, cell cycle analysis, apoptosis detection and multimer technology is provided, focusing on the appropriate application of these approaches as well as their limitations.

Instrumentation

The basic components of a flow cytometer, as shown in Fig. 94.1, include the illumination source, optical bench, fluidic system, electronics and computer.[1] Briefly, stained cells are focused and flow into single-file by the fluidic system, interrogated by a light source that generates light signals that are collected and directed by the optical system to the photodetectors, which convert light into electronic signals for storage and subsequent analysis. This process is discussed further in the section below.

The fluidic system lies at the heart of a flow cytometer and consists of isotonic sheath fluid that moves the sample stream containing the cells. This is accomplished by injecting the cell sample into the moving sheath fluid, establishing a hydrodynamically focused single-file flow of cells that move through the analysis point while maintaining this cell stream in a constant, central location.[2] The centrally focused cell stream insures that the illumination of all cells is virtually equivalent. Thus, the difference in magnitude of the emission signal(s) generated from each cell reflects biologic differences between the cells (rather than reflecting the variation in the illumination energy if the cells were not tightly focused). The use of hydrodynamic focusing has the additional advantage of producing little or no change in cell shape, although it may have an effect on cell orientation. The consistency in maintaining cell shape facilitates distinguishing "architectural" differences between specific leukocyte types (see Gating section).[3] However, this method can generate single-file cell rows with precision only up to a flow rate of 60 to 100 µl/min, which can lead to long acquisision times for the detection of very rare events. To overcome this problem, recently introduced flow cytometry instruments utilize acoustic focusing, which align cells through the use of sound waves, allowing sample flow rates of up to 1000 µl/min, without loss of signal quality.[4,5]

Illumination in standard clinical instruments is generated by two or three lasers each of which provide a specific monochromatic light source (e.g., a sapphire laser generates a 488 nm wavelength [blue] beam). Modern lasers are small and available in multiple wavelengths, including ultraviolet (350 nm), violet (405 nm), blue (488 nm), green (532 nm), yellow (560 nm), orange (610 nm), and red (633 nm), permitting the simultaneous use of multiple fluorochromes having different excitation requirements.[6,7] The point where the light illuminates the cell in analytical instruments occurs within a flow cell while in cell sorters the beam intersects cells flowing as a stream in air.

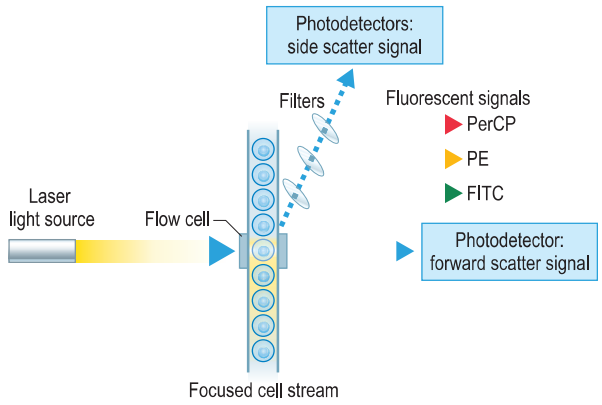

Fig. 94.1 Simplified design of a flow cytometer with one illumination source (laser) set up to collect five parameters. These include the two nonfluorescent parameters (blue light) forward and side scatter, as well as three fluorescent parameters, green (FITC), orange (PE), and red (PerCP) light.

The optical bench contains lenses that shape and focus the illumination beam to ensure consistent excitation energy at the analysis point.

The illumination of a cell generates both nonfluorescent and fluorescent signals that are collected and measured by optically coupling the signal to a detection system consisting of filters each linked to a photodetector. The filters are chosen to allow the nonfluorescent signals to be measured at the same wavelength as the excitation signal (e.g., 488 nm from a blue light source) for the forward- and side-scatter channels (see Gating section), whereas those for the fluorescence channels specific filters only allow passage of light with wavelengths specific to each fluorochrome (e.g., green, orange or red; see Fluorochrome section). The number and arrangement of the photodetectors allows for the simultaneous evaluation of multiple colors (parameters) for each cell and a recent report described a modified clinical instrument that was capable of evaluating up to 17 colors simultaneously from each cell contained within the sample.[8]

The internal electronics in the flow cytometer provides the system for converting analog light signals (photoelectrons) received at the photodetectors into digital signals for acquisition and storage in a computer. The intensity of these converted signals is measured on a relative scale that is generally set in either 256 or 1024 equal increments (referred to as channels) for display and analysis. A number of specialized analysis programs are available and results are depicted graphically as single-parameter histograms displaying specific light (fluorescence) intensity (*x*-axis) versus cell number (*y*-axis) (Fig. 94.2), or two-color displays where the *x*- and *y*-axis reflect the light intensity of the two colors while the cell numbers are represented via dot, pseudocolor, contour or density plots (Fig. 94.3). Most analysis programs enable the operator to evaluate the number and percentage of events, mean and/or median channel fluorescence, and selected statistical measures for each identified cell and these can be aggregated into specific populations and/or subpopulations of cells. Thus, a flow cytometer provides a platform with the capacity to assess multiple pieces of discrete information (parameters) generated from each individual cell contained within a large number of cells present in the test sample and these are typically accrued at rates of 1000–2000 (or more) cells per second.

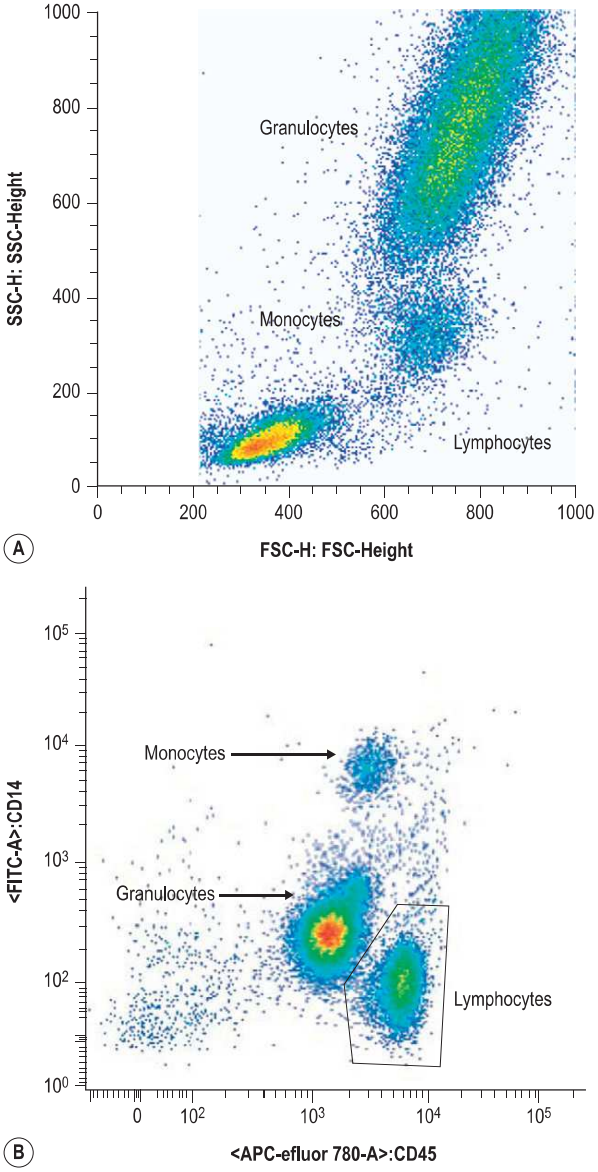

Fig. 94.2 (A) Forward- and side-scatter dot plots on a lysed whole blood sample, demonstrating the basic three-part leukocyte differential with lymphocytes, monocytes, and granulocytes. (B) Dot plot with DC45/CD14 gating reagents showing the fluorescence distribution of all the three leukocyte types identified to include lymphocytes, monocytes, and granulocytes, as well as a small number of nonlysed red blood cells and/or debris.

Fluorescence reagents

Standard monoclonal antibody reagents for clinical use are typically directly conjugated to a fluorochrome, a dye that absorbs and emits light of different wavelengths based on the energy lost during the return of excited electrons to their ground state associated with the illumination by a specific wavelength of light. Thus, the emitted light has a longer wavelength (lower energy) than the wavelength of the excitation beam. The number of commercially available fluorochromes has increased dramatically in the past few years, with the routine use of dye conjugates and instruments with three or more lasers.[7] Commonly used fluorochromes in

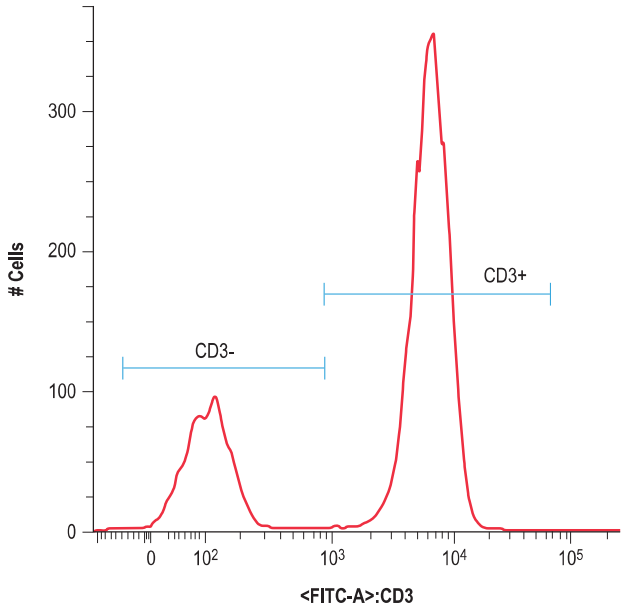

Fig. 94.3 Single-parameter histogram for CD3 expression on lymphocytes demonstrating the negative non-T-cell population (B cells, NK cells) and a positive T-cell population. Integrating the area under each curve would provide the numbers and percentage of cells present in each respective subpopulation.

clinical immunophenotyping include the organic dyes fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin chlorophyll protein (PerCP), and allophycocyanin (APC). Conjugations of PE and APC to cyanines (Cy5, Cy5.5, and Cy7) and Alexa Fluor dyes produce tandem dyes with additional emmision spectra, based on energy transfer from one fluorochrome to the second fluorochrome serving as the source of emitted light. This allows for the simultaneous evaluation of 6 to 8 colors in most current clinical instruments with only two or three lasers.

One recent advance in the field was the development of a new class of inorganic fluorescent semiconductor nanocrystals, named quantum dots (QDs).[9,10] These particles are perfectly suited for polychromatic flow cytometry, as they have broad excitation spectra (525-800 nm) and sharp, discrete emission spectra, that varies depending on their core size.[10] This means that quantum dots of different sizes (and consequently of different colors) can be excited by the same laser source, allowing simpler multiplexing.[11] In addition, quantum dots have high quantum yield, high molar extinction coefficients, and extraordinary resistance to photo- and chemical degradation. These qualities make them perfectly suitable for use in biological studies, including intracellular *in vivo* imaging, fluorescence resonance energy transfer (FRET) analysis and dynamic imaging of single proteins for longer periods of time.[9]

Additional dyes are available for functional studies and include calcium-sensitive dyes (e.g., fluo-3), glutathione-sensitive dyes (e.g., monochlorbimane) and H_2O_2-responsive dyes (e.g., dihydrorhodamine 123).[12,13] Assessment of DNA content can be performed with dyes that intercalate double-stranded DNA and RNA, including propidium iodide and ethidium bromide.[14] In addition there are ultraviolet-excited dyes that are highly specific for DNA, including Hoechst 33258 and 4,6-diamidino-2-phenylindole (DAPI); acridine orange is used for simultaneous staining of DNA/RNA.[15]

Data Analysis

Gating

KEY CONCEPTS

Gating

- Method for defining cell population of interest.
- Typically performed using forward and side scatter.
- Should be confirmed with gating reagents (anti-CD45 and anti-CD14 for lymphocytes and monocytes).

The proper assessment of specific cell types within a mixture requires initial identification of lineage-specific cells, an approach referred to as gating. In practical terms, immunophenotyping focused on lymphocytes requires minimizing the nonlymphocytes included in the evaluation and this is accomplished by lymphocyte gating. The standard sample for clinical studies is anticoagulated whole blood and directing the study to lymphocytes requires eliminating the great majority of non-lymphocytes from the collected data such that the expression of a percentage for a specific cell subpopulation is an accurate measurement. Without gating the data can also be negatively impacted by co-expression of surface antigens on different cell lineages (e.g., CD4 is found on lymphocytes and monocytes at differing density). In addition, nonspecific binding of monoclonal reagents through Fcγ receptors and the level of cytophilic human immunoglobulin varies between cell types, making appropriate gating crucial to generate valid data. These techniques are also used to focus the evaluation on other hematopoietic cells including monocytes, granulocytes, eosinophils, erythrocytes and platelets.

Initial gating to focus on a specific leukocyte population typically involves using the two nonfluorescent parameters, forward angle (low angle, FSC) and side (90°, SSC) light scatter (Fig. 94.2A).[3] Forward scatter is a reflection of cellular cross-sectional area (direct relationship to cell size) and refractile index, whereas side scatter is an indication of the cellular granularity and surface irregularity. The combination of these two nonfluorescent parameters provides a three part differential that distinguishes between normal lymphocytes, monocytes and granulocytes (in the absence of contaminating red blood cells and platelets). As can be seen in Fig. 94.2A, among leukocytes, lymphocytes have the lowest forward and side scatter, monocytes have higher forward and side scatter and granulocytes have the greatest side scatter. This method is effective in distinguishing a relatively pure population of lymphocytes under most circumstances. However, the presence of nucleated red cells, large platelets, basophils or other particulate debris can produce contaminating events (cells) within this "lymphocyte gate." Furthermore, malignant lymphoid cells may not fit into the previously outlined standard light scatter patterns.

A method for confirming the integrity of the light scatter-based lymphocyte gate uses the directly conjugated monoclonal reagents anti-CD45 and anti-CD14.[16] These "gating reagents" more accurately identify the three-part differential. Lymphocytes have the highest level of CD45 binding but are negative for CD14; granulocytes have a lower level of CD45 binding and an intermediate level of CD14 expression; and monocytes have high levels of both CD45 and CD14 expression (Fig. 94.2B). Importantly, non-leukocytes, including erythrocytes

and platelets, are negative for these markers. In addition, malignant leukocytes that have characteristics of early precursor cells often have altered CD45 and/or CD14 expression. Gating reagents provide a reliable means of checking the light scatter-based lymphocyte gate for the frequency of nonlymphocytes within the gate as well as the extent of lymphocyte exclusion from the gate. Guidelines for an acceptable degree of contamination within the lymphocyte gate, as well as the level of lymphocyte exclusion, are contained within the US Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) guideline for lymphocyte immunophenotyping.[17] With the expanded use of polychromatic flow cytometry, some centers now include anti-CD45 in every tube to refine the gate and prevent cell contamination that cannot be excluded using the standard non-fluorescent parameters (forward and side scatter) for gating.

Data display

KEY CONCEPTS

Data presentation

- Fluorescence intensity is plotted versus cell number.
- Can present cummulative data on more than one parameter for each cell.
- Multicolor data presentation can increase cell subpopulation resolution.

The simplest method for demonstrating flow cytometry data is the single-parameter histogram (Fig. 94.2), a graphic presentation of cell number on the *y*-axis versus fluorescence (light) intensity from a single fluorochrome on the *x*-axis. Integration of curve areas provides the number of cells and often there are two distinct distributions, one referred to as negative identifies cells that are not bound specifically by the monoclonal reagent and the second represents cells bound by the antibody. Negative actually reflects low-level fluorescence resulting from cellular autofluorescence together with any nonspecific binding of the monoclonal reagent(s), the magnitude of both phenomena varies between different cell types. The interpretation of the data is simplified when there are two distinct cell populations (i.e., negative and positive) while the evaluation of two overlapping distributions is more difficult.

Multicolor data can be evaluated using a series of single-parameter histograms that consider each fluorochrome independently. However, it is more informative to present two parameters simultaneously using a correlated display (Fig. 94.4), and two-color displays are recommended for clinical flow cytometry.[18] This approach enables the simultaneous visualization of four different populations: A^+/B^-, A^-/B^+, A^+/B^+ and A^-/B^-. More recently, these displays evolved to include a mixture of logarithmic (for higher intensity expression) and linear (for lower intensity expression) intensity for each axis in order to allow for better interpretation of events with very low, zero or negative fluorescence. This combined display approach resolves the previous problem of a large number of events being displayed compacted against the axes even with properly compensated samples and will be used in the illustrations throughout this chapter.[19]

The simultaneous use of n monoclonal reagents can identify a total of 2^n subpopulations. These different subsets can be identified sequentially by first dividing the cells into those that are positive versus those that are negative with one reagent and then evaluating the defined subpopulations for the remaining two reagents using a two-color approach. Alternatively, more modern software can represent multiple populations as polychromatic plots, which can simplify data analysis.[20] The polychromatic approach can provide a means to further resolve subpopulations, and has been particularly useful in the evaluation of cellular differentiation, activation and functional correlates as well as clarifying overlapping cell subpopulations.

Positive–negative discrimination

The evaluation of clinical immunophenotyping data requires establishing criteria for the boundaries between negative or nonstained cells and positive (stained) cells. A commonly used approach involves using directly conjugated control monoclonal antibodies of the appropriate class or subclass (e.g., IgG1, IgG2a, IgG2b, or IgM) that do not specifically react with human lymphocyte surface antigens (commonly called "isotype controls"). The marker (discriminator) is set at the fluorescence histogram channel number such that it includes 98 to 99% of the negative cells (Fig. 94.5A).

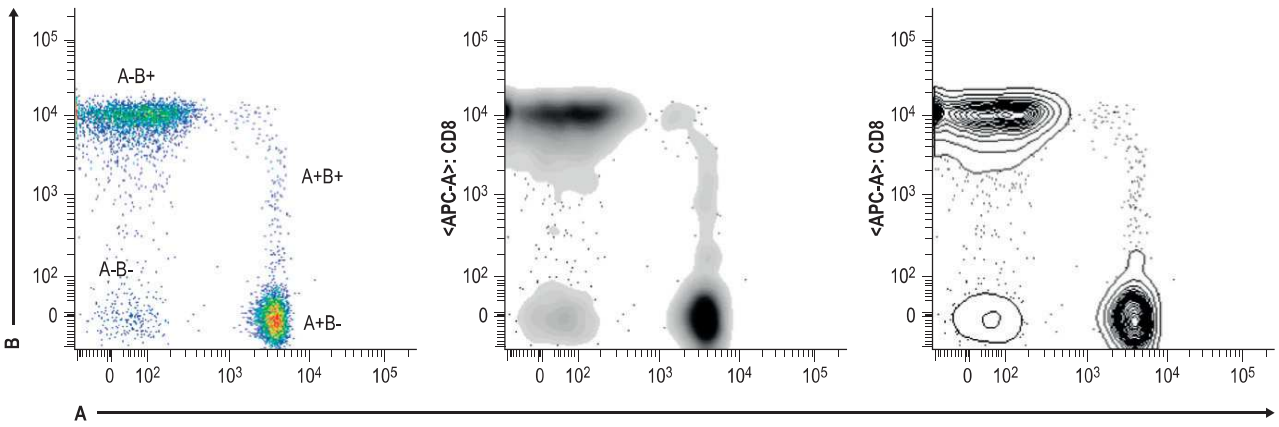

Fig. 94.4 Examples of dot pseudocolor (left), density (middle), and contour (right) displays based on the same two-color data. All three techniques enable simultaneous evaluation of both parameters, in this case evaluating the expression of markers A and B. These plots identify four populations of cells, those expressing only A or B, those expressing both A and B (very few), and those expressing neither A nor B.

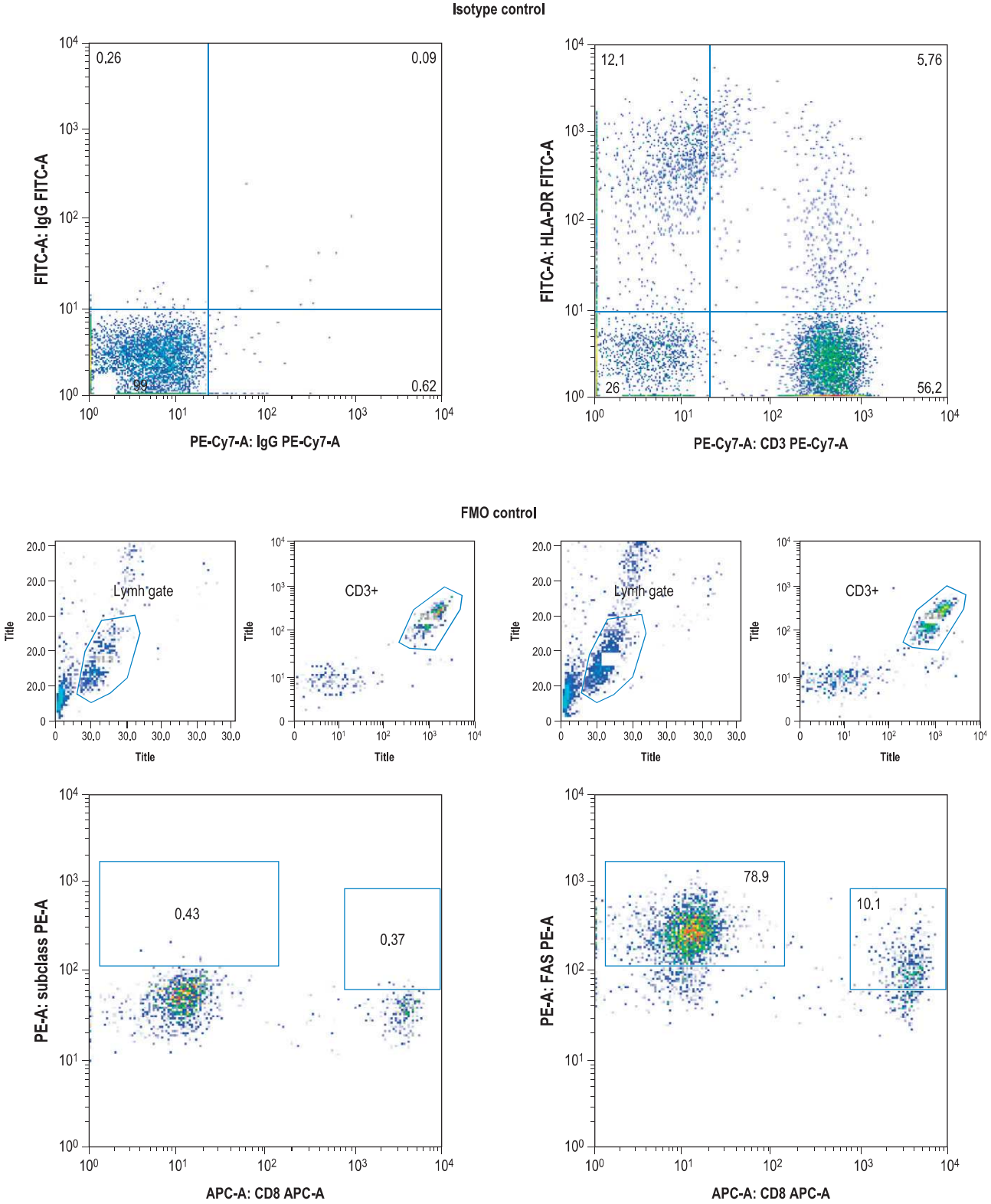

Fig. 94.5 Positive–negative discrimination strategies. (A) Non-specific antibodies (isotypes) were used to stain the sample shown on left and the positivity threshold determined and applied to the sample on the right. (B) The positivity threshold was determined by staining the sample on the left with the population-specific markers (CD3, TCR$\alpha\beta$, and CD8) and omitting FAS. The panel on the right contains FAS. Note that the positivity threshold is slightly different for $CD8^{+}$ and $CD8^{-}$ cells.

As previously noted, negative refers to the aggregate of baseline cellular autofluorescence plus nonspecific reagent binding, and this can vary according to the cell type. For this reason, the use of isotype controls may not correctly identify the positive-negative threshold for specific cell types, particularly when staining dimly expressed proteins. Additionally, to perfectly mimic the specific antibody used, the isotype control would need to have the same antibody to fluorochome ratio and brightness, something that is not easily accomplished. To overcome these difficulties, an alternative method for positive-negative discrimination has been developed and called Fluorescence-minus-One (FMO).[21]

FMO involves the staining of the sample with all the antibodies of interest, except the one targeted for positive-negative threshold. As an example, if one wanted to define the negative threshold for the protein FAS (CD95) in $CD8^+$ and $CD8^-$ T cells, the FMO control tube would include the cell subset-specific markers (CD3, TCRαβ, and CD8), and omit anti-FAS. After appropriate gating on that population, the threshold can be adequately defined, and it is different for the two exemplified populations (Fig. 94.5B, right panel). One obvious limitation of this method is the higher cost, given that multiple control tubes need to be set up for each sample.

Compensation

The fluorescence signals emitted by different fluorochromes are not completely separated by the filters. This can lead to signal overlap that is corrected by electronic subtraction of the overlapping signal, a process referred to as compensation. The overlap is particularly significant when using multiple fluorochromes, each with different spectral properties.[21] The compensation process involves subtraction of the "spillover" signal detected by the photodector generated by samples stained with only one fluorochromre. Currently, most flow cytometry analysis software allows for off-line compensation, where the single reagent stained tubes are used to create a compensation matrix that is then applied to all the tubes in the experiment. This allows for much simplified compensation procedures, without the need for any hardware compensation during data collection.

Quality control

Quality control is a critical component of clinical flow cytometry to ensure optimal results.[22] This includes monitoring instrument setup and performance, optimizing sample preparation and reagents and standardizing controls and data interpretation. Quantitative flow cytometry based on a fluorescence standard curve provides quantitative data in units referred to as molecular equivalents of soluble fluorochrome (MESF). When properly constructed standard curves are used, quantitative data for different reagents can be generated and compared. Finally, participation in interlaboratory proficiency testing surveys, such as the triannual samples provided by the College of American Pathologists (CAP), is an important additional measure to monitor laboratory performance, and this is mandated in US clinical laboratories by the Clinical Laboratory Improvement Amendment of 1988 (CLIA 88).

Methods

Whole-blood lysis represents the most common technique used for sample preparation and consists of mixing a fixed volume of anticoagulated whole blood (or bone marrow) with one or more directly conjugated monoclonal antibodies, followed by incubation at a designated temperature and time.[23,24] Next the red blood cells are lysed, the sample is washed and then run into the flow cytometer, usually following fixation in paraformaldehyde to reduce the infectious risk. The nonlysed cells remaining include all peripheral blood leukocytes, as well as any nonlysed red cells, platelets and debris. The heterogeneity of the sample necessitates careful lymphocyte gating (see section above) in order to generate accurate immunophenotyping data. The advantages of the whole-blood lysis method include fewer preparation steps, less sample handling, and a lower likelihood of differential lymphocyte loss. This last point can occur when density gradient techniques are used to prepare mononuclear cells for analysis. Alternative sources of cells (e.g., bronchial alveolar lavage fluid, fine-needle aspirates) can also be evaluated with flow cytometry.[25] Patient studies must be determined with the same methods and reagents as used in the determination of the control ranges to ensure comparability. The number of events (cells) analyzed typically ranges from 10 000-20 000 in routine clinical studies but must be increased when evaluating very small subpopulations of cells in order to produce statistically relevant data necessary in rare event analysis.

The application of control ranges must take into account the fact that significant changes take place in lymphocyte distribution and development during childhood, as well as changes in lymphocytes that occur among the very elderly.[26,27] There are also immunophenotypic differences induced by drugs, including tobacco products, such that information on current medications should be obtained whenever possible.[28] Other factors can also have an impact on lymphocyte distribution, including race, gender, diurnal variation and recent or intercurrent infection.[29]

The choice of immunophenoptyping reagents depends on the cells being targeted for study and the question being asked. However, regardless of the specific set-up, the inclusion of a tube with gating reagents (anti-CD45 and anti-CD14) to confirm the integrity of the standard lymphocyte gate is recommended.[16] In addition, control reagents should be included to establish the fluorescence intensity of negative cells. Important controls that provide an internal control include a pan-T-, B-, and NK-cell marker for every sample (Table 94.1), based on the principle that the whole is the sum of its parts. Thus, the total percentage of lymphocytes in the gate determined by the gating reagents, should approximately equal the sum of the percentages for T, B, and NK cells. A technical or biological explanation must be identified when this relationship does not hold. Biological

Table 94.1 Selected lymphocyte surface antigens for immunophenotyping

T cells
Pan-T cell: CD3, CD2, CD7, CD5 Major T-cell subset: CD4, CD8 Surface antigens associated with function: CD28, CD38, CD45RA, CD45RO, CD62L Activation antigens: CD25, CD40L, CD69, CD71, HLA-DR
B cells
Pan-B cell: CD19, CD20, surface immunoglobulin Major B-cell subset: CD5, CD21 Surface antigens associated with function: CD27, CD40 Activation antigens: CD23, CD25
NK cells
Pan-NK cell: CD16, CD56 NK subset: CD2, CD8, CD57

explanations for a significant difference would include the presence of immature or malignant cells that were not identified by standard pan-T-, B-, and NK-cell reagents. In addition, if the gating reagents (CD45/CD14) had not been included, contaminating cells (e.g., myeloid precursors, nucleated red blood cells, large platelets) with forward- and side-scatter characteristics similar to those of lymphocytes could not be ruled out. Potential technical problems include reagent or fluorochrome degradation, failure to add a reagent, and a host of others. Evidence for any major technical error should result in repeating the study.

Additional data that can be used for controls depend on the set-up. For example, the availability of multiple antibodies that identify a similar cell sub-population can serve as a useful check (e.g., total T cells by comparing CD3 and CD5 or CD2; total B cells by comparing CD19 and CD20). In addition, the use of specific reagents in more than one tube enables comparison between the repeat values as a measure of consistency. The application of internal checks should be performed by the flow operator as a simple means of confirming the validity of the data. Insights regarding unusual biological findings may also be uncovered through this type of evaluation (e.g., the presence of an increased population of $CD4^-/CD8^-$ double-negative T cells).[30]

The challenge in performing immunophenotyping is to accurately identify cells with specific surface characteristics (antigens). As previously noted, the capacity to discriminate cell subpopulations is often enhanced through the directed use of antibody combinations. The typical data generated consist of the percentage of negative versus positive cells when using one reagent, and multiple subpopulations when using more than one reagent. Regardless of the experimental design, it is important to consider not only the percentage of cells within each subpopulation, but also the absolute number of cells. This is most commonly obtained by multiplying the relevant percentage from the flow cytometer by the absolute lymphocyte count obtained using a white blood count and differential. For example, when assaying for CD4 T-cell counts, the percentage of $CD4^+$ cells is multiplied by the peripheral absolute lymphocyte count to yield the absolute CD4 count. A potential problem with this approach is the requirement for two separate procedures (i.e., dual platforms) to generate the final result. This introduces the possibility of additive error, based on the inherent errors of the two different methods. It also has fueled a search for approaches that facilitate performing both tasks by flow cytometry (i.e., a single platform). One alternative involves the inclusion of a fixed number of fluorescent beads (in a defined volume) in each tube as a reference standard to generate absolute numbers without requiring the use of the complete blood count and differential to generate a lymphocyte count. A recently introduced approach involves the use of impedance based cell counting in the flow cytometer to generate an absolute lymphocyte count (dependent on a fixed volume of sample being run) and then generating both percentage and absolute number of cells in each specific population or subpopulation. Regardless of the approach, the reporting of both percentages and absolute numbers is necessary when immunophenotyping peripheral lymphocytes.

The objective of evaluating malignant cells is often to characterize the lineage and differentiation level of the abnormal cells, rather than quantifying subpopulations. The pattern of reactivity combined with fluorescence intensity is often useful in identifying leukemic patterns, whereas the absolute number of cells may not be required. However, flow cytometric detection and quantitation of rare abnormal cells can be useful in evaluating for minimal residual disease in lymphoproliferative disorders.

Practical applications of flow cytometry

Immunophenotyping studies

CLINICAL RELEVANCE

Immunophenotyping studies

- Can be used to identify cell subsets, lineage, stage of cell differentiation, state of cell activation, and clonality.
- Lymphocyte results should be checked with T cells + B cells + NK cells = 100%.
- Immunophenotyping studies are not the equivalent of lymphocyte function studies.

The majority of immunophenotyping studies are directed at quantifying specific cell subpopulations, evaluating for the presence or absence of particular surface antigens, identifying the differentiation level of specific cells, determining cell lineage, evaluating for functional correlates based on specific antigen expression, examining for evidence of cell activation, and/or establishing monoclonality.

Quantification of a particular cell subpopulation can be readily accomplished with flow cytometry. The evaluation of absolute CD4 T-cell counts has formed the basis for monitoring patients infected with HIV.[31] The quantitation of $CD34^+$ hematopoietic stem cells in donor peripheral blood or bone marrow is used in many cellular reconstitution protocols.[32] Subpopulation characterization can also be useful in the evaluation of patients with clinical history and laboratory findings suggestive of immune deficiency.[33] These studies identify the presence or absence of cells and surface proteins associated with specific functional attributes, but do not assess the actual functional status of the cells. This point is clearly illustrated by the finding of normal B-cell numbers in most patients with common variable immune deficiency despite the fact that these patients fail to produce immunoglobulins normally.[34] However, changes in the characteristics of the B cells, particularly relative to memory B cells, provides potential insight into different phenotypes of this disorder and provides additional support for heterogeneity of patients with this disorder.[34] Due to the limitations of immunophenotyping, it is common practice when evaluating the status of the immune system to perform cell function testing in parallel.

Flow cytometry can be used to test for the presence or absence of a specific cell surface antigen. An example of this type of application is in the evaluation of a patient with a history of recurrent skin infections, delayed wound healing and persistent granulocytosis, which suggests a diagnosis of leukocyte adhesion deficiency type 1.[33,35] This disorder results from a defect in the gene encoding CD18, preventing the expression of three different heterodimeric adhesion molecules (β2 integrins) each containing CD18 (Chapter 21). This disorder can usually be diagnosed by studying granulocytes (and lymphocytes) for the expression of CD18 (as well as the three isoforms of CD11). Patients often have decreased rather than absent CD18 expression and confirmation of the diagnosis can be accomplished by demonstrating a failure of CD18 (and CD11a, 11b, 11c) upregulation following granulocyte activation.[33]

The directed use of a panel of monoclonal reagents can help address questions regarding the level of cell differentiation.

Antibodies specific to proteins expressed by early (precursor) cells represent one approach, and would include evaluating for the thymocyte marker CD1, or the pre-B-cell marker CD10 (CALLA). However, many surface antigens are expressed throughout differentiation. Examples of these include CD2 and CD7 found on thymocytes and circulating T cells, or CD19 and HLA-DR found on pre-B cells as well as B cells. Thus the pattern of surface antigen expression can also help distinguish the level of differentiation. Defining the developmental level of a particular cell population or subpopulation is best accomplished using a panel of reagents that span the natural history of the cell lineage. This approach represents the standard for testing leukemias and lymphomas enabling the improved classification of the malignant cells relative to prognosis and therapy. Focusing on the presence or absence of specific antigens also involves evaluating the level of expression, which may be altered in the abnormal cells. In addition, malignant cells may express antigens associated with different lineages, such that lineage-directed studies can provide insight into shared expression of specific antigens. Malignant cells may also have altered forward- and side-scatter characteristics, as well as diminished or absent CD45 expression. Thus, the approach to gating may have to be modified when studying hematopoietic malignancies.

Issues of monoclonality can be dealt with using flow cytometry when analyzing B cells, and in some circumstances when studying T cells. Normally B cells are a heterogeneous mixture of mutually exclusive κ or λ light-chain-positive cells. The distribution of light-chain expression can be evaluated for clonal excess using a method called the Komogorov–Smirnov D value comparison.[36,37] Light-chain clonal excess will usually be accompanied by an excess in B cells expressing one particular heavy chain (α, γ, μ, or ε). The capacity to evaluate T-cell monoclonality by flow cytometry is less definitive and consists of using T-cell antigen receptor β-variable (Vβ) chain-specific reagents looking for evidence of significant over representation of one Vβ chain family. This approach currently consists of setting up a number of tubes each with three different Vβ family-specific monoclonal antibodies, one conjugated with FITC, one with PE, and the third with FITC plus PE. This combination enables distinguishing the frequency of each of the three different Vβ families per tube (green$^+$, orange$^+$, green$^+$/orange$^+$) and represents a flow cytometric method to complement PCR-based spectratyping.[38]

The state of lymphoid activation can be addressed by evaluating for the presence of surface antigens that either are found only on activated cells or are upregulated following activation. These include receptors for specific growth factors (e.g., IL-2 receptor α chain, CD25), receptors for critical elements required for cell growth (e.g., transferrin receptor, CD71), ligands that are critical for cell–cell communication following activation (CD40 ligand [CD152] on activated CD4 T cells), and surface antigens that are upregulated as a result of activation (e.g., adhesion molecules, HLA-DR, CD69). In addition, the memory status of both T cells and B cells can be assessed based on differential surface molecule expression associated with prior antigen encounter. This enables a distinction to be made between naïve T cells that express CD45RA, CD62L and CXCR7 from memory T cells that express the alternative CD45 isoform, CD45RO (and varied CD62L or CXCR7, depending on whether the cells are central or effector memory cells).[39] In addition, memory B cells can be detected by the expression of CD27 and be further divided into isotype-switched and non-switched memory cells based on their pattern of surface immunoglobulin expression.[34,40]

Intracellular evaluation

Cellular activation

CLINICAL RELEVANCE

Intracellular flow cytometry

- Activation-directed studies:
  - Calcium flux
  - Intracellular protein phosphorylation
  - Oxidative burst: neutrophils
- Intracellular cytokine studies:
  - Clarify the Th1/Th2/Th17 status of an immune response
  - Can be assessed in an *in vitro* antigen-specific response
  - Can be combined with evaluation of cell surface studies

Ligand binding and transmembrane signal transduction resulting in cellular activation can be evaluated using flow cytometry. Changes in intracellular ionic calcium concentration (Ca^{2+}) are frequently used to monitor cell activation after ligand binding. These changes are associated with the activation of phospholipase C and protein kinase C. In general, three reagents have been used to measure Ca^{2+}: quin 2, indo-1, and fluo-3. Quin 2 has a low excitation coefficient and is not useful for flow cytometry; indo-1 requires ultraviolet excitation; fluo-3 can be excited by 488 nm but does not permit ratiometric analysis. Nevertheless, because of its ease of use fluo-3 is currently the most widely used probe for intracytoplasmic Ca^{2+} evaluation by flow cytometry. Strict attention must be paid to loading conditions, the presence or absence of free Ca^{2+} in the medium, experimental temperature, baseline measurements and calibration. This approach can be combined with cell surface marker or cell cycle evaluation.[12]

Intracellular pH changes related to cellular activation also can be evaluated. The most useful probe for pH is SNARF-1.[12] This probe can be excited at 488 nm and allows for ratiometric analysis with detection wavelengths set for 575 and 640 nm. Glutathione (glutamylcysteinylglycine, GSH) is an important antioxidant generated during cell activation that can be measured by flow cytometry.[12] The fluorescent probe monochlorobimane is commonly used for this measurement, but it is complicated by the need to determine GSH by an independent method such as HPLC.

Additional approaches to evaluate cellular activation include assessment of intranuclear markers (Ki-67, PCNA) as well as surface proteins that are upregulated following cellular activation (e.g., CD69, CD25, CD71).[41] Actual cell division can be evaluated using lipophilic membrane dyes (e.g., PKH26, CFSE) also referred to as cell tracking dyes that lose 50% of their fluorescence with each round of cell division.[42] This approach has become more common in the clinical assessment of lymphocyte function due to the capacity to evaluate specific lymphocyte subpopulations responding to mitogenic and antigenic stimuli. Lipophilic membrane dyes also can be used to label target cells in cell-based cytotoxicity assays.[43] Recently, an approach to evaluate lymphocyte proliferation following cell stimulation has been described using the thymidine analog, EdU. Detection of DNA sythesis induced by the different activating agents is measured using a copper-catalyzed click chemistry, which results in EdU being covalently bonded to a fluorescent azide.[44] This approach allows the assessment of cell proliferation at the cell population or

subpopulation (e.g., CD4, CD8) level and can be used in association with mitogen and recall-antigen stimulation.

Functional evaluation of cell activation can be accomplished with flow cytometry directed detection of the generation of phosphorylated intracellular proteins associated with specific activation signals. An example of this is the detection of phosphorylated STAT-1 following interferon-γ stimulation of monocytes, which has been found to be more sensitive than immunoblotting.[45] This type of assay requires fixation and permeabilization to allow the entry of the specific reagent and now has been extended to a number of additional intracellular proteins that are phosphorylated following exposure of selected cells to specific stimuli. Currently, a number of intracellular signaling proteins that undergo phosphorylation following a specific activation signal can be assessed with flow cytometry using commercially available reagents in kit form.

The assessment of oxidative burst following cell stimulation plays a central role in neutrophil function testing using the hydrogen peroxide-sensitive dye dihydrorhodamine 123 (DHR123). This procedure involves loading granulocytes with the dye, stimulating with phorbol myristate acetate (PMA), and evaluating for fluorescence by flow cytometry.[13,46] This test has proved to be extremely accurate in diagnosing patients with chronic granulomatous disease (CGD) and carriers of X-linked CGD.[46] A major advantage is its sensitivity, which allows the detection of one normal cell in a population of 1000 abnormal cells. This makes assessment of oxidative burst a useful tool in following allogeneic granulocyte survival after transfusion into patients with CGD, as well as a means of following donor chimerism in the setting of allogeneic stem cell transplantation and as a indicator of cell correction following gene therapy in CGD.[47]

Intracellular cytokine detection

Flow cytometry affords a platform to evaluate cytokine production at a single-cell level using cytokine-specific directly conjugated monoclonal antibodies following fixation and permeabilization of cells.[48] This approach allows for the simultaneous detection of two or more intracellular cytokines in combination with cell surface markers, or other intracellular markers. Important aspects of intracellular cytokine detection include the use of a protein transport inhibitor during activation, the use of proper controls and the choice of antibodies. As there is little or no spontaneous cytokine production in circulating human lymphocytes, intracellular cytokine detection requires *in vitro* activation. Initial experience was based on supraphysiological stimulation using PMA and ionomycin, but antigen-specific activation systems have also proven to be feasible. It should be emphasized that, regardless of the activation method, the duration of activation is an important variable, as individual cells reach maximum cytokine production at different times. In addition, different cytokines have different optimal periods of activation. It is recommended that a proper kinetic profile be established for the biological system or clinical condition being studied.[48]

To increase the amount of intracellular cytokines, inhibitors of intracellular protein transport (e.g., monensin or brefeldin) are commonly used, which lead to the accumulation of proteins within the cell. Nonspecific binding of the antibody reagents is an issue, as permeabilization allows access not only to the cytokine of interest, but also to other proteins present in much greater quantities than on the cell surface. In addition, fixation further increases nonspecific binding and the use of both a negative-control sample, which contains an excess of unlabeled or "cold" anticytokine antibody, and a subclass-matched or FMO-control sample provide the optimal control. When the conjugated anticytokine is added to the negative-control sample it can only bind to other proteins in a nonspecific manner, thereby providing a measure to discriminate between specific and nonspecific binding.[39] The use of directly conjugated anticytokine antibodies not only simplifies the staining procedure, but also provides the best distinction between specific and nonspecific binding. Because the fixation agent may change the native state of certain epitopes, it is also important to use antibodies that recognize antigens after fixation when combining cell surface characterization with intracellular cytokine evaluation.

One of the main applications of intracellular cytokine detection by flow cytometry has been the study and refinement of the Th1/Th2/Th17 paradigms. It has recently become clear that the regulated secretion of cytokines can be used to study

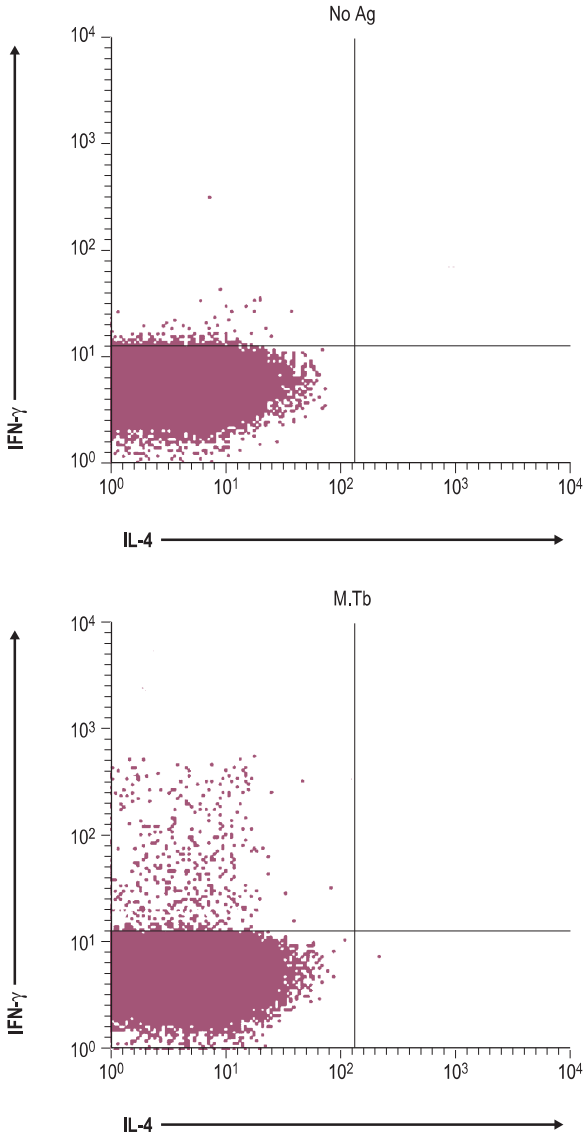

Fig. 94.6 Two-color dot plots of CD3$^+$ T cells evaluated for intracytoplasmic interferon-γ and IL-4 expression. The donor had a positive skin test to PPD and demonstated a Th1 pattern of cytokine expression (interferon-γ) in response to *Mycobacterium tuberculosis* antigen, with an absence of a Th2 cytokine pattern (IL-4). Courtesy of Calman Prussin, MD.

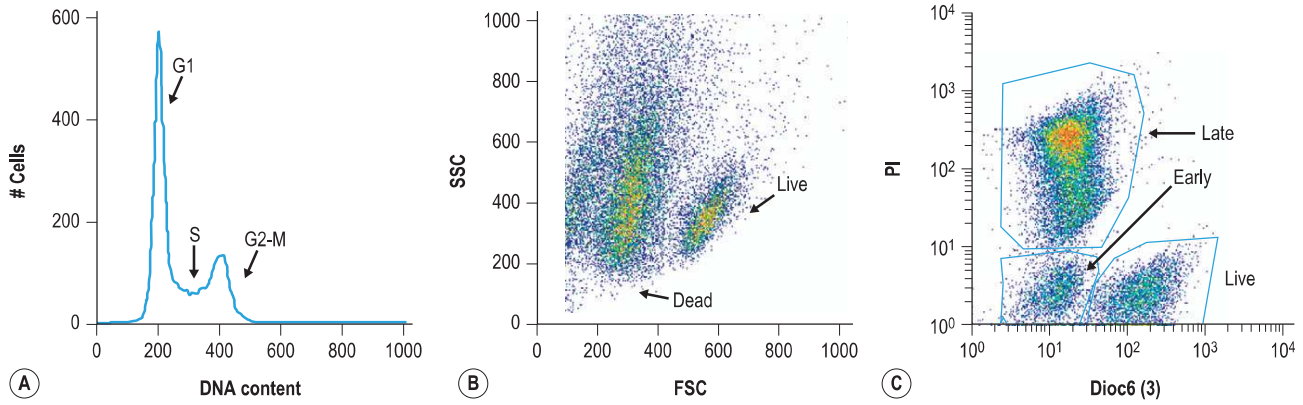

Fig. 94.7 (A) Assessment of DNA content as a reflection of cell cycle demonstrating cells in G_1, S, and G_2/M phases. (B) Assessment of live versus dead cells based on forward- and side-scatter characteristics. (C) Assessment of cell apoptosis using PI and Dioc6(3) identifying cells that recently initiated apoptosis (early), cells that are dead (late) and cells that are alive (live).

the response of individual T cells to both polyclonal stimuli and specific antigens. Measuring antigen-specific T-cell cytokine expression in response to specific antigen offers a useful alternative to the tetramer-based approach (discussed below) to quantify the frequency of antigen-specific T cells (Fig. 94.6).[49]

Cell cycle analysis

CLINICAL RELEVANCE

Cell cycle analysis

- Useful for screening percentage of S phase and aneuploidy
- Can be combined with cell surface studies
- Can be combined with markers of apoptosis

In addition to surface immunophenotyping and cytoplasmic characterization, flow cytometry is also used in cell cycle analysis. Propidium iodide (PI) is the most commonly used fluorochrome, owing to its optimal linear DNA-binding capacity in a variety of different cell types. Thus, a single-parameter histogram of DNA content using PI readily permits the determination of cell cycle compartments, expressed as the percentage of cells in G_0–G_1, S and G_2-M (Fig. 94.7A). In addition to these conventional parameters, the presence or absence of aneuploidy can be determined by inspection of the G_0–G_1 peak and/or use of a DNA index (ratio of abnormal DNA content to a diploid DNA standard). Also, elevation in the S and/or G_2-M phase can be detected. The optimal display of these data uses a combination of side scatter versus DNA content. Cells observed on the histogram in the area below the level of G_0–G_1 may be undergoing apoptosis.[50] When dealing with DNA staining, a consistent cellular source of DNA (e.g., chicken erythrocytes) should be used as an internal reference for evaluating DNA content and evaluating the cell cycle distribution.

It should be noted that several different computer algorithms have been developed to determine the relative proportion of each cell cycle compartment, and the selection of a software program is not a trivial process. The major instrument manufacturers supply cell cycle analysis programs, and there are also third-party programs available. Generally the optimal program should be capable of modeling two or more aneuploid populations, subtracting debris (particularly if formalin-fixed paraffin-embedded archival material is used) and accurately estimating S-phase cells.[51,52] The combination of a surface marker and cell cycle has been very useful in differentiating normal cell populations from tumor cell populations. One example is the use of anti-κ, anti-λ, or B-cell reagents to separate the aneuploid B-cell clone from the remaining normal, reactive B cells in a lymphoid cell mixture. Another uses cytokeratin as a marker to distinguish between the tumor cells and the inflammatory cells that are present.

The other major event that has occurred in cell cycle analysis has been the development of technology using the incorporation of bromodeoxyuridine.[53] This thymidine analog is used to directly determine the percentage of S-phase cells. Also, when used in kinetic studies it permits a determination of the individual times for the components of the cell cycle and a determination of the growth fraction. Finally, recent developments have resulted in the availability of two anticyclin reagents to evaluate cell cycle transition points in malignant cells.[54]

Apoptosis detection

Flow cytometry has become the method of choice for the detection and quantification of cellular apoptosis.[55] This is due in part to its capacity for rapid assessment of a large number of cells and samples. Many distinct features of an apoptotic cell can be evaluated by flow cytometry based on light scatter, plasma membrane changes, mitochondrial transmembrane potential, DNA content and DNA integrity.

The light scattering properties of a cell undergoing programed cell death are the simplest attributes that can be assessed by flow cytometry. Dying cells typically shrink, producing a loss in forward light scatter (FSC) and, despite an initial transient increase in side scatter (SSC), also ultimately demonstrate a decrease in SSC (Fig. 94.7B). The use of light scatter can be combined with cell surface staining to help characterize the dying cells. However, scatter changes alone are not specific to apoptosis and should be accompanied by an additional characteristic associated with cell death. Live cells have phospholipids asymmetrically distributed in the inner and outer plasma membrane, with phosphatidylcholine and sphingomyelin on the outer surface and phosphatidylserine (PS) on the inner side. Early during apoptosis cells lose asymmetry, exposing PS on the outside. Annexin V is a protein that binds preferentially to

negatively-charged phospholipids such as PS, and directly conjugated annexin V is a useful reagent for the specific detection of apoptotic cells.[55]

Another characteristic of plasma membranes associated with live cells is that they exclude charged cationic dyes such as propidium iodide (PI) and 7-amino-actinomycin-D (7-AMD). Consequently, only cells in a late stage of apoptosis, with ruptured cell membranes, will take up these dyes. Thus, the combined use of cationic dyes (e.g., PI) with annexin V allows the discrimination between live cells (annexin V negative/ PI negative), early apoptotic cells (annexin V positive/ PI negative) and late apoptotic cells (annexin V positive/ PI positive).

Assessment of mitochondrial transmembrane potential ($\Delta\psi m$) is yet another technique used to identify apoptotic cells. Cells decrease $\Delta\psi m$ very early in the apoptotic process, before rupture of the plasma membrane, losing the ability to accumulate potential-dependent dyes such as rhodamine 123, JC-1 or 3,3''-dihexyloxacarbocyanine iodide ($DiOC_6^3$). These dyes can also be used with PI to detect cells in the different stages of apoptosis (Fig. 94.7C).

Measurement of DNA content can also be employed to distiguish live from dead cells, as described above (see Cell Cycle Analysis). This kind of analysis has to be done using a linear scale, not logarithimic, in order to discriminate dying cells from debris. DNA cleavage also exposes -OH termini associated with the DNA breaks and these can be detected via the attachment of fluorochrome-conjugated deoxynucleotides, in a reaction catalysed by exogenous TdT, a technique called TUNEL.

Peptide–MHC multimers

KEY CONCEPTS

Peptide–MHC tetramers

- Useful for assessing the number of antigen-specific T cells
- Can be directed at both $CD4^+$ and $CD8^+$ T cells
- Requires information about the antigenic peptide and HLA (MHC) restriction

In contrast to B cells, direct visualization of antigen-specific T cells *in vivo* has, until recently, been unsuccessful. In 1996, Altman et al. introduced a novel flow-cytometry-based methodology that enables the direct visualization and quantification of antigen-specific T cells.[56] By generating soluble peptide–MHC multimers, such that multiple TCRs are engaged at the same time, the avidity of these multimeric ligands for the peptide-specific TCR is greatly increased. The methodology involves engineering a biotinylation recognition sequence on the -COOH terminus of the extracellular domain of one chain of the MHC molecule that, after combining with a specific antigenic peptide, is bound by avidin or streptavidin. As both avidin and streptavidin have four biotin-binding sites, the result is a tetrameric peptide–MHC complex that serves as a ligand for T cells specific for both the peptide and MHC. Flow-cytometric detection is achieved by labeling streptavidin with a fluorochrome. The major pitfall to this approach is the need to know the antigen-derived peptide and its HLA-restrictions, as well as the HLA type of each subject studied. Since the initial report, an increasing number of tetramer-based studies have appeared. Most have focused on the MHC class I-mediated immune response, in both mice and humans, to a variety of infectious agents, including cytomegalovirus (CMV), HIV, Epstein–Barr virus and others. Since the initial description with class I-restricted recognition, detection of antigen-specific CD4 T cells with tetramers of soluble MHC class II molecules and covalently linked peptide has also been reported.[57]

In addition to demonstrating the feasibility of this approach, the published studies have provided several new insights into the MHC class I-mediated immune response (Chapter 5). For example, it has become clear that the extent of the MHC class I-mediated cellular response is much greater than previously estimated. Furthermore, the extensive proliferation of CD8 T cells during an acute infection is not the result of bystander activation, but represents an expansion of antigen-specific CD8 T cells. One possible explanation is that, in contrast to previously used techniques such as the limiting dilution assay, tetramer-based assays do not depend on *in vitro* cell division and survival. Peptide–MHC tetramer assays have also shown promise in the study of the kinetics of primary and secondary immune responses, as well as in a better understanding of concepts such as immunodominance and clonal exhaustion.

An obviously attractive aspect of this technology is that tetramer staining can be combined with a variety of cell surface and intracellular phenotypic and functional markers. Already there are indications that the phenotype of antigen-specific T cells varies between individuals and between different phases of the immune response. In addition, tetramer-positive T cells can be sorted for further analysis, such as cytotoxicity assays or *in vitro* expansion. The tetramer-based technology has not only proved useful for the study of the immune response to infectious agents, it has also been applied to the study of oral tolerance, autoimmune conditions and tumor immunology. It is likely that this highly sensitive and specific technology and other approaches that define antigen specific response will find many more applications and will lead to new discoveries and a reassessment of certain existing concepts.[58]

Conclusion

ON THE HORIZON

- The recent development combining flow cytometry and mass spectrometry has an enormous potential for expanding the number of cell markers analyzed simultaneously (more than 50).
- This combined methodology could allow an unprecedented investigation focused on cell subsets combined with functional markers applied in the setting of human health and disease.

Flow cytometry has become readily available in clinical laboratories and the application of this technology has moved forward in parallel to significant improvements in instrumentation and the availability of an array of monoclonal reagents. Properly performed, flow cytometry can provide rapid and accurate lymphocyte subpopulation identification. The primary clinical indications of immunophenotyping remain quantifying CD4 T-cell counts in HIV infection, lineage assignment in leukemias and lymphomas, and assessing CD34 expression to identify stem cells for transplantation. Additional uses include characterizing immune deficiency disorders, evaluating immune-mediated inflammatory diseases, and assessing patients following organ transplantation. The real utility of these newer applications

remains to be fully clarified and, in most settings, immunophenotyping does not represent a diagnostic procedure but rather plays a part in the evaluation and understanding of complex disorders and the longitudinal evaluation of immunomodulatory therapy.

It is critical to recognize that immunophenotyping is a means of identifying cells, but it is not directed at cell function. The expansion of flow-cytometric techniques to evaluate intracellular characteristics, assess intracellular changes associated with activation, characterize apoptosis and identify antigen-specific T cells is moving this platform into the cell function arena. These newer approaches are expanding the utility of flow cytometry as a valuable tool for the characterization of immune function.

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