# 4

# Diagnostic Potential of Functional Measurements

J. Paul Robinson and L. Keith Plocki

Probably the most common use of flow cytometry is the phenotypic analysis of cell populations. This procedure allows the identification of cells with specifically identifiable surface antigens. This generally does not indicate the functional status of the receptor or the cell. There are several approaches in determining the functional integrity of the cell using flow cytometry. This chapter describes these techniques and relates them to potential use in diagnostic pathology. Only a small number of these techniques are presently used in clinical flow cytometry laboratories; however, several are likely to become generally available in the near future.

Flow cytometry allows one to make measurements on individual cells. Therefore, it is possible to identify populations of cells with varying degrees of functional activity within a heterogeneous population. Alternatively, using a bulk measurement system (for example, measuring superoxide of neutrophils using SOD inhibitable cytochrome C reduction),[4] there is no way to account for dead cells, poorly or highly reactive cells, or different cell types that may have been included in the cell suspension.[4,30] These cells can be excluded from calculations in flow cytometry by a process known as selective gating. This process uses a multiparametric approach whereby unwanted cells can be electronically marked and excluded from the analysis. This will be demonstrated later by the measurement of $H_2O_2$ in human neutrophils.

## Functional Measurements

There are several categories of measurements that can be classified as functional. For a complete review on flow cytometric evaluation of leukocyte function see Stelzer and Robinson.[47] The ability to identify a cell type with a surface marker does not indicate the viability of the cell. Similarly, a viable cell may not be metabolically responsive. Therefore determination of functional parameters requires more information about the cell than is usually collected for phenotypic classification. The following is a summary of many of the approaches that can be taken for assessment of cell function.

## MEMBRANE STUDIES

### Membrane Integrity (Viability)

Probably the most commonly used function assay in flow cytometry is measurement of cell viability using dyes such as propidium iodide or fluorescein diacetate. While there are no particular reasons why the flow cytometric method is better than any other, there are definite advantages in being able to determine viability directly in assay samples. Further, nonviability can be a useful parameter to gate out dead cells from analysis,[11] particularly important in functional assays.

### Enzyme Content/Activity

Dolbeare[20,21] has demonstrated the presence of phosphatases and glucuronidases using naphthol derivatives as fluorogenic substrates. Similarly, cellular enzyme activity can be measured by flow cytometry[52,57] as can esterase activity using dyes such as dichlorofluorescin diacetate.[6,32,37] These latter assays are more useful as indicators of the presence of esterases. Some use can be made of this activity to differentiate cell populations as well as to indicate normal metabolic function. Carboxyfluorescein diacetate is useful in this regard since it can be hydrolyzed directly to the fluorescent compound, carboxyfluorescein. This is a rapid process in most cells (1–5 min). Use of such techniques for clinical diagnostics will become important as part of a multiparametric assessment of phenotypic and functional characteristics.

### Membrane Fluidity

The use of diphenylhexatriene (DPH) or related probes has been described as a useful tool in measuring alterations in membrane fluidity of cells.[33,40,48] The basis of these measurements is that the dye is taken into the more hydrophobic regions of the cells, particularly the lipid bilayer in the cell membrane. By measuring light in different degrees of polarization, variations can be observed in the structural integrity of the membranes. Possible uses could be for identifying old, deformed, or degenerated cells, since cells increase rigidity with age.[45] Neutrophil function[39] and other functions[22] have also been described. While this concept has potential in the clinical arena, it presently has little diagnostic utility.

### Receptor Expression

*CR3 Receptor.* CR3 receptor is a glycoprotein associated with cell adhesion that regulates the adherence and phagocytosis of particles opsonized with iC3b. Arnaout *et al.*[2] have demonstrated the activation of neutrophils (via increased CR3 expression) by complement fragments generated by new hemodialysis membranes. Thus measurement of this receptor may have clearly identifiable clinical utility.

*Chemotactic Defects.* A deficiency in a surface glycoproteins LFA-1 and p150,95 and CR3 can lead to chemotactic defects. This syndrome has been identi-

fied by Arnaout *et al.*[3] and Ross.[41] This is a genetic abnormality in the synthesis of these molecules and patients with the defects suffer recurrent infections.

***IL-2 Receptor.*** The quantitation of IL-2 receptors has been used as a measure of T cell response to mitogen and antigen. Prince and John[34] have used this method for determining such responses in patients with immunodeficiency disorders.

## METABOLIC STUDIES

### Oxidative Burst

***Superoxide.*** NBT reduction has been measured by Blair.[15] The method is a variation of an age-old method requiring a microscope and a glass slide.[5] Use of this method has been mostly replaced by current studies utilizing measurements of $H_2O_2$.

***Hydrogen Peroxide.*** The assay depends upon the incorporation of 2′-7′, dichlorofluorescin diacetate (DCFH-DA) into the hydrophobic lipid regions of the cell, where the acetate moieties are cleaved by hydrolytic enzymes to a non-fluorescent molecule 2′-7′, dichlorofluorescin (DCFH)[6,26] which is trapped within the cell due to its polarity. Upon cell activation, NADPH oxidase catalyzes the reduction of $O_2$ to $O_2^-$ which is further reduced to $H_2O_2$. The oxidative potential of $H_2O_2$ and peroxidases are able to oxidize the trapped DCFH to 2′-7′,-dichlorofluorescein (DCF) which is fluorescent (530 nm). The green fluorescence produced is proportional to the amount of $H_2O_2$ generated. It is possible to calibrate this assay to allow the expression of the intracellular production of $H_2O_2$ in neutrophils in terms of attomoles/cell. We have used this assay to compare the activation states of resting and stimulated neutrophils from both rats and humans.[29,37,47] Figure 4.1 shows the use of this assay as a clinical tool in the diagnosis of chronic granulomatous disease (CGD). Conversion of the fluorescence channel on the flow cytometer is accomplished by use of a calibration curve generated based on data obtained from spectrophotometric and flow cytometric measurements according to the methods of Bass *et al.*[6] This test is easily adapted to the clinical cytometry laboratory. An example of this method is described in Table 4.1.

### Intracellular pH

Visser[56] and Bassoe[8] have both developed methods for determination of intracellular pH. Valet *et al.* have also described a method for determining pH alteration by ratioing the green and blue emission of an ultraviolet dye, 1,4-diacetoxy-2,3-dicyanobenzene.[54] These methods, however, may not be suitable for routine clinical applications. As new fluorescent dyes become available, rapid measurements of intracellular pH based upon these approaches will provide valuable biochemical information on the metabolic status of cells. Such an approach may be available using 2′,7′-bis-carboxyethyl-5(6)-carboxyfluorescein (BCECF), which has dual emission spectra upon activation at 488 nm. Using this dye, Alcover *et al.*[1] demonstrated that

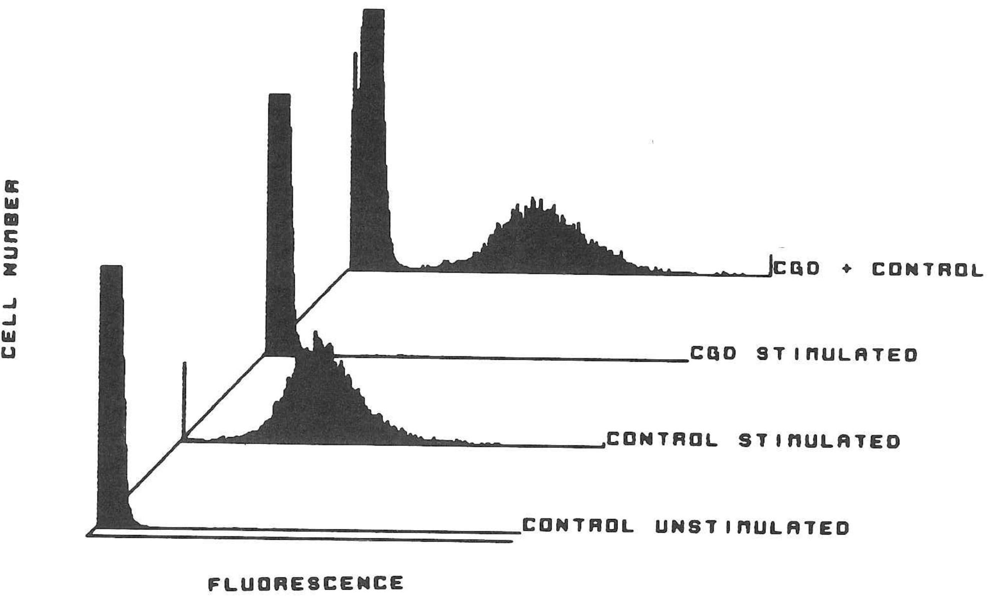


**Figure 4.1.** The oxidative burst assay using flow cytometry. Cells were prepared using the procedures outlined in Table 4.1. Fluorescence of gated neutrophils (whole blood with lysed RBC) was measured for both the CGD and the control. The PMA stimulated CGD cells failed to respond whereas the stimulated control cells showed a significant increase in mean channel linear fluorescence. A mixture of the CGD and control cells shows a bimodal distribution. The increase in fluorescence is proportional to the production of $H_2O_2$ by the neutrophils.

loaded helper T lymphocytes demonstrated early detectable pH changes when stimulated by antibodies to CD3 but not to CD4 or CD8. Thus the ability to not only differentiate lymphocytes subsets but also determine their functional integrity provides significantly more information about the immunological status of these cells.

## Degranulation

Degranulation is an important functional parameter for neutrophils. Patients with abnormal degranulation patterns have been described, and several assays have been developed to measure this phenomenon using flow cytometry.[31,46]

## Intracellular Calcium Redistribution

Calcium mobilization results from phospholipid degradation subsequent to ligand binding. A number of fluorescent dyes are now available to monitor the changing calcium flux inside the viable cell. Flow cytometric techniques for monitoring intracellular calcium redistribution have been developed for T cells,[35] B cells,[36] neutrophils,[25,43] and other cells.[55]

**Table 4.1**
**Rapid Clinical Method for Oxidative Burst by Flow Cytometry**

**Method**

1. In a 1.5-ml conical centrifuge tube, place 1 ml of standard 1.077 to 1.086 g/ml Ficoll-Paque or LSM. (Must be at room temperature before use.) Carefully overlay 0.5 ml of undiluted heparinized blood onto the Ficoll.
2. Leave upright and undisturbed for 20–25 min on the bench (room temperature). Red blood cells will agglutinate and sediment leaving essentially RBC free platelets and leukocytes in plasma.
3. Remove exactly one half of the amount of blood added to the Ficoll, i.e., 250 µl, by skimming from the top.
4. Be careful not to disturb the interface. The topmost layer should contain relatively few RBC and at least 50% of available leukocytes.
5. Place into 12 × 75 mm tube and add 2 ml PBS (containing 2% FCS), wash at 250 × *g*, for 10 min, and resuspend in PBS-glucose. (Platelets should be gone.)
6. Count on Coulter counter and resuspend whole cells at approximately $2 \times 10^6$ leukocytes/ml.

**DCF Assay**

1. Add at least 1 ml of above cell suspension to a 12 × 75 mm tube. Prepare for each patient to be tested plus appropriate control samples.
2. Add 1 µl of 20 mM DCFH-DA per ml of cells to "load" these cells. Gently mix.
3. Incubate at 37°C for 15 min.
4. Place on ice after 15 min OR immediately aliquot 100 µl into two 12 × 75 mm tubes for the stimulated measurements. Always keep at least 50–100 µl of DCFH-DA loaded cells for the unstimulated control.
5. For the stimulated samples, add an equal volume of warmed PMA solution (200 ng/ml to give a final concentration of 100 ng/ml).
6. Incubate at 37°C for 15 min and immediately run on cytometer for 15 min determination. (Measurements can be made at 30–40 min if more convenient.)
7. One to two minutes before each 15 min reading, the unstimulated control sample should be run.

**Results**

1. Collect linear green fluorescence using the same filter set-up as for FITC. If log signals are collected, data needs to be transformed into linear fluorescence.
2. The stimulated samples should increase from 10 to 30 times that of the unstimulated samples.
3. If no increase occurs, this may indicate either assay error or be diagnostic for CGD. In this case ALWAYS repeat the whole assay with a new blood sample and new control.

## Cell Cycle Analysis

*In vitro* lymphoproliferative response to mitogen stimulation using measurements of DNA is a commonly used functional assay for lymphocytes.[53] The obvious benefits of not using radioactive isotopes as well as a more objective analytical approach are clear. Other techniques for assessment of lymphocyte activation such as membrane potential alterations[17,44] or radio-frequency opacity,[24] while offered as promising techniques, have failed to excite clinical laboratories of their veracity or

potential usefulness. Despite this stoicism, measurement of proliferative response using flow cytometry is an excellent assay offered by clinical cytometry laboratories. The basis of cell cycle analysis is that the degree of the dye fluorescence retained by the cell is directly proportional to the DNA content. Thus, a normal lymphocyte has a diploid amount of DNA; if it is stimulated to proliferate, it will contain twice the DNA content immediately prior to mitosis demonstrated by a proportional increase in fluorescence. Measurements of the cell cycle can be useful in determining actively proliferating cells. Additionally, if cells are cultured in the presence of bromodeoxyuridine (BrdU), the BrdU will be incorporated into newly synthesized DNA.[19,42] Alternatively, by using a fluorescence conjugated anti-BrdU antibody, such cells can also be easily detected by flow cytometry[16] using standard immunofluorescence methods.

## PHYSICAL PHENOMENA

### Phagocytosis

Measurement of phagocytosis of bacteria has been extensively developed for flow cytometry primarily by Bassoe *et al.*[7-10,12-14,50] These measurements can be valuable in trauma such as thermal injury or in recurrent infections where particular organisms, isolated from patients, can be used for assessment of immune function. If the coefficient of variation of the phagocytosed particle is small, the numbers of ingested particles can be determined from the fluorescence intensity.[23] If a simple index of uptake is required, fluorescent latex particles can be used. A method using trypan blue has also been developed whereby internalized particles can be discriminated from those attached but not ingested.[11] *Immune complexes* can also be measured using methods similar to those for bacteria.[51]

### Pinocytosis

Pinocytosis can also be a useful measure of cell function, and several well-defined assays have been developed by Davis *et al.*[18] These assays are relatively easy to perform, particularly in the operating environment of the clinical cytometry laboratory.

## AUTOANTIBODIES

### Antineutrophil Antibodies

Antineutrophil antibody measurements by flow cytometry have become rapidly acceptable as a standard clinical assay by flow cytometry.[38] Children with neutropenia are prime candidates for this flow method since only 1 or 2 ml of blood is required. Additionally, there are usually sufficient cells available to perform qualitative testing of neutrophils using a simple $H_2O_2$ screen.

### Immune Thrombocytopenia

Measurement of antiplatelet antibody has also made its mark on clinical flow cytometry laboratories. Several very good flow cytometric techniques have now been described for measurement of antiplatelet antibodies.[27,49] The added benefit of the flow cytometric method is that measurements can be made using either patient serum (indirect method) or patient platelets (direct method) even under conditions of severe thrombocytopenia. This is due to the small number of platelets required for the assay and is therefore an attractive alternative to isotopic assays for immune thrombocytopenia.[28]

## Conclusions

While there are a plethora of functional measurement systems for use in flow cytometry laboratories, few are presently used as diagnostic tools. The two most common are probably lymphocyte proliferation and neutrophil metabolism (oxidative burst and phagocytosis). A number of clinical laboratories offer these tests as routine procedures. These currently available clinical tests are not yet widely familiar to clinicians and therefore not requested in vast numbers. One test that has been growing in clinical interest is antineutrophil antibody detection by flow cytometry. Initially included as a functional test, it is used extensively in studying neonatal neutropenia, in which it is possible to measure both the oxidative burst capacity of neutrophils as well as to determine the presence of antineutrophil antibodies on the surface of the patient's own neutrophils or to detect such antibodies in serum. Minute quantities of blood are required even in fairly severe neutropenic patients. Since flow cytometry can utilize very few cells, both determinations can be made on less than 100,000 total neutrophils. This combination can provide rapid useful clinical information not otherwise available.

There have been many new developments in flow cytometry, particularly the ability to perform complex multiparametric analysis on cell populations. In this way, we have placed more attention on the heterogeneous nature of many cell populations allowing discrimination between *functional* and *nonfunctional* characteristics. As we better understand the relationship between antigenic markers and the corresponding function, the assays described in this chapter will be in greater demand and probably better understood in terms of recognized clinical syndromes.

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