CHAPTER 28

Oxidative Product Formation Analysis by Flow Cytometry

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I. Introduction

Prior to flow cytometric methods, measurement of oxidative function was labor intensive and often very difficult to quantitate. The *Staphylococcus aureus* killing methods (Alexander *et al.*, 1968) required a large volume of blood and at least 48 hr to complete. More recent methods such as chemiluminescence, while quantitative and rapid, have been difficult to interpret (DeChatelet *et al.*,

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1982; Cheung *et al.*, 1983). The advantages of the flow-based methods are that a significantly reduced cell number is required, the procedure is relatively easy, and, if a flow cytometer is already available, the procedure is inexpensive to perform. A significant feature of the flow-based methods not available using any other technique is the capability to determine heterogeneity of response (Taga *et al.*, 1985; Neill *et al.*, 1985). Subpopulations defined by either light scatter or fluorescence intensity can be identified and quantified. Thus unresponsive or poorly responsive cell populations are easily discerned.

II. Oxidative Burst

A. Application

A variety of reactive oxygen species is produced by several cells. Our principal application for measuring oxidative systems is for functional evaluation of "oxidative or respiratory bursting" in neutrophils. The respiratory burst results from activation of the membrane-bound NADPH oxidases via an electron transfer reaction. Two electrons are transferred from NADPH through an FAD-flavoprotein utilizing cytochrome b_{-245} to oxygen. Superoxide anion is produced and then dismutates to hydrogen peroxide (H_2O_2) either spontaneously or by superoxide dismutase (SOD). The reactive oxygen species (ROS) and the hydrogen peroxide produced are necessary for normal bactericidal mechanisms in the neutrophil. These oxidative mechanisms exist in many cells and we have used these techniques to evaluate a variety of cell types including neutrophils, macrophages, HL-60 cells (human leukemia-60 cells, see Vol. 42, chapter 25), and endothelial cells.

A calibration curve can be generated based upon spectrophotometric data and flow cytometric measurements. This allows for conversion of flow cytometry fluorescence channels into quantitative estimations of H_2O_2, if necessary (Bass *et al.*, 1983).

The assays described below utilize two dyes. In the first assay, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which is freely permeable, is incorporated into hydrophobic lipid regions of the cell (Bass *et al.*, 1983). The acetate moieties are cleaved off leaving the nonfluorescent 2′,7′-dichlorofluorescin (DCFH). Hydrogen peroxide and peroxidases produced by the cell oxidize DCFH to 2′,7′-dichlorofluorescein (DCF) which is fluorescent (530 nm). The green fluorescence produced is thus proportional to the H_2O_2 produced.

The second assay utilizes hydroethidine (HE) which can be directly oxidized to ethidium bromide by superoxide anions produced by the cell. The third assay incorporates both dyes and has been used to detect selective defects in phagosomal oxidation following lysosomal degranulation as has been reported to occur in sepsis (Rothe and Valet, 1990). There are several advantages of HE over the DCF assay.

B. Materials

1. Hanks' balanced salt solution (HBSS)

   Stock HBSS 10× concentration: NaCl, 40 g; KCl, 2.0 g; Na_2HPO_4, 0.5 g; $NaHCO_3$, 0.5 g; q.s. to 500 ml.

   Stock Tris 1.0 *M:* Tris base, 8.0 g; Tris–HCl, 68.5 g; q.s. to 500 ml, pH to 7.3.

   Preparation of 100 ml HBSS:

Stock HBSS, 10×	10 ml
Distilled water	80 ml
Tris, 1.0 *M*	2.75 ml
$CaCl_2$, 1.1 *M*	170 μl
$MgSO_4$, 0.4 *M*	200 μl
Dextrose	220 mg

   adjust pH to 7.4 and q.s. to 100 ml.

2. PBS gel
   - Stock PBS gel

EDTA (disodium salt), 0.2 *M*	7.604 g
Dextrose, 0.5 *M*	9.0 g
10% Gelatin (Difco)	10 g
Distilled water	100 ml

   - Heat water to 45–50°C and slowly add gelatin while mixing with a magnetic stirrer. Continue stirring and add EDTA and dextrose. Do not exceed 55°C because gelatin and glucose will "caramelize." Store in 1.2-ml aliquots at −20°C.
   - Working solution PBS gel (make daily as needed). Warm 1 ml gel to 45°C. Add 95 ml warm PBS (phosphate buffered saline) and mix. Adjust pH to 7.4 and q.s. to 100 ml.

3. Erythrocyte lysing solution (preparation of 100 ml):

NH_4Cl, 0.15 *M*	0.8 g
Na HCO_3, 10 m*M*	0.084 g
EDTA (disodium), 10 m*M*	0.037 g
Distilled water	95 ml

   adjust pH to 7.4 and q.s. to 100 ml.

4. DCFH-DA (MW 487.2) [Molecular Probes, Inc., Eugene, OR], 20 m*M* solution:
   - Weigh 2–9 mg of DCFH-DA and place in a foil-covered 12 × 75-mm tube.
   - Add absolute ethanol in a volume equivalent to the weight in milligrams of the DCFH-DA divided by 9.74.
   - Cap the tube, mix, cover in foil, and store at 4°C until use.

5. HE (MW 315) [Molecular Probes Inc., Eugene, OR], 10 m*M* solution:
   - Stock solution, 10 m*M* in dimethylformamide (3.15 mg/ml).
6. PMA (phorbol 12-myristate 13-acetate) [Sigma Chemical Co., St. Louis, MO]: PMA is toxic and carcinogenic; additionally dimethyl sulfoxide (DMSO) is readily absorbed through the skin. Wear gloves while handling solutions, prepare solutions in a hood, and be extremely cautious!

   Stock PMA (2 mg/ml in DMSO): Mix well and aliquot 15–20 μl of stock PMA in small capped polypropylene bullets. Store at −20°C.

   Working PMA solution (make daily as needed): 5 μl PMA stock in 10 ml PBS gel (1000 ng/ml PMA solution). A final PMA concentration of 100 ng/ml will predictably result in maximal cell stimulation (e.g., 900 μl of cells in solution and 100 μl of working PMA solution).

C. Instrumentation

Excitation is at 488 nm for both the above probes. Emission filters should be 525 nm for DCF and 590 nm for HE. Collection of forward light scatter and 90° scatter, as well as both fluorescence wavelengths, is necessary. Where possible collect list-mode data for further analysis. If performing a kinetic assay, time may be required.

D. Methods

DCFH-DA Assay (using whole blood):

1. Place 2 ml of preservative-free heparinized whole blood in a 50-ml conical tube.
2. Add 48 ml of erythrocyte lysing solution.
3. Gently mix solution for 10 min at 25°C on a hematology rotator.
4. Centrifuge for 10 min at 350*g* and 4°C.
5. Decant supernatant and resuspend in 5 ml of PBS gel (working solution).
6. Centrifuge for 10 min at 350*g* and 4°C. Decant supernatant and resuspend in 2.5 ml HBSS.
7. Count leukocytes and adjust cell suspension to 2.0×10^6 cells/ml.
8. Add 1 μl 20 m*M* DCFH-DA per ml of cell suspension to be loaded.
9. Incubate loaded cells at 37°C for 15 min.
10. Stimulate cells with PMA: add 100 μl PMA (working solution) to 900 μl of cell suspension (final PMA concentration 100 ng/ml). Reserve some loaded, unstimulated cell suspension for a control.
11. Maintain cell sample at 37°C and run stimulated and unstimulated samples every 10 min on the cytometer for a total of 40 min.

Hydroethidine Assay: Procedures 1–7 are identical (as above).

8. Add 1 μl HE per ml of cell suspension to be loaded.
9. Incubate loaded cells at 37°C for 15 min.

Procedures 10 and 11 are identical (as above)

Combined DCFH-DA and Hydroethidine Assay: Procedures 1–7 are identical (as above)

8. Add 1 μl 20 m*M* DCFH-DA per ml of cell suspension to be loaded.
9. Incubate loaded cells at 37°C for 5 min.
10. Add 1 μl HE per ml of cell suspension to be loaded.
11. Incubate loaded cells at 37°C for an additional 15 min.
12. Stimulate cells with PMA: add 100 μl PMA (working solution) to 900 μl of cell suspension (final PMA concentration 100 ng/ml). Reserve some loaded, unstimulated cell suspension for a control.
13. Maintain cell sample at 37°C and run stimulated and unstimulated samples every 10 min on the cytometer for a total of 40 min.

E. Critical Aspect

Clumped cells are obviously detrimental to the assay and may potentially plug the flow cell. Additionally, clumped cells cannot be measured as "functionally normal" and must be eliminated. Clumping may be related to highly activated cells and may therefore provide important information regarding the context of the assay. Keeping the cell sample at 4°C (on ice) and using PBS gel will help prevent clumping of these reactive cells. Additionally, the dextrose, calcium, and magnesium in the HBSS are necessary for optimal cell function. Preservative-free heparin is also important to prevent any alteration in normal cell function. Dimethylformamide, used for preparation of the hydroethidine solution, will dissolve plastics and should be stored in glass. Alternatively DMSO can be used to prepare the hydroethidine solution. Another crucial aspect of these assays is that an unstimulated cell sample must be used for comparison at all time points.

There is a steady increase in oxidation of the DCFH-DA and hydroethidine even in unstimulated cells. This spontaneous oxidation varies with the cell type and with the dye and is primarily mitochondrial in origin. Mitochondrial oxidation can be blocked with azide or cyanide. In addition to measuring an increase in red fluorescent ethidium bromide, a decrease in blue fluorescent hydroethidine (excitation 350–380 nm, emission 418–500 nm) can be simultaneously measured in cells. One of the advantages of hydroethidine is the measurement of a ROS, superoxide anion, which occurs earlier in the cascade of oxidation events than hydrogen peroxide. An advantage of the combined assay is the identification of different subpopulations of neutrophils with disparate oxidative function.

F. Results

Cells must be preloaded for the correct time before activation. The time period varies with the cell type. Neutrophils are usually fully "loaded" within 15 min at 37°C.

Figures 1–3 show a typical histogram of an unstimulated and a stimulated population of human neutrophils. A 4- to 20-fold increase in green fluorescence is expected and evident above.

III. Phagocytosis

A. Application

Phagocytosis involves a series of stages by a cell progressing from particle attachment to ingestion. Any step in the phagocytic process can potentially fail. By using both opsonized and unopsonized particles in the assay we can determine whether abnormal phagocytosis is due to defective ingestion or opsonization. The technique described below employs the use of opsonized and

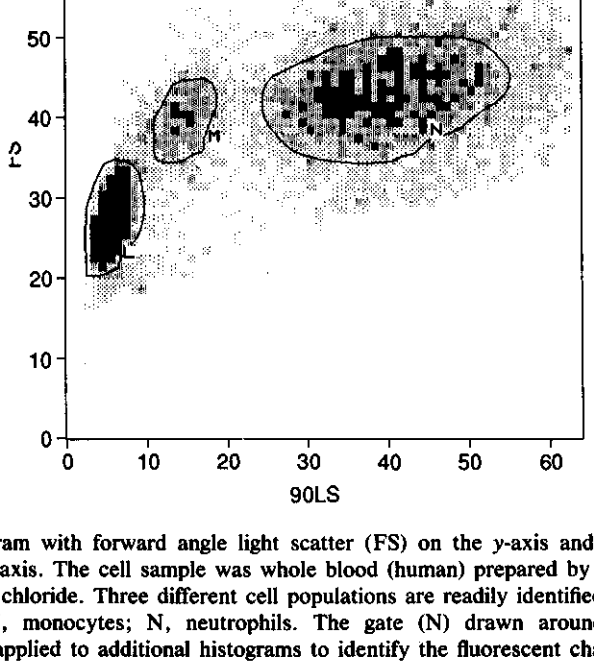

Fig. 1 Histogram with forward angle light scatter (FS) on the *y*-axis and 90° light scatter (90LS) on the *x*-axis. The cell sample was whole blood (human) prepared by erythrocyte lysis with ammonium chloride. Three different cell populations are readily identified and labeled: L, lymphocytes; M, monocytes; N, neutrophils. The gate (N) drawn around the neutrophil population was applied to additional histograms to identify the fluorescent changes in only the neutrophils.

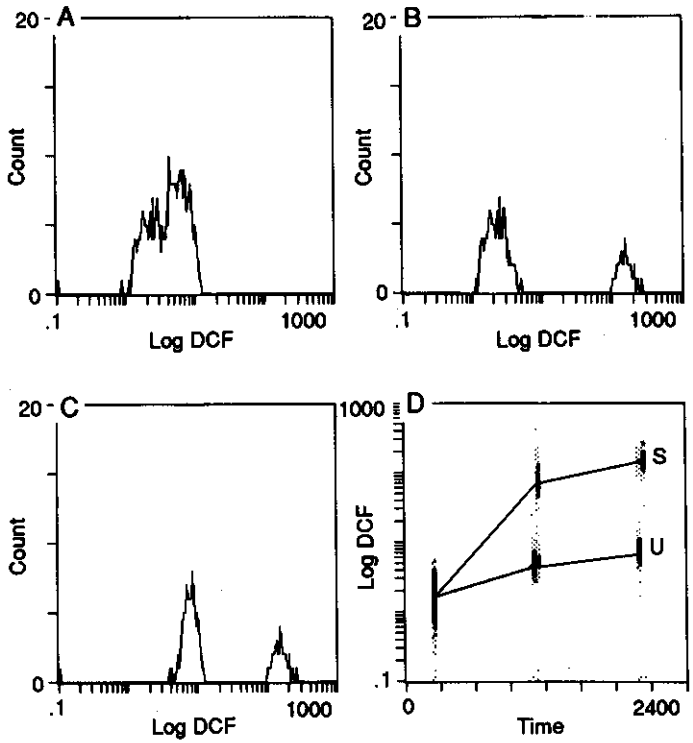

Fig. 2 (A) Histogram showing the increase in green fluorescence (log DCF) from 0 to 40 min in unstimulated neutrophils. (B) Similar histogram to A except the neutrophils are stimulated with PMA. (C) Histogram indicating the difference in fluorescence (log DCF) at 40 min between unstimulated and PMA-stimulated neutrophils (a 4- to 20-fold increase in fluorescence is expected in normal neutrophils). (D) Overview histogram of three time points (time in seconds) versus green fluorescence (log DCF on *y*-axis) for both unstimulated (U) and PMA-stimulated (S) neutrophils. The lines are drawn through the mean channel fluorescence of each time point. As indicated in the text, unstimulated neutrophils will oxidize (primarily mitochondrial oxidation) some DCFH-DA to DCF and increase the intensity of green fluorescence. Nevertheless, the change in fluorescence is much greater in PMA-stimulated neutrophils.

unopsonized bacteria (Bjerknes and Bassoe, 1984). Alternatively, fluorescent beads may be used or FITC (fluorescein isothiocyanate)-labeled yeast. After phagocytosis of the FITC-labeled yeast, ethidium bromide can be added to stain the extracellular yeast red so they can be differentiated on the flow cytometer.

A related version of this assay can also be used for evaluating phagocytosis in macrophage populations. This has particular importance in evaluating lavage fluids such as peritoneal lavage or bronchoalveolar lavage.

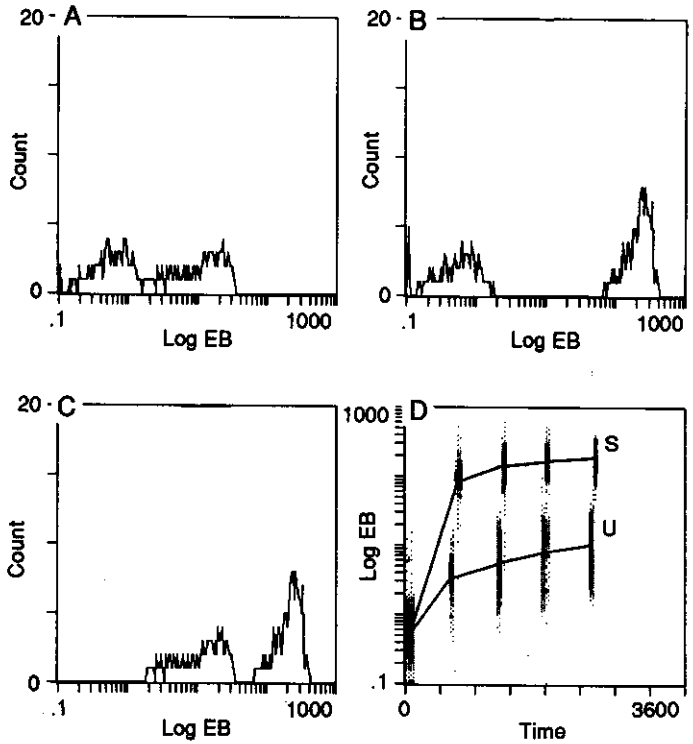

Fig. 3 (A) Histogram similar to that of 2A except hydroethidine is oxidized to ethidium bromide (EB) in unstimulated neutrophils. (B) Similar histogram to A except the neutrophils are stimulated with PMA. The change in red fluorescence (log EB) is evident from 0 to 40 min in PMA-stimulated neutrophils. (C) Difference in red fluorescence (log EB) between unstimulated and PMA-stimulated neutrophils at 40 min. (D) Overview histogram showing five time points (time in seconds) versus EB fluorescence for unstimulated and PMA-stimulated neutrophils. The lines are drawn through the mean channel fluorescence of each time point. Similar to the changes with DCF, an increase in EB fluorescence is evident in unstimulated neutrophils. However, the fluorescence change is much greater for PMA-stimulated neutrophils. More time points were collected for the HE assay than the DCF assay (Fig. 2) but the result is the same.

B. Materials

Bacterial culture
Blood agar plate
Brain–heart infusion broth
Brain–heart infusion agar slant
Neutrophil isolation medium (NIM) [Cardinal Associates, Santa Fe, NM]
HBSS (as above)
Pooled human serum

Glycerin

Carbonate/bicarbonate buffer (pH 9.5):

Na_2CO_3, 0.5 *M* (5.3 g/100 ml)	1 volume
$NaHCO_3$, 0.5 *M* (4.2 g/100 ml)	3 volumes

FITC: 0.02 mg/ml in carbonate/bicarbonate buffer (make a concentrated solution and dilute).

0.9% NaCl–0.02% EDTA:

NaCl	900 mg
EDTA disodium	20 mg
Distilled water, q.s. to	100 ml

Trypan blue (Gibco Laboratories, Grand Island, NY):

Stock, 4 mg/ml in saline

Working solution, 3 mg/ml (dilute stock 3:4 with saline).

C. Methods

1. Bacterial Culture

Bacteria are cultured in a blood agar plate and then subcultured to obtain discrete colonies. A discrete colony is then cultured on a brain–heart infusion agar slant and again subcultured. Using a sterile loop, some of the colonies are transferred into broth and cultured overnight in a 37°C incubator.

2. Labeling Bacteria with FITC

The bacteria are washed with HBSS and centrifuged at 10,000*g* for 10 min and the supernatant is decanted. The bacterial slurry is heat killed for 1 hr at 60°C. Enumeration of the bacteria is achieved by serial dilutions and subsequent plating and correlation using spectrophotometry so that the final concentration is approximately 10^9/ml. The bacteria are resuspended in carbonate/bicarbonate buffer such that the absorbance is, for example, 0.35 at 620 nm (this may differ depending upon the bacterial species and the media composition). The volume of the bacterial suspension is doubled with 0.02 mg/ml FITC in carbonate/bicarbonate buffer and incubated at 37°C with end-over-end rotation for 30 min. The bacteria are washed three times with HBSS, counted, and resuspended at a final concentration of 1×10^8/ml. The bacteria are aliquoted into sterile Eppendorf microcentrifuge tubes at a volume of 1 ml/vial; a drop of sterile glycerin is added to each vial and frozen at −70°C. The labeled bacteria will last for several years at −70°C.

3. Phagocytosis Assay (Using a Purified Population of Neutrophils)

1. Overlay 5 ml of EDTA or preservative-free heparinized whole blood on NIM.

2. Centrifuge for 30 min at 400*g* and 25°C.

3. Remove neutrophil layer and wash in PBS gel; centrifuge 250*g* for 10 min at 4°C.

4. Remove supernatant and resuspend neutrophil pellet in HBSS.

5. Count neutrophils and adjust cell suspension to 1×10^6 cells/ml.

6. Thaw one vial of bacteria; sonicate five times for 10-sec intervals at approximately 75% power. Cool bacteria on ice between cycles.

7. For opsonization, dilute 1 ml of pooled human serum with 3 ml HBSS (1:4 dilution).

8. Mix 1 ml of bacterial solution wth 4 ml of diluted human serum and incubate at 37°C for 15 min with end-over-end rotation.

9. Mix 5 ml of neutrophil solution with opsonized bacteria (adjust volumes to approximate a 20:1 bacteria:neutrophil ratio) and immediately remove 1 ml of the mixture and place into a 12 × 75-mm tube containing 1 ml 0.9% NaCl–0.02% EDTA solution at 4°C to stop phagocytosis.

10. Maintain the remaining neutrophil/bacteria mixture at 37°C and remove 1-ml aliquots at 10, 15, 30, and 60 min each time mixing the aliquot with 1 ml cold 0.9% NaCl–0.02% EDTA solution at 4°C in a 12 × 75-mm tube to stop phagocytosis.

11. For a control with unopsonized bacteria, mix 100 μl of bacterial solution with 400 μl of HBSS. Incubate for 15 min at 37°C, then add 500 μl of neutrophil solution and incubate the tube for 30 min at 37°C. Add 1 ml cold 0.9% NaCl–0.02% EDTA solution at 4°C in a 12 × 75-mm tube to stop phagocytosis.

12. Run on cytometer measuring green fluorescence at 525 nm emission.

13. Immediately after measuring the green fluorescence of each tube, add 1 ml of trypan blue (3 mg/ml) and repeat measurements on the cytometer. The trypan blue quenches the fluorescence of the extracellular bacteria and thus allows for measurement of only the intracellular bacteria.

D. Critical Aspects

As with the oxidative burst, proper cell handling and care are necessary to prevent clumping. Since phagocytosis is an active functional assay, dextrose, calcium, and magnesium in the HBSS are necessary for normal cell function. This assay can be performed with a leukocyte mixture instead of a pure neutrophil suspension. The neutrophil population is relatively easy to separate on the cytometer using forward angle and 90° light scatter. However, it may be difficult to separate the neutrophil phagocytosis from the monocyte phagocytosis. The optimal ratio of bacteria to neutrophils is 20:1. Small particles such as bacteria may be difficult to see using light scatter. It is necessary to trigger using green fluorescence to detect these small particles (1 μm or less).

IV. Controls and Standards

It is important to establish a standard procedure for running oxidative burst assays. This can be achieved by finding a fluorescent bead which falls generally within the range of fluorescence of activated cells. This bead is then used to set up the flow cytometer each time setting the HV of the PMTs based upon the bead fluorescence. If a full calibration is performed, the mean channel fluorescence can then be equated with the quantity of H_2O_2 formed per cell.

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