



# Flow Cytometry for Measuring Neutrophil Function

# 18

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## Abstract

Neutrophils are crucial cells for host defence. In particular, their phagocytic function is a major mechanism through which the immune system eliminates extracellular pathogenic microorganisms. The production of reactive oxygen species (ROS) by neutrophils, referred to as the ‘oxidative burst’, is a critical step in the destruction of these phagocytosed bacteria. Flow cytometry is an ideal technology to use for the measurement of these functions. In this paper I describe methods for doing this that can be applied to the study of bacteria-neutrophil interactions and used in the clinical diagnosis of neutrophil defects and as a measure of an individual’s immune system status.

## Keywords

Flow cytometry · Oxidative burst · Host defence · Neutrophil defects

## 18.1 Introduction

Neutrophils are the primary cell involved in the initial host defence to pathogenic microorganisms (Nathan 2006). They have evolved a number of mechanisms to kill phagocytosed pathogens, of which the most important is the respiratory oxidative burst, where the nicotinamide dinucleotide phosphate (NADPH) oxidase complex produces reactive oxygen species (ROS) to kill the phagocytosed microorganisms (Condliffe et al. 2006). Deficiency of the neutrophil respiratory oxidative burst is

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associated with impaired neutrophil killing (Quie et al. 1967) and recurrent infection (Lekstrom-Himes and Gallin 2000). The most well-recognised deficiency of neutrophil function is the condition chronic granulomatous disease (CGD) which occurs due to an abnormality of one of the four NADPH sub-units resulting in an absent (or more rarely reduced) oxidative burst (Lekstrom-Himes and Gallin 2000).

Given its single cell measurement capabilities, flow cytometry is an ideal technology to measure neutrophil ROS production and phagocytic function. To measure ROS, neutrophils can be loaded with the non-fluorescent dihydrorhodamine 123 (DHR 123). Any stimulus that causes these cells to produce reactive oxygen species can then be measured by the oxidation of the DHR123 to the fluorescent Rhodamine 123 (Emmendorffer et al. 1990). Neutrophil phagocytosis can be measured using fluorescently labelled particles or organisms such as propidium iodide (PI)-labelled *Staphylococcus aureus* (Pansorbin), with the amount of neutrophil associated fluorescence proportional to their phagocytic activity (Bohmer et al. 1992). In this paper methods to do this are described in detail. Using these techniques it is possible to analyse bacteria-neutrophil interactions, diagnose defects such as chronic granulomatous disease (Jirapongsananuruk et al. 2003), and measure immune system status (Hutchinson et al. 2003).

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## 18.2 Using Flow Cytometry to Measure Neutrophil Function

Three assays are presented that measure human peripheral blood neutrophil ROS production and phagocytosis function, two using whole blood and the other using purified or isolated neutrophils.

### 18.2.1 Whole Blood Phagocytosis and Reactive Oxygen Species Production Assay

This assay was adapted from Perticarari et al. (1994) and Bohmer et al. (1992). We have published on using it to assess neutrophil function in bronchiectasis (King et al. 2009) and kidney transplant patients (Hutchinson et al. 2003).

#### 18.2.1.1 Specimen Requirements

The whole blood assay to measure ROS production and phagocytosis function of neutrophils requires at least 4 mL of Li heparin blood.

#### 18.2.1.2 Reagents

##### Stock Solutions

Dihydrorhodamine 123 5 mM (1.73 mg/mL) in DMSO [THERMOFISHER Cat # D23806]. Aliquot and store at  $-20^{\circ}\text{C}$ .

Propidium iodide 1.0 mg/mL solution in water [THERMOFISHER Cat# P3566]. Keep covered from light and store at  $4^{\circ}\text{C}$ .

Pansorbin cells—10%W/V [Merck Millipore 507,858]. Keep covered from light and store at 4 °C.

### 18.2.1.3 Method

- To make enough Pansorbin-PI solution for 1 sample, add 200  $\mu\text{L}$  of Pansorbin (10% W/V suspension, 0.1% Az in buffer) to 200  $\mu\text{L}$  of 100  $\mu\text{g}/\text{mL}$  propidium iodide in PBS. Incubate at room temperature for 30 min, covered from light.
- Add 1 mL of PBS. Spin at 500  $g$  for 4 min. Repeat wash. Resuspend in 400  $\mu\text{L}$  PBS (final concentration of PI-Pansorbin 5% W/V).
- Setup 4 aliquots of blood (Li Hep), 450  $\mu\text{L}$  each. To two of them, add 50  $\mu\text{L}$  of PI-Pansorbin. Incubate all tubes in 37 °C water bath. Important: Always check water bath temperature is exactly 37 °C before use.
- After 20 min add 1  $\mu\text{L}$  DHR123 stock (final concentration—10  $\mu\text{M}$ ) to all of the + PI-Pansorbin and no Pansorbin-PI aliquots; mix thoroughly and then incubate in 37 °C water bath for a further 10 min.
- After the 30 min of total incubation, remove 100  $\mu\text{L}$  of blood from each sample. Add to 2 mL of FACS lysing solution (Becton Dickinson, New Jersey). Incubate for 10 min at 25 °C to lyse the red blood cells. Run on flow cytometer.

The type of controls used for the experiment depends on the application, but as a minimum use the no PI-Pansorbin sample to serve as the negative control for the fluorescence measurements.

*NOTE:* The time of addition of DHR123 and the lysing of samples is crucial to this assay; do not vary the times by more than 5%.

### 18.2.1.4 Data Analysis

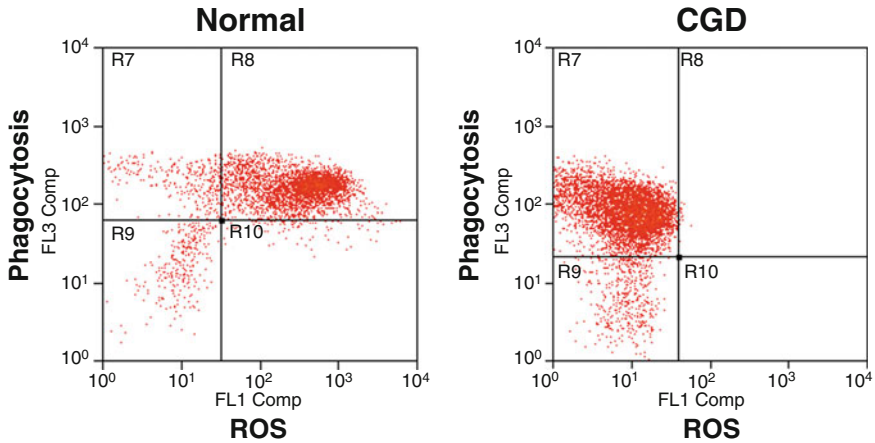
Exclude doublets by forward and side scatter height vs. pulse histograms. Identify the neutrophils by forward and side scatter, and then gate to measure and quantify (using mean fluorescence intensity) the level of fluorescence of the probes for ROS and phagocytosis function (Fig. 18.1).

## 18.2.2 Separated Neutrophil Phagocytosis and Reactive Oxygen Species Production Assays

Use density gradient separation medium Polymorphprep (Progen, Germany) to isolate neutrophils from whole blood.

### 18.2.2.1 Specimen Requirements

This assay requires at least 6 mL of Li heparin or EDTA blood.



**Fig. 18.1** Neutrophil ROS and phagocytosis function. Example histograms of neutrophil ROS and phagocytosis staining for a normal (top) and chronic granulomatous disease (bottom) donors after using the whole blood phagocytosis and ROS production assay. Neutrophils were gated by forward and side scatter

### 18.2.2.2 Method

#### Isolating Neutrophils

- Up to 5.5 mL of blood is layered on top of 3.5 mL of Polymorphprep in a 12 mL round-bottomed Falcon tube. It is then centrifuged for 35 min at 450 g at room temperature.
- Remove the neutrophils from the second layer of cells. Add an equal amount of 0.45% NaCl solution to the recovered neutrophils, and spin down for 10 min at 450 g.
- To lyse the residual red cells, remove supernatant and resuspend the cell pellet in 1 mL distilled water. Leave for 30 s only, and then add 0.9% saline solution (NaCl), and fill up to 10 mL. Centrifuge for 5 min at 450 g.
- Remove supernatant, and resuspend pellet in 10 mL of 0.9% NaCl. Then determine cell concentration by doing cell count.
- Centrifuge cells for 5 min at 450 g. Remove supernatant, and resuspend cells in PBS/5% normal human serum at a concentration of  $0.5 \times 10^6$  cells/mL.

#### Reactive Oxygen Species Production

- Make up a Phorbol Myristate Acetate (PMA) solution of 20  $\mu\text{g/mL}$  by diluting 20  $\mu\text{L}$  of PMA stock (2 mg/mL in DMSO, stored at  $-20^\circ\text{C}$ ) in 2 mL PBS/5% FCS. This must be done fresh on the day of the assay.
- Setup two tubes with 1 mL of the isolated neutrophils. To one tube add 5  $\mu\text{L}$  of the 20  $\mu\text{g/mL}$  PMA solution (final concentration 100 ng/mL PMA), and to the other add nothing. Incubate both at  $37^\circ\text{C}$  for 10 min.

- Add 1  $\mu\text{L}$  of DHR123 (final concentration—5  $\mu\text{M}$ ) to both and incubate again at 37 °C for 10 min. After this place samples on ice, and run all samples on flow cytometer within 10 min.

### Phagocytosis

1. Add 100  $\mu\text{L}$  of Pansorbin from Calbiochem (10% W/V suspension, 0.1% Az in buffer) to 100  $\mu\text{L}$  of 100  $\mu\text{g}/\text{mL}$  propidium iodide in PBS. Incubate at room temperature for 30 min, covered from the light. This must be done fresh on the day of the assay.
2. Add 1 mL of PBS. Spin at 500  $g$  for 4 min. Repeat wash. Resuspend in 200  $\mu\text{L}$  PBS (final concentration of Pansorbin-PI 5% W/V).
3. Set up 3 tubes with 0.9 mL of the isolated neutrophils. To two of the tubes, add 100  $\mu\text{L}$  of the Pansorbin-PI solution, and add nothing to third tube. Incubate one PI-Pansorbin tube and the no PI-Pansorbin tube at 37 °C; the other PI-Pansorbin tube incubate on ice. After 30 min place 37 °C tubes incubated on ice. Run on flow cytometer within 10 min.

#### 18.2.2.3 Data Analysis

Exclude doublets by forward and side scatter height vs. pulse histograms. Identify the neutrophils by forward and side scatter, and then gate to measure and quantify (using mean fluorescence intensity) the level of fluorescence of the probes for ROS and phagocytosis function.

The type of controls used for the experiment depends on the application, but as a minimum use the PMA sample as the negative for the DHR123 measurement and the 4 °C sample and no PI-Pansorbin samples as the negative for the phagocytosis measurement.

### 18.2.3 No Lyse No Wash Whole Blood Phagocytosis and Reactive Oxygen Species Production Assay

In this assay pHrodo BioParticles from ThermoFisher are used as the targets for phagocytosis by the neutrophils. These particles are inactivated, unopsonized *E. coli* reagents that are conjugated to the pHrodo dye. This dye becomes much more fluorescent as the surrounding pH becomes more acidic. Therefore, we can measure the ingestion of this particle based on the increased fluorescence due to the acidic environment of the phagolysosome. At the same time, we can measure the induced oxidative burst with the DHR123 dye. By adding the DyeCycle Ruby stain at the end of the phagocytosis incubation, which binds to the DNA of the leukocytes, we can distinguish the white blood cells from the red blood cells by triggering on the red fluorescence signal from the red laser and thus eliminate the need to lyse the RBCs before flow cytometric analysis.

Materials recommended but not provided with the pHrodoBioParticle kit:

- Whole blood sample collected in sodium or lithium heparin collection tube.
- Water bath or incubator set to 37 °C.
- Ice bath/bucket.
- Analysis tubes for your flow cytometer.
- Water bath sonicator.
- Flow cytometer with 488 nm, 561 nm and 640 nm excitation wavelengths.

### 18.2.3.1 Specimen Requirements

The whole blood assay to measure ROS production and phagocytosis function of neutrophils requires at least 4 mL of Li heparin blood.

### 18.2.3.2 Method

#### Preparing the BioParticles<sup>®</sup> Solution

1. Bring the Lysis Buffer A (Component A) to room temperature before use.
2. Add 2.2 mL Buffer B (Component B) to the vial containing the lyophilized product to resuspend the pHrodo<sup>™</sup> BioParticles<sup>®</sup> conjugate. This provides sufficient pHrodo<sup>™</sup> BioParticles<sup>®</sup> conjugate in a 20 µL aliquot for a 20:1 particle-to-phagocyte ratio.
3. Vortex for 1 min. Sonicate for 5 min until all the fluorescent particles are homogeneously dispersed.
4. Store the pHrodo<sup>™</sup> BioParticles<sup>®</sup> solution on ice for ~10 min prior to use.

Please note: It has been found that excess unused pHrodo dye can be frozen at -20 °C in aliquots, but avoid repeat freeze thawing (Fine et al. 2017).

#### Phagocytosis and Staining Protocol

1. Transfer 50 µL aliquots of whole blood (Li heparin) to each of two flat bottom 96-well plates; the number of wells will depend on the number of assay conditions. One plate will be kept at 37 °C and 5% CO<sub>2</sub> and the other plate at 4 °C as a negative control.
2. Add the concentration indicated in the product insert of the pHrodo *E. coli* BioParticles conjugate to the wells (10 µL). Set up samples as detailed in Table 18.1.
3. Bring volumes up to 100 µL per well with RPMI-1640 Medium; incubate one plate at 37 °C and 5% CO<sub>2</sub> and the other plate at 4 °C for 10–20 min.
4. Add DHR123 (final concentration—10 µM). Pipette up and down to mix the well, and incubate one plate at 37 °C and 5% CO<sub>2</sub> and the other plate at 4 °C for 5–10 min.
5. Prepare flow cytometry tubes with 500 µL of RPMI 1640 Medium and 1 µL of Vybrant DyeCycle dye.
6. Transfer 5 µL blood from each well of the 96-well plate into the tubes prepared in step 4; incubate all tubes for 15 min at 37 °C and 5% CO<sub>2</sub>.

**Table 18.1** Sample setup

37 °C, 5% CO <sub>2</sub>			
Time: 15 min – pHrodo – DHR123 <sup>a</sup>	Time: 15 min – pHrodo + DHR123 <sup>a</sup>	Time: 15 min + pHrodo – DHR123 <sup>a</sup>	Time: 15 min + pHrodo + DHR123 <sup>a</sup>
Time: 30 min – pHrodo – DHR123 <sup>b</sup>	Time: 30 min – pHrodo + DHR123 <sup>b</sup>	Time: 30 min + pHrodo – DHR123 <sup>b</sup>	Time: 30 min + pHrodo + DHR123 <sup>b</sup>
4 °C			
Time: 15 min – pHrodo – DHR123 <sup>a</sup>	Time: 15 min – pHrodo + DHR123 <sup>a</sup>	Time: 15 min + pHrodo – DHR123 <sup>a</sup>	Time: 15 min + pHrodo + DHR123 <sup>a</sup>
Time: 30 min – pHrodo – DHR123 <sup>b</sup>	Time: 30 min – pHrodo + DHR123 <sup>b</sup>	Time: 30 min + pHrodo – DHR123 <sup>b</sup>	Time: 30 min + pHrodo + DHR123 <sup>b</sup>

<sup>a</sup> Add DHR123 at  $T = 10'$ <sup>b</sup> Add DHR123 at  $T = 20'$ 

- Dilute the samples to 4 mL with RPMI 1640 Medium in each flow cytometry tube.
- Acquire the samples on flow cytometer.

### Flow Cytometer Setup

Trigger off Red Laser APC/Alexa 647 detector signal (DyeCycle Ruby).

DyeCycle Ruby—Red Laser 640 nm APC/Alexa 647 detector.

pHrodo Red—Yellow Green Laser 561 nm PE detector.

DHR123—Blue 488 nm Laser FITC/GFP detector.

#### 18.2.3.3 Data Analysis

For the no lyse no wash whole blood method, make sure the trigger or threshold signal is set to the DyeCycle Ruby channel and the level set so that positive cells are visible, but the negative cells are not. Exclude doublets by forward and side scatter height vs. pulse histograms. Next use forward and side light scatter to identify the neutrophils. Then gate to measure and quantify (using mean fluorescence intensity) the level of fluorescence of the probes for ROS and phagocytosis function.

The correct controls to use does depend on the purpose of the experiment, but using the listed controls in Table 18.1 will enable you to determine the negative background staining of the pHrodo and DHR123 probes.

## 18.3 Clinical Applications of these Assays

Patients have been identified with defects in the intracellular killing of phagocytosed microorganisms, usually due to failure of production of superoxide anion, singlet oxygen and hydrogen peroxide (ROS). This has been called chronic granulomatous disease (CGD) and is characterised by recurrent life-threatening opportunistic

infections and uncontrolled inflammation, often with granuloma formation (Mauch et al. 2007). The flow cytometry assays described here can be used to determine this defect in neutrophils and help diagnose patients with CGD.

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## 18.4 Summary

Neutrophils are a vitally important component of the immune system, and defects in their function can have serious consequences for an individual. This paper details methods for the measurement of neutrophil ROS and phagocytosis function using flow cytometry. The methods offer relatively simple and cost-effective ways to measure neutrophil function in both the clinical and research setting.

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