Neutrophil Oxidative Burst Assay: A Dihydrorhodamine (DHR) based testing of Chronic Granulomatous Disease (CGD) with CytoFlex Flow Cytometer

APPLICATION NOTE

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IN THIS PAPER YOU WILL LEARN

To be introduced to a method to measure oxidative burst in eukaryotic cells

Proper controls for an oxidative burst assay

A method to quantitate oxidative burst by flow cytometry

Principal of the Technique

Background:

Chronic granulomatous disease (CGD) is an inherited disease that leads to recurrent life threatening infections and widespread granulomatous inflammation in tissue [1]. This inherited disorder is caused by the deficiencies in oxidative burst of neutrophils. The neutrophils of individuals with this condition are unable to assemble nicotinamide dinucleotide phosphate (NADPH) oxidase complex which leads to difficulty in forming superoxide anions (O_2^-) and reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). The lack of proper ROS production leads to deficiencies in phagocytes ability to kill pathogens as well as inflammatory damage to the individual's tissue.

Introduction:

The nitroblue-tetrazolium (NBT) test is the original and most commonly used test to diagnose this condition. However, the dihydrorhodamine (DHR) based flow cytometry test for CGD is more sensitive, less laborious, and is the method of choice. DHR Flow Cytometry test is an indirect detection of the reduced levels of ROS, specifically hydrogen peroxide. In this application note, the ease and robustness of this assay is showcased by using a CytoFLEX Flow Cytometer.

Procedure:

Dihydrorhodamine123 (DHR123) (Molecular Probes, Eugene, OR) (2500 µg/mL in DMSO) is diluted to 15 µg/mL with phosphate buffered saline with azide (PBA)(Ca and Mg free with 2.5 % bovine serum albumin and 0.2 % sodium azide). The phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St Louis, MO) (100 µg/mL in DMSO) is diluted to 300 ng/mL with PBA.

Three tubes are set up for each sample:

1. Blood only
2. Blood + DHR
3. Blood + DHR + PMA

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To all tubes, 100 µL of heparinized blood is diluted 1:10 with PBA. 25 µL DHR123 (375 ng/ml final concentration) is added to tubes 2 and 3. All tubes are incubated in a 37°C water bath for 15 minutes. This allows for the DHR123 to be loaded into the cells. Following incubation, 100 µL of the prepared PMA solution (30 ng/mL final concentration) is added to tube 3 then all tubes are incubated an additional 15 minutes at 37°C. This step allows the neutrophils to undergo the oxidative burst thereby oxidizing the DHR123 to rhodamine which fluoresces when excited by 488 nm laser. After washing and centrifugation, the samples were stained with anti-human CD45 Krome Orange antibody (Beckman Coulter, Miami, FL) according to manufacturer's recommendations and lysed with ammonium chloride (Pharm Lyse, Becton Dickinson, Mountainview, CA) for 10 minutes in the dark, followed by centrifugation, washing, and fixing in 1 % formalin. The samples are then acquired using a CytoFLEX Flow Cytometer (Beckman Coulter, Miami, FL) and subsequently the data was analyzed using CytExpert Software (Beckman Coulter, Miami, FL). Fluorescence is quantitated by mean peak channel fluorescence. Results are expressed as oxidative index of neutrophils which is the ratio of mean fluorescence of PMA to mean fluorescence of unstimulated sample. Oxidative index values over 100 are considered normal, while values below 100 are considered abnormal. Although neutrophils (found in the granulocyte gate of the SSC vs CD45 dotplot) are the cells of interest when studying oxidative burst, we are able to identify and gate internal controls for oxidative burst from the same dotplot: monocytes, which serve as low-level controls and lymphocytes which serve as negative controls, lacking this enzyme system activity. In addition to these gating controls strategies, a blood only control is also run for each assay to discern autoflourescence. The DHR flow cytometry test can detect CGD patients, carriers, and can suggest the genotype of the CGD patients.

Laser	405nm					488nm					638nm		
Fluor	Krome Orange	Pacific Blue	V610	V660	V780	FITC	PE	ECD	PC5.5	PC7	APC	APC AF700	APC AF750
Marker	CD45					DHR							

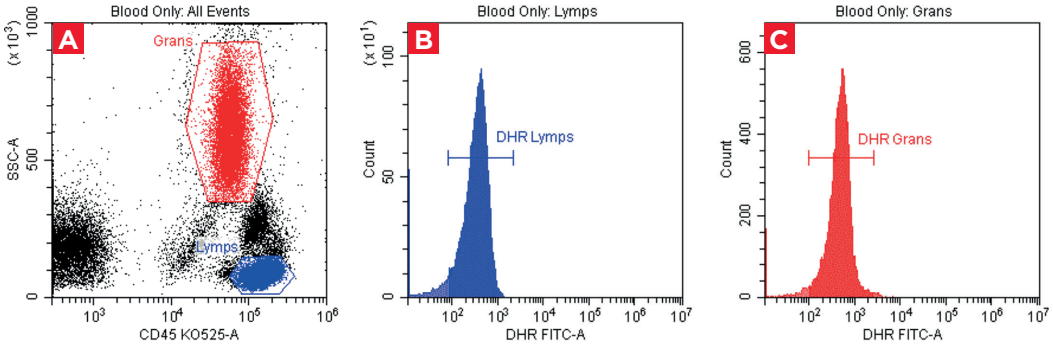
Results

Oxidative burst is the term used to describe the phagocytic response of neutrophils to produce ROS. DHR flow cytometry assay can detect reduced oxidation of dihydrorhodamine, making it a very robust assay to measure the oxidative burst of neutrophils. When oxidized, dihydrorhodamine123 is converted to rhodamine123 which then fluoresces when excited by a 488nm laser. In normal blood, the oxidative burst of neutrophils can be triggered by incubation with PMA. While neutrophils show increased oxidative burst with PMA stimulation, lymphocyte populations lack the oxidative burst components making this population a good internal negative control.

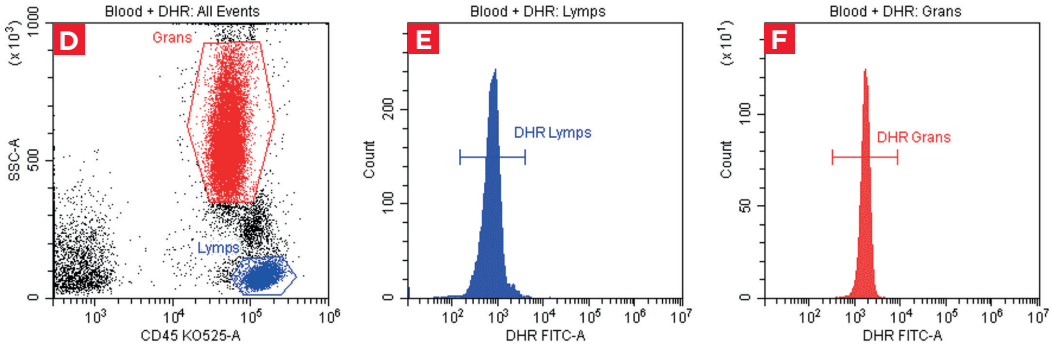
In Figure 1, blood only samples are used as negative control for DHR staining. When we compare the mean fluorescence from this sample with blood + DHR sample, we did not detect any significant increase in either neutrophil gated or lymphocyte gated population. However, the increase in DHR fluorescence is evident in the PMA stimulated sample in the granulocyte population (I), while the lymphocytes show no sign of DHR fluorescence (H) upon PMA stimulation.

In Figure 2, the oxidative index of neutrophils is calculated by dividing the mean fluorescence of DHR in PMA stimulated sample by unstimulated sample. The result was 367.0, which is above the cutoff for normal functional ratio of 100. The oxidative index of lymphocytes is calculated by dividing the mean fluorescence of DHR in PMA stimulated sample by unstimulated sample. The result was 1.2 which is significantly below 100, which makes this population an adequate internal negative control.

Blood only



Blood + DHR



Blood + DHR + PMA

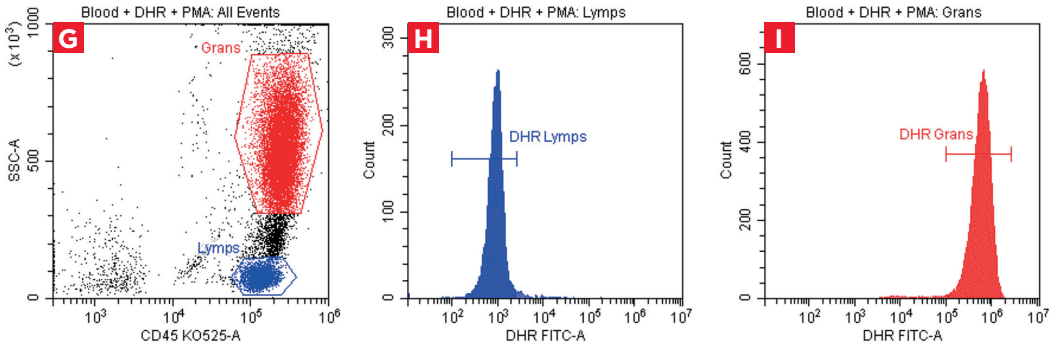


Figure 1:

Figures A-C display the data from Blood alone sample without any stimulants or DHR.

Figures D-F show the data from Blood + DHR sample stained with DHR without any stimulants.

Figures G-I show the data from Blood + DHR + PMA sample stained with DHR and in addition, stimulated with PMA.

In plots A, D, G; the SSC vs. CD45 plot is used to detect the 3 leukocyte populations; lymphocytes, monocytes and granulocytes. Neutrophil and lymphocyte populations are then gated. Lymphocyte gate is used to gate DHR histogram (B, E, H) to detect the DHR fluorescence. Neutrophil gate is used to gate DHR histograms (C, F, I) to detect the DHR fluorescence.

Tube Name: Blood Only

Population	% Total	Mean DHR FITC-A	CV DHR FITC-A
DHR Grans	20.87 %	542.8	55.05 %
DHR Lymps	39.55 %	390.3	47.94 %

Tube Name: Blood + DHR

Population	% Total	Mean DHR FITC-A	CV DHR FITC-A
DHR Grans	55.82 %	1764.3	24.40 %
DHR Lymps	19.01 %	823.8	48.36 %

Tube Name: Blood + DHR + PMA

Population	% Total	Mean DHR FITC-A	CV DHR FITC-A
DHR Grans	62.04 %	647401.1	41.66 %
DHR Lymps	21.41 %	952.7	37.25 %

	DHR Mean PMA stim	DHR Mean PMA unstim	Oxidative Index
Neutrophils	647401.1	1764.3	367.0
Lymphocytes	952.7	823.8	1.2

Figure 2:
The Oxidative Index of Neutrophils is calculated by dividing the mean fluorescence of DHR in PMA stimulated sample by unstimulated sample.

References

1. Golightly, Marc G. ICCS eNewsletter. 2011 Vol II(1)

Notes

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFlex Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow cytometers.

For Research Use Only. Not for use in diagnostic procedures.

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