Assessment of Oxidative Metabolism

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Summary

Oxidative metabolism is one of the central physiological processes that regulate multiple functions in a cell including cell death and survival, proliferation, gene transcription, and protein modification. There are multitudes of techniques that are used to evaluate oxidative activity. Here, we summarize how to measure oxidative activity by flow cytometry. This versatile technique allows the evaluation of the level of oxidative activity within heterogeneous populations of cells and in cell culture. Flow cytometry is a quick method that yields highly reproducible results with small-size samples. Therefore, it is an ideal technique for evaluating changes in oxidative activity in samples from small animals.

Keywords: Oxidative metabolism, oxidative activity, flowcytometry, fluorescent dyes, whole blood, cell culture.

1. Introduction

The evaluation of oxidative metabolism efficiency is a useful measure of physiological activity in the cell. The oxidative state of a cell regulates fundamental

processes of cell metabolism including proliferation, survival, inflammation, DNA damage/repair, and cell death. Not surprisingly, many diseases are associated with defects in regulation of oxidative metabolism, which include neurodegeneration, carcinogenesis, diabetes, and atherosclerosis (1). For example, mutation of the gene encoding the SOD protein results in increased oxidation in motor neurons and leads to Amyotrophic lateral sclerosis (ALS)-like pathology. On the other hand, a defect in neutrophil associated NADPH oxidase, which leads to deficiency in the production of superoxide and hydrogen peroxide is an underlying mechanism of chronic granulomatosis disease. Oxidative activity can be described as a sum concentration of oxidative reagents in the cell at any given time. Most prominent contributors of oxidative activity are reactive nitrogen species (RNS) and reactive oxygen species (ROS). ROS consists of hydrogen peroxide (H_2O_2) and of substances containing unpaired electrons, which include superoxide ,□□□□□x□□ , peroxyl, and hydroperoxyl.

The generation of ROS is dependent on NADPH oxidases, which consists of multiple members of the *NOX* gene family (Nox1-5). RNS are comprised of peroxynitrites (ONOO-), which are downstream of nitric oxide syntheses. The level of oxidative activity can be measured by the amount of damaged cell material, such as DNA, RNA, protein, and lipids (2-4). Increased oxidative activity is counterbalanced by increased expression of the reducing enzymes that protect cells from the harmful environment of reactive oxygen and nitrogen species. Therefore, another way to measure oxidative activity is to measure the expression of antioxidants that accumulate in a cell in response to the presence of ROS and RNS (5).

Several methods that measure oxidative activity have been developed. For example, the Nitroblutetrasolium precipitation reduction test is a crude method that evaluates high levels of oxidative activity and it requires large sample volumes(6). Likewise, chemiluminescence is a highly sensitive and easily quantified method that can be used with small 20 to 100µl sample sizes (7). Chemiluminescense can measure the level of oxidative activity in a homogeneous cell population (8). Lastly, flow cytometry is a very sensitive way to evaluate oxidative activity in heterogeneous cell populations, as well as, in homogeneous cell populations. This review will describe one of the ways that flow cytometry can be utilized to measure the oxidative activity of a cell.

Several detection dyes have been developed that are useful to measure oxidative activity by flow cytometry. Upon oxidation, dyes such as 2'7'-dichlorofluorescin (DCFH), DCFH diacetate (DCFH-DA), and dihydrorhodamine 123 (DHR), fluoresce when excited with a 488nm laser. From our experience, DHR 123 typically yields the most accurate and reproducible results. In addition, DHR easily penetrates into live cells *in vivo* and can be used to evaluate the oxidative state within tissues using microscopy, flourimetry, and flow cytometry. For this reason, this review will concentrate on the protocols using DHR to measure oxidative activity using flow cytometry. This assay can be used to quantify the oxidative activity of cells in whole blood, bone marrow, spleen, and other tissues. Also, we and others have successfully utilized this technique to measure the oxidative activity of THP-1 monocytic cell lines, RAW mouse monocytic cell lines, primary mouse astrocytes, and mouse embryonic fibroblasts (9-11).

2. Materials

-Ammonium chloride (NH_4Cl)
-Deionized ddH_2O
-Dihydrorhodamine (DHR)
-Dimethyl sulfoxide (DMSO)
-Ethylenediaminetetraacetic acid (EDTA)
-Fetal bovine serum (FBS)
-Human and rodent blood samples/ human and mouse cell lines
-Liquid nitrogen
-RPMI-1640
-Paraformaldehyde powder
-Phosphate buffered saline (PBS)
-Phorbol 12-myristate 13-acetate (PMA)
-Potassium bicarbonate ($KHCO_3$)
-Sodium hydroxide NaOH

Amonium chloride (NH_4CL

2-1. Animals

Herein described procedure has been successfully performed using human and rodent blood samples, and multiple human and mouse cell lines. Only 100µl of blood is required to measure oxidative activity of neutrophils, monocytes, T cells, and B cells. Half a million to a million cells was used when oxidative activity was quantified in different cell lines.

2-2. Supplies

12x75 mm test tubes

Heparinized syringes

2-3. Equipment

Cell culture centrifuge capable to spin at 300xg

Water bath 37 **C**

Flow cytometer equipped with an argon laser and a photomultiplier tube PMT with a 525nm band pass filter

2-4. Reagents

1. 10X Lysis buffer: 82.5g of ammonium chloride (NH_4Cl), 10 g of potassium bicarbonate($KHCO_3$), 0.37 g of Ethylenediaminetetraacetic acid (EDTA), and bring the volume up to 1 liter using ddH2O (***see Note 1)***.
2. Fixation buffer: 1.7% formaldehyde and 2% Fetal bovine serum (FBS) in Phosphate buffered saline (PBS) (***see Note 2***).
3. Dihydrorhodamine 123 (DHR): Add 2.5mg/ml of DHR in DMSO for the stoke concentration and divide this stock into small aliquots of 30-50µl. Store the aliquots in liquid nitrogen ***(see Note 3)***. Before an experiment, prepare a 1/1000 working solution (2.5µg/ml) in RPMI 1640. Prepare 100µl of working DHR solution for each 400µl sample.
4. Phorbol 12-myristate 13-acetate (PMA)(Sigma): Dissolve 1mg/ml of PMA in DMSO and divide this stock solution into small aliquots of 30 – 50µl. Freeze each aliquot at -80°C. Prepare a 1/100 working solution and prepare to use 5 µl per sample.
5. Prepare 4% formaldehyde from powder. To completely dissolve paraformaldehyde heat the solution to 60°C on a stir plate. Several drops of NaOH may help to clear the solution.

3. Methods

1. Prepare DHR, PMA, and reagent of interest to specified concentrations in separate tubes (12x75 mm test tubes). Each tube should contain 300 µl of RPMI 1640 and 100µl of DHR ***(see Note 4)***. The tubes should be labeled as

follows: Tube #1 “Cells only”, which will contain 100μl of RPMI 1640 instead of DHR; Tube #2 “Cells DHR ice”; Tube #3 “Cells DHR”; Tube #4 “Cells DHR + PMA”; Tube #5 “Cells DHR + reagent of interest”. The concentration of each reagent of interest should enable 5μl of the reagent to be added to each sample ***(see Note 5)***.

2. Put the “DHR ice” tubes on ice, the rest of the tubes can be placed at 37°C (***see Note 6***).
3. Collect blood from experimental mice by heart stick into a heparinized syringe and aliquot 100μl into each of the test tubes. Make sure to thoroughly re-suspend the samples (***see Note 7***). Incubate each sample for 15 min at 37°C.
4. Spin cells for 5 min at 280 x g. Re-suspend the cell pellet in 1 ml of lysis buffer and incubate for 5 min at room temperature (***see Note 8***). Following the incubation, add 3ml of ice cold PBS.
5. Centrifuge the cells at 4°C for 5 min at 280 x g and re-suspend the cells in fixative buffer. If any surface staining is desired then cells should be re-suspended in blocking buffer (***see Note 9***).
6. Surface staining is performed according to routine protocol (note 10)
7. Scan cells using a flow cytometer ***(see Note 11)***.
8.

1. Cells incubated on ice with DHR may have high levels of non-physiologically relevant background staining. Therefore, special care must be taken to use

quiescent cells. Incubation with PMA shows the oxidative capacity of cells. The concentration of PMA used in this protocol will push cells to the near maximum oxidative activity, which is also referred to as an oxidative burst. While staining with DHR only gives an idea of the level of oxidative activity at rest.

2. The intense brightness of the staining with DHR123 usually results in a skewed readout of the population of cells (Figure 1B). Therefore, the geometrical means florescence intensity should be used for analysis rather then mean florescence intensity.
3. In the case when several peaks of fluorescence (Figure 1C) can be distinguished within one population, this population can be separated into "high" and “low" populations. When working with small changes (Figure 1D), the percent of positive cells in the specifically assigned gate might be a more useful measure then the geometrical mean of fluorescent intensity.

9. Data are to be analyzed using multiple comparison tests ***(see Notes 12).***

4. Notes

1. This 10x formulation is stable at room temperature for at least 6 months. Prepare fresh 1x buffer before each experiment.
2. The concentration of 1.7% was determined as optimal concentration of fixative for intracellular and surface staining(12).
3. DHR 123 is easily oxidized. Storage in liquid nitrogen minimizes batch-to-batch variation over time. Once the aliquot of DHR123 has been thawed, it can be stored at -20°C for several weeks with repeated freeze-thaw cycles.

4. It is very important to use the same batch of regents within the same series of experiments in order to increase the reproducibility of the data.
5. The negative control cells (Cells incubated on ice -ice negative control), the DHR only treated cells, and the positive control PMA stimulated cells are all required conditions for each experiment that may reveal mishandling of cells during the procedure. Also, these conditions allow the user to identify the relative oxidative activity of a cell compared to its oxidative potential.
6. The sample size can be reduced depending on the amount of cells in the population of interest. At least 10,000 events in the gate of interest must be collected. In our experience, working with mouse neutrophils, 50µl of whole blood is sufficient to collect the necessary number of events.

   When working with cells that have been activated for a prolonged period of time (for example, blood samples after an infection or primed cells from a cell culture, the DHR on ice control cells should always be used on non-activated/non-primed cells.
7. Use a water bath rather than a forced air exchange incubator because water has a superior level of heat exchange. Gently mix samples every 5 minutes. The incubation time may be adjusted according to the desired experimental conditions. For example, cells can be primed first with one or a combination of reagents and then fluorescence can be measured using the above protocol.
8. The formation of clots in blood samples will activate cells. Therefore, every sample with any level of clotting must be discarded.
9. Spinning cells faster than 280 x g may result in poor red blood cells lysis.

10. General cell surface staining protocols typically include 5 min of blocking in 30ul of PBS with 5% FBS. This blocking is then followed by incubation with an antibody cocktail specific for the desired cell surface markers for an additional 15 min. Cells are then washed and fixed. Staining with surface markers should be performed on ice after DHR staining as antibody binding may change the level of oxidative activity in a cell. In this case, cells should be fixed after surface staining is complete.
11. Ideally, samples should be analyzed by the flow cytometer immediately after staining. This will allow the fixation step to be skipped. However, when working with human samples, the fixation step is required. In addition, fixing cells allows samples to be scanned within 24 or even 48 hrs.
12. Usually in each experiment, an n of at least 4 should be performed. This should be followed by an ANOVA statistical analysis. A T-test is not suitable since more that two conditions are compared with each experiment.

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Figure legends:

Figure 1. Examples of data. A) Side scatter and forward scatter of mouse blood sample. B), C), and D) overlay histograms of 4 samples (the thin dashed line represents unstained cells; the thin line represents DHR on ice; the thick dashed line represents DHR only; and the thick line represents DHR+PMA) of B) neutrophils from gate, C) neutrophils from a different experiment, and D) human THP1 cells.