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An *In vitro* Assay to Quantify Nitrosative Component of Oxidative Stress

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Abstract

Oxidative stress is a major contributing factor in a variety of neurodegenerative and vascular diseases. In vitro assessment of a major oxidant reactive nitrogen oxide species (RNOS) using dihydrorhodamine 123 (DHR 123) is a useful assay to quantify the reactive oxygen species in a cell. DHR 123, non-fluorescent laser dye freely penetrates the cell membrane and stains the mitochondria. Density of staining varies with the level of peroxynitrite ($O=NOO^-$); as a result of interaction of superoxide anion (O_{2-}) and nitric oxide (NO). The fluorescence is read using a spectrophotometer. Cells are seeded in 24 or 96 well plate and DHR 123 working solution is added after appropriate treatment. The fluorescence is read after 60 minutes of incubation at 485/528 nm with spectrophotometer. This assay is more sensitive and forms a stable end product than comparable assays and takes 90 minutes to complete.

Keywords: NO; RNOS; ROS; DHR 123

Introduction

Oxidative damage resulting from the accumulation of reactive oxygen species (ROS) such as superoxide anion (O_{2-}), hydrogen peroxide, peroxynitrite ($O=NOO^-$), nitric oxide (NO) and organic hydroperoxides are a major contributing factor to a variety of ocular, vascular, neurodegenerative and arthritic disorders [1,2].

In aerobic respiration ROS are produced via a variety of metabolic and physiological activities. Metabolic pathways like mitochondrial electron transport chain, arachidonic acid cascade or nitric oxide cascade contribute to production of ROS [3]. NO is synthesized enzymatically from L-arginine by nitric oxide synthase (NOS) during electron transfer from nicotinamide adenine dinucleotide phosphate (NADPH). In normal conditions, NO functions as an intracellular messenger and transmit signals from one cell to another. NO reacts with superoxide anion (O_{2-}) or molecular oxygen and forms di-nitrogen trioxide (N_2O_3) and peroxynitrite ($O=NOO^-$) which creates nitrosative stress (RNOS) [4,5]. ROS/RNOS are interrelated to each other and play an important role in intracellular signaling, gene regulation, expression, cellular proliferation and apoptosis [6,7].

When physiological mechanisms are altered in the diseased state, one of the three different forms of nitric oxide synthase is up regulated with consecutive increase in RNOS/nitrosative stress [5]. Measuring nitrosative stress is useful in assessment of the oxidative damage in diseases such as respiratory burst, neurodegenerative and vascular diseases, pharmacological/toxicological studies [8-10]; as well as in radiation induced late normal tissue injury [11]. Detection of intracellular ROS is a challenging goal and relies on chemiluminescence or fluorescence. 2', 7' dichlorofluorescein diacetate (DCFDA) based assay that is used currently is oxidized by hydrogen peroxide and produces an unstable fluorescent end product dichlorofluorescein (DCF) [12-14].

We report a superior and more sensitive method using non-toxic dihydrorhodamine 123 (DHR123). Dihydrorhodamine 123 probe, an uncharged molecule freely penetrate through cell membrane, converts to positively charged rhodamine 123 derivative after oxidation by ROS such as nitric oxide and peroxynitrite and stains mitochondria inside a live cell [3,15]. Since mitochondria is a major source of ATP and ROS production, measuring RNOS/ROS using DHR123 either with flow cytometry [12] or spectrophotometry is a more potent and predictable method to estimate nitrosative stress.

This method is applicable to all primary cell cultures or cell lines of human, primate and other origin (endothelial cells, neuronal cells, epithelial cells, epidermal keratinocytes and fibroblast cells) [3,15-17]. DHR123 assay is simple and easy to perform and produce consistent results.

Materials

Reagents

- Primary cells or cell lines
- Respective media for different cell types for cell cultivation
- 0.05%Trypsin-EDTA (Gibco, cat no. 25300)
- Hank's Buffered Salt Solution (HBSS)/Phosphate buffer Solution (PBS) (HBSS; Gibco, cat no. 14175)
- Dihydrorhodamine 123 (DHR123; Anaspec, cat no. 85711)
- Respective serum and pH free media

Equipment

- Tissue culture hood and 95% air/5% CO_2 incubator for culture set-up.
- Inverted phase contrast microscope.
- Plate Reader (Biotek Synergy HT) adjustable to different culture plates (for eg. 6-well, 12-well, 24-well and 96-well plates) or spectrofluorimeter ideal with excitation and emission wavelength of 485 and 528 nm, respectively.
- Centrifuge.

- Hemocytometer or Vi-cell XR cell counter (Beckman coulter) or any cell viability analyzer.
- 8 or 12-channel pipette.
- Ensure that other required materials like sterile pipettes, sterile test tubes, culture flasks and culture plates are at hand.

Reagent Setup

Stock solution 10 mg of DHR123 is dissolved in DMSO to get a concentration of 10 mM. Once the stock solution is made, aliquot into small volumes such as 100 uL in to micro centrifuge tubes and stores them at -20°C.

Critical: Both DHR123 chemical and the solution are light sensitive; once the stock solution is made, it is very sensitive to air and light sensitive; cover them with aluminum foil and store them at -20°C.

DHR123 working solution (10 μM) Take 10 uL from the stock concentration and dilute to 10 mL of serum and pH free respective media in an air tight sterile test tube.

Critical Step: This working concentration should be prepared on the day of use in a complete dark room with minimal light source or without light source.
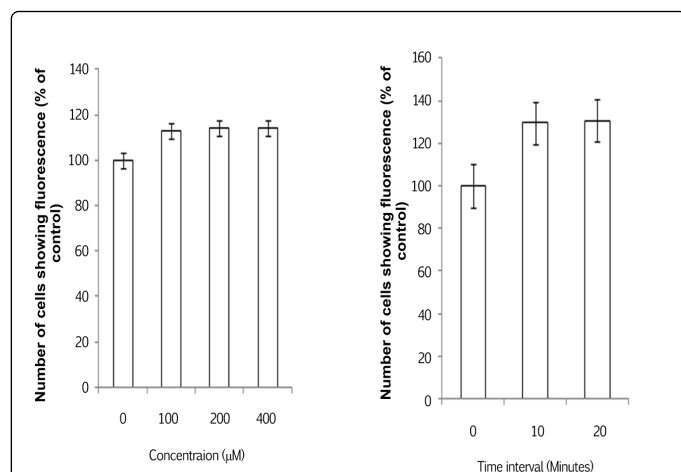
Figure 1: Detection of RNOS/ROS on different treatment condition; a) treatment with 100 μM, 200 μM and 400 μM concentration of hydrogen peroxide. b) Treatment with 10 and 20 minutes exposure to ultraviolet light (UV); RGC-5 rat retinal ganglion cells were plated in 24-well cell culture plate and incubated for 48 hrs and serum starved for 24 hrs. Cells were treated with different concentration of H_2O_2 and 10 and 20 minutes time points of UV in a separate experiments and analyzed for RNOS/ROS after 24 hrs using DHR123 at excitation /emission wavelength of 485/525 nm. Results are normalized against controls (Mean ± SE; n=4)

Procedure

Plating the cells (Timing: Day 1-30 minutes)

- Remove the medium from the flask where the cells are growing in and rinse it with 3-5 mL of HBSS

- Add 2-3 mL of trypsin-EDTA and incubate the flask in the CO_2 incubator for 2-5 minutes until the cells are rounded off and completely detached from the flask
- Stop the trypsinization by adding 2-5 mL of respective media
- Collect the cells and spin down and resuspend in a respective media
- Count the number of cells in a sample of cell suspension using a Vi-cell XR counter or hemocytometer
- Dilute the cells based on the cell count and dispense them in a 96-well plate at the rate of 1000 cells/well using a multichannel pipette. This cell number will be approximately adequate for most types of cells, it may vary in different culture plates such as in 24-well plate, 12 or 6-well plates
- Incubate the cells at a humidified atmosphere in presence of 5% CO_2 and 95% air for 24-48 hrs
- During this incubation, cells will adhere and start proliferating exponentially
- Check the cell growth in an inverted phase contrast microscope until it reaches your target growth

### Culture Treatment (•Timing: Day 2–30 minutes; it can vary based on treatment condition)

- On day 2 or 3, different treatments can be made to the cell culture plates
- Prepare the test solutions immediately before use and maintain in a sterile condition
- Always prepare different concentrations of test solution while using the test solution for the first time to identify the increased production or stability of RNOS/ROS. In Figure 1, an example of the different treatment condition and the yield of RNOS/ROS is displayed
- Use an internal control for every treatment

RNOS/ROS measurement (•Timing: Day 3–90 minutes)

- Remove the test solution/treatment media by gentle aspiration once the desired treatment time period is over
- Add freshly prepared working solution of DHR123 using multichannel pipette to each well. It is recommended to use 100 uL per well in case of 96-well plate and 500 uL per well for 24-well plate

Caution: Always prepare the working solution before use and avoid any exposure of the solution to light and air

- In addition to test control, always keep an assay control with DHR123 alone without any treated culture cells
- Incubate the cells for 90 minutes at the appropriate culture conditions
- Measure the fluorescence after 20 minutes of incubation and every 10 minutes after the first measurement to identify the maximal production of RNOS/ROS. Depending upon the treatment condition, it varies from one cell type to another. In Figure 2, a typical example of the increased production of RNOS/ROS on different time interval for RGC-5 rat retinal ganglion cells is presented
- Fluorescence should be measured no later than 90 minutes
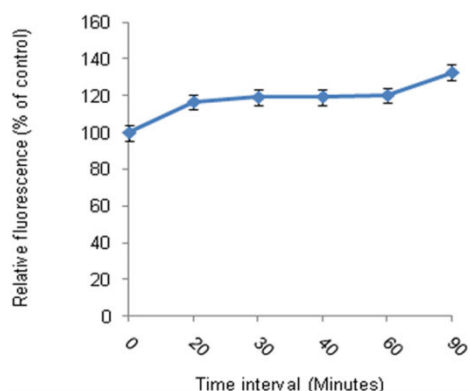
Figure 2: Relationship between the incubation time in presence of DHR 123 and the fluorescence at 485/525 nm after treatment. RGC-5 cells were seeded on 24-well culture plate and incubated for 48 hrs and serum starved for 24 hrs. After treating the cells with 200 µM of H_2O_2 for 2 hrs, DHR123 is added and RNOS/ROS is evaluated on different time points and is normalized against controls (Mean ± SE; n=4)
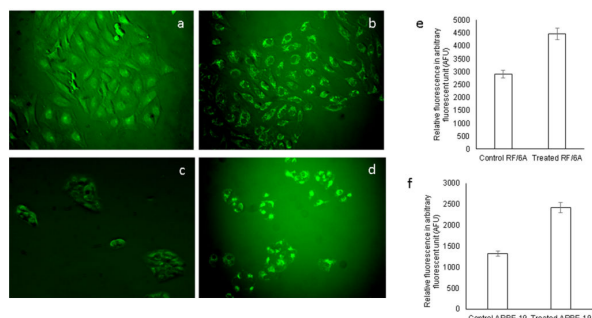
Figure 3: Fluorescent photomicrographs and quantification of RNOS/ROS production in RF/6A and ARPE-19 cells. a) RF/6A and ARPE-19 cells were plated in 24-well plate and after 48 hours of incubation cells were exposed to 10 minutes of xenon light radiation; a) Control RF/6A; b) Treated RF/6A cells; c) Control ARPE-19 cells; d) Treated ARPE-19 cells; e) and f) Histogram shows the respective quantification of RNOS/ROS using microplate reader at 485/525 nm. Representative data shows fluorescence after 60 minutes of DHR123; x-axis represents the condition and y-axis represents relative fluorescence in arbitrary fluorescent units (AFU).

Troubleshooting

- In the case of measuring the direct correlation of your test solution or treatment condition and RNOS/ROS stress, the DHR123 probe can be added to the cells before treatment. In those cases,

Once you achieve your target cell growth, discard the media and add freshly prepared DHR123 and incubate the cells at the culture set-up for no longer than 30 minutes

During this incubation, DHR123 will penetrate the cell membrane and accumulates in the mitochondria

Wash the cells using HBSS/PBS thrice and treat the cells using your test solution or different treatment condition. Since the mitochondria is the major producer of ROS, after treatment, quantify the fluorescence of oxidized R123 which stain the mitochondria based on RNOS/ROS

- Measure the fluorescence using either with spectrofluorimeter or plate reader with excitation and emission wavelengths of 485 and 528 nm, respectively
- In the case of plate reader, data will be expressed as mean relative fluorescent units (RFU) and standard deviation, coefficient of variation will also be displayed
- Data can be saved in an excel sheet in the case of plate reader or in an appropriate format in the case of spectrofluorimeter
- Any graphical method can be used to interpret the data (Figure 3); draw a curve which will give you the increased or decreased production of RNOS/ROS Vs concentration of your test solution/ treatment condition; in case of different treatment concentrations, ANOVA (analysis of variance) statistical method can be used to interpret the data

Troubleshooting

As an alternative to measure the fluorescence using plate reader/ spectrofluorimeter, the fluorescence can be evaluated by imaging the cells within the time period

In order to achieve the consistent results, experiments must be replicated at least thrice.

Anticipated Results

The results of this DHR123 assay are based on two different factors. First, they are dependent on treatment condition or test solution concentration which is direct or inversely proportional; second, the results are based on the incubation time interval and should be optimized for each cell type for different treatment condition.

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