NCL Method ITA-31

Detection of Nanoparticle-Mediated Total Oxidative Stress in T-Cells Using CM-H2DC-FDA Dye

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

The purpose of this protocol is to understand the propensity of nanomaterials to induce oxidative stress. Induction of oxidative stress is a common toxicity associated with nanoparticles. Generation of reactive oxygen species (ROS) and its control by antioxidant systems is a part of normal cellular metabolism [1]. However, disturbance of redox homeostasis and accumulation of ROS damages cellular organelles and DNA, eventually leading to cell death [2]. Another consequence of excessive ROS generation is activation of stress-related pathways and release of pro-inflammatory cytokines [1].

2. Principles

T-lymphocytes are isolated from human blood anti-coagulated with Li-heparin using RosetteSep™ reagent from STEMCELL Technologies, followed by gradient separation using Ficoll-Paque Plus solution. The blood is obtained from three healthy donor volunteers. Specimens from each donor are processed separately. Isolated T-cells are incubated with test-nanoparticles, and the generation of the ROS is detected using CM-H_2DC-FDA reagent. CM-H_2DC-FDA reagent is a cell-permeable fluorogenic probe which is non-fluorescent when in resting state but emits a strong fluorescent signal in FITC fluorescence upon oxidation.
Note: Cell lines/PBMC can be used instead of T-cells.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of suppliers listed below; their inclusion is for informational purpose only. Equivalent supplies from alternative vendors can be substituted.

3.1 Reagents

1. Phosphate buffer saline (PBS) (HyClone, AQB 22934)
2. Ficoll-Paque Premium (GE HealthCare, 17-5442-03)
3. Fetal bovine serum (Hyclone, SH30070.03)
4. RPMI1640 (Invitrogen, 11875-119)
5. Pen/Strep solution (Invitrogen, 15140-148)
6. L-glutamine (Hyclone, SH30034.01)
7. Cellometer ViaStain AOPI Staining Solution in PBS (Nexcelom Biosciences, CS2-0106; or other reagent for distinguishing between live and dead cells)

8. Albumin, from bovine serum (BSA) (Sigma, A4503)
9. CM-H_2DC-FDA reagent (Molecular Probes, C6827)
10. Hank's balanced salt solution (HBSS) (Invitrogen, 24020-117)
11. Dimethyl sulfoxide (DMSO) (Sigma, D2650)
12. RosetteSep™ (STEMCELL technologies, 15061)
13. tert-butyl hydroperoxide (TBHP) (Acros organics, AC180340050)
14. N-Acetyl-L-(+)-cysteine (Fisher Chemical, O1049-25)

3.2 Materials

1. Pipettes, 0.05 to 10 mL
2. Polypropylene tubes, 50 and 15mL
3. 24/48 well cell culture plates (Corning, 3524, 3548)
4. Polystyrene round bottom 12 x 75 mm^2 tubes (Falcon tubes, 352058)

3.3 Equipment

1. Refrigerator, 2-8°C
2. Freezer, -20°C
3. Cell culture incubator with 5% CO_2 and 95% humidity
4. Biohazard safety cabinet approved for level II handling of biological material
5. Vortex
6. Cellometer, Source or Hemacytometer to perform cell count
7. Flow Cytometer (e.g., FACSCalibur)
8. Centrifuge, 1200xg

4. Reagent and Control Preparation

4.1 Complete RPMI

The complete RPMI medium should contain 10% FBS (heat-inactivated), 4 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulphate. Store at 2-8°C protected from light for no longer than 1 month. Before use, warm the medium in a 37°C water bath.

4.2 Heat-inactivated fetal bovine serum

Thaw a bottle of FBS at room temperature or overnight at 2-8°C and allow to equilibrate to room temperature. Incubate 30 minutes in a 56°C water bath, mixing

every 5 minutes to heat-inactivate it. Single-use aliquots may be stored at 2-8°C for up to one month or at a nominal temperature of -20°C indefinitely.

4.3 Positive Control (tert-butyl hydroperoxide (TBHP)

Prepare a 50 mM intermediate dilution of TBHP by adding 3.22 µL of the 70% stock (7.78 M) to 496.8 µL of complete RPMI. Add 4 µL of intermediate TBHP dilution to appropriate wells and adjust sample volume to 1 mL.

4.4 Negative Control

Use PBS as a negative control.

4.5 Inhibition Control (5 mM solution of N-acetyl-L-cysteine (NAC))

The working concentration of NAC is 5 mM. Prepare a 250 mM interim stock by dissolving 40.8 mg NAC in 1 mL DMSO. Use 16 µL of the prepared stock solution of NAC in the appropriate wells containing 800 µL cells/media (1:50 dilution).

4.6 CM-H2DC-FDA Reagent

Briefly centrifuge the CM-H2DC-FDA vial before opening. Prepare 100 µM intermediate dilution by adding 100 µL of DMSO to CM-H2DC-FDA reagent. Add 5 µL of the intermediate CM-H2DC-FDA dilution to 1 mL of cell suspension. Alternatively, use 300 µL of medium with 5 µM CM-H2DC-FDA per sample.

4.7 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used are trehalose, sucrose, and albumin. However, other reagents and materials are also used alone or in combination. Vehicle control should match formulation buffer of the test-nanomaterial by both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

5. Preparation of Nanoparticles

When the experiment is conducted in 24-well plates, the assay requires 1.5 mL of nanoparticles dissolved/re-suspended in complete culture medium at a concentration 5X higher than the highest final test concentration. Test concentrations are based on the calculated plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol this concentration is called “theoretical plasma concentration”. Considerations for

estimating theoretical plasma concentration have been reviewed elsewhere [3] and are summarised in Box 1 below.

Box 1. Example Calculation to Determine Nanoparticle Theoretical Plasma Concentration

In this example, we are assuming a known efficacious mouse dose of 123 mg/kg. Therefore, the scaled equivalent human dose would be:

$$\text{human dose} = \frac{\text{mouse dose}}{12.3} = \frac{123 \text{ mg/kg}}{12.3} = 10 \text{ mg/kg}$$

The blood volume of a human is approximately 8% of the body weight. Therefore, an average human of 70 kg body weight has approximately 5.6 L of blood. Assuming all the nanoparticle injected goes into the systemic circulation, this provides a rough approximation of the potential maximum nanoparticle concentration in a human. The theoretical plasma concentration, i.e. in vitro test concentration, is calculated by:

$$\text{theoretical plasma concentration} = \frac{\text{human dose}}{\text{human blood volume}} = \frac{70 \text{ kg x } 10 \text{ mg/kg}}{5.6 \text{ L}} = 0.125 \text{ mg/mL}$$

This assay will evaluate four concentrations: 10X (5X if 10X cannot be achieved, or 100X or 30X when feasible) of the theoretical plasma concentration, the theoretical plasma concentration, and two 1:5 serial dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, 1 mg/mL is used as the highest concentration. Alternatively, the highest reasonably achieved concentration can be used if 1 mg/mL is unattainable.

For example, if the theoretical plasma concentration to be tested is 0.2 mg/mL, a stock of 10 mg/mL is prepared. This sample is then diluted 10-fold (1.0 mg/mL), followed by two 1:5 serial dilutions (0.2 and 0.04 mg/mL). When 0.2 mL of each of these sample dilutions are combined in a culture plate with 800 µL cells, the final concentrations of nanoparticles are 0.008, 0.04, 0.2, and 2 mg/mL. Each nanoparticle concentration is plated two times.

6. Collection and Handling of Whole Blood

Collect whole blood from three healthy donor volunteers who have been not on medication and who have been clear from infection for at least 2-weeks before blood donation. Use Li-heparin tubes and discard the first 10 cc. For best results, whole blood should be used

within 1 hour after collection. Prolonged storage (> 2 hr) of whole blood will lead to a decrease in cell function.

7. Preparation of T-Cells

7.1. Place freshly drawn blood into 15 or 50 mL conical centrifuge tubes. Add 50µL RosetteSep per mL of blood, mix well, and incubate the tubes at room-temperature for 20 minutes.

7.2. Add an equal amount of room-temperature PBS with 2% FBS to blood and mix well.

7.3. Slowly layer the Ficoll-Paque solution underneath the blood/PBS mixture by placing the tip of the pipet containing Ficoll-Paque at the bottom of the blood sample tube. Alternatively, the blood/PBS mixture may be slowly layered over the Ficoll-Paque solution. Use 1 mL of Ficoll-Paque solution per 2 mL of blood/PBS mixture. For example, 15 mL Ficoll-Paque is required for 15 mL blood diluted with 15 mL PBS with 2% FBS in a 50 mL tube.

Note: To maintain Ficoll-blood interface it is helpful to hold the tube at a 45° angle.

7.4. Centrifuge 20 min at 1200xg, 18-20ºC, without brake.

Note: Depending on the type of centrifuge, one also may need to set acceleration speed to minimum.

7.5. Using a sterile pipet, remove the upper layer containing plasma and platelets and discard it.

7.6. Using a fresh sterile pipet, transfer the T-lymphocyte cell layer into another centrifuge tube.

7.7. Wash cells by adding an excess of HBSS and centrifuging for 10 min at 400xg, 18-20ºC. The HBSS volume should be ~3 times the volume of the mononuclear layer.

Note: Usually 20 mL of blood/PBS mixture results in ~ 1 mL of the T-cell layer and requires at least 3 mL of HBSS for the wash step. We use 5 mL of HBSS per each 1 mL of cells.

7.8. Discard supernatant and repeat wash step once more.

7.9. Resuspend cells in complete RPMI-1640 medium. Dilute cells 1:2 with AOPI dye and load 20 µL in Cellometer slide. Report cell count and viability displayed by Cellometer. If viability is at least 90%, proceed to step 8.

Note: Similar assay can be applied for the PBMC isolated according to ITA-10.

8. Treatment with Nanoparticles and Controls

8.1. Adjust cell count to 1 x 10^6 cells/mL.

8.2. Aliquot 400 µL of cell suspension prepared in step 8.1 per well of 48-well plate.

8.3. Pretreat "Inhibition Control" wells with NAC for 1 hr at 37°C/5% CO_2 before treatment with nanoparticles. Do not wash cells after incubation.

8.4. Add 100 µL of test-nanoparticles and control to appropriate wells and incubate for appropriate length of the assay at 37°C/5% CO_2. (Add 100 µL of complete media to "unlabeled cells" wells).

Note: For the positive control inhibition, it is essential to perform PC treatment immediately after NC treatment for an additional 30 minutes. Longer incubation after NC treatment is ineffective when tested for inhibition on PC.

8.5. Wash cells once with 3 mL of PBS.

8.6. Add the CM-H_2 DC-FDA reagent to all samples except "unlabeled cells" and incubate for 30 minutes at 37°C/5% CO_2.

8.7. Wash cells once with 3 mL of PBS.

8.8. Reconstitute cells at 1 x 10^6 cells/mL with PBS and analyze samples by flow cytometry.

9. Flow Cytometry

At the NCL, we use FACSCalibur. The procedure below is based on our experience with this instrument. If you are using another cytometer, please follow the procedure specific to that instrument.

9.1 Switch on the instrument. Make sure the sheath tank is full and the waste tank is empty.

9.2 To get consistent results, run BD FACSComp software using BD CaliBRITE beads.

9.3 Adjust FSC vs. SSC dot plot using unlabeled cells to get the population of cells in the plot. See Figure 1 below for example.

9.4 Set the threshold to remove most of the debris.

9.5 Gate lymphocyte population. See Figure 1 below for example.

Note: The purified T cells do not need to be gated unless the cells show multiple dense clusters on the FSC vs. SSC plots.

9.6 Create FL1 histogram for each analyzed live cell population. See Figure 2 below for example.

9.7 Analyze shift of FL1 fluorescence in treated samples comparing to NC.

9.8 Acquire and save the data.

9.9 Follow the instrument closing procedure.

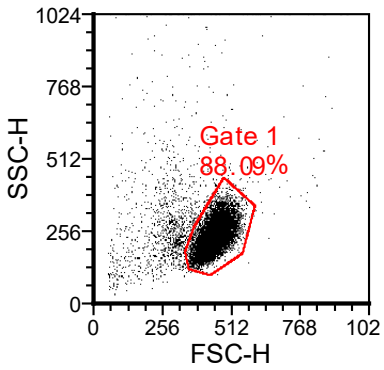

Figure 1. Set up gates for lymphocytes: gate 1.

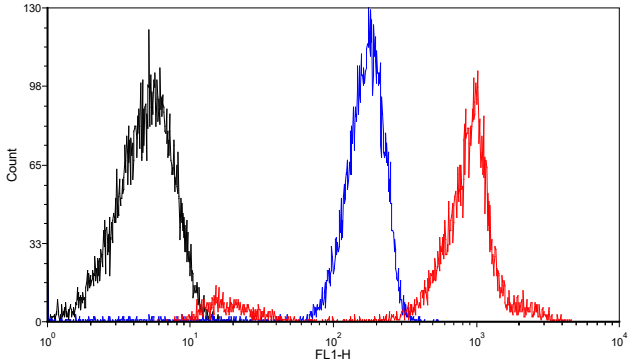

Figure 2. Detection of total oxidative stress in lymphocytes from gate 1 in FL1 histogram (black–US, blue–NC, red–PC).

10. Data Analysis and Report

Use appropriate software to analyze the data acquired in step 9.8. CellQuest or other flow cytometry software can be used. Here, FCSExpress from De Novo Software Solutions, Inc. was used. Report the data as GeoMean of treated samples/GeoMean (NC). Alternatively, percent positive cells can be reported if Geo Mean is not statistically different.

11. Acceptance Criteria

11.1 The run is acceptable if GeoMean (PC)/GeoMean (NC) ≥1.5.

11.2 The run is acceptable if GeoMean (PC+NAC)/GeoMean (PC) <1.

12. References

1. Finkel T, Holbrook NJ: Oxidants, oxidative stress and the biology of ageing. *Nature* 408(6809), 239-247 (2000).
2. Kohen R, Nyska A: Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol* 30(6), 620-650 (2002).
3. Dobrovolskaia MA, McNeil SE: Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. *J Control Release* 172(2), 456-466 (2013).

13. Abbreviations

DMSO – dimethyl sulfoxide

FL – fluorescence

HBSS – Hank's balanced salt solution

NAC – N-acetyl-L-cysteine

NC – negative control

PBS – phosphate buffered saline

PC – positive control

RPMI – Roswell Park Memorial Institute

TBHP – tert-butyl hydroperoxide

TS – test samples

VC – vehicle control

14. Appendix

Example Plate Map

	1	2	3	4	5	6
A	NC	VC	PC	PC + NAC	TS (0.008 mg/mL)	TS +NAC (0.008 mg/mL)
B	TS (0.04 mg/mL)	TS +NAC (0.04 mg/mL)	TS (0.2 mg/mL)	TS +NAC (0.2 mg/mL)	TS (2 mg/mL)	TS +NAC (2 mg/mL)
C	Unlabeled Cells					
D						

NC – negative control, VC – vehicle control, PC – positive control, NAC – N-acetyl-L-cysteine, TS – test sample; shaded cells – inhibition control