NextGEM

Standard Operating Procedure @ CNR- IREA

PROTOCOL	**Intracellular ROS measurement by flow cytometer in HaCaT cells**
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1. Purpose

This procedure describes the materials and the protocol used for flow cytometric analysis of ROS formation with H2DCF-DA staining in HaCaT cells.

2. Background

2',7'-Dichlorodihydrofluorescein diacetate (H2DCF-DA) is used to assess the overall oxidative stress. H2DCF-DA crosses the cell membrane and is deacetylate by intracellular esterases, resulting in 2',7'-dichlorodihydrofluorescein (H2DCF). H2DCF reacts with reactive oxygen species (ROS) to give the fluorescent 2',7'-dichlorofluorescein (DCF), which is measured by flow cytometry.

3. Procedure

3.1. Equipment

- Cell culture incubator (Thermo Scientific Forma, Model 311)
- Refrigerated centrifuge (Heraeus Sepatech Minifuge RF)
- FACSCalibur flow cytometer (BD Biosciences) equipped with 488 nm laser

3.2. Materials

- 35 mm cell culture dish (Corning, cod. 430165)
- 5 mL polystyrene round bottom FACS tube (Falcon, cod. 352052)

3.3. Reagents

- H2DCF-DA (Sigma, cod. 6883): 10 mM stock solution is prepared in DMSO. Aliquots of 100 µl are prepared and stored in the dark at -20°C
- H_2O_2 (Sigma, cod. S86968-359): Hydrogen Peroxide 30 wt. % solution in water, 8820 mM stock solution. Aliquots of 2ml are prepared and stored in the dark at +4°C
- DMEM (Dulbecco's Modified Eagle Medium), supplemented with 4.5 g/L glucose, sodium pyruvate and sodium bicarbonate (Microgem, cod. AL007) is stored at +4°C
- 1X PBS (Dulbecco's Phosphate Buffered Saline) w/o Calcium w/o Magnesium (Microgem, cod. TL1006) is stored at +4°C
- Trypsin – EDTA 1X in solution w/o Calcium w/o Magnesium w/ Phenol Red (Microgem, cod. L0930) is stored at -20°C

3.4. Experimental procedure

The following procedure has been optimized for HaCaT human keratinocyte cells (CLS, Cat. No. 300493, Eppelheim, Germany). Procedure for cell maintenance is detailed in Annex 6.

$3x10^5$ cells are seeded in 3 ml complete medium in a 35 mm cell culture dish and the assay is performed after 72 h of growth.

3.4.1. H_2O_2 treatment

H_2O_2 is a ROS inducer and 1 mM for 30 min is used as positive control in setting up the procedure to evaluate ROS formation in HaCaT cells. The aim is to verify the adequacy of the procedure.

3.4.2. UVB exposure

UVB exposure (Annex 8) is used as treatment for co-exposure in the 5G radiofrequency experiments (Annex 7).

3.4.3. Sample preparation

1) Remove the culture medium and replace it with 3 ml of DMEM base medium with 10 µM H2DCF-DA (incubation medium), along with H_2O_2 where required

2) Incubate for 30 min at 37°C

3) Collect the incubation medium into a FACS tube

4) Wash the adherent cells with PBS (1 ml) and detach by 12 min trypsin treatment (300 µl) at 37°C

5) Collect the cells by using the incubation medium and add cold PBS (1 ml)

6) Centrifuge (4°C, 1200 RPM, 5 min)

7) Discard the supernatant and wash the cell pellet with cold PBS (2 ml)

8) Centrifuge (4°C, 1200 RPM, 5 min)

8) Resuspend the pellet in 500 µl of cold PBS

9) Analyze by flow cytometer

Note: the whole procedure is performed in the dark.

3.4.4. Sample acquisition

CellQuest software is used for sample acquisition and data storage.

For each sample, 15000 events are acquired. Forward scatter (FSC) and side scatter (SSC) dot plot are selected to identify the cell population. FL1 channel is selected to detect the DCF fluorescence in log scale.

The following graphs are displayed:

1° Dot Plot: Acquisition, FSC (x-axis) and SSC (y-axis)

2° Dot Plot: Acquisition, FL1 (x-axis) and FSC (y-axis)

1° Histogram: Acquisition, FL1

3.4.5. Sample analysis

The DCF fluorescence histograms are analyzed by the Flow Jo analysis program (TreeStar, OR, USA). The percentage of DCF positive cells is quantified considering a threshold fluorescence level (expressed as arbitrary units) set on the basis of the background fluorescence in the control cell population (about 10^2).