Standard Operating Procedure @ CNR- IREA

PROTOCOL	**Apoptosis detection by flow cytometer in SH-SY5Y cells**
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1. Purpose

This procedure describes the materials and the protocol used for flow cytometric analysis of apoptosis measurement in SH-SY5Y cells.

2. Background

Apoptosis, the physiological process of cell death, is fine-tuned. Phosphatidylserine (PS) is a cell membrane phospholipid that translocates to the outside-facing side of the membrane when apoptosis is initiated. Annexin V, a protein binding PS with high affinity, is used to detect apoptotic cells by flow cytometry when labeled with FITC. If the staining protocol with Annexin V-FITC is combined with propidium iodide (PI), it is also possible to screen late apoptotic and dead cells:

Annexin V-FITC stained cells identify early-stage apoptotic cells, the double stained Annexin V/PI identify late-stage apoptotic cells, while PI stained ones are necrotic.

3. Procedure

3.1. Equipments

- Cell culture incubator (Thermo Scientific Forma, Model 311)
- Refrigerated centrifuge (Heraeus Sepatech Minifuge RF)
- Automated cell counter (Luna II)
- 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

- DMSO (Dimethylsulphoxide, LABSCAN, cod A3534) is stored at room temperature (RT)
- Menadione (Sigma, cod. M5625): 5.8 mM stock solution is prepared in DMSO. Aliquots of 100 µl are prepared and stored in the dark at -20°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
- Trypan blue stain, 0.4% (Logos Biosystems, cod. TB0BJC2301) is stored at room temperature (RT)
- Apoptosis Detection kit (Leinco Technologies, cod. A432) containing: i) Binding Buffer, ii) Annexin V-FITC, iii) Propidium Iodide (PI) is stored at 4°C

3.4. Experimental procedure

The Annexin V-FITC Apoptosis Detection kit is used following the manufacturer's instruction and the described procedure has been optimized for SH-SY5Y human neuroblastoma cells (ATCC, Cat. No. CRL2266, Rockville, MD, USA). Procedure for cell maintenance is detailed in Annex 1.

$1x10^6$ 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. Menadione treatment

Menadione (MD) 20 µm is used as positive control.

MD (5 and 20 µm) is used as agent for co-exposure in the 4G LTE radiofrequency experiments (see SOP_4G LTE exposure_CNR).

3.4.2. Sample preparation

1) Collect cell growth medium into FACS tube

2) Wash the adherent cells with PBS (1 ml) and detach by 3 min trypsin treatment (300 µl) at 37°C

3) Collect the cells by using the growth medium and add cold PBS (500 µl)

4) Centrifuge (4°C, 1200 RPM, 5 min), and remove the supernatant

5) Resuspend the cell pellet with DMEM complete medium (1 ml)

6) Count the cells by trypan blue stain

7) Transfer 300000 cells into a clean FACS tube

8) Add cold PBS (2 ml), centrifuge (4°C, 1200 RPM, 5 min) and remove the supernatant. Repeat this step once

9) Resuspend the pellet in Binding Buffer (100 µl)

10) Add Annexin V-FITC (5 µl), PI (5 µl) and mix by gentle pipetting

11) Incubate for 10 min at RT

12) Add cold PBS (400 µl) and mix by gentle pipetting

13) Analyze by flow cytometer

Note

- Before starting the first experimental run, the calibration of the instrument is required and is achieved by setting up the following samples: i) unstained cells to assess the level of autofluorescence, ii) cells stained only with Annexin V-FITC and iii) cells stained only with PI to define the boundaries of each population.
- Be careful during trypsinization to avoid damaging the cells. Moreover, if trypsin EDTA is used, it is necessary to completely remove the EDTA by washing the cells twice before staining to avoid chelating the calcium needed for Annexin binding.
- Be sure that steps 9 to 13 are performed in the dark.

3.4.3. 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 plots are selected to identify the cell population. FL1 and FL2 channels are selected to detect the FITC and the PI fluorescence (log scale) respectively.

The following graphs are displayed:

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

2° Dot Plot: Acquisition, FL1 (x-axis) and FL2 (y-axis)

1° Histogram: Acquisition, FL1

2° Histogram: Acquisition, FL2

3.4.4. Sample analysis

FlowJo software (TreeStar, OR, USA) is used for sample analysis.

Apoptotic cells are displayed in the right quadrants of the FL1/FL2 dot plot, with early apoptotic cells at the bottom (Annexin V- FITC positive) and late apoptotic cells at the top (Annexin V- FITC and PI positive). Necrotic cells (PI positive) are displayed on the top left. The percentage of cells in each quadrant is calculated by the software.