

Multiparametric Detection of Apoptosis (1)

Background

This method is used for detection of apoptotic cells via four different morphological and biochemical changes in the cell: loss of cell membrane asymmetry by phosphatidylserine externalisation, loss of cell membrane integrity, loss of mitochondrial membrane potential, and degradation of DNA. It's a combination of Annexin V and DAPI staining, the detection of mitochondria membrane potential changes using TMRE, and DNA degradation based on subG1 analysis, all together in the same sample tube.

Materials

1. **TMRE** (40 μ M in PBS)
2. **Annexin V-Alexa647 conjugate**
3. **Annexin V Binding buffer:** HEPES buffer: 10mM HEPES/NaOH, (pH 7.4), 150mM NaCl, 5mM KCl, 5mM MgCl₂, and 1.8mM CaCl₂, **can be bought pre-made from BD, cat. No: 556454)**
4. **1x PBS**
5. **DAPI solution** (200 μ g/ml)
6. **Propidium Iodide solution** (50 μ g/ml in PBS)
7. **70% ethanol** (in DI water)
8. **RNase A** (100 μ g/ml)
9. **SubG1 Phosphate-Citrate buffer:**
 - 0.2M Na₂HPO₄ (disodium phosphate, 2.84g /100ml)
 - 0.1M Citric Acid (2.10g /100ml)(Mix 192 parts 0.2M Na₂HPO₄ and 8 parts 0.1M citric acid and adjust to pH 7.8)

Equipment

1. **Centrifuge.**
2. **Pipettes.** You will need one in the range of 2-10 μ l, one in the range of 10-100 μ l, and another ranging from 100-1000 μ l.
3. **12x75 mm polystyrene/polypropylene tubes.** Depending on which machine you wish to use (LSR II prefers polystyrene while the CyAn prefers polypropylene).
4. **Ice bucket with cover.** Generally, cells are more stable and tolerate insult better when they're cold. The cover keeps light out, which could bleach the fluorochromes.
5. **Flow cytometer.** We have a variety of machines at your disposal including a BD LSR II, BD FACSAria and a Beckman Coulter CyAn. Each machine has different capabilities, strengths and weaknesses so be sure to check with us which machine is suited for your needs.

Additional Considerations

1. **Single colour control:** when combining fluorochromes with overlapping fluorescence emission, a single colour positive control should be prepared for each dye in the experiment.
2. **Negative control:** unstained cells can be used to set the base line of each parameter.

Procedure

1. Harvest cells in the appropriate manner and wash in PBS.
2. Resuspend one million cells in 1ml PBS and add TMRE at 40nM final concentration and incubate the cells at 37°C in the dark for 10 minutes.
3. Centrifuge and decant to remove supernatant. Be careful not to lose the pellet. A slight amount of liquid can remain (usually you will see the cell pellet at the bottom).
4. Dilute the Annexin V conjugate 1 in 100 in Annexin V binding buffer (if staining more than one sample, prepare a batch solution for all samples and mix well).
5. Resuspend pellet in 500µl Annexin V conjugate/binding buffer mixture incubate for 20 minutes at room temperature in the dark.
6. Transfer cells onto ice and add 10µl of DAPI solution.
7. Run the samples on LSR II probing for Annexin V binding (660 off Red laser), DAPI (450 off UV laser) and TMRE (580 off Blue laser) staining. Save 10 to 30,000 cells.
8. Centrifuge at 4°C the rest of each sample and decant supernatants.
9. Fix in cold 70% ethanol for at least 30 minutes on ice. Add drop wise to the cell pellet while vortexing. This should ensure fixation of all cells and minimise clumping. Cell can be kept in ethanol overnight or for longer.
10. Wash twice in 1ml sub G1-Phosphate-citrate buffer. Spin down and be careful to avoid cell loss when discarding supernatant especially after spinning out ethanol.
11. Add 50µl of RNase A to the pellet.
12. Add 200µl propidium iodide.
13. Analyse by flow cytometry for subG1 accumulation of late apoptotic cells.

Cautions:

TMRE emits at around 580 nm. For subG1 analysis, use 670 filter for PI detection to avoid any residual TMRE staining. When analysing samples for subG1, be sure to collect PI in linear scale. Use a dot plot showing Area vs. Height (LSRII)/Peak (CyAn) or Width (LSRII) to gate out doublets and clumps and analyse at a low flow rate under 400 events/second.