Technical Resources DSF·UNIPD

Flow Cytometry and Cell Sorting Facility
Department of Pharmacological Sciences
University of Padua
Italy

Cell cycle staining with propidium iodide in GFP transfected cells

Background

Propidium iodide (PI), a fluorescent dye that intercalates with DNA, is used to stain each cell. The fluorescence intensity of PI dye integrated into cells is directly proportional to the amount of DNA in cells. Because the dye cannot penetrate living cells, the latter are treated with a fixative before staining. This approach works well in assessing the cell cycle in cells fixed with 70% ethanol. However, in response to such ethanol treatment, the soluble cytoplasmic GFP seeps out of the cells, causing its loss. Conversely, when aldehydes are used as fixatives (such paraformaldehyde), the cells retain GFP, but coefficients of variation (CV) of G1/G0 peaks are unacceptably high. This protocol can be used to overcome these issues.

Specimen

GFP transfected cells

Reagents and materials

- 5 ml flow cytometry test tubes
- refrigerate centrifuge
- sterile-filtered phosphate-buffered saline (PBS), 4 °C
- 2 % w/v paraformaldehyde fixation solution in PBS pH 7.2, 4 °C
- 70 % ethanol, ice-cold
- 40 μg/ml propidium iodide (PI) + 100 μg/ml RNAse A solution in PBS

Controls

- Untreated GFP transfected cells without staining
- Untreated untransfected cells stained with PI

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Procedure

1. Harvest cells in the appropriate manner, the objective is to get approximately 10^6 cells in each test tube.

2. Centrifuge cells at the appropriate conditions (likely 300 x g for 5 min, 4°C), discard supernatant, resuspend in 3 ml PBS and centrifuge again.

3. Discard the supernatant and resuspend the pellet in 500 µl PBS ice-cold. Mix gently.

4. Add 500 µl 2 % paraformaldehyde. Mix gently. Incubate 1 hr at 4 °C. Note that could be necessary to identify the best concentration of paraformaldehyde and fixation time. Add drop wise to cell pellet while vortexing. This should ensure fixation of all cells and minimise clumping.

5. Centrifuge cells 5 min at 300 x g, 4°C. Discard the supernatant being careful not to aspire the pellet. Wash once with 3 ml PBS. Discard the supernatant.

6. While vortexing, add 1 ml cold 70 % ethanol dropwise to the cell pellet. Incubate cell suspension for at least 2 hours on ice (overnight can be reached). Vortexing cells gently during the addition of ethanol can reduce the formation of cell clumps; however, avoid extensive vortexing, because it can lead to cell disruption. Samples can be left at this step for several weeks.

7. Centrifuge cells at higher speed compared to live cells for 5 minutes at 4°C (ethanol-fixed cells become more floating), discard the supernatant being careful not to aspire the pellet.

8. Wash twice with cold PBS. Facultative: the addition of 0.1 % saponin and 3 % BSA to PBS can help to improve both GFP and DNA staining.

9. Add 1 ml of PI/RNase solution and mix well. Incubate at room temperature for 5 to 10 minutes. For optimal staining, certain cells, such as fibroblasts, may require an extended incubation time, typically overnight.

9. Analyse samples by flow cytometry. Remember to analyse PI in a linear scale. Record at least 20,000 doublets-excluded events. Use a low flow rate to reduce CV of the peaks.

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