Cell cycle analysis and apoptosis determination by flow cytometry in unfixed cells

1. Background and purpose of the procedure

When stained with a cell cycle reagent, DNA in the cells bind the dye stoichiometrically (in proportion to the amount of DNA present in each cell). The flow cytometric analysis of cell count versus linear fluorescence is used to create a histogram of the DNA content distribution across the steps of the cell cycle. There are standard modeling algorithms that can then be employed to determine the breakdown of cells in the G0/G1 phase versus S phase, G2, or polyploidy state of the cell population. Hoechst 33342 is a DNA dye that can detect differences in chromatin condensation. Furthermore, this dye can penetrate by active transport into live cells. Propidium iodide (PI) is a DNA dye that is unable to penetrate live cells and can be used as a probe for membrane damages. By using a staining combination of Hoechst 33342 and PI, we can discriminate intact cells, cells undergoing apoptosis, and dead cells in the same sample.

2. Materials and equipment

- Adherent cells treated with pharmacological or radiological agent
- D-PBS, without Ca^{++} and Mg^{++} (Gibco, 14190144)
- 0.25% Trypsin, 0.1%EDTA (Corning, 25053CI)
- Medium for cell culture containing 2% FBS
- FACS Buffer (*see 3. Recipes*)
- Propidium iodide stock solution (Thermo Fisher Scientific, 1 mg/mL, P3566)
- Hoechst 33342/propidium iodide staining solution (*see 3. Recipes*)

- Flow cytometer equipped with 350/488 nm lasers and able to read emission at 461/575 nm
- Ice bucket
- Ice

3. Recipes

a. FACS Buffer

2% Fetal Bovine Serum (Gibco, 10437028) *[2 mL for 100 mL]*
D-PBS D-PBS, without Ca^{++} and Mg^{++} (Gibco, 14190144) *[98 mL for 100 mL]*

Solution can be stored at 4˚C for up to 3 weeks.

b. Hoechst 33342 stock solution

1 mg/mL Hoechst 33342 (Thermo Fisher Scientific, H1399) *[10 mg for 10 mL]*
DMSO qsp 10 mL (Fisher Chemical, Certified ACS, D128-1)

Aliquot stock solution in 1.5 mL amber microtubes and store at -20˚C until use. Solution can be stored at -20˚C for up to 12 months and is stable for few weeks at 4˚C.

c. Hoechst 33342/propidium iodide staining solution

5 µg/mL Hoechst 33342 *[5 µL of Hoechst 33342 stock solution (b) for 1 mL]*
1 µg/mL Propidium iodide *[1 µL of PI stock solution for 1 mL]*
FACS Buffer (a) qsp 1 mL

Staining solution cannot be stored and must be prepared no more than 15-25 min prior to incubation of cells.

4. Method

a. Gently aspirate the cell culture media from the tissue culture plate or dish
b. Rinse cells using D-PBS without Ca^{++} and Mg^{++}
c. Detach cells with Trypsin-EDTA *[300 µL for 12-well plate, 500 µL for 6-well plate, 1mL for 10cm dish]*
d. Incubate at 37˚C for 1 to 3 min
e. Add cell culture media containing 2% FBS to inhibit Trypsin *[600µL to 3 mL]*
f. Transfer to a centrifuge tube *[1.5 mL or 15 mL depending on total volume]*
g. Pellet cells by centrifugation at 350 g for 5 min at room temperature
h. Resuspend pellet in FACS buffer *[100µL to 500 µL]*
i. Pellet cells by centrifugation at 350 g for 5 min at room temperature
j. Resuspend cells in Hoechst 33342/propidium iodide staining solution *[150µL to 500 µL]*
k. Incubate cells on ice for 30 min
l. Analyze fluorescence by flow cytometry at λ_{ex} 350 nm and λ_{em} 461 nm (DAPI channel) and λ_{ex} 488 nm and λ_{em} >575 nm (TRITC or Texas Red channel)
m. Analyse data using Flowjo or OMIQ software

5. References

Belloc F, Dumain P, Boisseau MR, Jalloustre C, Reiffers J, Bernard P, Lacombe F. A flow cytometric method using Hoechst 33342 and propidium iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells. Cytometry. 1994 Sep 1;17(1):59-65. doi: 10.1002/cyto.990170108. PMID: 7528124.

The Molecular Probes Handbook, Thermo Fisher Scientific/Invitrogen, Chapter 15 - Assays for Cell Viability, proliferation and Function

6. Revision history

Revision #	Date	Prepared by
1.0	**2021-09-25**	**Elie Besserer-Offroy**
Summary of modifications		
Initial version of the protocol		