Cell Cycle in GFP Positive Cells

The simultaneous measurement of GFP and propidium iodide (PI) fluorescence is difficult since PI staining requires cell permeabilization for DNA staining. The use of ethanol causes the leaking out of the GFP molecules accumulated in the cytoplasm. In contrast, cell fixation with formaldehyde leads to GFP retention and DNA histograms with high CVs for peaks G0/G1.

An appropriate cell preparation for GFP and PI detetection requires a balance between GFP retention and adequate cell cycle profile. Alternative, the use of Hoechst 33342 that enter live cells can prevent problems associated with cell membrane permeabilization (Reviewed in (1)).

Protocol N1: PI Staining
In this protocol, cells are fixed with 1% formaldehyde, permeabilized with 70% ethanol, and then stained with PI in the presence of ribonuclease A.

1. Recover cells from the culture plate and count them
2. Transfer 1x106 cells into a tube and wash the cells with cold (4°C) PBS1X
3. Spin the cells at 300g, 5 min, 4°C
4. Resuspend the cell pellet in 500µL of PBS 1X, mix gently. Add 500µL of fixation solution cold (4°C) and mix again
5. Incubate 1 hour at 4°C
6. Spin the cells at 300g, 5 min, 4°C. Remove the supernatant carefully and wash the cells once with cold PBS1X
7. Spin the cells at 300g, 5 min, 4°C
8. Add 1ml of 70% ethanol at -20°C dropwise to the cell pellet with the tube sitting on a vortex mixer.
9. Incubate cell suspension from 2h to over-night at 4°C
10. Spin the cells at 300g, 5 min, 4°C
11. Resuspend the cell pellet with 1ml of PI working solution
12. Incubate cell suspension 30 min in a 37°C water bath in the dark
13. Acquire the samples

***If target cells express red fluorescent proteins (mCherry,RFP, DsRed) PI should be replaced by DAPI.**

Protocol N2: Hoechst 33342 Staining
In this protocol cells are stained at 37°C with the cell-permeant DNA dye Hoechst 33342 for combined GFP and DNA content analysis.

1. Recover cells from the culture plate and count them
2. Transfer 1x106 cells into a tube and spin the cells at 300g, 5 min, room temperature.
3. Remove supernatant and resuspend the cell pellet with 500ul of the same medium that was used for growing the cells, prewarmed to 37°C. Add 5ul of Hoechst stock solution (1mg/ml) and mix.
4. Incubate 45min at 37°C
5. Acquire the samples

* The optimal Hoechst 33342 dye concentration and staining time may vary between different cell types, as dye uptake depends on cellular metabolic rates; therefore, both have to be determine empirically. In general, dye concentrations between 1 and 10µg/ml, and incubation times between 20 and 90 min, will produce DNA histograms with acceptable coefficients of variations. Because Hoechst DNA staining is performed on unfixed cells, it is possible to use other nonvital DNA dyes (PI, 7AAD), for concurrent dead cell discrimination.

Reagents

Fixation solution:
For 100ml of 2% paraformaldehyde, add 80 mL of 1X PBS to a glass beaker on a stir plate in a ventilated hood. Heat while stirring to approximately 60 °C. Take care that the solution does not boil.
Add 2g of paraformaldehyde powder to the heated PBS solution. The powder will not immediately dissolve into solution. Slowly raise the pH by adding 1N NaOH dropwise from a pipette until the solution clears. Once the paraformaldehyde is dissolved, cool to room temperature and adjust pH to 7.2 with 0.1 M NaOH or 0.1 M HCl. Adjust the volume of the solution to 100ml with 1X PBS. The solution can be aliquoted and frozen or stored protected from light at 2-8 °C for up to one month (R&D systems protocols webpage).

Hoechst 33342 stock solution, 1 mg/ml
Dissolve 1 mg Hoechst 33342 powder (Molecular Probes) in 1 ml distilled water. Store at 2 to 8°C protected from light for up to 1 month.

Propidium iodide (PI) stock and working solutions
Stock solution: Dissolve 1mg PI in 1 ml PBS. Add 2.5mg DNase-free ribonuclease A (Sigma-Aldrich) to the solution. Store at 2 to 8°C protected from light for up to 1 month.
Working solution: Dilute PI stock solution 1:25 with PBS 1X. Final concentration of PI is 40 µg/ml. Final concentration of ribonuclease A is 100 µg/ml. Make fresh before staining. Protect from light.

References

1. Currents Protocols in Cytometry (1997) 7.6.1-7.6.10 Copyright ©1997 by John Wiley & Sons, Inc.