

Internal Antigen Labeling and Cell Cycle Profile

Background

0.5-2% Paraformaldehyde cross linking is the fixation of choice for optimal antigen preservation and staining but gives sub-optimal DNA profiles. 70% ethanol is the optimal fixative for DNA staining but it's not convenient for small internal antigen detection. However, after aldehyde cross-linking, an alcohol treatment can be used to improve DNA profile. Permeabilisation with a detergent like Saponin also helps improve internal antigen staining and DNA profile. Therefore some preliminary tests may be required to optimise your staining.

Here we suggest a method for staining small internal antigens against cell cycle profile; that is, detection of internal antigens of molecular weight lower than 50KDa and staining for DNA content. This allows the user to correlate internal antigen expression and cell cycle.

This method can be preformed with both direct and indirect staining. Here indirect staining is outlined. For bigger internal antigens (>50KDa), refer to protocol for detection of mitotic cells.

Materials

1. **Antibodies.** For simultaneous labeling, choose fluorochromes carefully. You want the weakest signal antibody labelling the antigen with the greatest density (most receptors per cell).
2. **2% Paraformaldehyde (in PBS)**
3. **70% Ethanol (in DI water)**
4. **PBS + 0.1% Saponin + 3% BSA**
5. **PI 50µg/ml (Sigma P4170)**
6. **RNase 100µg/ml (Sigma, Product N. R6513-10MG)**

Additional Considerations

1. **Single stain control:** If you're planning to label cells with 2 or more antibodies simultaneously, you need a positive control for each fluorochrome. If you have a limited number of cells there are alternatives that use beads, just ask and we can assist you.
2. **Negative Sample:** An amount of unstained cells can be used to initially adjust voltages on the machine.

Equipment

1. **Centrifuge.**
2. **Pipettes.** You will need one in the range of 2-10µl, one in the range of 10-100µl, and another ranging from 100-1000µl.
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.

Procedure

1. Harvest cells in the appropriate manner and wash in PBS.
2. Fix with 2% paraformaldehyde (PFA) for 30 min on ice (PFA concentration and fixation time may need optimisation). Add drop wise to cell pellet while vortexing. This should ensure fixation of all cells and minimise clumping.
3. Pellet and wash once with PBS.
4. Fix in 1ml cold 70% ethanol. Add drop wise to cell pellet while vortexing. This should minimise clumping.
5. Fix for at least 30 minutes on ice. Specimens can be left at this stage for several weeks (make sure you seal the tubes for long term storage).
6. Pellet cells at higher speed for 5 minutes; decant the supernatant being careful not to lose the pellet. Note that ethanol-fixed cells require higher centrifugal speeds to pellet compared to unfixed cells since they become more buoyant upon fixation.
7. Wash twice with PBS + 0.1% Saponin + 3% BSA.
8. Pour off supernatant and add primary antibody directly to the pellet (may require optimisation). Mix well and incubate at room temperature for 30-45 minutes.
9. Wash twice with PBS + 0.1% Saponin + 3% BSA.
10. Add directly to the pellet 5µl fluorochrome conjugated secondary antibody (may require optimisation). Mix well and incubate at room temperature in the dark for 30 minutes.
11. Wash twice with PBS.
12. Add 50µl RNase A and 200µl PI and incubate for 5-10 minutes at room temperature in the dark.
13. Analyse for cell cycle profile and internal antigen expression, saving at least 20,000 single cells.

Flow analysis:

Keep the cells at RT covered until your scheduled time on the flow cytometer.

When analysing samples, be sure to collect PI in linear scale. Use a dot plot showing PI parameter 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. A dot plot showing PI in X-axis vs. fluorochrome conjugated secondary antibody in Y-axis should show the relation between internal antigen expression and cell cycle.