Protocol II: Indirect cell labeling for flow cytometry

Introduction

This protocol describes the procedure for labeling of cells using purified and Biotin conjugated antibodies. It can be applied to a large variety of cell types. Keep in mind that in addition to antibodies detecting the cell markers of interest, matching isotype and FMO (fluorescence minus one) controls need to be included in the experiment. When target cells are treated in a specific manner prior to flow analysis, make sure to include appropriate negative control cells in the experiment (e.g. cells treated with substance X vs. cells untreated). Also think about compensation samples when needed.

The labeling protocol can be performed either in 96 well plates (U bottom shape) or appropriate flow cytometry tubes. As an example, the use of $2*10^5$ human PBMCs per sample as target cells in a staining volume of 100ul is described here. Optimal cell number depends on frequency of cells of interest and may vary in other experimental settings. Before starting your experiment, always make sure that your cytometer is equipped with the adequate set of lasers and filters to analyze the fluorophores you wish to use.

Procedure

All samples must be kept on ice during the procedure. Set centrifuge temperature to 4°C.

1. Suspend PBMC in 10ml cold staining buffer in a 15ml tube. Recommended staining buffer 1x PBS, 4% FBS, 0.09% NaN_3.
2. Centrifuge cells at 300 – 450 x g for 5 minutes at 4°C.
3. Discard supernatant, dislodge pellet with mild vortex, and prepare a cell suspension at $4x10^6$ cells/ml with ice-cold staining buffer.
4. Filter the cell suspension through a nylon mesh or a cell strainer.
5. Add appropriate volume of staining buffer (50ul – recommended antibody volume) per well into a 96well U bottom plate.

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6. Add recommended test volume (e.g. 5 ul) of each antibody to be tested to each well, mouse immunoglobulin isotype control staining (negative control) must be used in separate wells. If using a panel of several antibodies, the use of a mastermix cocktail is highly recommended.
7. Add 50ul of ice cold PBMCs in each well, carefully pipette up and down 2-3 times to ensure thorough mixing. Always keep an aliquot of unstained cells for gating and/or compensation.
8. Incubate for 30 minutes on ice, protect from light.
9. In the meantime, prepare dilution of appropriate secondary antibody or SA-PE in staining buffer. Calculate with 100ul per sample. Typical dilution for SA-PE is 1:200, while secondary antibody dilution can be expected between 1:1000 and 1:3000 depending on the antibody.
10. After incubation, add 150ul of staining buffer and spin samples down at 300 – 450 x g for 5 minutes at 4°C.
11. Remove supernatant by gentle flicking, vortex and repeat wash one more time by adding 250 ul staining buffer per well. Spin samples down at 300 – 450 x g for 5 minutes at 4°C.
12. Remove supernatant by gentle flicking, vortex and resuspend cells stained with primary antibody in 100µl secondary antibody dilution per sample.
13. Incubate for 20 minutes on ice, protect from light.
14. Add 150ul of staining buffer and spin samples down at 300 – 450 x g for 5 minutes at 4°C.
15. Remove supernatant by gentle flicking, vortex and repeat wash one more time by adding 250 ul staining buffer per well.
16. Spin samples down at 300 – 450 x g for 5 minutes at 4°C.
17. Remove supernatant by gentle flicking, vortex and resuspend cells in 80µl staining buffer per well. Place samples on ice in the dark until ready to run in the flow cytometer.
18. Run samples in your flow cytometer. Always control your instrument settings to obtain reliable data.
19. Analyze the data using appropriate software.

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