Simultaneous Measurement of Cell Surface Markers with Cellular Proliferation and Protein Phosphorylation

Reagents and Antibodies

Full Name	Short Name	Catalog Number
BD Falcon™ polystyrene 12 x 7-mm tubes	BD Falcon tubes	352058
RPMI-1640 containing 10% FCS	RPMI	
BD Pharm Lyse™ solution	Lysing Solution	555899
BD™ APC BrdU Flow Kit	BrdU Flow Kit	552598
BD Pharmingen™ stain buffer	Stain Buffer	554657
BD Cytofix™ buffer	Fixation Buffer	554655
BD Phosflow™ perm buffer III	Perm Buffer III	558050
DNAse diluted in DPBS		
BD Cytofix/Cytoperm™ Plus buffer	Permeabilization Buffer	561651
NA/LE anti-mouse CD3e, clone 145-2C11	CD3	553057
NA/LE anti-mouse CD28, clone 37.51	CD28	553294
Anti-mouse CD4-FITC	CD4-FITC	553047
Anti-human Stat5 PerCP-Cy™5.5	Stat5	560118
DAPI (1 mg/mL stock)		

Procedural Notes

- This assay enables the simultaneous measurement of cellular proliferation (using BrdU incorporation), cell signaling (BD Phosflow™ technology), and cell surface markers.
- In this example protocol, the cells were prepared from mouse spleen (Balb/c mice (♀), Simonsen Laboratories). The mice were sacrificed, spleen was harvested, and the red blood cells were lysed with Lysing Solution. The unstimulated and stimulated cells were pulsed with 50 µM of BrdU for 1 hour prior to harvest at 37°C in 5% CO_2.
- Cells can be bulk fixed and prepared for staining at a concentration of 1 x 10^7 cells per mL.
- Be sure that the PBS used to wash cells before permeabilization does not contain FBS or BSA, since buffer containing protein is not suitable before permeabilization with methanol.

Procedure, stimulation

1. Coat a sterile 6-well tissue culture plate with CD3 at a concentration of 10 µg/mL in sterile 1X PBS. Incubate overnight at room temperature (RT).
2. The next day, remove the CD3 and wash once with 2 mL of RPMI.
3. Prepare splenocytes from mouse spleen. Lyse the red blood cells for 2 min at RT, using 2 mL of Lysing Solution for each spleen.
4. Add 18 mL of RPMI and centrifuge at 1,200 rpm for 5 to 10 min. Remove the supernatant.
5. Resuspend the cells and plate 5–7 x 10^6 cells per well into a 6-well plate.
6. Add NA/LE CD28 at a concentration of 2 µg/mL and incubate for 48 h at 37°C in 5% CO_2.
7. Harvest the cells after 48 h and proceed to the staining protocol.

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Procedure, BrdU labeling

1. Label the cells with BrdU according to the BrdU Flow Kit Instruction Manual.
2. Wash the cells once in 5 mL of Stain Buffer.

Procedure, staining

1. Fix cells with 2 mL of Fixation Buffer at a concentration of 1 x 10^7 cells per mL for 15 min at RT. Alternately, bulk fix the cells (see Procedural Notes).
2. Pellet cells by centrifugation at 1,400 rpm for 5 to 10 min. Remove the supernatant.
3. Wash the cells with 5 mL of PBS without FBS or BSA.
4. Pellet the cells by centrifugation at 1,400 rpm for 5 to 10 min. Remove the supernatant.
5. Vortex the pellet to loosen the cells.
6. Permeabilize the cells by adding 3 mL of ice cold Perm Buffer III (kept at -20°C), at a concentration of 1 x 10^7 cells per mL, for 30 min on ice. Gently vortex the pellet while adding the Perm Buffer to prevent cells from clumping.
7. Wash the cells once with PBS (1 x 10^6/mL) followed by washing twice with 5 mL of Stain Buffer.
8. Pellet the cells by centrifugation at 1,400 rpm for 5 to10 min. Remove the supernatant.
9. Vortex the pellet to loosen the cells.
10. If bulk cell fixation was done, aliquot the cells into BD Falcon tubes (1 x 10^6 cells per tube).
11. Treat the cells with Permeabilization Buffer, using 100 µL per tube and incubating for 10 min at RT.
12. Wash the cells with 1 mL of Stain Buffer.
13. Pellet by centrifugation at 1,400 rpm for 5 to 10 min. Remove the supernatant.
14. Re-fix the cells in 100 µL of Fixation Buffer for 5 min at RT.
15. Wash the cells with 1 mL of Stain Buffer.
16. Pellet by centrifugation at 1,400 rpm for 5 to 10 min. Remove the supernatant.
17. Resuspend the cells with 100 µL per tube of DNase (diluted to 300 µg/mL in DPBS) (the equivalent of 30 µg of DNase in each tube).
18. Cover the tubes with foil and incubate at 37°C for 1 hour.
19. Wash the cells with 1 mL of Stain Buffer.
20. Pellet by centrifugation at 1,400 rpm for 5 to 10 min. Remove the supernatant.
21. Stain the cells with fluorochrome-conjugated anti-BrdU and BD Phosflow antibodies and surface antibodies at their appropriate concentrations for 60 min at RT, in the dark.
22. Wash the cells with 1 mL of Stain Buffer.
23. Pellet by centrifugation at 1,400 rpm for 5 to10 min. Remove the supernatant.
24. To stain cells for total DNA for cell cycle analysis, incubate cells with DAPI (1 µg/mL in 400 µL) for 30 min at RT, in the dark.

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Flow Cytometric Analysis of Stained Cell Samples

Flow cytometric analysis of the samples can be performed by using a flow cytometer equipped with three lasers: a 488-nm blue laser, 635-nm red laser, and 407-nm violet laser or 355-nm UV laser. In this example, the staining was done using Stat5-PerCP-Cy5.5, CD4-FITC, BrdU-APC, and DAPI.

The compensation was performed using the automatic compensation procedure in BD FACSDiva™ software (see the *BD FACSDiva Software Reference Manual* for more details). The following single-stained controls were acquired to determine the optical spillover:

1. Unstained cells
2. CD4-FITC stained cells
3. pStat5-PerCP-Cy5.5 stained cells
4. BrdU-APC stained cells
5. DAPI stained cells

A linear scale was used for the acquisition of the DAPI, whereas a logarithmic scale was used for the fluorochromes FITC, PerCP-Cy5.5, and APC. The sample acquisition in this example was done by using the BD™ LSR II flow cytometer, and the analysis using BD FACSDiva software. The 488-nm blue laser was used to detect FITC (BP 530/30) and PerCP-Cy5.5 (BP 695/40), the 633-nm red laser was used to detect APC (BP 660/20), and the 355-nm UV laser was used to detect DAPI (BP 450/50).
(see the data on the next page)

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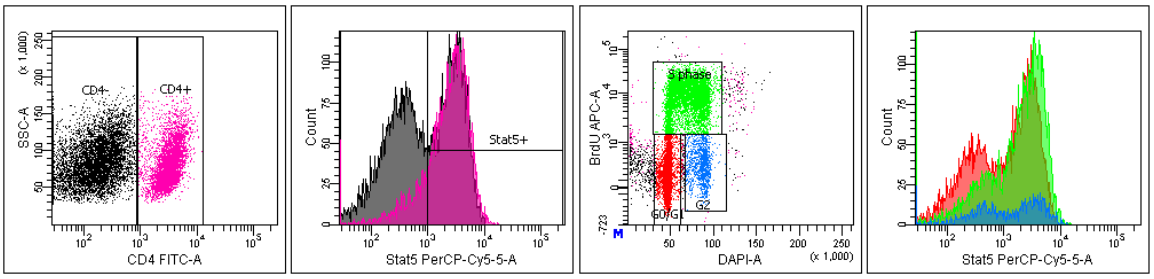
A.



B.

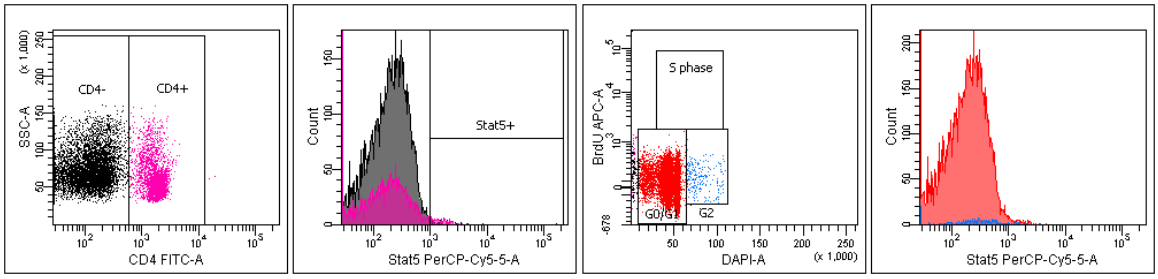


Figure 1. The cells were stimulated with anti-CD3 and anti-CD28 antibodies for 48 hours. NA/LE anti-mouse CD3ε was coated onto sterile tissue culture plates at a concentration of 10 µg/mL in sterile 1X PBS. Soluble NA/LE anti-mouse CD28 was used at 2 µg/mL (Figure 1A). The unstimulated cells were used as a negative control (Figure 1B). Eighty-three percent of the $CD4^+$ cells were Stat5 positive, whereas only 46% of the $CD4^-$ cells were Stat5 positive.