Intracellular Cytokine FACS-Staining Protocol

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Materials	Qty	Order Info
PMA	1mg	Phorbol 12-Myristate 13-Acetate: Sigma P-8139
Ionomycin		Ionomycin: Sigma I0634
Monensine		Monensine: Sigma M5273
KB 8301 (matrix metalloproteinase inhibitor)	0.5mg	KB 8301: Pharmingen Cat. 66131D
PBS/BSA/Azide		
Permeabilization buffer		Saponin: Sigma S-7900
Ethidium Monoazide Bromide (EMA)		EMA: Molecular Probes E-1374
4% Formaldehyde		
0.5% Paraformaldehyde		

Protocol prepared by D.K. Mitra, S.C. De Rosa, N. Watanabe

Cell Preparation

1. Harvest cells into conical tubes and place them on ice at the end of culture period
2. Pellet cells
3. Wash the cells once with ice-cold PBS/BSA/Azide (2mL)

Notes on Stimulation

Surface staining:

1. Suspend cells in ice-cold PBS/BSA/Azide (50µl for each test = 1-2 x 106 cells)
2. Transfer cells to 96-well plate containing the surface antibodies (if using EMA to exclude dead cells, include with surface stains, final conc. = 5µg/ml; this is 1:1000 of 5mg/ml stock; typically 10 µl of stock diluted with 490 µl of media, then use 5 µl per 100 µl final stain volume - minimize exposure to light until step 12 below). If using PI (e.g., with perforin), include PI with the surface stains; use 50x, twice the usual concentration.
3. Stain for 15 min on ice in dark
4. Add 150µl PBS and spin. Wash once with 200 µl PBS and spin.
5. Resuspend in 100µl PBS (containing no protein). If using EMA, expose to light for 5 to 10 minutes on ice at this point before fixation.

Fixation:

1. Add 100µl of 4% Formaldehyde and mix well (final conc. = 2%)
2. Let the cells stand at RT in dark for 20 min
3. Pellet the cells
4. Wash the cells twice with ice-cold PBS/BSA/Azide buffer
5. Pellet the cells and resuspend in 150 ml of permeabilization buffer. Mix gently with multichannel pipette
6. Incubate cells at room temperature for 10 min
7. Pellet cells (aspirate very carefully, or flick out supernatant)

Intracellular staining

1. Resuspend in 25 ml per well of permeabilization buffer containing intracellular Abs(cytokine, perforin)
2. Incubate cells at RT for 30 min in the dark
3. Wash twice (2.5x) with 150-200 ml of permeabilization buffer. (Note that one wash may be sufficient, but more washes may decrease the background).
4. Wash twice with 200 ml of PBS/BSA/Azide buffer (no Saponin)
5. Suspend cells in 200 ml of PBS/BSA/Azide buffer (no Saponin) and transfer to FACS tubes. For HIV samples, this final resuspension should be in staining media (without serum) containing 0.5% paraformaldehyde.
6. FACS analysis

Reagents and Solutions

1) PMA, Ionomycin

Stock solutions. (frozen separately in 10-20uL aliquots)

1mg/ml PMA (DMSO)
2 mM Iono (DMSO)

2) Monensine, KB 8301 (metalloproteinase inhibitor)

Stock solutions.

2 mM Monensine (ethanol)

10 mM KB 8301 (DMSO), as sent supplied by BD PharMingen, dissolve 0.5mg powder in 120µl DMSO. Store frozen.

3) Permeabilization buffer:

Mix: 5 ml 10% Saponin in PBS + 95 ml PBS/BSA/Azide buffer

a)10% Saponin

Mix: 5 g Saponin (Sigma) with 50 ml PBS, pH7.4

Place at 37°C until the saponin has dissolved completely
Sterile filter the mixture (0.22 ul)
Store at 4°C

b) PBS/BSA/Azide buffer

Mix: 50 ml 10x PBS, pH 7.4 with 450 ml Cell culture grade H2O and 0.5 ml 1 M Azide
Total: 500 ml
Layer 2.5 g of BSA on top of liquid mixture
Allow BSA to dissolve at RT without stirring
Sterile filter the mixture
Store at 4°C

4) Ethidium Monoazide bromide, EMA (Molecular Probes E-1374)

Prepare stock as 5 mg/ml in DMSO, freeze in single-use aliquots of 20µl in dark vials in a dessicator

5) Paraformaldeyhde (4%)

Paraformaldehyde is very toxic and aerates easily. Avoid breathing in the powder. Use a fume hood if necessary.

1. Mix required amount of paraformaldehyde (4g/100ml) to 2/3 final volume in ddH2O.
2. Heat to 60C while stirring in a fume hood (monitor temperature with thermometer).
3. Add 1 drop 2N NaOH to clear the solution.
4. Remove heat and add 1/3 vol 3x PBS.
5. Let cool and adjust to pH 7.2 with HCL.
6. Filter.

Notes on Stimulation

1. Separate PBMC using Ficol-Paque and wash 2.5 times with 10 ml culture medium

2. For prestaining with CD4 and CD62L, suspend cells (at least 2 x 106, but 4 x 106 is better) in 100µl culture medium, add stain, and incubate for 15 min at RT.

a) Half the cells will be unstimulated: add 50µl of stained cells to 950µl of culture medium in 24 well plate

b) Half the cells will be stimulated: add 1µl of metalloproteinase inhibitor (1:1000=10µM, stock is 10mM) to the 50µl of remaining cells; mix gently, and incubate a few minutes. In the meantime, to approx. 950 µl of culture medium in the 24 well plate, add 50µl PMA (diluted 1:1000 from 1mg/ml stock), 5µl ionomycin (of 1:10 dilution of 2 mM stock), 5µl monensin (of 1:10 dilution of 2 mM stock), and mix. If you choose to culture instead in 2ml, then double these amounts. Then add the cells, gently mix using P1000 pipetteman, and incubate at 37°C for 6 hours.

3. For prestaining with gd reagents, the procedure is the same except that after the staining, the cells must be washed once with large volume (3-5ml). Also, metalloproteinase inhibitor is not used. **Special note:** The unstimulated control cultures should not be prestained with the gd reagents; instead the gd reagents should be included with the other surface reagents at the time of staining. (The prestained unstimulated gd cells lose staining over 6 hrs in culture.)

4. For the cells that will be stained for **perforin**, no stimulation or prestaining is required. Set aside an appropriate number of cells and leave them on ice or at room temp during the 6 hr time period so that all cells can be stained simultaneously.