FACS Staining of phosphorylated antigens

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ABSTRACT

This is a protocol for the detection of phosphorylated antigens via flow cytometry without the use of commercially available kits. It is adapted from the publication linked after the description.

MATERIALS

- Fetal Bovine Serum Gibco - Thermo Fischer Catalog #10270106
- Methanol J.T. Baker Catalog #9093-03
- Paraformaldehyde Sigma Aldrich Catalog #P6148
- Phorbol 12-myristate 13-acetate Sigma Aldrich Catalog #P8139
- Dulbecco's Phosphate Buffered Saline Sigma Aldrich Catalog #D5652
- Dimethyl sulfoxide (DMSO) Sigma Aldrich Catalog #D2650
- IMDM Gibco - Thermo Fischer Catalog #12440053
- Gentamicin Gibco - Thermo Fischer Catalog #15750037
- 2-Mercaptoethanol (50 mM) Gibco - Thermo Fischer Catalog #31350010
- L-Glutamine (200 mM) Gibco - Thermo Fischer Catalog #25030081
- Ionomycin from Streptomyces conglobatus Sigma Aldrich Catalog #I9657
- Albumin Bovine Fraction V Serva, Germany Catalog #11924
SAFETY WARNINGS

Wear gloves and safety goggles while handling 8% PFA and methanol. Incubation with methanol only under a fume hood.

BEFORE START INSTRUCTIONS

Prepare IMDM (complete):

Add to the 500 ml IMDM Bottle:
25 ml fetal bovine serum (final concentration: 5%)
5 ml L-Glutamine (final concentration 2 mM)
500 µl Gentamicin (final concentration 50 µg/ml)
500 µl 2-Mercaptoethanol (final concentration 50 µM)

Prepare PBS with 5% BSA. Dilute part of it to 0.5%.

Prepare 1 mM Ionomycin stocks in DMSO.
Prepare 10 µg/ml PMA Stocks in DMSO.
Prepare 8% PFA Solution in 1xPBS.

Cool methanol to 4 °C.
Prepare ice bath under the fume hood for incubation with methanol.

Cool down the centrifuge.

Incubation

1. Resuspend cell suspension at a concentration of 5*10^6 cells/ml. Incubate for 2 h at 37 °C and 5% CO₂.
   - 02:00:00 First Incubation
   - 10 mL IMDM (complete) per Sample
   - 37 °C and 5% CO₂

2. Pipette 1 ml of single cell suspension in each of 6 FACS-Tubes. Label them accordingly (0 Minutes, 5 Minutes, 15 Minutes, 30 Minutes, 60 Minutes, 120 Minutes).
   - 02:00:00 longest incubation
   - 37 °C and 5% CO₂

3. Incubate the cells for the allotted time. Immediately after incubation take the samples out and add 200 µl 8% PFA (final concentration 1.5% PFA). Incubate at room temperature for 10 minutes.
00:10:00 for each sample
200 µL 8% PFA
20 °C

Permeabilization and Staining

4 Centrifuge for 5 minutes at 520 g and 4 °C. Decant supernatant. Resuspend samples in the return flow.

00:05:00 centrifugation
4 °C

5 Add 1 ml of ice cold methanol. Incubate for 30 minutes on ice.

1 mL Methanol per sample
00:30:00 Incubation on ice
4 °C on ice

6 Add 3 ml PBS/0,5% BSA to each sample. Centrifuge for 5 minutes at 520 g and 4 °C. Decant supernatant and Resuspend in 300 µl PBS/5% BSA.

300 µL PBS/5% BSA per sample
00:05:00 centrifugation
4 °C
3 mL PBS/0,5% BSA per sample

7 Transfer 75 µl of sample into a new FACS-Tube. Add 25 µl antibody mastermix.

Example for 10 stainings:

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dye</th>
<th>Clone</th>
<th>Volume/Sample</th>
<th>Volume in mastermix</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8a</td>
<td>PerCP</td>
<td>53-6.7</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>CD4</td>
<td>APC</td>
<td>RM4-5</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>TCRgd</td>
<td>FITC</td>
<td>eBioGL3</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>STAT3P</td>
<td>v450</td>
<td>4/P-STAT3</td>
<td>20</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PBS/5 % BSA</td>
</tr>
</tbody>
</table>

Incubate for 30 minutes at room temperature in the dark.

25 µL Mastermix per sample
20 °C
8 Add 3 ml PBS/0.5% BSA. Centrifuge for 5 Minutes at 520 g and 4 °C. Decant supernatant.

9 Repeat Step 8. Add 200 µl of PBS/0.5% BSA for measuring.