The Fleischman Lab

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Phospho Flow Staining

Introduction

Phosphoflow is a modification of intracellular staining techniques that allows the detection of phosphorylated proteins. Cells are stimulated to initiate signaling cascades, then fixed, permeabilized, and stained for intracellular phosphoproteins.

Materials

1. Cells for stimulation
2. Stimulant (e.g. LPS, R848, IFNα, IFNγ)
3. MicroFACS tubes
4. PFSB (PBS + 0.5% BSA)
5. 16% paraformaldehyde
6. Ice-cold methanol
7. Phosphoflow antibodies
8. Micropipettes and tips
9. 37°C 5%CO_2 incubator with ≥95% humidity
10. Swinging bucket centrifuge with plate carriers or microcentrifuge
11. Flow cytometer (BD Accuri)

Protocol		*Notes*
	A. Stimulate Cells	
1.	Add 10µl of each stimulant to microFACS tubes.	*Keep everything at 37°C until fixation, including stimulants.*
2.	Add 90µl of cells to each tube.	
3.	Incubate at 37°C for 15 minutes to 2 hours.	*Length of time may vary depending on your experiment.*
	B. Fix Cells	
1.	Add 10µl 16% paraformaldehyde to each tube.	*Solution should turn yellow in color.*
2.	Incubate at room temperature for 10 minutes.	
	C. Permeabilize Cells	
1.	Add 1ml of ice-cold methanol per tube.	*Methanol is stored in the -20°C kneehole freezer.*
2.	Incubate at 4°C for 15 minutes.	
3.	Centrifuge for 10 minutes at 1500rpm (plate spinner) or 500g (microcentrifuge).	
	D. Stain Cells	
1.	Fill tubes with 1ml PFSB.	
2.	Centrifuge for 10 minutes at 1500rpm (plate spinner) or 500g (microcentrifuge).	
3.	Aspirate supernatant and repeat steps 1 and 2.	

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<tr><td>4.</td><td>After second wash, aspirate all but 100µl of supernatant. Vortex to resuspend cells.</td><td></td></tr>
<tr><td>5.</td><td>Prepare a master mix containing your antibodies:<table><tr><th>Reagent</th><th>For 1 tube</th><th>For X tubes</th></tr><tr><td>PFSB</td><td>50µl</td><td>50(x+1)</td></tr><tr><td>Antibody #1</td><td>2µl</td><td>2(x+1)</td></tr><tr><td>Antibody #2</td><td>2µl</td><td>2(x+1)</td></tr><tr><td>Antibody #3</td><td>2µl</td><td>2(x+1)</td></tr></table></td><td>Prepare enough master mix for at least 1 additional tube. Exclude single-stain controls from your calculations.</td></tr>
<tr><td>6.</td><td>For controls, prepare a new set of tubes containing:<ol type="a">
Unstained – add 50µl PFSB
Single stains – add 50µl PFSB and 2-5µl of the appropriate antibody.</td><td></td></tr>
<tr><td>7.</td><td>For sample tubes, prepare a new set of tubes and aliquot 50µl per tube.</td><td></td></tr>
<tr><td>8.</td><td>Add 75µl of cells to each sample tube.</td><td></td></tr>
<tr><td>9.</td><td>Pool the remaining cells from each tube into a new tube labeled “compensation mix.” Aliquot 75µl of mixture to all control tubes.</td><td></td></tr>
<tr><td>10.</td><td>Vortex tubes to mix and incubate at room temperature for 30 minutes.</td><td>Place cells in a drawer to keep dark!</td></tr>
<tr><td>11.</td><td>Fill tubes with 1ml PFSB.</td><td></td></tr>
<tr><td>12.</td><td>Centrifuge for 10 minutes at 1500rpm (plate spinner) or 500g (microcentrifuge).</td><td></td></tr>
<tr><td>13.</td><td>Aspirate all but 100µl of supernatant and vortex to resuspend cells. Cells are ready to be analyzed on the cytometer.</td><td></td></tr>
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