The Fleischman Lab

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Title: General Cell Surface Staining and Annexin V Staining

Introduction

Annexin V (AV) is a marker of apoptosis in cells that can be combined with propidium iodine (PI) to distinguish between healthy (AV-), apoptotic (AV+ PI-), and necrotic (PI+) cell populations. AV staining can be combined with standard cell surface staining methods to examine apoptosis and necrosis in specific cell types. Cell surface staining is performed first, followed by AV staining in a specific AV binding buffer.

Materials

1. Cells (e.g. mononuclear cells)
2. FACS tubes
3. PFSB (PBS + 0.5% BSA)
4. Cell surface antibodies
5. 1x Annexin V binding buffer (dilute from 10x stock)
6. Annexin V-PE
7. Propidium iodine (PI) – we have diluted PI in the FACS refrigerator
8. Transfer pipettes
9. Micropipettes and tips
10. Swinging bucket centrifuge
11. Flow cytometer (BD Accuri)

Protocol	A. Prepare Cells	*Notes*
1.	For Annexin V staining, treat cells with drug or UV to induce apoptosis.	*The specific treatment will depend on your experiment.*
2.	Harvest cells into FACS tubes and fill tube with PFSB.	
3.	Centrifuge at 1200rpm for 10 minutes. Discard supernatant.	
4.	Label FACS tubes for staining. Be sure to include single-stain controls (e.g. unstained, FITC alone, AV-PE alone, etc.)	
5.	Resuspend pellets in 100-200µl PFSB and transfer a small amount from each condition to a single, pooled FACS tube. Vortex to mix.	*The final volume of the pooled sample should be large enough to distribute amongst unstained and single stain control tubes.*
6.	Evenly distribute the pooled cells between all control tubes (i.e. unstained and single stain tubes).	
7.	Add 1ml PFSB per tube.	
8.	Centrifuge at 1200rpm for 10 minutes. Discard supernatant.	

<table>
<tr><td></td><td colspan="2">B. Cell Surface Staining</td><td></td></tr>
<tr><td>1.</td><td colspan="2">Prepare a master mix containing all cell surface antibodies; for example:
<table>
<tr><th>Reagent</th><th>For 1 tube</th><th>For X tubes</th></tr>
<tr><td>PFSB</td><td>100µl</td><td>100x</td></tr>
<tr><td>Antibody #1 (e.g. FITC)</td><td>1-2µl</td><td>1-2x</td></tr>
<tr><td>Antibody #2 (e.g. APC)</td><td>1-2µl</td><td>1-2x</td></tr>
</table></td><td>Certain antibodies are brighter than others. For a dim antibody, use 2µl per tube. For a bright antibody, use only 1µl per tube.

Prepare enough master mix for at least 1 additional tube.</td></tr>
<tr><td>2.</td><td colspan="2">For control tubes:
a. Unstained – add nothing
b. Single stains – add 100µl PFSB and 2µl of the appropriate antibody
c. Intracellular single stains (e.g. Annexin V) – add nothing</td><td></td></tr>
<tr><td>3.</td><td colspan="2">For sample tubes, add 100µl of staining master mix.</td><td></td></tr>
<tr><td>4.</td><td colspan="2">Incubate all tubes for 30 minutes at 4°C.</td><td></td></tr>
<tr><td>5.</td><td colspan="2">Add 1ml PFSB to each tube.</td><td></td></tr>
<tr><td>6.</td><td colspan="2">Centrifuge at 1200rpm for 10 minutes. Discard supernatant.</td><td>Cells may be resuspended in a small volume of PFSB and analyzed on the cytometer at this point.</td></tr>
<tr><td></td><td colspan="2">C. Annexin V Staining</td><td></td></tr>
<tr><td>1.</td><td colspan="2">Prepare a master mix containing Annexin V:
<table>
<tr><th>Reagent</th><th>For 1 tube</th><th>For X tubes</th></tr>
<tr><td>1x AV Binding Buffer</td><td>200µl</td><td>200x</td></tr>
<tr><td>Annexin V-PE</td><td>2µl</td><td>2x</td></tr>
</table></td><td>Prepare enough master mix for at least 1 additional tube. Include Annexin V sinlge stain control tube in your calculations.</td></tr>
<tr><td>2.</td><td colspan="2">Add 200µl master mix per tube.</td><td></td></tr>
<tr><td>3.</td><td colspan="2">Incubate in the dark at room temperature for 15-30 minutes.</td><td>Tubes can be placed in an empty drawer to keep them dark.</td></tr>
<tr><td>4.</td><td colspan="2">Add 1ml 1x AV binding buffer to each tube.</td><td></td></tr>
<tr><td>5.</td><td colspan="2">Centrifuge at 1200rpm for 10 minutes. Discard supernatant.</td><td></td></tr>
<tr><td>6.</td><td colspan="2">Resuspend cells in a small volume of AV binding buffer.</td><td></td></tr>
<tr><td>7.</td><td colspan="2">Using a transfer pipette, add 2 drops of diluted PI per tube and vortex to mix. Cells are ready to be analyzed on the cytometer.</td><td>Be careful not to touch the transfer pipette to the sides of the tubes.</td></tr>
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