Apoptosis Assay

- Daniela Janevska

List of Reagents:

FITC-Annexin-V – BD Biosciences (cat#556420)

10X Annexin V Binding Buffer – BD Biosciences (cat# 556454)

DAPI – ThermoFisher Scientific (cat# 62248)

Recombinant human, rh IL-5 – R&D Systems (cat# P05113)

IgG 1 isotype control – Made for the Bochner lab, not commercially available

AutoMACS Running Buffer (FACS Buffer) – Miltenybiotech (cat#130-091-221)

Experimental Protocol:

1. Following isolation, prime eosinophils in 30 ng/mL rhIL-5 overnight
2. In a 96 well plate, plate 200,000 eosinophils per well
3. To appropriately labelled wells, add 2.5 μg/mL 2C4 (430 μg/mL stock) or IgG1 isotype control (960 μg/mL stock)
4. Gently mix plate to distribute antibody
5. Incubate plate at 37°C for 18 – 24 hrs
6. Following overnight incubation, transfer cells into FACS tubes and wash cells with 500 μL of cold FACS buffer (1X).
7. Spin cells down at 150 x g, 4°C for 5 min. Decant supernatant, being careful not to disturb the cell pellet.
8. To each tube resuspend the cells in 100 μL Annexin V/DAPI solution:
    a. 10 μL 10X binding buffer
    b. 5 μL Annexin V – FITC
    c. 85 μL dH_2O
    d. DAPI (stock 14.2 mM)
9. Incubate cells in the dark for 15 min at room temperature
10. Following incubation, add 400 μL of 1X Annexin V binding detection buffer (10X stock)
11. Analyze apoptosis using FACS

ROS – ID Total ROS Assay

- **Daniela Janevska**

Reagents:

ROS Assay kit (cat# Enzo Life Sciences, ENZ-51011)

FACS tube - Corning (cat# 352052)

BD LSR II flow cytometer

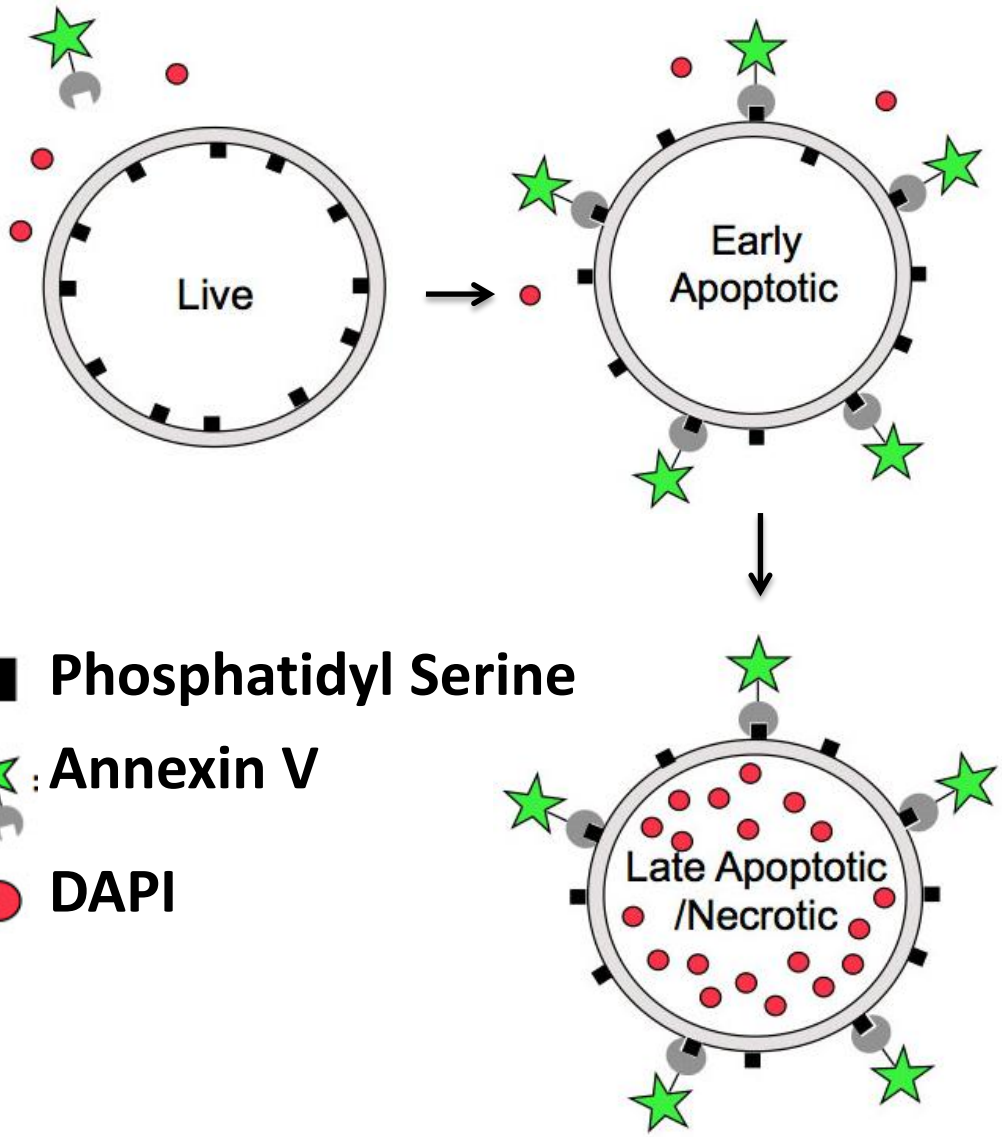
Experimental Protocol:

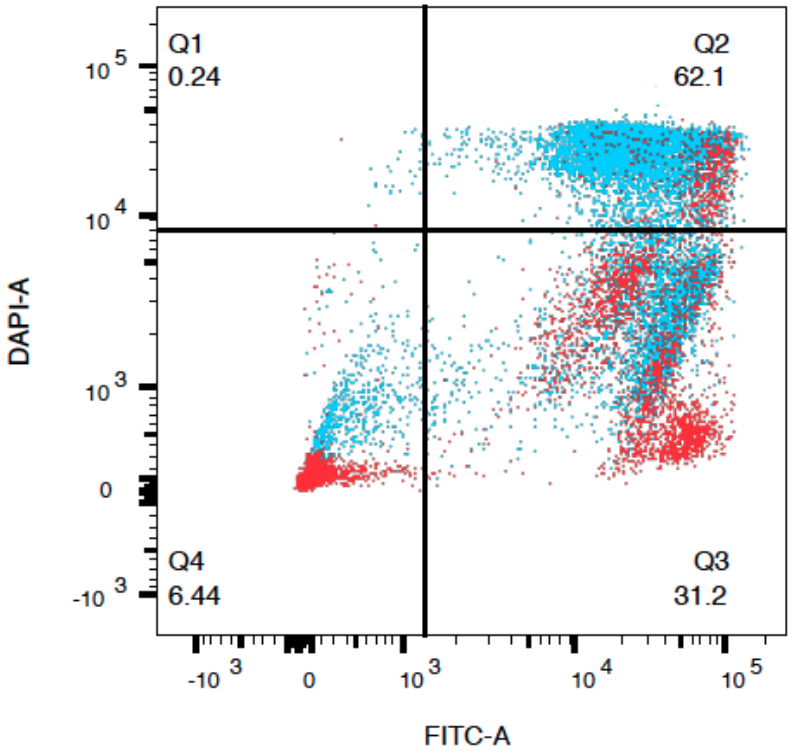
1. Following isolation, prime eosinophils in 30 ng/mL rhIL-5 overnight
2. Add 500,000 eosinophils per FACS tube
3. To appropriate tubes, add 2.5 µg/mL 2C4 (430 µg/mL stock) or IgG1 isotype control (960 µg/mL stock)
4. Incubate cells at 37°C for 1 hour
    a. As a positive control, add 200 µM Pyocyanin (10mM stock) and incubate for 30 mins at 37°C
5. Following incubation, add 1 mL 1X PBS (provided with kit)
6. Spin cells down at 150 x g, 4°C for 5 min. Decant supernatant, being careful not to disturb the cell pellet.
7. Resuspend cells with 400µL of ROS Detection Solution
    a. To make ROS Detection solution, add 2 µL of Oxidative stress Detection Reagent to 10 mL of 1X PBS
8. Load cells at 37°C for 30 min
9. Analyze ROS levels using FACS

*** Note: This protocol was altered to fit our needs. Manufacturers protocol was only used as a guideline.

***For a more detailed protocol provided by the manufacturer and other ways to use this kit to measure ROS, please refer to product manual **http://static.enzolifesciences.com/fileadmin/files/manual/ENZ-51011_insert.pdf** – ROS-ID Total ROS Detection Kit for fluorescence microscopy, flow cytometry and microplate assay.

Apoptosis Assay





	Sample Name	Subset Name	Count
	EosViability_IgG1_002.fcs	non-debris	10086
	EosViability_2C4_003.fcs	non-debris	10132

ROS – ID Assay

- Directly monitors global levels of intracellular reactive oxygen species (ROS), e.g. mitochondrial ROS
- Distinguishes between different ROS, such as hydrogen peroxide, peroxynitrite and hydroxyl radicals.
- Kit can detect ROS using IF imaging, FACS, and plate reader.
- In our experiments we stimulate with:
- IgG1 – Isotype control
- 2C4 – mouse monoclonal, anti-Siglec-8
- Pyocyanin – A bacterial toxin that enhances oxidative metabolism and increases the formation of Intracellular ROS.