

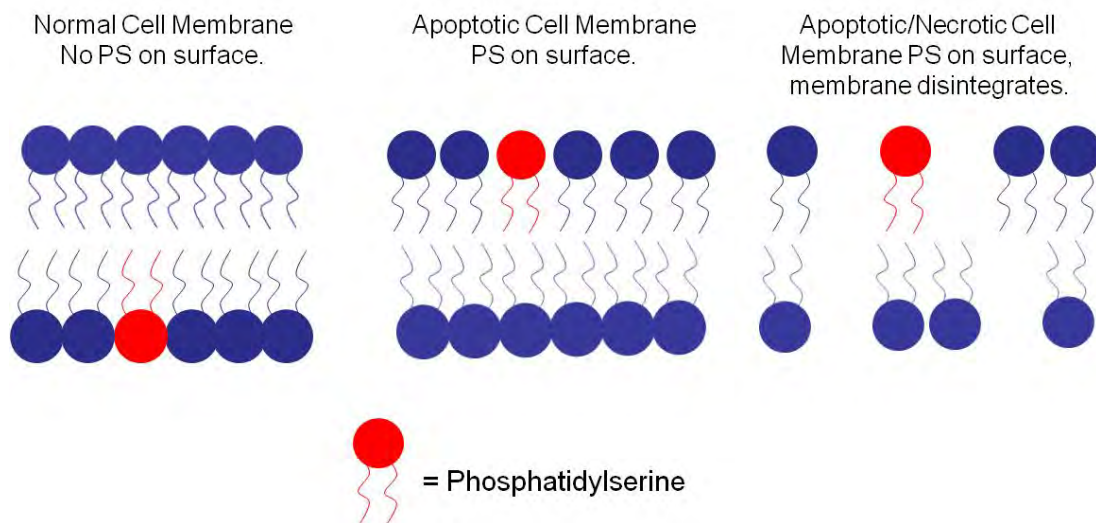
The Annexin V Apoptosis Assay

Development of the Annexin V Apoptosis Assay:

- 1990 Andree et al. found that a protein, Vascular Anticoagulant α , bound to phospholipid bilayers in a calcium dependent manner. The protein was later renamed Annexin V.
- 1992 Fadok et al. discovered that macrophages specifically recognize phosphatidylserine (PS) that is exposed on the surface of lymphocytes early in the apoptotic process. This PS is normally on the inner leaflet of the membrane.
- 1994 Koopman et al. developed a flow cytometric assay using Annexin V conjugated to FITC to measure Annexin V binding to apoptotic cells. The authors stained control and serum starved cells with ethidium bromide and Annexin V-FITC. Binding of Annexin V correlated with other changes consistent with apoptosis, including changes in nuclear morphology, DNA fragmentation and membrane leaflet symmetry. Only cells that stained with both Annexin V and ethidium bromide demonstrated chromatin condensation, a late indicator of apoptosis.

Principles of the Annexin V Apoptosis Assay:

- Phospholipids of the cell membrane are asymmetrically distributed between the inner and outer leaflets of the membrane. Phosphatidylcholine and sphingomyelin are exposed on the external leaflet of the lipid bilayer, while phosphatidylserine is located on the inner surface.
- During apoptosis, this asymmetry is disrupted and phosphatidylserine becomes exposed on the outside surface of the plasma membrane. Because the anticoagulant protein Annexin V binds with high affinity to phosphatidylserine, fluorochrome-conjugated Annexin V can be used to detect apoptotic cells by flow cytometry.



Reagents and Protocol (adapted from Invitrogen):

Annexin V is commercially available, conjugated to most common fluorochromes.

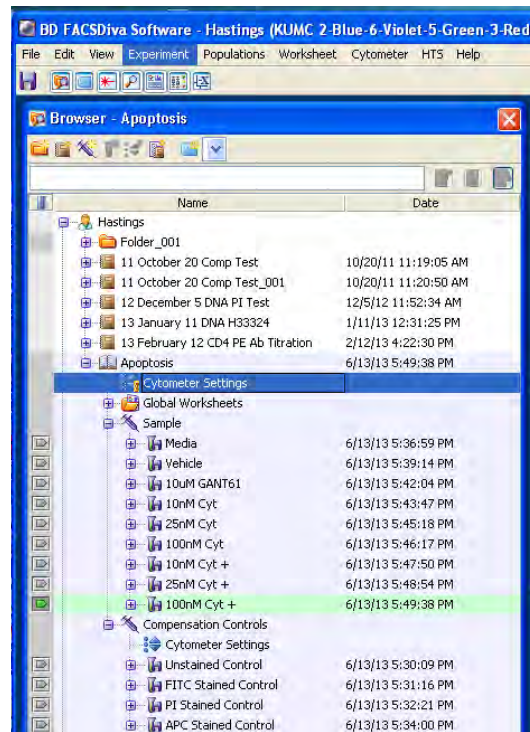
Propidium iodide (PI)

Binding buffer: 10 mM Hepes adjusted to pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂

Always titer the Annexin V and PI to determine the correct concentrations for your assays.

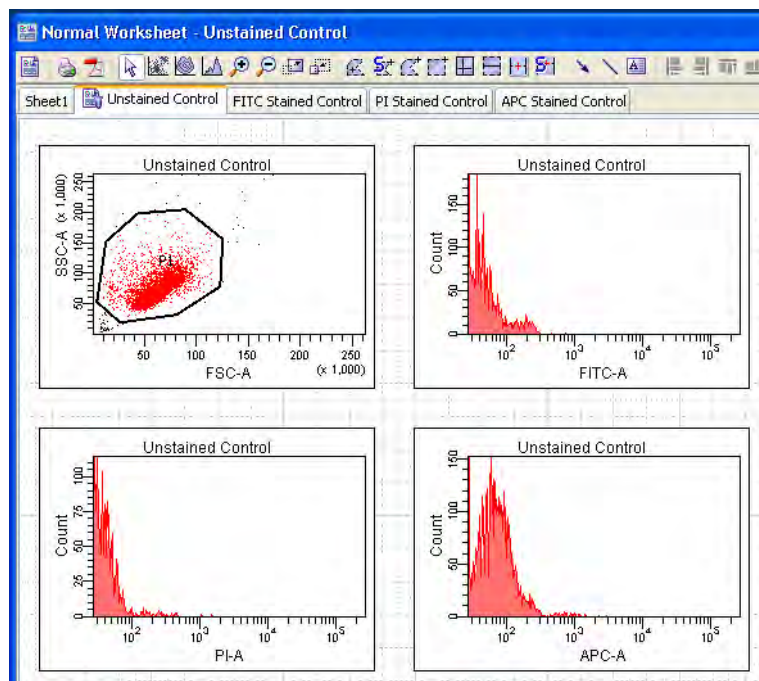
1. Induce apoptosis in cells using the desired method.
 - A. Prepare a negative control by incubating cells in the absence of inducing agent.
 - B. Prepare a positive control by inducing cell death with a potent method, such as incubating cells at 55°C for 20 min. Because this is such a potent method for inducing cell death, mix the positive control cells with some negative control cells so that positive and negative peaks are detectable.
2. Prepare 1X Annexin-binding buffer. All Invitrogen Annexin V kits, come with Annexin V binding buffer.
3. Prepare a 100 µg/mL working solution of PI by diluting 5 µL of the 1 mg/mL PI stock solution in 45 µL 1X Annexin-binding buffer. Store the unused portion of this working solution for future experiments.
4. Harvest the cells after the incubation period and wash in cold phosphate-buffered saline (PBS). Re-suspend the cells in 1X Annexin-binding buffer at a concentration of $\sim 1 \times 10^6$ cells/mL, preparing a sufficient volume to have 100 µL per assay.
5. Add Annexin V and < 1 µL 100 µg/mL PI working solution (prepared in step 4) to each 100 µL of cell suspension.
6. Incubate the cells at room temperature for 15 minutes in the dark.
7. After the incubation period, add 400 µL 1X Annexin-binding buffer, mix gently, and keep the samples on ice. As soon as possible, analyze the stained cells by flow cytometry.

Running samples on the LSR II:

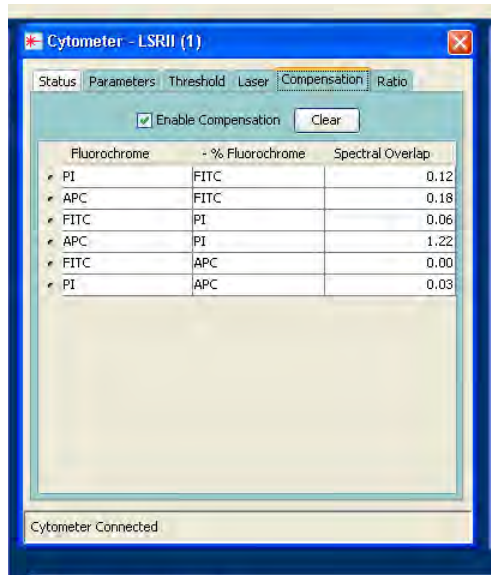


Set up experiment and the compensation controls.

Run the compensation controls, in this experiment, the cells were simultaneously stained with Annexin V-FITC and an antibody conjugated to APC.

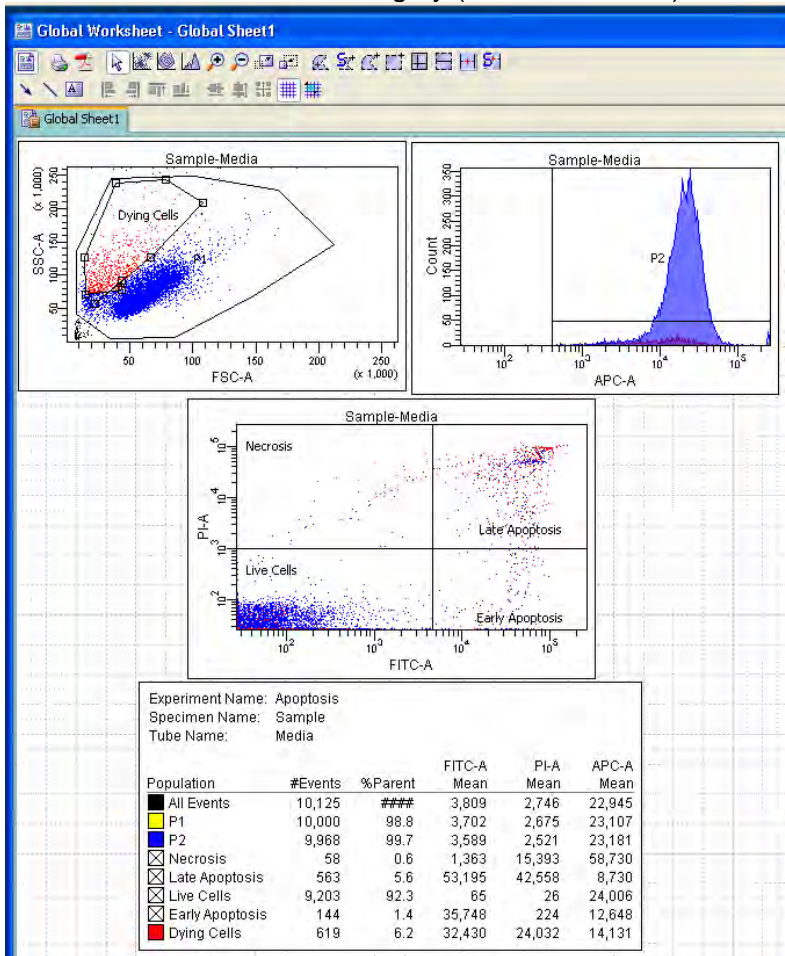


Because APC is excited by the Red (637 nm) laser, FITC is excited by the Blue (488 nm) laser, and PI is excited by the Green (552 nm) the compensation values are minimal.



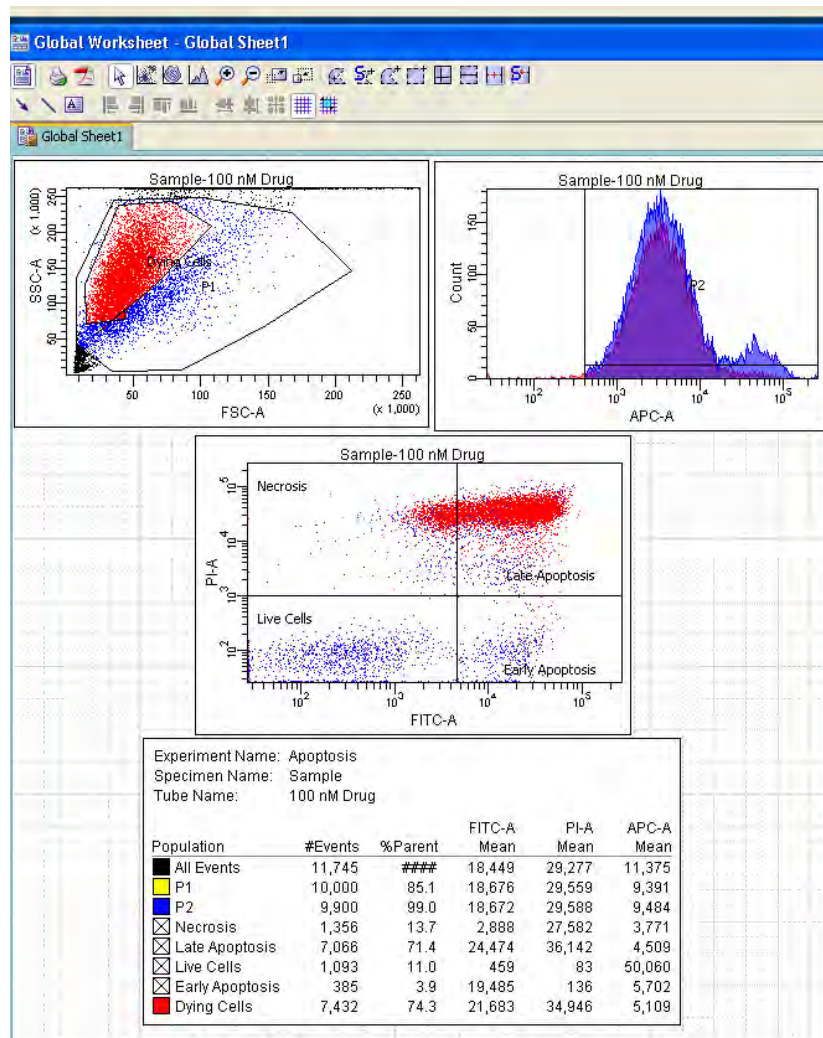
Representative media-only control data:

Apoptotic cells shrink and lose their membrane integrity (illustrated in red).



Representative apoptotic cell data:

This drug treatment induces apoptosis. The cells shrink and their membranes lose integrity. First, Annexin V staining becomes detectable and then, as the cells become permeable, PI gains access to the DNA.



References:

- Andree, H. A. M. et al. (1990) Binding of Vascular Anticoagulant α (VAC α) to Planar Phospholipid Bilayers. *The Journal of Biological Chemistry* 265, 9, 4923-4928.
- Fadok, V. et al. (1992) Exposure of Phosphatidylserine on the Surface of Apoptotic Lymphocytes triggers specific recognition and removal by Macrophages. *The Journal of Immunology* 148, 2207-2216.
- Koopman, G. et al. (1994) Annexin V for Flow Cytometric Detection of Phosphatidylserine Expression on B cells undergoing Apoptosis. *Blood* 84, 1415-1420.