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Annexin V and Propidium Iodide Labeling

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Abstract

Staining with annexin V and propidium iodide (PI) provides researchers with a way to identify different types of cell death—either necrosis or apoptosis. This technique relies on two components. The first, annexin V, is a protein that binds certain phospholipids called phosphatidylserines, which normally occur only in the inner, cytoplasm-facing leaflet of a cell's membrane, but become "flipped" to the outer leaflet during the early stages of apoptosis. The second component is the DNA-binding dye molecule PI, which can only enter cells when their membranes are ruptured —a characteristic of both necrosis and late apoptosis.

This video article begins with a review of the concepts behind annexin V and PI staining, and emphasizes how differential patterns of staining can be used to distinguish between cells progressing down different death pathways. We then review a generalized protocol for this technique, followed by a description of how researchers are currently using annexin V and PI staining to better understand cell death.

Transcript

Annexin V and propidium iodide (PI) labeling of cells is a technique used to identify cell death, and distinguish between its different pathways: apoptosis, or programmed cell death, and necrosis. Cells undergo distinct morphological changes depending on the pathway. By identifying the specific conditions that lead a cell to undergo apoptosis or necrosis, scientists gain insight into cellular physiology and the pathophysiology of disease. Annexin V and PI labeling, followed by flow cytometry, has been established as one of the most efficient methods to categorize the type of cell death.

Today, we'll describe how this assay helps in identification of cell death pathways, how to perform this technique, and some exciting example experiments that use this method to answer important questions in the field of cell biology.

First, let's take a look at the principles behind annexin V and PI staining.

As mentioned earlier, apoptotic cells exhibit distinctive morphological features. These include cell shrinkage, membrane blebbing, nuclear fragmentation, and the appearance of apoptotic bodies. In addition, following induction of apoptosis—that is, at the early apoptotic stage—certain characteristic changes appear on the membrane. A normal cell membrane has all the phosphatidylserine or PS residues on the inner leaflet. However, in early apoptotic cells some of these PS residues are translocated to the outer surface—but how?

To understand that, let's take a step back. During apoptosis, it is well known that cytosolic caspases are activated. These enzymes in turn activate a membrane-bound enzyme called scramblase. Scramblase "scrambles" the cell membrane by translocating PS residues from the cytoplasmic side to the outer surface. In contrast, during necrosis PS residues remain where they are, but the membrane itself ruptures. Annexin V and PI labeling capitalize on these differences in membrane alterations of the two types of cell death.

Annexin V conjugated with fluorescent dye, such as fluorescein isothiocyanate—commonly known as FITC—is a membrane impermeable, natural ligand for PS, and therefore easily identifies early apoptotic cells by binding to the externalized PS. However, following membrane rupture, which occurs during necrosis and also during later stages of apoptosis, annexin can also bind to PS on the inner face and yield false positives.

To avoid this, scientists use PI. This DNA binding dye molecule is again membrane impermeable, and can only enter a cell when the membrane is ruptured. Therefore, if a cell is only annexin stained it is early apoptotic, whereas a cell that is both PI and annexin stained could be either necrotic or late apoptotic.

Now that you've learned the basic principles behind this assay, let's go through the procedure of staining and analyzing cell death.

First, cells are harvested and centrifuged at low speed to prevent any morphological disruption, and resuspended in physiological washing buffer such as phosphate buffered saline, also known as PBS. Following another centrifugation step, cells are resuspended in annexin V binding buffer containing calcium, which is necessary for annexin binding to PS. Annexin V conjugated with FITC is then added to the solution, which is incubated at room temperature in the dark to allow annexin and PS association. In the next step, PI is directly added to the annexin staining cell solution and incubated in the dark. After incubation, cells are washed in PBS by centrifugation, resuspended in washing buffer, and kept on ice. Now they are ready for analysis.

Fluorescence activity of cells is analyzed by flow cytometry, a laser-based technique that captures fluorescence data from single cells suspended in a stream of fluid. After fluorescence calibration using cells separately stained with each dye, data from dual stained cells are acquired. Fluorescence signals from the cell population are used to create a plot where annexin-bound FITC intensity is plotted on the logarithmic X-axis, and PI on the logarithmic Y-axis. Cells that are annexin-FITC positive and PI negative will cluster on the lower right side of the plot, representing the early apoptotic cells, and cells double positive for annexin-FITC and PI will occur on the upper right, representing the late apoptotic or necrotic cells. The cells that remain unstained or insignificantly stained for both annexin and PI are live cells.

Since you now know why cell biologists rely on this technique to study cell death, why don't we review some of its applications.

Using this procedure, scientists can follow which intracellular proteins are involved in apoptosis. In this video, researchers analyzed the effect of cisplatin, an anti-cancer drug, on normal kidney cells to see if it induces apoptosis. One set of cells was transduced to overexpress an active form

of the enzyme protein kinase C, or PKC, and the other set was transduced with a non-functional form—dominant negative PKC. Cells expressing PKC and treated with cisplatin underwent apoptosis over time, but cells expressing the inactive dominant negative PKC were resistant to apoptosis, indicating that the enzyme is a key player in the cisplatin-induced apoptosis pathway.

Researchers also use these stains to test the cytotoxic potential of specific T cells. Cytotoxic T cells can recognize specific membrane proteins on its target cell, and induce its death upon binding to it. Here, researchers incubated antigen-specific T cells with target tumor cells and annexin V, and then observed induction of tumor cell apoptosis by T cell engagement. Flow cytometry data revealed the percentage of target cell death in the presence and absence of cytotoxic T cells.

Lastly, scientists use this method to evaluate cell viability following gene delivery. In this case, researchers wanted to assess cell survival after nucleofection—a method that uses a combination of chemicals and an applied electric field to create transient pores in cellular and nuclear membranes, thereby facilitating gene delivery. By using annexin V plus a dye called 7-aminoactinomycin D or 7-AAD, which, similar to PI binds to DNA, they showed that nucleofection caused low cell death by either apoptosis or necrosis.

You've just watched JoVE's video on annexin V and PI labeling. In this video, we briefly discussed the distinguishing features of apoptotic and necrotic cells, reviewed the principles behind this method, watched a generalized procedure for this commonly used technique, and previewed some applications. Given the importance of understanding how different cells die under different conditions, annexin V and PI labeling serves as an important tool for cell biologists interested in this phenomenon. As always, thanks for watching!