Tools for Assessing Cell Events

Apoptosis, Cell Cycle, and Cell Proliferation

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Life, Death, and Cell Proliferation

The balance of cell proliferation and apoptosis is important for both development and normal tissue homeostasis. Cell proliferation is an increase in the number of cells as a result of growth and division. Cell proliferation is regulated by the cell cycle, which is divided into a series of phases. Apoptosis, or programmed cell death, results in controlled self-destruction.

Several methods have been developed to assess apoptosis, cell cycle, and cell proliferation. BD Biosciences offers a complete portfolio of reagents and tools to allow exploration of the cellular features of these processes.

Over the years multicolor flow cytometry has become essential in the study of apoptosis, cell cycle, and cell proliferation. Success of the technology results from its ability to monitor these processes along with other cellular events, such as protein phosphorylation or cytokine secretion, within heterogeneous cell populations. BD Biosciences continues to innovate in this area with new products such as the BD Horizon™ violet cell proliferation dye 450 (VPD450) and popular reagents such as antibodies to cleaved PARP and caspase-3 available in new formats and for different types of applications.

In addition to flow cytometry products, BD Biosciences carries a broad portfolio of reagents for determination and detection of apoptotic and proliferative events by ELISA, immunohistochemistry, cell imaging, or Western blot.

As part of our commitment to maximize scientific results, BD Biosciences provides a variety of tools to assist customers in their experimental setup and analysis. These include a decision tree to guide in the selection of the most suitable methods for a specific study.

BD Biosciences carries high-quality reagents in the latest formats to examine cell cycle, proliferation, and apoptosis across a variety of platforms, in applications from basic research to drug screening.

Cell Cycle and Cell Proliferation: An Overview

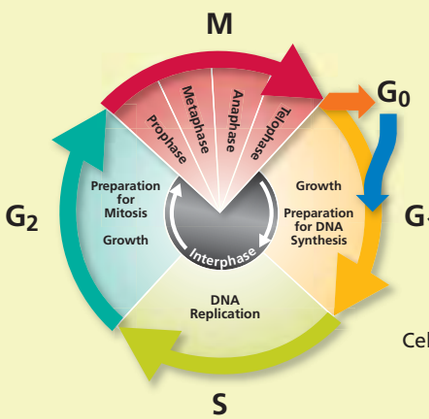
To help researchers better understand the fundamental cellular mechanisms involved in immunity, inflammation, hematopoiesis, neoplasia, and other biological responses, BD Biosciences offers a range of tools including antibodies, kits, and systems to measure proliferative responses. Using flow cytometry, immunofluorescence, or immunohistochemistry, researchers can quickly and accurately determine the cell cycle status or tissue localization of individual cells within proliferating populations. These tools include:

- DNA dyes, propidium iodide (PI), 7-aminoactinomycin D (7-AAD)
- Antibodies against cyclins, retinoblastoma, and phosphorylated histone H3

In adaptive immunity, specific T and B lymphocytes undergo clonal expansion (division, proliferation, and differentiation) in response to foreign antigenic stimulation. Cell growth, replication, and division in eukaryotic cells occur according to a highly controlled series of events called the cell cycle.[1]

The Cell Cycle

The cell cycle has two major phases: interphase, the phase between mitotic events, and the mitotic phase, where the mother cell divides into two genetically identical daughter cells. Interphase has three distinct, successive stages. During the first stage called G1, cells "monitor" their environment, and when the requisite signals are received, the cells synthesize RNA and proteins to induce growth. When conditions are right, cells enter the S stage of the cell cycle and "commit" to DNA synthesis and replicate their chromosomal DNA. Finally in the G2 phase cells continue to grow and prepare for mitosis.



Cell cycle phases

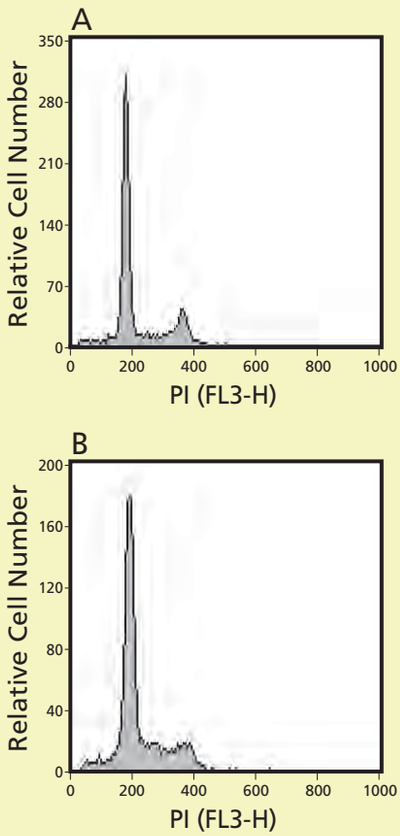
Measures	Reagents	Mechanism	Technology	Sample Types
DNA	Propidium Iodide (PI), 7-aminoactinomycin D (7-AAD)	Interaction into DNA double strands	Flow cytometry	Fixed, permeabilized, and for live/dead discrimination in intact cells
Cell Dyes	BD Horizon violet proliferation dye 450 (VPD450)	Diffuses into live cells and is hydrolyzed by intracellular non-specific esterases to become fluorescent products.	Flow cytometry	Live proliferating cells
Newly Synthesized DNA	BrdU and antibodies to BrdU	Bromodeoxy uridine replaces thymidine (T) in dividing DNA. It is then detected by antibodies to BrdU.	Flow cytometry, cell imaging, immunohistochemistry	Fixed and permeabilized cells, treated tissues (cell imaging, immunohistochemistry only)
Protein Level	Antibodies to Ki67, PCNA	Levels increase as a result of proliferation.	Flow cytometry, bioimaging, immunohistochemistry, Western blot	Fixed cells, tissues, and extracts
Protein Level	Antibodies to cyclins, retinoblastoma (Rb), other cell cycle markers	Levels go up and down at different stages of the cell cycle.	Flow cytometry, bioimaging, immunohistochemistry, Western blot	Fixed cells, tissues, and extracts
Protein Modification	Antibodies to phosphorylated histone H3, cyclin dependent kinases (cdk)	Proteins become phosphorylated as a result of proliferation or changes to the cell cycle.	Flow cytometry, bioimaging, immunohistochemistry, Western blot	
BD™ CBA (for quantitative detection)	Fixed cells, tissues, and extracts			

Methods for the study of cell cycle and proliferation

Analysis of Cellular DNA Content
BD Biosciences offers a wide variety of reagents to study the cell cycle. Reagents include DNA dyes such as propidium iodide (PI) and 7-amino actinomycin D (7-AAD). In addition, the BD Cycletest Plus reagent kit includes PI and other reagents to degrade proteins and RNA to allow more precise DNA measurement. The samples are subsequently analyzed using flow cytometry to assess ploidy, identify abnormal DNA stemlines, and estimate the DNA index (DI) and cell cycle phase distributions of stemlines.

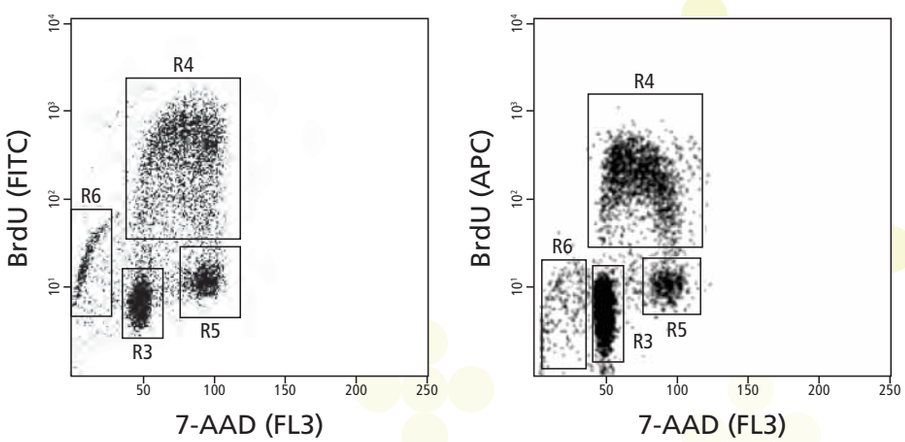
During the cell cycle phases, DNA levels change, facilitating the use of DNA dyes such as 7-AAD to generate characteristic cellular DNA content profiles (see the figure below).

As cells go through the phases of the cell cycle, proteins such as histone H3 Ser28 become modified or change in expression.[2] To facilitate DNA replication the histone is modified, opening the chromatin to allow entry of replication machinery. To further support the study of cell cycle, BD Biosciences carries antibodies to these proteins to use for imaging or flow cytometry applications.



Measurement of DNA content using PI. Two mouse T-cell lines MGG3 (A) and C20.4 (B) were treated with 100 µg/mL RNase A and then stained with 10 µg/mL of PI.

Cell cycle analysis of a population stained for incorporated BrdU and total DNA levels (7-AAD).
Human PBMCs were stimulated with anti-CD3/CD28 for 48 hours and re-stimulated with PMA+ionomycin for 4 hours, and BrdU was added for the final 1 hour. Cells were then harvested and stained using the BrdU staining protocol.



New tool to determine cell divisions

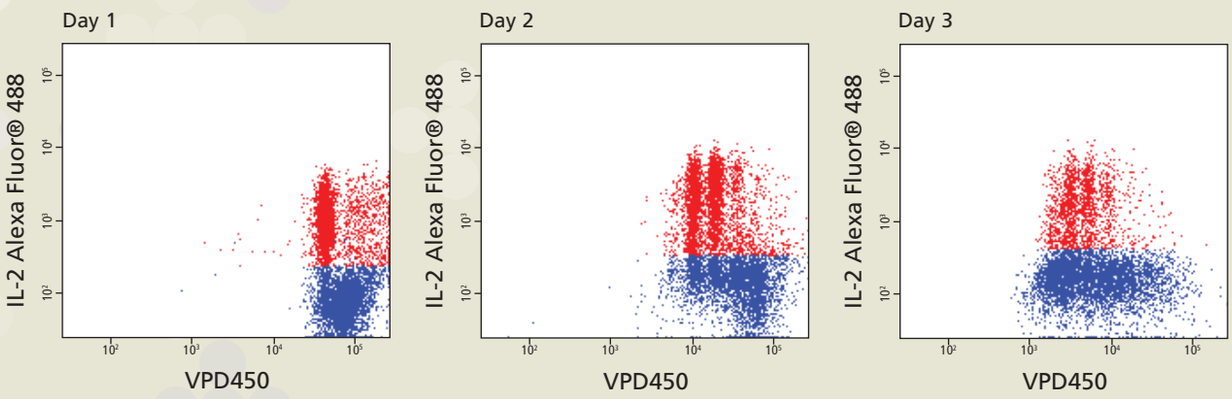
Tools and Techniques to Study Cell Proliferation

Cell proliferation can occur in response to many stimuli such as cytokine exposure or a variety of other processes. BD has a new product to help researchers study cell proliferation.

BD Biosciences offers Violet Proliferation Dye 450 for the detection of cell proliferation with the violet laser, which facilitates the use of larger panels. This allows the determination of more data from limited samples using multicolor flow cytometry.

VPD450 is a nonfluorescent esterified dye. The ester group allows the dye to enter the cell. Once the dye is inside the cell, esterases cleave off the ester group to convert the dye into a fluorescent product and trap it inside the cell. With each replication event the amount of dye in the cell is decreased, leading to a characteristic pattern.

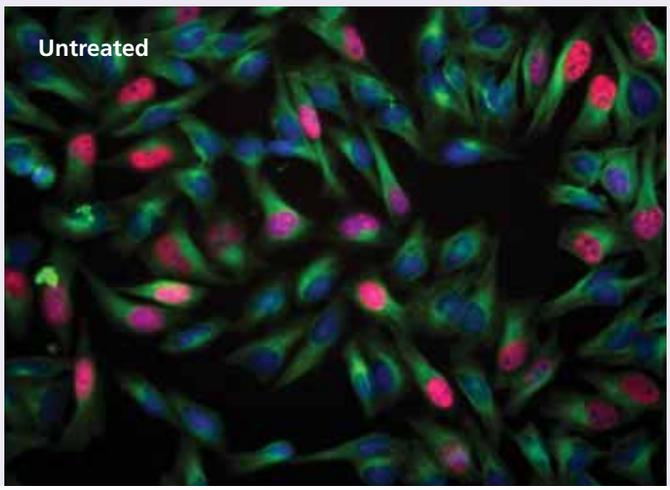
The use of VPD450 to correlate cell proliferation with IL-2 production.
$CD4^+$ enriched mouse splenocytes were loaded with 1 µM VPD450 for 10 minutes. Cells were then stimulated with anti-CD3/CD28 and harvested at the indicated times. Approximately 4 to 6 hours prior to harvest, cells were stimulated with PMA/ionomycin in the presence of BD GolgiStop™ protein transport inhibitor. Cells were fixed and permeabilized, stained for IL-2, and analyzed on a BD™ LSR II flow cytometer.

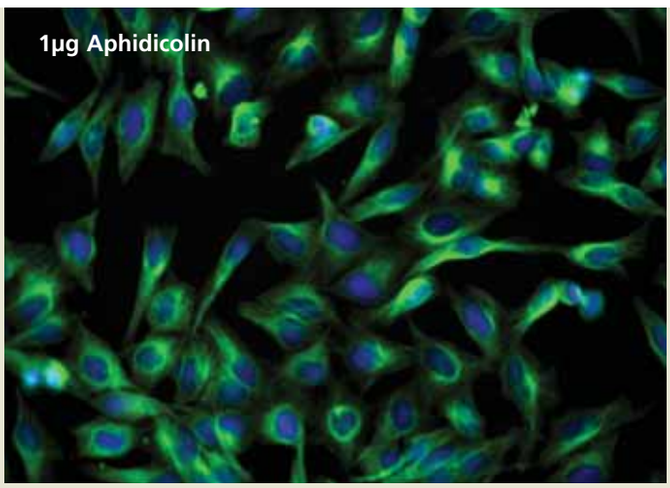


Tools for BrdU Analysis

BD Biosciences carries a series of antibodies and kits designed for the detection of proliferating cells by measurement of bromodeoxyuridine (BrdU), an analog of the DNA precursor thymidine used to measure de novo DNA synthesis. During the S phase of the cell cycle (DNA synthesis) BrdU is incorporated into the newly synthesized DNA and can be readily detected by anti-BrdU specific antibodies. BD antibodies and kits designed for the detection of BrdU are available for both intracellular flow cytometry and immunohistochemistry and include BD Horizon™ V450 and PerCP-Cy™ 5.5 formats.

In addition to DNA increases, levels of certain proteins also rise as a result of cell proliferation. For example, Ki67 is an antigen that is expressed in the nucleus of dividing cells. However, during the G0 phase of the cell cycle it is not detected. Ki67 can be combined with other proliferation markers such as BrdU and VPD450 for added confidence. These markers can also be combined with cell surface and other types of markers to gain additional information about cell subsets and their signaling pathways.





Cell cycle analysis of HeLa cells treated with Aphidicolin (DNA polymerase inhibitor) monitored by BrdU staining. The images were captured on a BD Pathway™ 855 bioimaging system with a 20x objective and merged using BD Attovision™ software.

Hoechst	– Blue
BrdU	– Red
Histone H3 (pS28)	– Yellow
Tubulin	– Green

The importance of tissue homeostasis

Techniques to Study Apoptosis—Programmed Cell Death

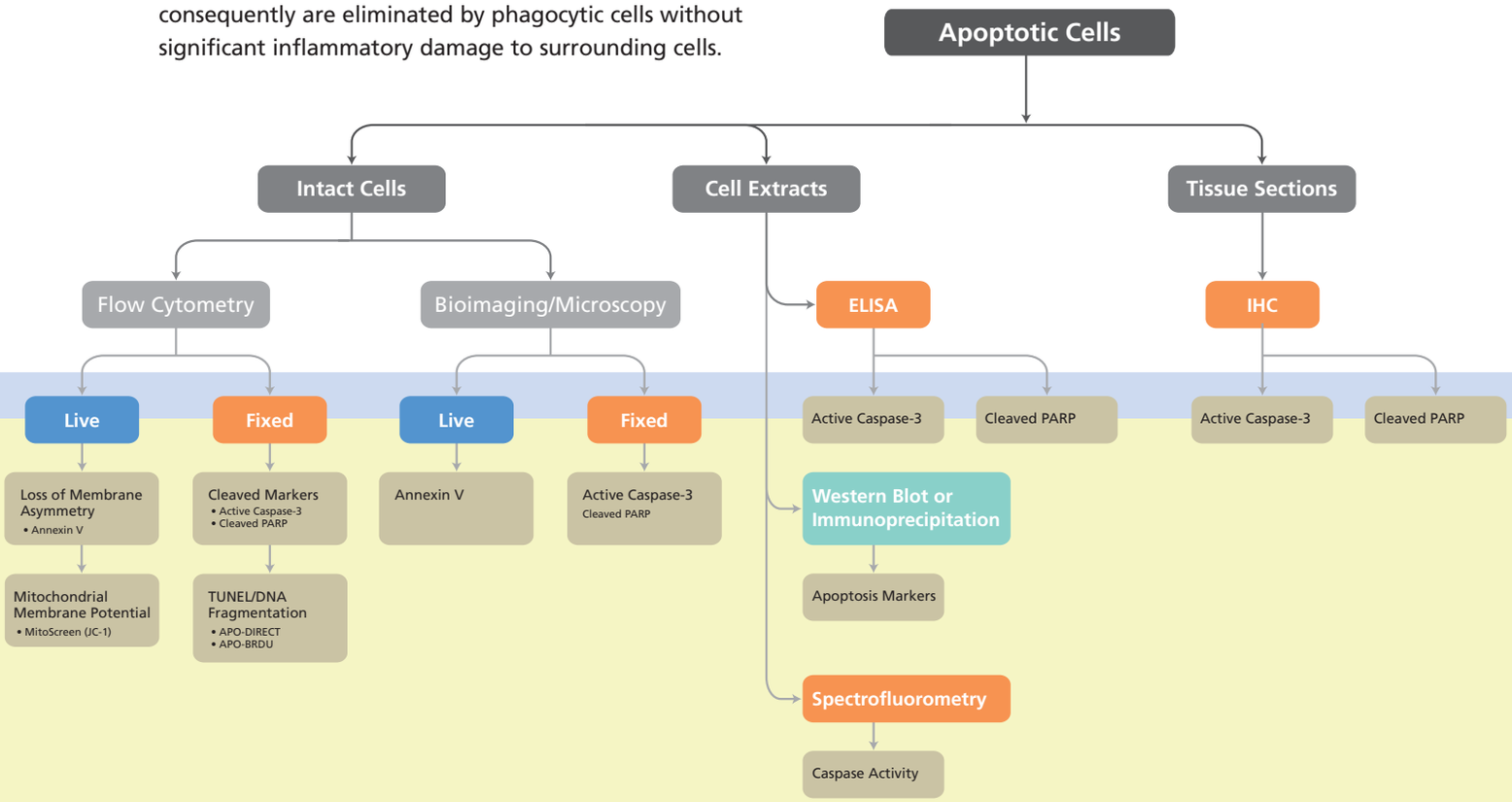
As cells become damaged or are no longer needed, they undergo apoptosis or programmed cell death, a normal physiological process that occurs during embryonic development and tissue homeostasis maintenance.

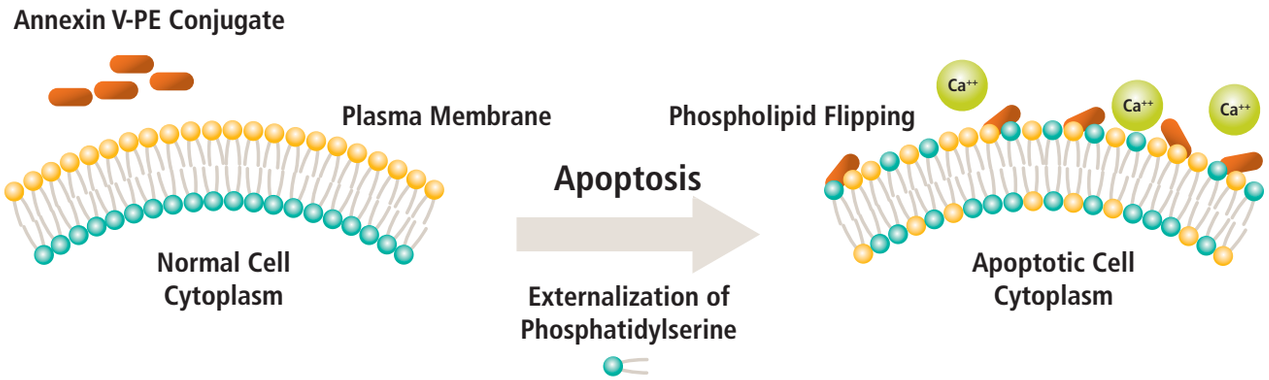
Apoptosis is an organized process that signals cells to self destruct for cell renewal or to control aberrant cell growth. Apoptosis controls the orderly death of damaged cells, whereas necrosis occurs as a result of tissue damage, causing the loss of both damaged and surrounding cells.[3]

The apoptotic process is characterized by certain morphological features. These include changes in the plasma membrane (such as loss of membrane symmetry and loss of membrane attachment), a condensation of the cytoplasm and nucleus, protein cleavage, and internucleosomal cleavage of DNA. In the final stages of the process, dying cells become fragmented into "apoptotic bodies" and consequently are eliminated by phagocytic cells without significant inflammatory damage to surrounding cells.

However, some cell types do not display characteristic features of apoptosis. In those cases multiple aspects of apoptosis might need to be analyzed to confirm the mechanism of cell death.

To support this spectrum of requirements, BD Biosciences offers a full range of apoptosis detection tools and technologies for measuring indicators at different stages across the apoptotic process. BD Biosciences tools use multiple methodologies including flow cytometry, bioimaging, and microscopy (for live and fixed cell analysis) as well as ELISA, IHC, Western blot, and spectrofluorometry.

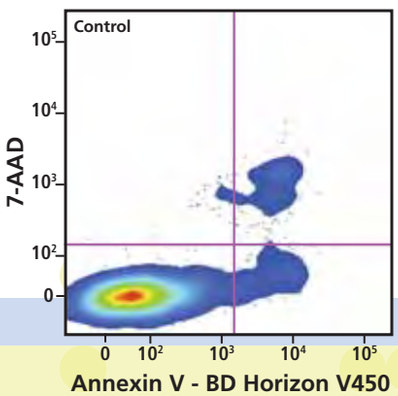




Annexin V—A Key Protein in Apoptosis Signaling
Changes in the plasma membrane are one of the first characteristics of the apoptotic process detected in living cells. Apoptosis can be detected by the presence of phosphatidylserine (PS), which is normally located on the cytoplasmic face of the plasma membrane. During apoptosis PS translocates to the outer leaflet of the plasma membrane and can be detected by flow cytometry and cell imaging through binding to fluorochrome-labeled Annexin V when calcium is present.

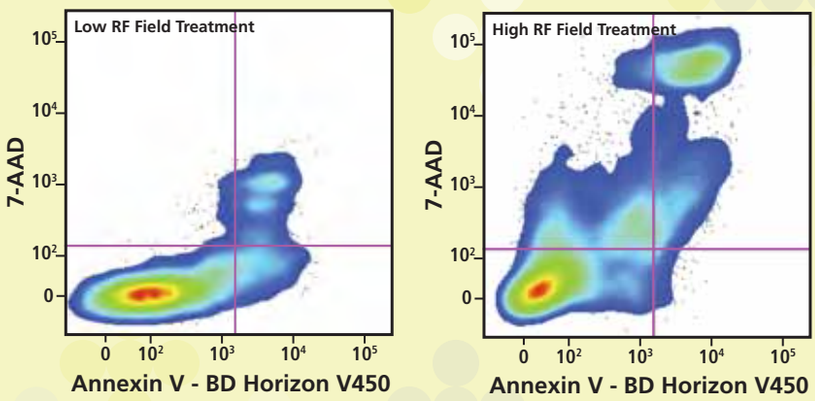
BD Biosciences offers Annexin V in several common formats such as FITC, PE, and BD Horizon V450 for the violet laser. With the addition of these new formats, more complex assays can be developed to look at apoptosis within heterogeneous cell subsets.

Since intracellular Annexin V is also exposed if the plasma membrane is compromised, a membrane-impermeant dye such as 7-AAD is commonly used to distinguish between apoptotic and dead cells to exclude the dead cells. The populations of cells that are stained with Annexin V only represent the apoptotic cell populations.



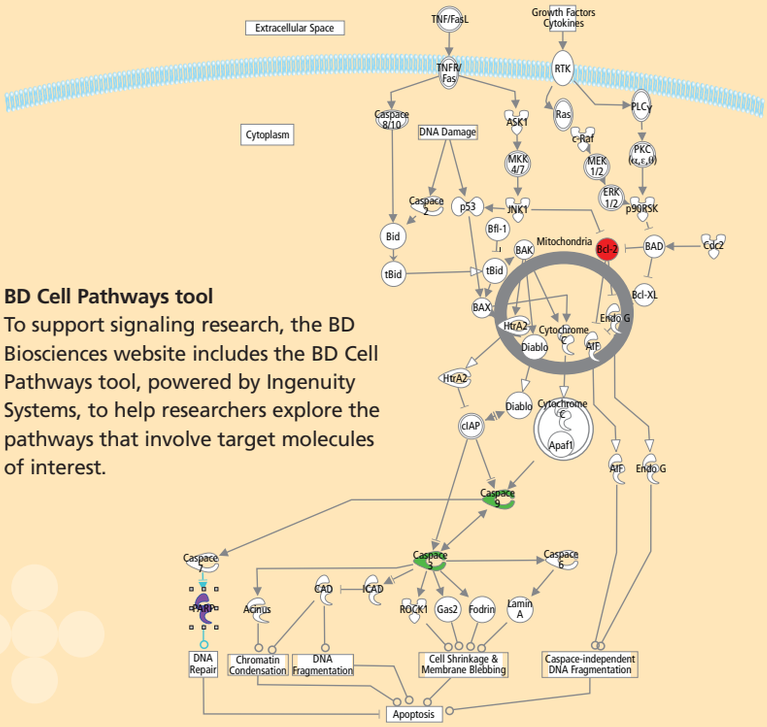
Radio frequency dose dependent apoptosis, necrosis, and cell death monitored by Annexin V-BD Horizon V450 in pancreatic carcinoma cell lines treated with a low dose of cetuximab targeted gold nanoparticles. As the RF field power increases, the temperature increases, and a shift from apoptosis (lower right quadrant) to frank necrosis (upper left quadrant) is seen.

Data courtesy of ES Glazer and SA Curley, MD Anderson Cancer Center.



Additional Techniques for the Detection of Apoptosis

There are many apoptosis triggers including certain cytokines, protein-protein interactions, and chemicals. Once apoptosis starts, changes in the mitochondria membrane potential can be measured by flow cytometry using the BD™ MitoScreen (JC-1) flow cytometry kit.



BD Cell Pathways tool
To support signaling research, the BD Biosciences website includes the BD Cell Pathways tool, powered by Ingenuity Systems, to help researchers explore the pathways that involve target molecules of interest.

Increases in mitochondrial membrane potential lead to increased mitochondrial membrane permeability and the release of soluble proteins such as cytochrome c and pro-caspases.

Caspases are a series of proteases activated upon cleavage at aspartate residues during earliest stages of apoptosis. Active caspases can then cleave many proteins including Poly-ADP ribose polymerase (PARP) and other caspases.

DNA fragmentation is one of the last phases in apoptosis resulting from the activation of endonucleases during the apoptotic process. There are several established methods for the study of DNA fragmentation including isolation and separation of DNA fragments by agarose gel electrophoresis and end labeling.

Feature Measured	Assays	Key Features
Plasma Membrane Alterations (Phosphatidylserine Exposure)	Annexin binding assay • Single conjugates • Annexin V kits	• Detects early apoptosis markers • Quick and easy • Flow cytometry or immunofluorescence application
Mitochondrial Changes	• BD MitoScreen Kit	• Fast, easy, single cell resolution by flow cytometry or fluorescent microscopy
Caspase Activation	• Caspase Activity Assay Kits and Reagents	• Quick and easy, uses spectrofluorometry
	• Active Caspase-3 immunoassays	• ELISA, flow cytometry, or Western blot
DNA Fragmentation	• APO-BrdU TUNEL Assay • APO-DIRECT TUNEL Assay	• Works with adherent cells, single cell resolution in conjunction with cell cycle analysis by flow cytometry

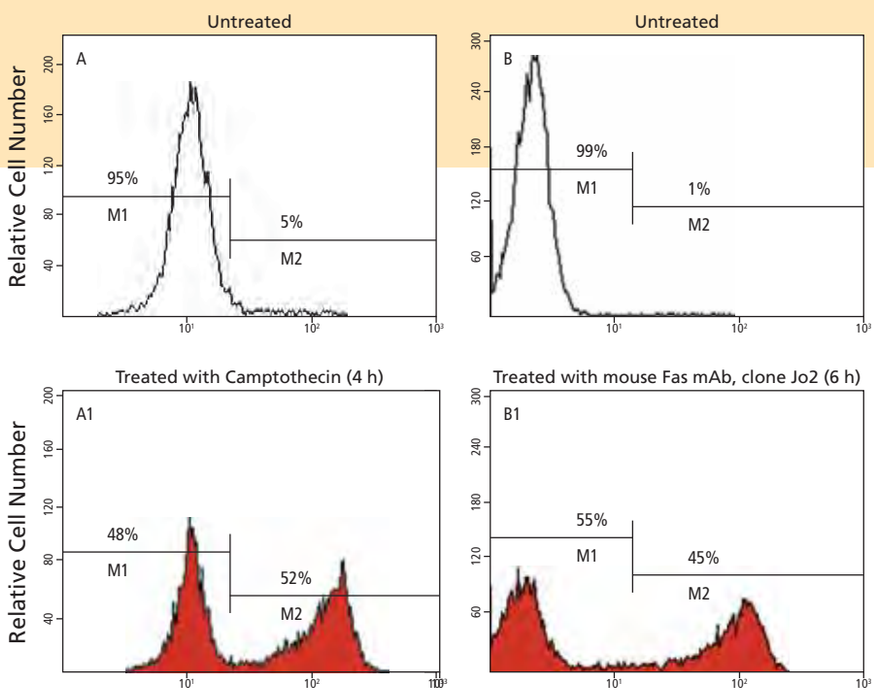
With an overwhelming number of available techniques and products, selecting the most appropriate method is often difficult. To help make this choice easier, the overview above summarizes commercially available assays from a biological perspective.

Caspase-3 cleavage/inhibition reactions
Active caspase-3 binds to the fluorogenic Ac-DEVD-AMC substrate and cleaves it between asparatic acid (D) and AMC, releasing the fluorescent AMC. AMC fluorescence is quantified by UV spectrofluorometry. The Ac-DEVD-CHO aldehyde inhibitor binds strongly to the caspase-3 active site and blocks substrate binding. Hence, Ac-DEVD-AMC is not cleaved and fluorescence is not emitted.

Measurement of Cleaved Caspases and PARP
Caspases are important initiators of apoptosis. One of the earliest and most consistently observed characteristics of apoptosis is the activation of a series of cytosolic proteases, called caspases. When apoptosis is activated, caspases cleave multiple protein substrates en masse, which leads to the loss of cellular structure and function, and ultimately results in cell death.[4] In particular, caspases -8, -9, and -3 have been implicated in apoptosis: caspase-9 in the mitochondrial pathway, caspase-8 in the Fas/CD95 pathway, and caspase-3 more downstream, activated by multiple pathways.

BD Biosciences carries a variety of reagents to measure caspases, particularly caspase-3. They include antibodies directed exclusively against the active form of the caspase. These antibodies are available in a variety of formats and can be used for flow cytometry, imaging, ELISA, and Western blot.

BD Biosciences offers a range of tools for caspase activity assays from individual fluorogenic peptide substrates and inhibitors, to kits, to ready-to-use assay plates. All are based on the use of synthetic tetrapeptide substrates[5] that are designed such that proteolytic cleavage by active human or mouse caspases results in release of a fluorophore or chromophore. The individual synthetic tetrapeptide substrates, together with the caspase inhibitors and active caspase enzymes, offer flexibility in the experimental design of a caspase activity assay.



Flow cytometric analysis of apoptotic and non-apoptotic populations using anti-active caspase-3 antibodies.
Jurkat T cells (A, A1) or mouse thymocytes (B, B1) were left untreated (A, B) or treated for 4 h with camptothecin (A1) or a mouse Fas monoclonal antibody, clone Jo2 (Cat. No. 554254) to induce apoptosis (B1). Cells were permeabilized and then stained with PE-conjugated active caspase-3 antibodies (Cat. No. 557091). Untreated cells were primarily negative for the presence of active caspase-3, whereas about half of each population of cells induced to undergo apoptosis had detectable active caspase-3.

Obtain the complete picture

Additional Proteins for the Study of Apoptosis

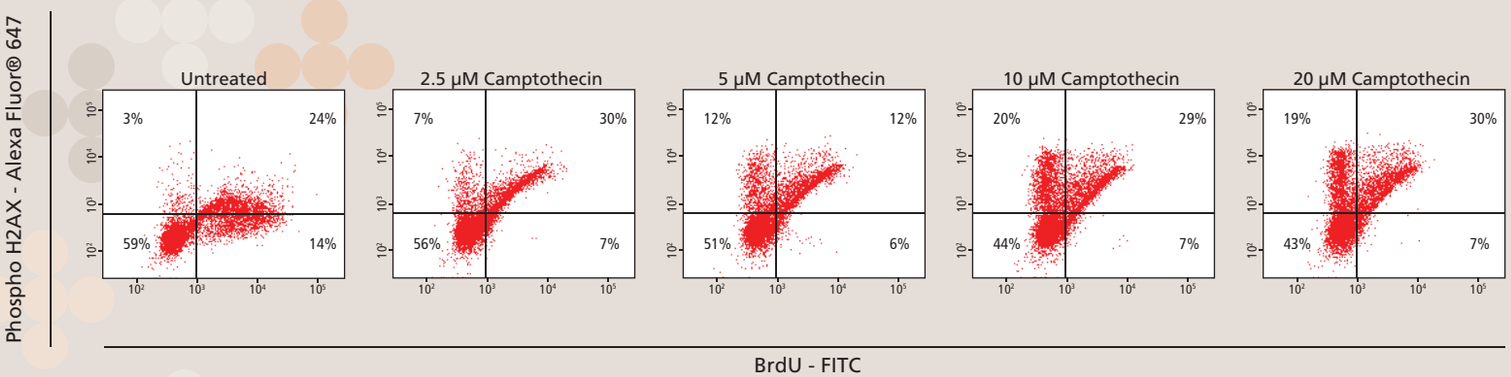
In addition to caspases and Annexin V, there are several other proteins important for the study of apoptosis, including the Bcl-2 family, tumor necrosis factor receptor (TNFR) family, PARP, and other signaling molecules. Bcl-2 family members, identified by the presence of conserved BCL2 homology (BH3) domains, are versatile key regulators of apoptosis. Bcl-2, for example, protects cells from apoptosis by associating with the mitochondrial membrane and preventing the release of cytochrome c from the mitochondria. In contrast other Bcl-2 family members such as Bax promote apoptosis. Increased levels of Bcl-2 have been reported in cancer.[6]

The TNFR family contains many members, including CD95, that can be divided into three major groups based on structure. Signaling through the TNFR pathway leads to apoptosis.[7]

PARPs are DNA repair enzymes that are activated by DNA strand breaks. Cleavage of PARP by caspase-3 into 24- and 89-kDa fragments inactivates the PARP enzyme.

BD Biosciences carries antibodies specific for cleavage products of PARP that are useful markers of apoptosis. These antibodies are available in a variety of formats and can be combined with other markers to gain additional information about the cell.[8,9]

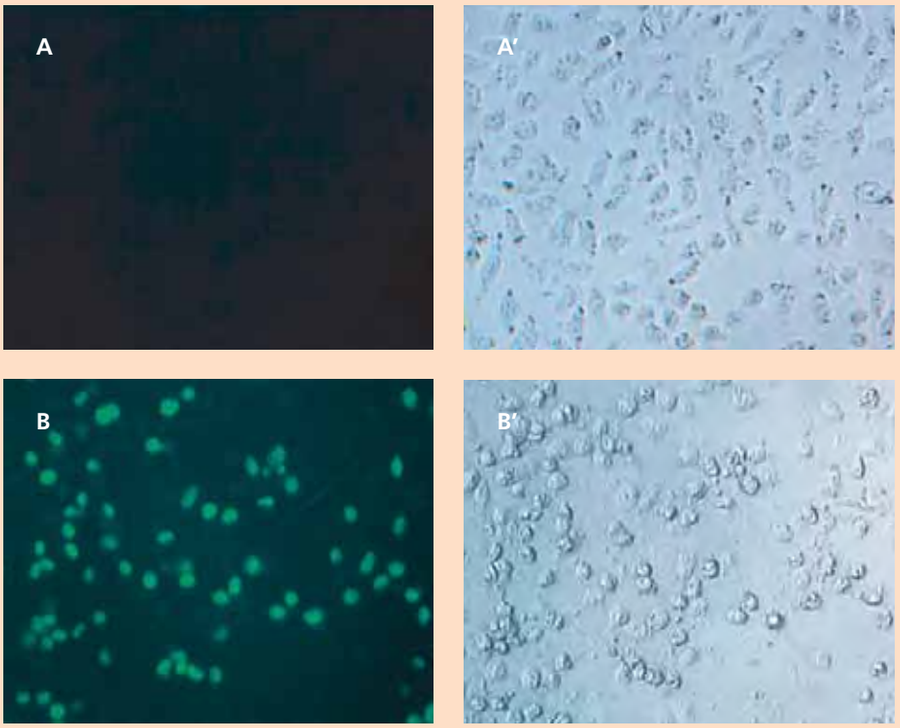
In this experiment, Jurkat cells were treated with camptothecin, a potent inhibitor of topoisomerase I and apoptosis inducer. Phosphorylation of H2AX, a protein important for maintaining genome integrity, has been shown to correlate with levels of DNA damage.[10] Using multicolor flow cytometry, cell proliferation (BrdU), apoptosis (cleaved PARP), and DNA damage (histone H2AX pS140) were evaluated in the same experiment.



Simultaneous Studies of Apoptosis, Cell Cycle, and DNA Damage

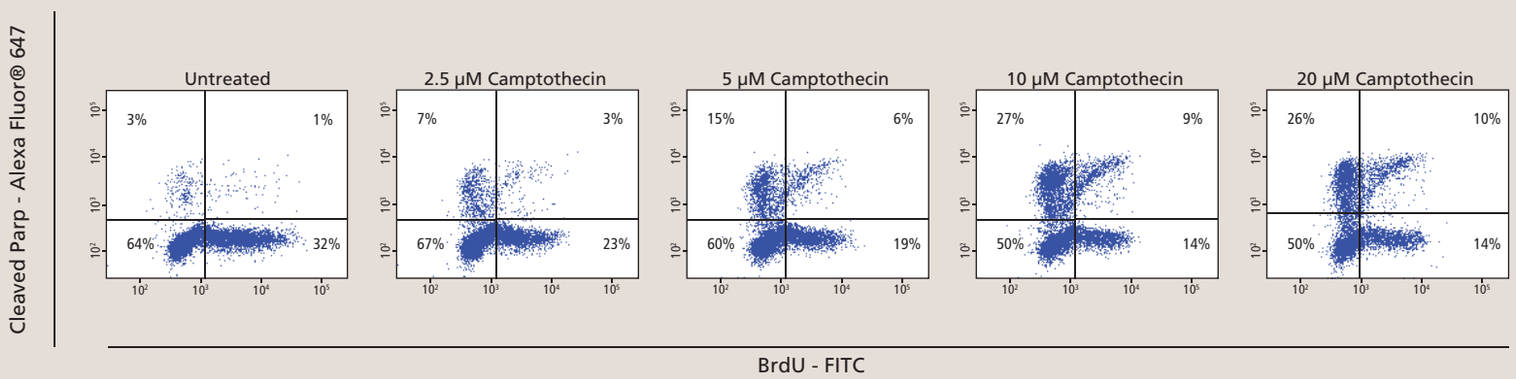
Apoptosis and cell proliferation assays are particularly useful for basic cancer research and drug discovery. Comparing data across different experiments can be challenging due to variability introduced by sample handling, timing, and variability within the sample.

Multicolor flow cytometry addresses these challenges and is an excellent tool to study apoptosis and cell proliferation. Relevant markers can be combined with cell phenotyping markers to look at events within subpopulations of cells. Antibodies to phosphoproteins can be used to examine phosphorylation events.



Immunofluorescence of cleaved PARP

HeLa cells grown were either left untreated (A) or treated with staurosporine (1.0 mM, 4 h) to induce apoptosis (B). Cells were then fixed with 3.7% formaldehyde (15 min on ice), then permeabilized in 0.25% Triton™ X-100/3% BSA/PBS (15 min on ice). Cells were then washed twice with 3% BSA/PBS and stained with 4 µL/mL of FITC-labeled anti-PARP in 3% BSA/PBS (1 h at RT). Cells were washed twice with 3% BSA/PBS and then visualized by immunofluorescence microscopy. A′ and B′ represent phase correlates of A and B, respectively. The results indicate that untreated cells were primarily negative for cleaved PARP (A), whereas a significant percentage of the staurosporine-treated population is positive for cleaved PARP.



Services

BD Biosciences instruments and reagents are backed by a world-class service and support organization with unmatched flow cytometry experience. Our integrated approach combines high content bioimaging and flow cytometry instrumentation with trusted, certified reagents, and advanced applications. The BD Biosciences tools enable our customers to discover more and obtain the most complete picture of cell function, and at the same time experience improved workflow, ease of use, and optimal performance.

Researchers come to BD Biosciences not only for quality products, but as a trusted lab partner. Our repository of in-depth, up-to-date knowledge and experience is available to customers through comprehensive training, application and technical support, and expert field service.

For example, our website, bdbiosciences.com, now incorporates BD Cell Pathways, a collection of detailed, interconnected, interactive maps of biological signaling and metabolic pathways. Researchers can look up specific genes or molecules in the knowledge database, trace the pathways that involve them, and find BD products related to them.

Technical Applications Support

BD Biosciences technical applications support specialists are available to provide field- or phone-based assistance and advice. Expert in a diverse array of topics, BD technical application specialists are well equipped to address customer needs in both instrument and applications support.

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