Tools for Assessing Cell Events

Apoptosis, Cell Cycle, and Cell Proliferation

BD

Helping all people live healthy lives

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™ cell proliferation dyes (VPD450 and CFSE), BD Horizon™ fixable viability stains, 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 immunohistochemistry, cell imaging, and 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:

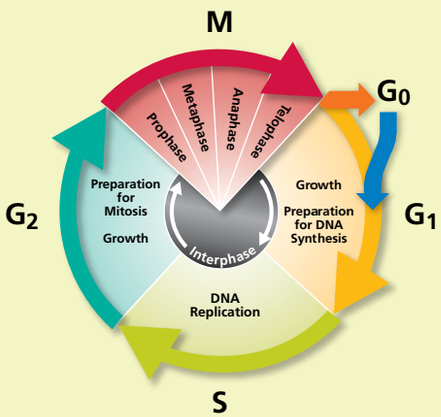
- BD Biosciences reagents and the BD Cycletest™ Plus reagent kit for the analysis of cellular DNA content
- DNA dyes such as propidium iodide (PI), 7-aminoactinomycin D (7-AAD), Hoechst 33342, and DAPI
- Antibodies against cyclins, retinoblastoma, and phosphorylated histone H3
- BrdU kits and antibodies for the detailed analysis of cell cycle
- Cell proliferation dyes for the violet and blue lasers to track cell division across multiple generations

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 G_1, 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 G_2 phase, cells continue to grow and prepare for mitosis.

Cell cycle phases



Methods for the study of cell cycle and proliferation

Measures	Reagents	Mechanism	Technology	Sample Types
DNA	PI, 7-AAD, DAPI, Hoescht, DRAQ5™, DRAQ7™	Interaction into DNA double strands.	Flow cytometry	DNA content in live (Hoechst, DRAQ5) or fixed (PI, 7-AAD, DAPI, DRAQ7) cells, and viability discrimination in live cells (PI, 7-AAD, DAPI, DRAQ7)
Proliferation dyes	BD Horizon Violet Proliferation Dye 450 (VPD450), CFSE	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	Bromodeoxyuridine (BrdU) replaces thymidine (T) in dividing DNA. It is then detected by antibodies to BrdU.	Flow cytometry, bioimaging, immunohistochemistry	Fixed and permeabilized cells and treated tissues (bioimaging and immunohistochemistry only)
Protein level (proliferation)	Antibodies to Ki67 and PCNA	Levels increase as a result of proliferation.	Flow cytometry, bioimaging, immunohistochemistry, Western blot	Fixed cells, tissues, and extracts
Protein level (cell cycle)	Antibodies to cyclins, retinoblastoma (Rb) and 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 and cyclin-dependent kinases (cdk)	Proteins become phosphorylated as a result of proliferation or changes to the cell cycle.	Flow cytometry, bioimaging, immunohistochemistry, Western blot	Fixed cells, tissues, and extracts
Quantification of cytokines and modified proteins	BD™ Cytometric Bead Array (CBA)	Beads capture target protein in extracts and supernatant, allowing quantification.	Flow cytometry	Supernatants and cell extracts

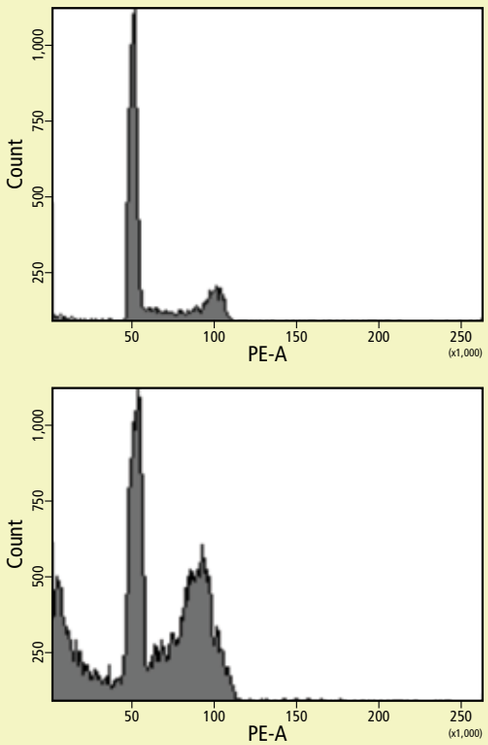
Analysis of Cellular DNA Content
BD Biosciences offers a wide variety of reagents to study the cell cycle. Reagents include DNA dyes such as PI, Hoescht, DAPI, and 7-AAD. In addition, the BD Cycletest Plus reagent kit includes PI and other reagents to degrade proteins and RNA to allow more precise DNA 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 at the right).

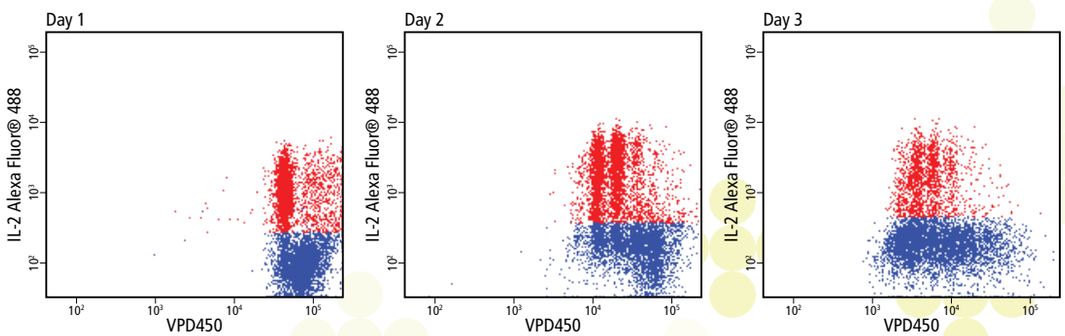
As cells go through the phases of the cell cycle, proteins become modified or change in expression, such as the phosphorylation of histone H3 at Ser28.[2] To facilitate DNA replication, the histone is modified, opening the chromatin to allow entry of replication machinery. To further support the study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

Tracking Cell Proliferation
Cell proliferation can occur in response to many stimuli such as cytokine exposure or a variety of other processes. BD has products to help researchers study cell proliferation. BD Biosciences offers BD Horizon™ Violet Proliferation Dye 450 (VPD450) and CFSE for the detection of cell proliferation with the violet and blue lasers.

BD cell proliferation dyes are nonfluorescent esterified dyes. 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 later generations of cells exhibiting lower fluorescence.



DNA content in untreated and colcemid-treated mouse splenocytes.
CD4-enriched mouse splenocytes were cultured with anti-CD3/CD28, IL-2, and IL-4 for 6 days. Cells were harvested and treated with 10 ng/mL of IL-2 plus 1 µg/mL of colcemid for 4 (top) or 24 (bottom) hours and stained with the BD Cycletest Plus DNA reagent kit. Cells treated with colcemid (which is known to depolymerize microtubules and arrest cells in metaphase) show an increase in cells with 4N DNA content.



The use of VPD450 to correlate cell proliferation with IL-2 production.
$CD4^+$ enriched mouse splenocytes were loaded with 1 µM of 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. IL-2 production increases in later generations of cells.

Additional Tools and Techniques to Study Cell Proliferation

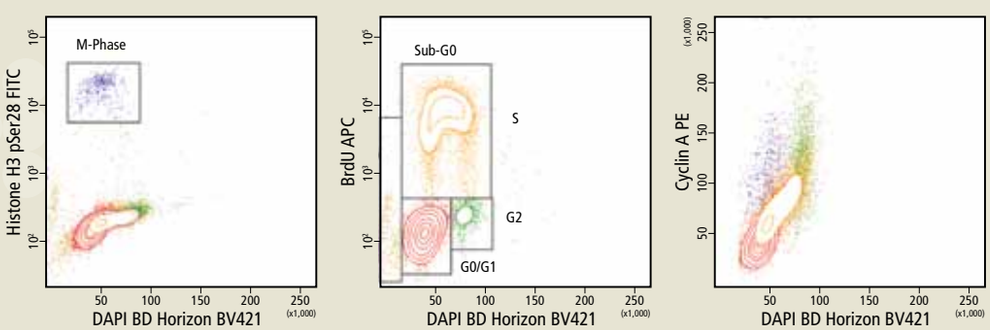
Tools for BrdU Analysis

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, BD Horizon™ BV510, and PerCP-Cy™5.5 formats. The variety of colors available facilitates easier construction of multicolor flow cytometry panels.

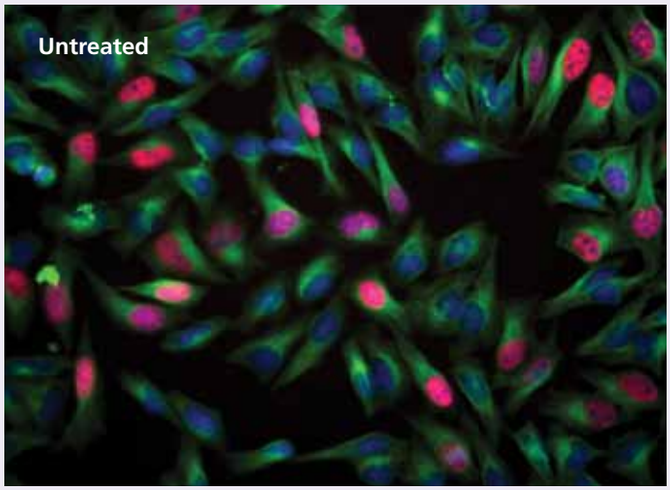
When analysis of BrdU incorporation and DNA content is combined, the G_0/G_1, S, and G_2/M phases of the cell cycle can be discriminated. These compartments can be further analyzed for expression of other cell cycle–related proteins, such as cyclins. This technique allows the tracking of cell cycle–related protein expression throughout the various phases of the cell cycle.

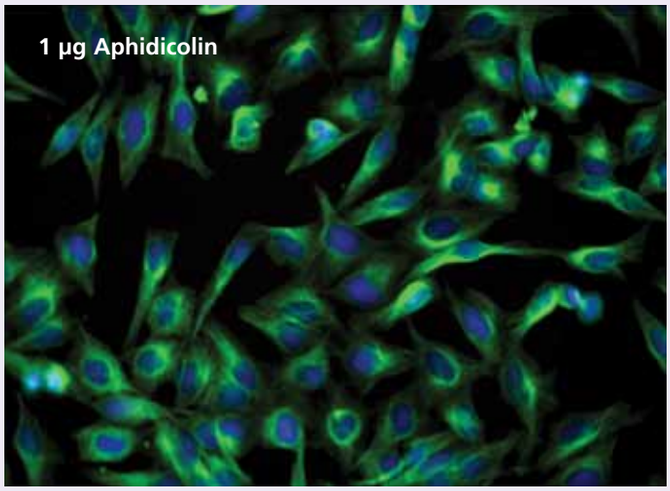
Cell cycle and cyclin status in HeLa cells

HeLa cells were pulsed with BrdU and then fixed and permeabilized according to the BD Pharmingen™ APC BrdU Flow Kit protocol. Cells were stained with APC anti-BrdU, Alexa Fluor® 488 anti-Histone H3 pSer28, and PE anti-Cyclin A. After washing, cells were stained with 1 µg/mL of BD Pharmingen™ DAPI solution. Cyclin A content increases over the course of the cell cycle, peaking in the G_2 and M phases.



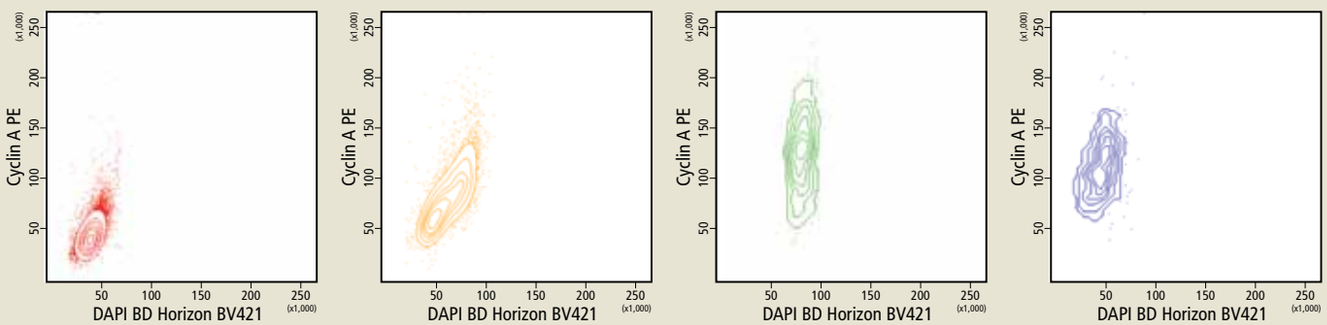
In addition to DNA increases, levels of certain proteins also rise as a result of cell proliferation. For example, Ki67 is an antigen that is expressed in the nucleus of dividing cells. However, during the G_0 phase of the cell cycle it is not detected. Similarly, histone H3 becomes phosphorylated only during the M phase of the cell cycle. Thus, histone H3 pSer28 can be used as a specific marker for M-phase cells and combined with other proliferation tools to further segment cell cycle compartments. Markers like Ki67 and histone H3 pSer28 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
Aphidicolin treatment blocks cells from entering early S phase.

Target	Pseudo-Color
Hoechst (DNA)	Blue
BrdU	Red
Histone H3 (pSer28)	Yellow
Tubulin	Green



The importance of tissue homeostasis

Techniques to Study Apoptosis—Programmed Cell Death

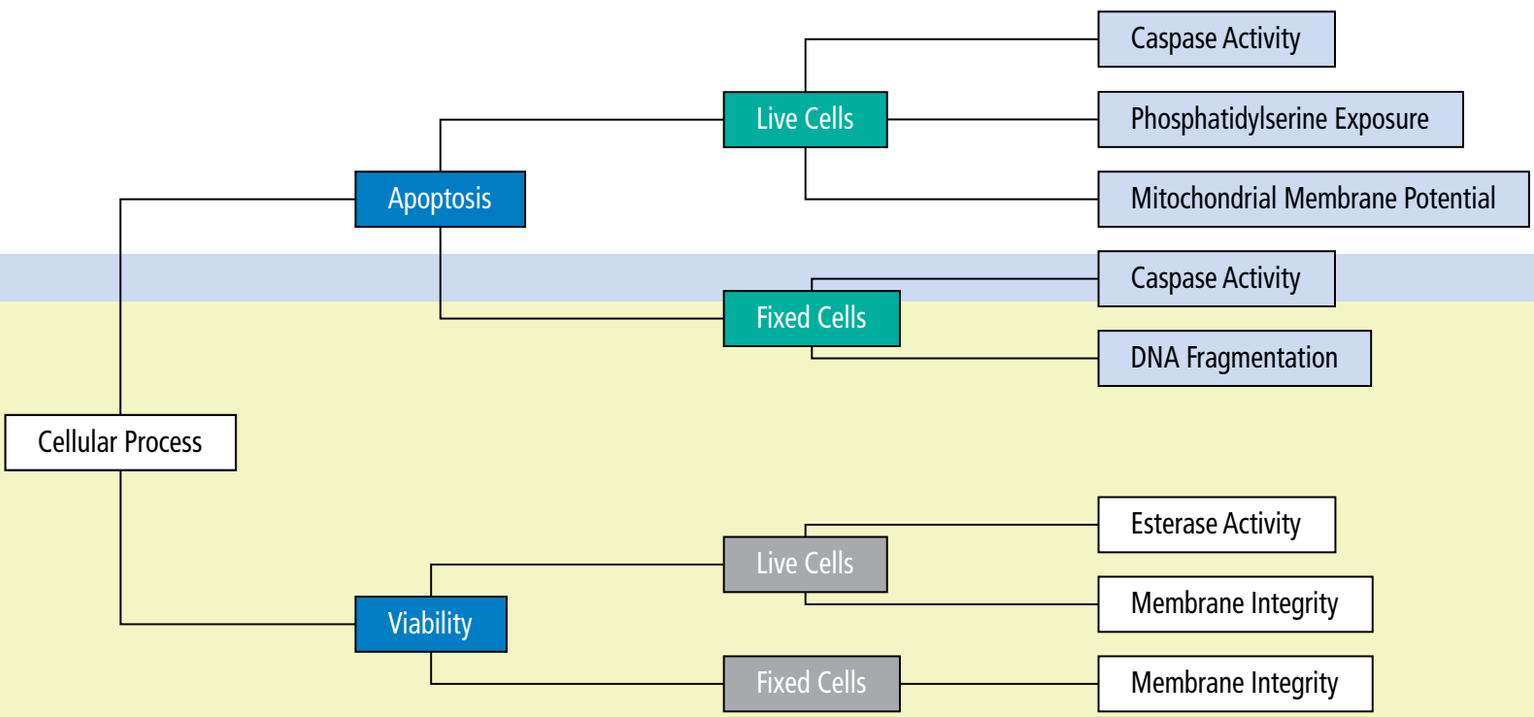
As cells become damaged or are no longer needed, they undergo apoptosis, or programmed cell death, a normal physiological process that occurs during embryonic development and tissue homeostasis maintenance.

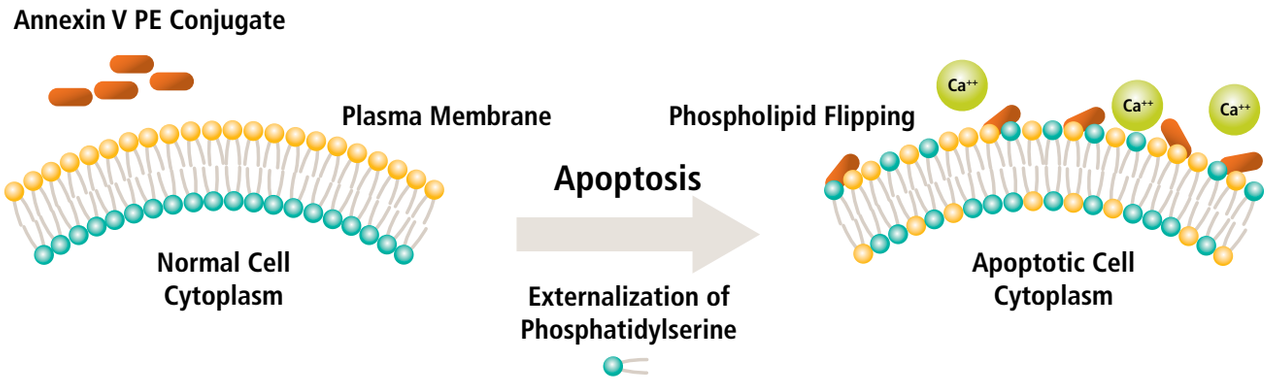
Apoptosis is an organized process that signals cells to self destruct for cell renewal or to control aberrant cell growth. Apoptosis controls the orderly death of damaged cells, whereas necrosis occurs as a result of tissue damage, causing the loss of both damaged and surrounding cells.[3]

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.[4]

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.[5]

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 IHC and Western blot.

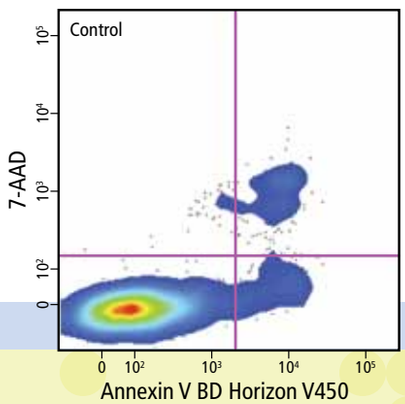


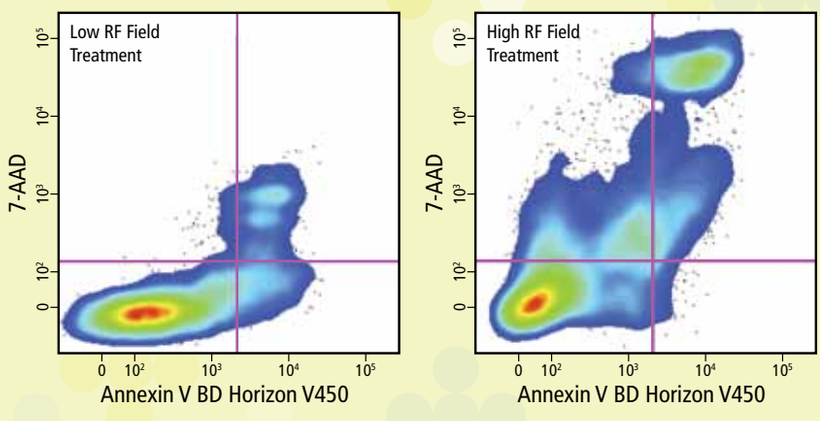


Annexin V: A Key Protein in Apoptosis Signaling
Changes in the plasma membrane are one of the first characteristics of the apoptotic process detected in living cells. Apoptosis can be detected by the presence of phosphatidylserine (PS), which is normally located on the cytoplasmic face of the plasma 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 formats such as FITC, PE, BV421, and other BD Horizon Brilliant™ Violet formats for the violet laser, and BUV395 for the ultraviolet 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 only Annexin V represent the apoptotic cell populations.





Radio frequency (RF) 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.

Tools to streamline apoptosis research

Additional Techniques for the Detection of Apoptosis and Viability

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 or BD Pharmingen™ MitoStatus dyes.

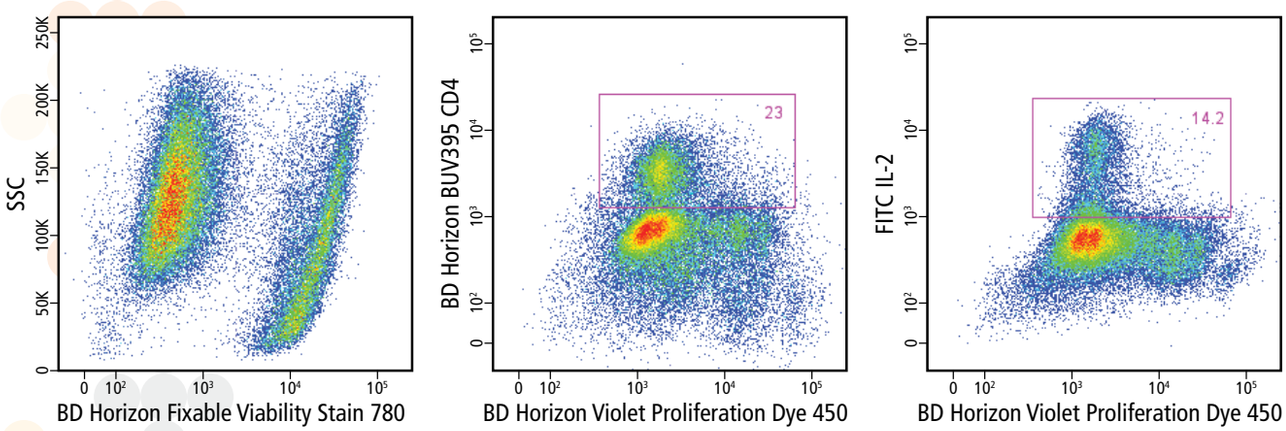
Increases in mitochondrial membrane potential lead to increased mitochondrial membrane permeability and the release of soluble proteins such as cytochrome c and pro-caspases.

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

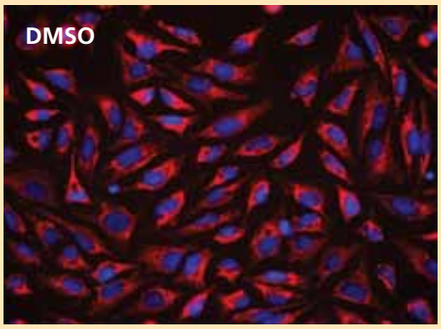
DNA fragmentation is one of the last phases in apoptosis resulting from the activation of endonucleases during the apoptotic process. There are several established methods for the study of DNA fragmentation including isolation and separation of DNA fragments by agarose gel electrophoresis and end labeling. The BD™ APO-BrdU kit uses end labeling or the terminal deoxynucleotidyl transferase (TdT) nick end labeling (TUNEL method) to support the study of DNA fragmentation. In this assay, TdT catalyzes a template-independent addition of bromolated deoxyuridine triphosphates (Br-dUTP) to the 3′-hydroxyl (OH) termini of double- and single-stranded DNA. After the Br-dUTP is incorporated, these terminal sites of double- and single-stranded DNA are identified using flow cytometry by staining cells with labeled anti-BrdU.

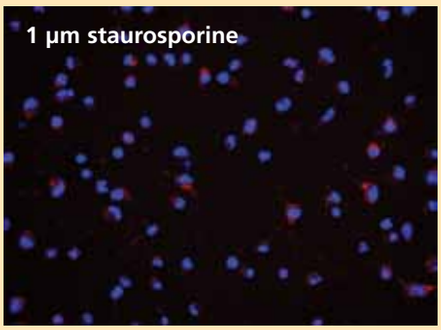
At the end of apoptosis, cells become completely nonviable as they lose membrane integrity and become permeable to membrane-impermeant dyes such as 7-AAD or fixable viability stains. These dyes allow the identification of dead cells for further analysis or for exclusion in multicolor panels where dead cells may non-specifically bind antibody, affecting experimental results. In the case of fixable viability stains, these reagents bind covalently to surface and intracellular amines, providing compatibility with protocols requiring fixation and permeabilization.[6] BD offers eight fixable viability stains for four lasers for ease of multicolor panel design.

Analysis of proliferating BALB/c splenocytes for surface and intracellular markers
BALB/c splenocytes were stained with VPD450 and then cultured with anti-CD3e/CD28. After 3 days, the cells were re-stimulated with PMA/ionomycin in the presence of BD GolgiStop Protein Transport Inhibitor. Cells were harvested, stained with FVS780, fixed and permeabilized, and stained with BD Horizon™ BUV395 anti-Mouse CD4 and FITC anti-Mouse IL-2. Dead cells were either included (left panels) or excluded (right panels) from analysis by FVS780, and the subsequent results on CD4 and IL-2 staining are shown. Dead cell exclusion results in more accurate estimation of CD4 and IL-2 positive populations. Plots were derived from gated events based on light scattering characteristics of BALB/c splenocytes.



Immunofluorescent imaging of TMRE in HeLa cells
HeLa cells were treated with 0.02% of dimethyl sulfoxide (DMSO) or 1 µM of staurosporine for 3 hours. Cells were stained with 200 nM of BD Pharmingen MitoStatus TMRE and 5 µg/mL of Hoechst. Staining media was removed and replaced with Dulbecco's phosphate-buffered saline (DPBS). Compared to the vehicle-treated control, staurosporine-treated cells show a decrease in mitochondrial staining with TMRE, as well as pyknotic nuclei characteristic of apoptotic cells.





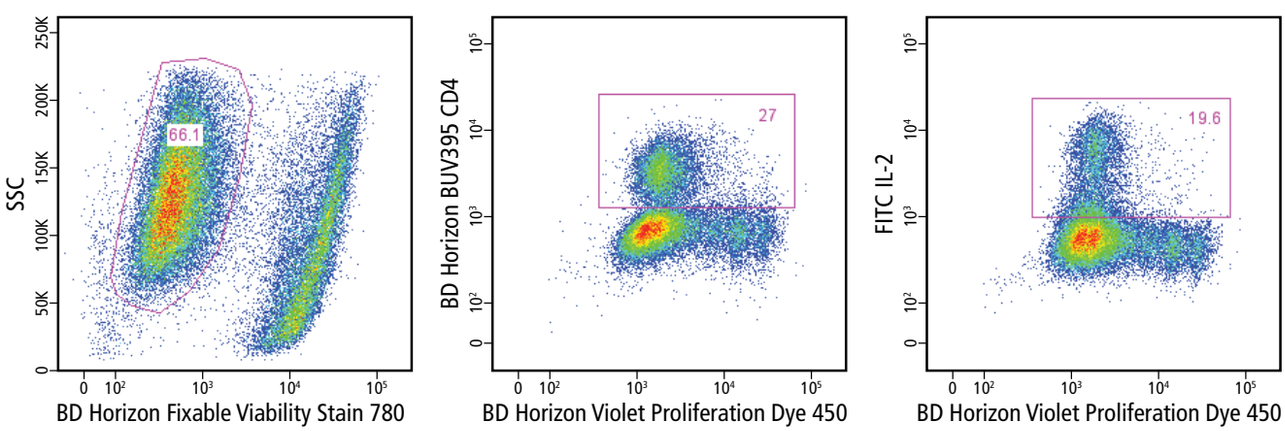
Measurement of Cleaved Caspases and PARP
Caspases are important initiators of apoptosis. One of the earliest and most consistently observed characteristics of apoptosis is the activation of a series of cytosolic proteases called caspases. When apoptosis is activated, caspases cleave multiple protein substrates en masse, which leads to the loss of cellular structure and function, and ultimately results in cell death.[7] 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, and Western blot.

BD Biosciences offers a range of tools for caspase activity assays from individual fluorogenic peptide substrates and inhibitors to kits. All are based on the use of synthetic tetrapeptide substrates[8] 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.

BD Horizon™ Fixable Viability Stain Reagents

Dye	Excitation Laser	Excitation (nm)	Emission (nm)	Fluorescence Channel	Size	Cat. No.
FVS450	Violet	406	450	BD Horizon™ BV421, BD Horizon™ V450, Pacific Blue™	100 µg	**562247**
FVS510	Violet	408	512	BD Horizon™ BV510, BD Horizon™ V500	100 µg	**564406**
FVS520	Blue	498	521	BD Horizon™ BB515, FITC, Alexa Fluor® 488	150 µg	**564407**
FVS570	Blue, Yellow-Green	547	573	PE	150 µg	**564995**
FVS620	Blue, Yellow-Green	523	617	BD Horizon™ PE-CF594, PE-Texas Red®	100 µg	**564996**
FVS660	Red	649	660	APC, Alexa Fluor® 647	100 µg	**564405**
FVS700	Red	657	700	BD Horizon™ APC-R700, Alexa Fluor® 700	100 µg	**564997**
FVS780	Red	759	780	APC-Cy™7	200 µg	**565388**



Obtain the complete picture

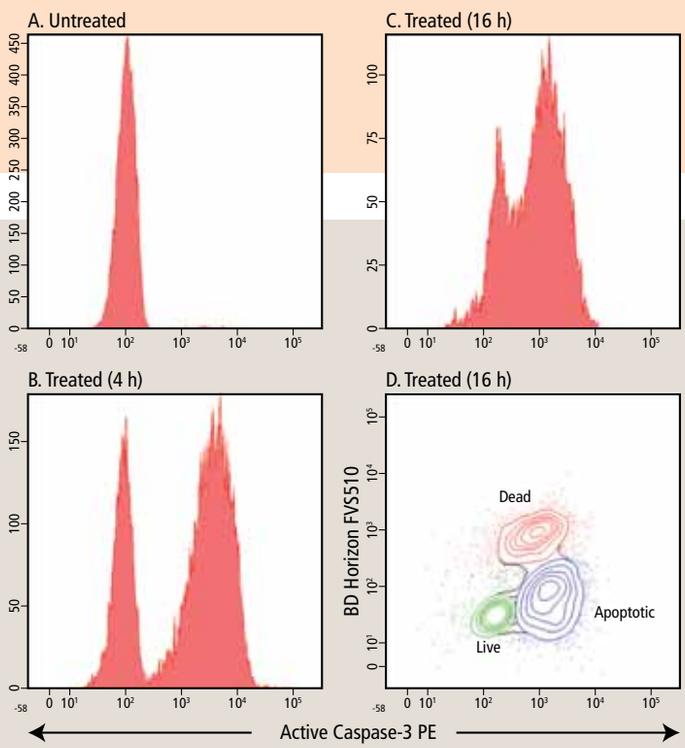
Additional Proteins for the Study of Apoptosis

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.[9]

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.[10]

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 cells.[11,12]

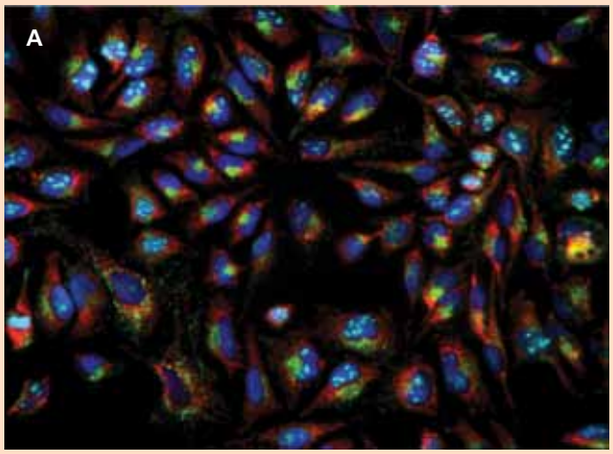


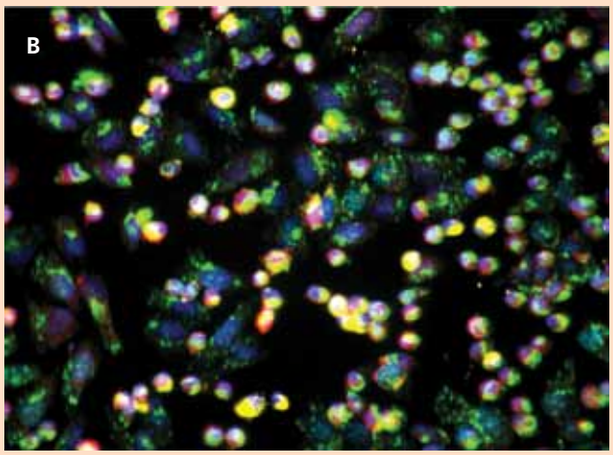
Flow cytometric analyis of apoptotic and dead cells using caspase-3 and BD Horizon™ Fixable Viability Stain 510 (FVS510) Jurkat cells were left untreated (A) or treated for 4 hours (B) or 16 hours (C, D) with camptothecin to induce apoptosis and death. Cells were then analyzed for viability by staining with PE anti-active caspase-3 with or without FVS510. At 4 hours, treated Jurkats show increased active caspase-3 expression compared to the unstained control. At 16 hours, many dead cells are present, which can express variable amounts of caspase-3. As a result, resolution of live and apoptotic populations by active caspase-3 staining is confounded by the dead cell population. Co-staining with FVS510 helps to better resolve live, apoptotic, and dead populations at the 16-hour time point. Live cells (green) are Caspase-3^{neg}FVS510^{neg}, apoptotic cells (blue) are Caspase-3^{pos}FVS510$^{neg\ to\ intermediate}$, and dead cells (red) are Caspase-3^{pos}FVS^{pos}.

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 and multicolor imaging address these challenges and are excellent tools 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.

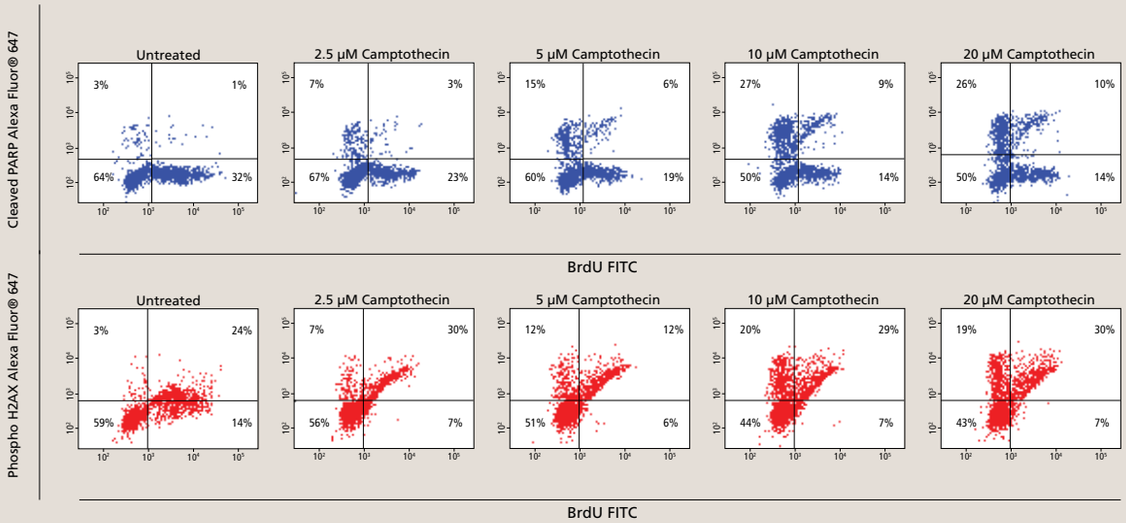




5-color immunofluorescent staining of apoptotic cells
HeLa cells were either left untreated **(A)** or treated with camptothecin (20 µM, 6 hours) to induce apoptosis **(B)**. Cells were then fixed, permeabilized, blocked, and stained with purified rabbit anti-active caspase-3 antibody. The second step reagent was BD Horizon™ BV480 goat anti-rabbit Ig. Cells were then stained with BV421 mouse anti-LAMP-1, Alexa Fluor® 488 mouse anti-Cytochrome c, and Alexa Fluor® 555 mouse anti-human Ki-67. The nuclear counterstain was DRAQ5. In treated cells **(B)** there are increased levels of activated caspase-3 and LAMP-1 while levels of Ki-67 are reduced and cytochrome c is released into the cytoplasm.

Sample filter configuration for standard epifluorescence microscope

Fluorochrome	Pseudo-Color	Excitation (nm)	Dichroic (nm)	Emission (nm)
BD Horizon BV421	Green	392/23	409	430/24
BD Horizon BV480	Yellow	438/24	458	485/20
Alexa Fluor® 488	Red	490/30	506	537/26
Alexa Fluor® 555	Cyan	543/22	562	593/40
DRAQ5	Blue	628/40	660	692/40



Monitoring proliferation, apoptosis, and DNA damage by flow cytometry
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.[13] Using multicolor flow cytometry, cell proliferation (BrdU), apoptosis (cleaved PARP), and DNA damage (histone H2AX pS140) were evaluated in the same experiment. Treated cells show increased apoptosis and DNA damage, as well as decreased proliferation.

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. 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.

Technical Application Support

BD Biosciences technical application 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 application support.

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