Multiparametric approaches for studying apoptosis

UNRAVELING THE SPATIAL AND TEMPORAL COMPONENTS OF PROGRAMMED CELL DEATH.

No single assay adequately characterizes apoptosis

The orderly progression toward cell death is fundamental to the life of a multicellular organism. Apoptosis—the genetically controlled ablation of cells—not only allows for proper growth and development by ridding the organism of unneeded cells and tissues, but also minimizes threats to the organism by destroying surplus cells of the immune system and virus-infected or DNA-damaged cells.[1,2]

Programmed cell death is morphologically and biochemically distinct from cell death by injury (necrosis). In contrast with necrotic cells, apoptotic cells exhibit compaction of the nuclear chromatin, shrinkage of the cytoplasm, and production of membrane-bound apoptotic bodies, as well as DNA fragmentation and cleavage or degradation of several cellular proteins. These differences can be selectively targeted with fluorescent probes in order to characterize the spatial and temporal components of apoptosis (Table 1). Incorrectly regulated apoptosis

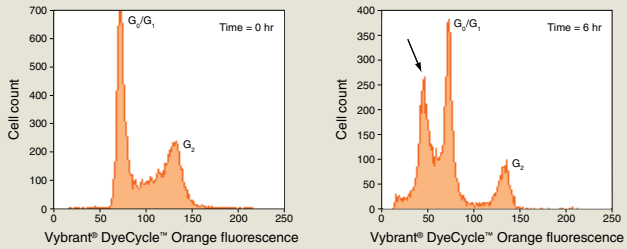
Table 1—Temporal relationships of apoptotic events in Jurkat cells treated with camptothecin.

Time after induction	Cell function changes	Reagents used	Cat. no.
Early	Mitochondrial membrane potential	MitoTracker® Red CMXRos	M7512
		MitoProbe™ $DiOC_2(3)$ Assay Kit	M34150
		MitoProbe™ $DiIC_1(5)$ Assay Kit	M34151
		MitoProbe™ JC-1 Assay Kit	M34152
	Mitochondrial transition pore opening	Image-iT® LIVE Mitochondrial Transition Pore Assay Kit	I35103
		MitoProbe™ Transition Pore Assay Kit	M34153
	Phosphatidylserine translocation to outer membrane	Alexa Fluor® 488 annexin V	A13201
		Allophycocyanin (APC) annexin V	A35110
	Caspase activity	Image-iT® LIVE Green Caspase-3 and -7 Detection Kit	I35106
		Image-iT® LIVE Green Caspase-8 Detection Kit	I35105
		Image-iT® LIVE Green Poly Caspases Detection Kit	I35104
		Image-iT® LIVE Red Caspase-3 and -7 Detection Kit	I35102
		Image-iT® LIVE Red Poly Caspases Detection Kit	I35101
		Vybrant® FAM Caspase-3 and -7 Assay Kit	V35118
		Vybrant® FAM Caspase-8 Assay Kit	V35119
		Vybrant® FAM Poly Caspases Assay Kit	V35117
	Metabolic activity	Vybrant® Cell Metabolic Assay Kit	V23110
	DNA condensation	Vybrant® DyeCycle™ Violet stain	V35003
		Hoechst 33342	H1399, H3570
	DNA fragmentation	APO-BrdU™ TUNEL Assay Kit	A23210
		Anti-BrdU antibody	A21300, A21303
		Vybrant® DyeCycle™ Orange stain	V35005
Late	Plasma membrane integrity	Propidium iodide	P1304MP, P3566
		SYTOX® Green dye	S7020

PRODUCT HIGHLIGHT

Detection of sub-G_1 DNA peak

DNA fragmentation is a relatively late apoptotic event that follows chromatin condensation and is characterized by the appearance of a sub-G_1 population in apoptotic cells. This population is typically detected by flow cytometry using propidium iodide following cell permeabilization and extensive washing. We have found that a new cell-permeant DNA stain—Vybrant® DyeCycle™ Orange stain—can directly detect a sub-G_1 population in cells with intact plasma membranes. Vybrant® DyeCycle™ Orange stain was used to label Jurkat cells at time points up to 6 hours after treatment with camptothecin, and a reagent such as SYTOX® Red dead cell stain or SYTOX® Blue dead cell discriminator was used to exclude cells with damaged membranes. The sub-G_1 population was initially absent in intact cells (left panel) and first appeared at 3–4 hours post-induction, increasing with additional time of induction (right panel).



Multicolor staining using Vybrant® DyeCycle™ Orange stain and SYTOX® Red dead cell stain. Camptothecin-treated Jurkat cells were stained with 10 µM Vybrant® DyeCycle™ Orange stain, excited with 488 nm light, and 10 nM SYTOX® Red dead cell stain, excited with 633 nm light. Analysis was gated on cells with intact membranes using SYTOX® Red dead cell stain. These cells showed a distinct peak to the left of the G_0/G_1 peak after 6 hours of induction. The sub-G_1 population was confirmed as apoptotic by co-staining with 1 µM PO-PRO™-1 iodide.

Product	Quantity	Cat. no.
SYTOX® Blue dead cell stain *for flow cytometry* *1000 assays* *1 mM solution in DMSO*	1 ml	S34857
SYTOX® Red dead cell stain *for 633 or 635 nm excitation* *5 µM solution in DMSO*	1 ml	S34589
Vybrant® DyeCycle™ Orange stain *5 mM solution in DMSO* *200 assays*	400 µl	V35005

is implicated in a number of disease states, including cancer, stroke, Alzheimer's disease, and several autoimmune diseases,[3–5] making these probes important tools for understanding these pathways.

As with cell viability, no single parameter fully defines cell death in all systems. Therefore, it is often advantageous to use a multiparametric approach when studying apoptotic events and their temporal relationships. Here we summarize a series of Molecular Probes® reagents and assays for flow cytometry and fluorescence imaging analyses, as well as a diverse group of antibodies that recognize important apoptosis-related antigens (Tables 2 and 3). The Vybrant® Apoptosis Assay Kits provide a convenient means of detecting multiple apoptotic parameters in a single assay (Table 2). These probes enable you to distinguish live cells, apoptotic cells, and necrotic cells, as well as to determine the relative stage of apoptosis in your particular experimental system. For more information on our complete line of apoptosis products, please visit **probes.invitrogen.com**. →

Table 2—Selected Vybrant® Apoptosis Assay Kits.

Kit name *	Vital cell stain	Apoptotic cell stain	Dead cell stain	Cell markers detected	Notes	Cat. no.
Vybrant® Apoptosis Assay Kit #2	NA	Alexa Fluor® 488 annexin V	Propidium iodide	Phosphatidylserine exposure and plasma membrane integrity	Two-color (green/red) assay for optimal separation of apoptotic and dead populations	V13241
Vybrant® Apoptosis Assay Kit #4	NA	YO-PRO®-1	Propidium iodide	Early loss of plasma membrane integrity	Two-color (green/red) assay for optimal separation of apoptotic and dead populations	V13243
Vybrant® Apoptosis Assay Kit #10	C_{12}-resazurin	Allophycocyanin annexin V	SYTOX® Green dye	Phosphatidylserine exposure, plasma membrane integrity, and cell vitality	Three-color (green/red/far-red) assay for optimal separation of viable, apoptotic, and dead populations	V35114
Vybrant® Apoptosis Assay Kit #11	MitoTracker® Red CMXRos	Alexa Fluor® 488 annexin V	NA	Phosphatidylserine exposure and mitochondrial membrane potential	Two-color (green/red) assay for optimal separation of viable and apoptotic populations	V35116
Vybrant® Apoptosis Assay Kit #12	NA	Vybrant® DyeCycle™ Violet stain	7-AAD	DNA fragmentation and membrane integrity	Two-color (blue/red) assay for optimal separation of apoptotic and dead populations	V35121

*For a complete list of Vybrant® Apoptosis Assay Kits, please visit **probes.invitrogen.com**. NA = not applicable; 7-AAD = 7-aminoactinomycin D.

Case in point: Multiparametric detection at work

Figure 1 illustrates how Molecular Probes® reagents and assay kits can be used for multiparametric analysis of apoptosis. We induced Jurkat cells with camptothecin and assayed over time for phosphatidylserine translocation to the outer membrane using fluorescent annexin V conjugates and for loss of membrane integrity using propidium iodide.[6] Flow cytometric analysis confirmed that the number of cells stained with green-fluorescent Alexa Fluor® 488 annexin V increased over time (green dots), while the number of cells stained with red-fluorescent propidium iodide remained relatively constant (blue dots) (Figure 1A, 1B, and 1C).

Table 3—Selected primary antibodies for studying apoptosis.

Target/ Antibody type	Clone/PAD	Western blotting	ELISA	Immuno-precipitation	Immuno-histochemistry	Immuno-fluorescence	Flow cytometry	Cat. no.
Annexin II—Specific for annexin II, and does not cross-react with other annexin proteins.								
Mouse IgG1	Z014	●	●		●			03-4400
ASPP2 (C-term)—Specific for ASPP2 (apoptosis-stimulating protein of p53 protein 2, p53-binding protein 2, 53BP2, Bcl2-binding protein (Bbp), PPP1R13A).								
Mouse IgG1 kappa	DX54.10	●	●	●		●		39-7000
BAG1—Specific for human BAG-1, BAG-1M, and BAG-1L proteins.								
Mouse IgG1 kappa	10B6H7	●	●	●	●	●		37-4000
Bax—Specific for the 21 kDa Bax protein.								
Mouse IgG1	2D2	●	●	●	●			33-6400, 18-0218
Mouse IgG1	6A7	●	●	●		●		33-6600
Bcl-2—Specific for the human Bcl-2 protein.								
Mouse IgG1 kappa	Bcl-2-100	●		●	●	●	●	13-8800, 18-0193Z
Bcl-XL—Specific for the Bcl-XL protein.								
Mouse IgG2a	2H12	●	●		●			33-6300, 18-0217
BrdU—Available unlabeled, as well as labeled with Alexa Fluor® 488, Alexa Fluor® 594, Alexa Fluor® 647, and Alexa Fluor® 680 dyes.								
Mouse IgG1 kappa	PRB-1				●	●	●	A21300, A21303, A21304, A21305, A31859
Caspase-3—Specific for human caspase-3.								
Mouse IgG2a	4-1-18	●			●			35-1600Z
Cytochrome *c*—Key component of the SelectFX® Alexa Fluor® 488 Cytochrome *c* Apoptosis Detection Kit.								
Mouse IgG					●	●		S35115
Lamin B1—Specific for the 68 kDa lamin B1 isoform; reactivity with other lamin isoforms has not been detected.								
Mouse IgG1 kappa	L-5	●	●	●		●		33-2000
Lamin B2—Specific for the 66 kDa lamin B2 isoform; reactivity with other lamin isoforms has not been detected.								
Mouse IgG1 kappa	E-3	●	●	●		●		33-2100
PARP—Specific for the 116 kDa full-length PARP protein, as well as its 85 kDa cleavage fragment. The immunoreactive epitope is contained within the C-terminal region of the 46 kDa DNA-binding domain of the PARP protein.								
Mouse IgG1 kappa	C-2-10	●	●			●		33-3100
RCAS1—Specific for the RCAS1 protein.								
Mouse IgG1 kappa	ZR001	●	●		●			39-2300
Smac/DIABLO—Specific for Smac/DIABLO, a 29 kDa mammalian mitochondrial protein that regulates apoptosis by binding apoptosis inhibitor proteins (IAPs).								
Mouse IgG1	17-1-87	●		●	●	●		35-6600

Using the Vybrant® FAM Poly Caspases Assay Kit in conjunction with fluorescent Alexa Fluor® 488 annexin V and propidium iodide probes, we could elucidate the temporal relationship between caspase activation, annexin binding, and loss of membrane integrity in identically induced Jurkat cells (Figure 1D, 1E, and 1F). The far-red–fluorescent allophycocyanin annexin V staining became prominent between 1 and 2 hours, while caspase activity increased after cells reacted with annexin V. Accordingly, cells with low caspase activity and increased annexin V binding were observed (arrow in Figure 1E). Similar results were seen with the Vybrant® FAM Caspase-3 and -7 Assay Kit and Vybrant® FAM Caspase-8 Assay Kit (data not shown), suggesting that phosphatidylserine translocation precedes caspase activation. A more detailed analysis of the temporal relationship of apoptotic events was obtained using additional probes, including MitoTracker® Red CMXRos and MitoProbe™ $DiIC_1(5)$ to detect changes in mitochondrial membrane potential, calcein to follow mitochondrial pore activation, and C_{12}-resazurin to monitor cell metabolism.[6] Multiparametric studies such as these can be used to determine the relative timing of apoptotic events and enable researchers to characterize specific apoptotic pathways. ■

References

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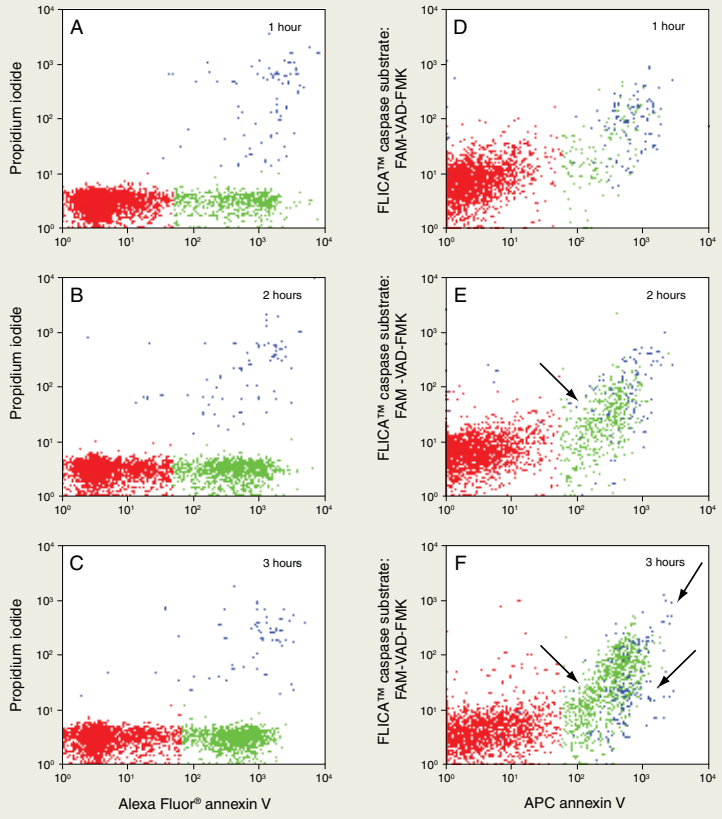

Figure 1—Flow cytometric analysis of multiple apoptotic events. Jurkat cells were treated with 10 µM camptothecin for 30 minutes to 3 hours to induce apoptosis, then stained with Alexa Fluor® 488 annexin V conjugate and propidium iodide (A, B, C) or with allophycocyanin (APC) annexin V conjugate, propidium iodide, and the FLICA™ caspase substrate FAM-VAD-FMK provided in the Vybrant® FAM Poly Caspases Assay Kit (D, E, F). The annexin V staining (indicating phosphatidylserine translocation to the outer membrane) became prominent between 1 and 2 hours, while caspase activity increased after the cells reacted with annexin V; cells with low caspase activity and increased annexin V binding are visible (arrow in Figure 1E). Cells stained with propidium iodide (indicating loss of membrane integrity) show both high and low polycaspase activity (arrows in Figure 1F). Blue dots represent propidium iodide–positive events (loss of membrane integrity); green dots represent annexin V–positive events (phosphatidylserine translocation); red dots represent live-cell events.