immunochemistry
TECHNOLOGIES

CELL VIABILITY ASSAYS AND REAGENTS

Bright Minds, Bright Solutions™

ASSAYS AND KITS

Fluorescent whole-cell assays
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Intracellular apoptosis detection
Cellular analysis

DETECTION CAPABILITIES

Apoptosis
Necrosis
Intracellular caspase activity
Cell-mediated cytotoxicity
Activated serine proteases
Autophagy
Oxidative stress
Mitochondrial membrane permeability
And many more!

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Apoptosis

Apoptosis is a process of programmed cell death that occurs in multicellular organisms. It is a highly regulated and controlled process that occurs normally during development and aging as a homeostatic mechanism to maintain cell populations. During early apoptosis, cell shrinkage and pyknosis occur. With cell shrinkage, the cytoplasm becomes dense and the organelles are more tightly packed. Pyknosis is the result of chromatin condensation and is a characteristic feature of apoptosis. At a later stage, apoptosis produces cell fragments called apoptotic bodies that phagocytic cells engulf and quickly remove before the contents can spill out causing inflammation.

There is a wide variety of stimuli and conditions that can trigger apoptosis. However, not all cells will necessarily die in response to the same stimulus. For example, irradiation or drugs used for cancer chemotherapy results in DNA damage, which can lead to apoptotic death through a p53-dependent pathway, but not in all cells. Other cells have a default death pathway that must first be blocked by a survival factor such as a hormone or growth factor. There is also the issue of distinguishing apoptosis from necrosis, which is a form of cell death that does not involve activation of a programmed cell death pathway. Apoptosis and necrosis are two processes that can occur independently, sequentially, as well as simultaneously. In some cases, it's the type of stimuli and/or the degree of stimulation that determines if cells die by apoptosis or necrosis. At low doses, a variety of injurious stimuli such as heat, radiation, hypoxia and cytotoxic anti-cancer drugs can induce apoptosis but these same stimuli can result in necrosis at higher doses. Finally, apoptosis is a coordinated and energy-dependent process that involves the activation of a group of cysteine proteases called caspases and a complex cascade of events that link the initiating stimuli to the final structured demise of the cell.

Pathways

Apoptosis can be initiated through one of three pathways. In the intrinsic or mitochondrial pathway, the cell kills itself because it senses cell stress, while in the extrinsic pathway the cell is instructed to kill itself through signal transduction stimulators from other cells. The Perforin/Granzyme pathway is mediated by cytotoxic T cells. In this third pathway, apoptosis is induced via either Granzyme B or Granzyme A. All three initiation pathways (apart from Granzyme A) induce cell death through the Execution pathway that involves the activation of caspase-3.

Intrinsic Pathway

Activation of the intrinsic pathway triggers changes in the inner mitochondrial membrane that result in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential, and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol.

The first group consists of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi. Cytochrome c binds and activates Apaf-1 as well as procaspase-9, forming an "apoptosome". The clustering of procaspase-9 in this manner leads to caspase-9 activation. Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting IAP (Inhibitors of Apoptosis Proteins) activity. IAP suppresses caspases.

The second group consists of AIF (Apoptosis-Inducing Factor), endonuclease G and CAD (Caspase-Activated DNAse). AIF transfers to the nucleus and causes DNA fragmentation into ~50–300 kb pieces and condensation of peripheral nuclear chromatin (also called stage I condensation). Endonuclease G also transfers to the nucleus where it cleaves nuclear chromatin to produce oligo-nucleosomal DNA fragments. AIF and endonuclease G both function in a caspase-independent manner. CAD is subsequently released from the mitochondria and transfers to the nucleus where, after cleavage by caspase-3, it leads to oligo-nucleosomal DNA fragmentation and a more pronounced and advanced chromatin condensation (also called Stage II condensation).

Extrinsic pathway

The Extrinsic initiation pathway involves receptors of the TNFR (Tumour Necrosis Factor Receptor) family. In the extrinsic (death receptor) pathway of apoptosis, ligation of death receptors on the cell surface leads to caspase activation. This pathway relies on the formation of a Death-Inducing Signaling Complex (DISC), which always includes FADD and caspase-8. The death receptors known so far are TNFR1 (ligand TNF-alpha), CD95 (ligand FasL), and TRAILR1 plus TRAILR2 (ligand TRAIL). In the case of ligated TNFR1, adapter TRADD (TNF Receptor type 1-Associated Death Domain) is first engaged, which in turn recruits FADD. TRADD is only required for apoptosis when induced by TNF-alpha. The other ligated death receptors engage FADD directly. Fas associated via death domain (FADD) protein is responsible for the recruitment of caspase-8 to form the DISC. The presence of the FLICE-like inhibitory protein (cFLIP) in the FADD-caspase-8-cFLIP complex determines if and how cells die. cFLIP comes in two major isoforms, the long isoform cFLIPL and the short isoform cFLIPS. The cFLIPS protein is an inhibitor of caspase-8 and blocks DISC-dependent procaspase-8 activation. The cFLIPL protein regulates the extent of activation and possibly substrate specificity of procaspase-8. Low levels of cFLIPL can enhance apoptotic signaling, whereas apoptosis is inhibited when cFLIPL levels are high.

CD95 and TRAIL-R ligation induces apoptosis by direct recruitment of FADD-caspase-8 in a complex called the DISC. As described above, isoform levels of cFLIP then determine whether apoptosis is blocked or engaged. Ligation of TNFR1 can both induce caspase-8-mediated apoptosis as well as block apoptosis via the NF-κB-induced expression of cFLIP in a feedback loop. Receptor-interacting serine/threonine kinase 1 (RIPK1) is key in regulating TNFR1-induced FADD-caspase-8-mediated apoptosis. TNFR1 ligation leads to the recruitment of TRADD, TRAF2, cIAP1/2, and RIPK1 (complex I). RIPK1 ubiquitination by cIAP1/2 mediates activation of NF-κB and the production of pro-inflammatory and pro-survival gene expression. RIPK1 is deubiquitinated by CYLD and leaves complex I to recruit FADD to form the ripoptosome (complex IIa) involving caspase-8 and cFLIPL. Homodimerization and activation of caspase-8 on FADD induces apoptosis. One of the expressed pro-survival genes, cFLIPL, heterodimerizes with caspase-8, resulting in inhibition of caspase-8 activation and apoptosis. Alternatively, RIPK1 interacts with RIPK3 to either stimulate RIPK3 oligomerization in the necrosome (complex IIb), or to suppress it. Oligomerized RIPK3 is a prerequisite to trigger phosphorylation of the downstream mediator Mixed-Lineage Kinase-Like (MLKL) that triggers necrosis. Thus, death receptor TNFR1 provides two separate pathways downstream from TRADD recruitment: deubiquitinated RIPK1 either allows the activation of DISC in the ripoptosome (complex IIa) igniting apoptosis, or it interacts with RIPK3 to activate MLKL in the necrosome (complex IIb). MLKL is the effector protein that, once activated, transfers to the plasma membrane where it induces rupture and subsequent cell death. This regulated form of necrosis is also known as necroptosis. The release of cellular components this way results in an inflammatory response.

Perforin/Granzyme pathway

One aspect of the adaptive immune system is recognizing and eliminating target cells through the induction of apoptosis, involving CD8+ cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. CTLs and NK cells execute similar effector functions to remove target cells. This is achieved through the release of cytotoxic granules containing perforin, granzymes, and granulysin, which work together to induce apoptosis in the target cells.

A key protein in the cytotoxic granule is perforin. Classically, perforin is known to form a pore in cell membranes, allowing passage of granzymes into the cell inducing apoptosis. Granzyme B can either directly, or through caspase-10 activation, convert pro-form caspase-3 into active caspase-3 to initiate the Apoptosis Execution pathway.

Execution pathways

Both extrinsic and intrinsic apoptosis induction pathways lead to an end-point execution phase, considered the final pathway of apoptosis. Execution caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7 function as effector or “executioner” caspases, cleaving various substrates including cytokeratins, PARP, the plasma membrane cytoskeletal protein Fodrin alpha, the nuclear protein NuMA and others, ultimately causing the morphological and biochemical changes seen in apoptotic cells. Caspase-3 is the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase-3 specifically activates the endonuclease CAD. In proliferating cells CAD is complexed with its inhibitor, ICAD. In apoptotic cells, activated caspase-3 cleaves ICAD to release CAD. CAD then degrades chromosomal DNA within the nuclei causing chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies through the cleavage of Gelsolin. The cleaved fragments of Gelsolin, in turn, cleave actin filaments in a calcium independent manner. This results in disruption of the cytoskeleton, intracellular transport, cell division, and signal transduction.

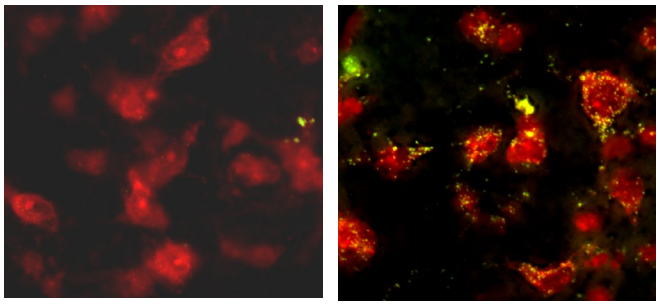
Phagocytic uptake of apoptotic cells is the last component of apoptosis. Phospholipid asymmetry and externalization of phosphatidylserine on the surface of apoptotic cells and their fragments is the hallmark of this phase. The appearance of phosphatidylserine on the outer leaflet of apoptotic cells facilitates noninflammatory phagocytic recognition, followed up quickly by their early uptake and disposal. This process of early and efficient uptake, with no release of intracellular constituents, eliminates the probability of inflammatory events occurring.

Detect caspase activity in vivo with FLIVO®

- *FLIVO®(FLuorescence in vIVO) is a powerful method for assessing caspase activity in vivo.*
- *FLIVO probes are non-cytotoxic fluorescent inhibitors of caspases, and contain the preferred binding sequence for many caspases, Val-Ala-Asp (VAD).*
- *FLIVO is a cell-permeant reagent that readily diffuses in and out of all cells that it encounters as it circulates through the body. If there are active caspase enzymes inside a cell, FLIVO will form an irreversible covalent bond with a reactive cysteine on the large subunit of the caspase heterodimer, thereby inhibiting further enzymatic activity. The bound FLIVO probe will remain inside the cell as long as the cell membrane is intact. Any unbound FLIVO is removed from the circulation of the animal.*
- *Apoptotic cells will retain a higher concentration of FLIVO and fluoresce brighter than non-apoptotic cells.*
- *FAM-FLIVO and SR-FLIVO are injectable inhibitors for labeling active caspases in tissues.*
- *NIR-FLIVO are injectable tracers for labeling caspase activity in whole animals. NIR-FLIVO tracers utilize near-infrared fluorescent labels for non-invasive detection of active caspases using whole animal imagers.*

FIGURE 1: FAM-FLIVO DETECTION OF CASPASE ACTIVITY IN RAT BRAIN

Using FAM-FLIVO to monitor cell death, there is a clear distinction between healthy and apoptotic neurons. In this live animal brain study of diabetes, Dr. Thomas Morrow at the University of Michigan VAMC Ann Arbor was able to assess neurodegeneration via caspase activity in control (left) and 8-week STZ diabetic rats (right). FAM-FLIVO (catalog #981) was injected intravenously to directly label caspase-positive apoptotic neurons 30 minutes prior to sacrifice. After sacrifice, 20 µm frozen sections of the periaqueductal gray (PAG) were prepared and counter-stained with red fluorescent Nissl to identify all neurons. Dying apoptotic neurons (left) exhibit dual staining with FAM-FLIVO (yellow/green) and Nissl (red). In this model, diabetic animals show greater levels of caspase activity in the PAG than control animals.



CONTROL RAT **DIABETIC RAT**

What are FLIVO® Poly Caspase Inhibitors?

FLIVO Poly Caspase Inhibitors are *in vivo* research tools for detecting caspase activation and apoptosis in excised animal tissues. ICT's FLIVO inhibitors are available in green (carboxyfluorescein or FAM) or red (sulforhodamine B or SR) fluorescence. FAM-FLIVO and SR-FLIVO are injectable caspase inhibitors that can be used to quantify cell death in living animal tissues. FAM-FLIVO and SR-FLIVO are direct stains that can be used to quantitate poly caspase activity in the tissues of live animals. Once labeled, tissues are ready for analysis and no further processing is necessary. FAM-FLIVO Poly Caspase Inhibitor optimally excites at 488-492 nm and has a peak emission at 515-535 nm. SR-FLIVO Poly Caspase Inhibitor optimally excites at 550-580 nm and has a peak emission at 590-600 nm.

FLIVO® PRODUCTS	SIZE	CAT. #
FAM-FLIVO In vivo Poly Caspase Assay	6 Tests	980
	24 Tests	981
SR-FLIVO In vivo Poly Caspase Assay	6 Tests	982
	24 Tests	983
NIR-FLIVO 690 Tracer In vivo Assay	20 Tests	9112
NIR-FLIVO 690 Free Dye Control Assay	20 Tests	9113
NIR-FLIVO 747 Tracer In vivo Assay	20 Tests	9114
NIR-FLIVO 747 Free Dye Control Assay	20 Tests	9115

What are FLIVO® Poly Caspase Tracers?

NIR-FLIVO Poly Caspase Tracers are *in vivo* research tools for detecting caspase activation and apoptosis in whole live animals. ICT's NIR-FLIVO Tracers utilize the near-infrared fluorescent labels Dylight® 690 and Dylight® 747 to enable researchers to illuminate caspase-positive, apoptotic cells and tissues in living animal models using whole animal imagers. NIR-FLIVO 690 Poly Caspase Tracer optimally excites at 690 nm and has a peak emission at 709 nm. NIR-FLIVO 747 Poly Caspase Tracer optimally excites at 747 nm and has a peak emission at 776 nm.

How is FLIVO used?

After control and experimental animal subjects are prepared for the investigation, FLIVO is intravenously injected and allowed to circulate for 6-18 hours. FLIVO is a direct stain; once labeled, apoptotic cells will fluoresce upon excitation and no further processing is necessary. If using FAM-FLIVO or SR-FLIVO, caspase

activity in cells and tissues can be detected using a window chamber system, or tissues can be excised and cells can then be analyzed by flow cytometry, fluorescence microscopy, or fluorescence plate reader. If using NIR-FLIVO, the Tracers can be non-invasively imaged with whole animal imagers, or tissues can be excised and cells can then be analyzed by flow cytometry, fluorescence microscopy, or fluorescence plate reader.

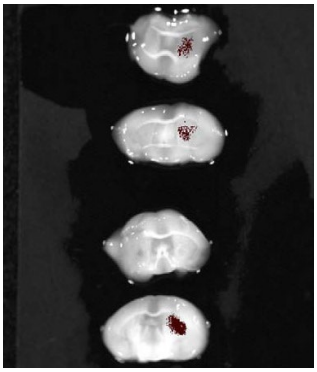
How does FLIVO work?

Our FLIVO products are cell permeant, non-toxic caspase inhibitors, allowing for the *in vivo* detection of caspase-mediated apoptosis in living animals. FLIVO Inhibitors and Tracers consist of the amino acid peptide sequence valine-alanine-aspartic acid (VAD), which is targeted by active caspase enzymes, a fluoromethyl ketone group that aids in the formation of a covalent bond with active caspases, and the fluorescent label for detection.

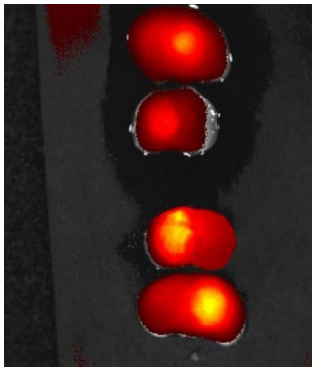
After intravenous injection, FLIVO diffuses in and out of all cells that it encounters as it circulates through the body. Active caspase enzymes will covalently bond with FLIVO and retain the fluorescent signal within the cell as long as the cell remains intact. Unbound FLIVO is rapidly cleared from the circulation of the animal, although it may be held in the liver for 4-6 hours, and it may accumulate in the bladder and the feces until excreted. FLIVO is very sensitive and will also detect naturally occurring, low-level apoptosis from normal cellular turnover.

FIGURE 2: DETECT APOPTOSIS IN MURINE BRAIN ABSCESSES USING NIR-FLIVO 690 TRACER

Brain abscesses were induced in mice following the intracerebral inoculation of live *Staphylococcus aureus*. Animals received intravenous injections of NIR-FLIVO 690 Tracer (catalog #9112) or NIR-FLIVO 690 Free Dye Control (catalog #9113) at 17 hours post-infection, whereupon signals were acquired 1 hour later from brain tissues *ex vivo* using an IVIS® Spectrum™ (Caliper Life Sciences). Strong caspase activity was associated with brain abscesses following administration of the NIR-FLIVO 690 tracer (right image), whereas minimal signal was detected in animals injected with the NIR-FLIVO 690 Free Dye Control (left image).



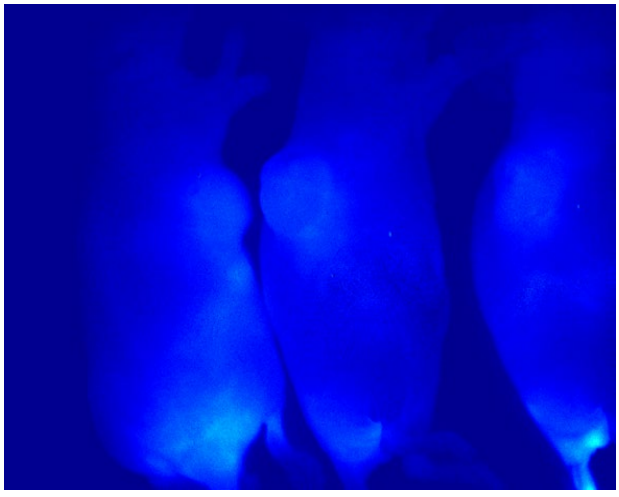
CONTROL DYE



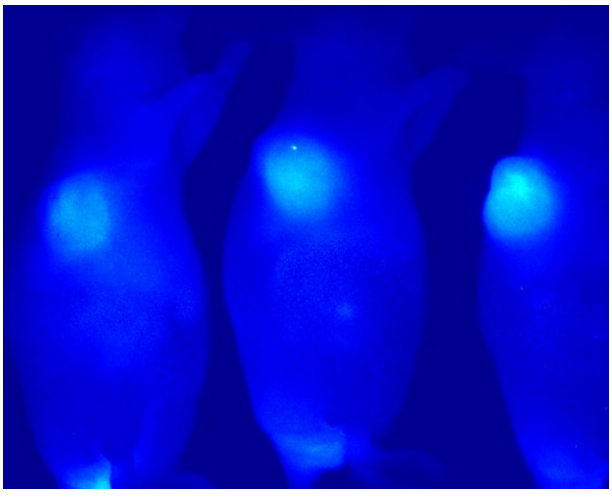
NIR-FLIVO TRACER

FIGURE 3: *IN VIVO* MONITORING OF APOPTOSIS DURING EXPERIMENTAL ANTI-CANCER TREATMENT

Apoptotic tissues in murine tumor models were imaged *in vivo* with NIR-FLIVO 747 Tracer (catalog #9114) using the CRi Maestro™ imaging system. NIR-FLIVO 747 Tracer was used to non-invasively monitor the efficacy and time kinetics of an experimental anti-cancer treatment. After receiving a placebo or an experimental treatment, negative control mice (left image) and experimentally treated mice (right image) were injected intravenously with NIR-FLIVO 747 Tracer and imaged non-invasively at various time points with the CRi Maestro™ imaging system. Apoptotic tumor tissues fluoresce bright blue in the experimental group subjects, indicating that the experimental treatment had an apoptotic effect.



NEGATIVE CONTROL



EXPERIMENTALLY TREATED

Detect caspase activity in vitro with FLICA®

- *FLICA (Fluorescent-Labeled Inhibitor of CAspases) are powerful tools for assessing programmed cell death by detecting apoptosis and pyroptosis in vitro.*
- *FLICA probes are non-cytotoxic, cell membrane permeant inhibitors that covalently bind to active caspase enzymes.*
- *FLICA measures the intracellular process of apoptosis instead of a side-effect, such as the turn-over of phosphatidyl serine, and eliminates the incidence of false positives that often plagues methods like Annexin V and TUNEL staining.*
- *FLICA can be used to label suspension or adherent cells and thin tissue sections.*
- *ICT has kits for the detection of: caspase-1 (YVAD) (also recognizes caspases 4 and 5), -2 (VDVAD), -3/7 (DEVD), -6 (VEID), -8 (LETD), -9 (LEHD), and -10 (AEVD).*
- *FLICA kits are available with a green, red, or far red fluorescent label.*

What are FLICA® Caspase Inhibitors?

FLICA Caspase Inhibitors are *in vitro* research tools for detecting caspase activation and apoptosis in cell culture. Each FLICA probe contains a 3 - 5 amino acid sequence that is targeted by different activated caspases. This target sequence is sandwiched between a fluorescent label and a fluoromethyl ketone (FMK).

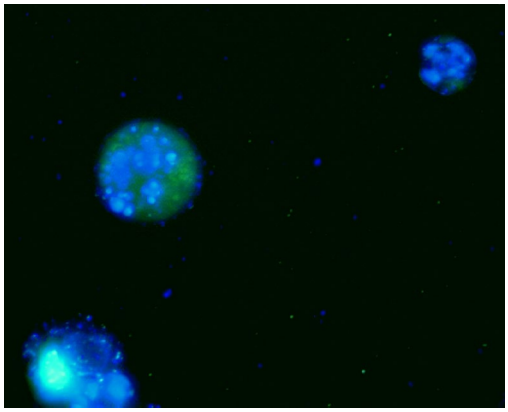
How does FLICA work?

Our FLICA products are cell permeant, non-toxic caspase inhibitors, allowing for the *in vitro* detection of caspase-mediated apoptosis in whole live cells. Simply add FLICA to the cell culture media and it will passively cross the cell membrane. A caspase enzyme cannot cleave the FLICA inhibitor probe; instead, it forms an irreversible covalent bond with the FMK on the target sequence and becomes inhibited from further enzymatic activity. If there is an active caspase enzyme inside the cell, it will covalently bind to FLICA and retain the fluorescent signal within the cell. There is no interference from procaspases nor inactive forms of the enzyme. FLICA probes constantly fluoresce; therefore, any unbound reagent must be washed out to remove any background fluorescence. No further processing is necessary. FLICA is very sensitive and will also detect naturally occurring, low-level apoptosis from normal cellular turnover.

FLICA can be used to label suspension or adherent cells and thin fresh or frozen tissue sections. After labeling with FAM-FLICA, cells can be fixed or frozen. For tissues that will be paraffin-embedded after labeling, use ICT's red sulforhodamine SR-FLICA probes; do not use the green FAM-FLICA probes as the FAM dye will be quenched during the paraffin embedding process.

FIGURE 4: POLY CASPASE ACTIVITY IN STAUROSPORINE-TREATED THP-1 MONOCYTES

Human monocytic leukemia THP-1 cells were dually stained with ICT's green FAM-FLICA poly caspase inhibitor reagent, FAM-VAD-FMK (catalog #92), and a blue DNA stain, Hoechst 33342. Cells were incubated with 1 µM staurosporine for 3 hours at 37°C to induce apoptosis. Cells were then labeled with FAM-VAD-FMK for 60 minutes at 37°C. Cells were washed, then Hoechst stain was added and incubated for 5 minutes. Wet-mount slides were prepared and two photos were taken and superimposed. Caspase activity (green) was detected using a band pass filter (excitation at 488 nm; emission at 520 nm). Nuclear staining by Hoechst 33342 was revealed using a UV-filter (excitation at 365 nm, emission at 480 nm). Only one cell of the three cells is apoptotic (middle) – it is stained positive for caspase activity with FLICA FAM-VAD-FMK. It also has many bright blue spots from the Hoechst stain, indicating that the DNA is breaking down and the cell is beginning to die. The lack of green staining and the concentrated blue DNA in the lower left cell indicate it is alive (not apoptotic). The upper right cell is necrotic (no green, scattered blue).



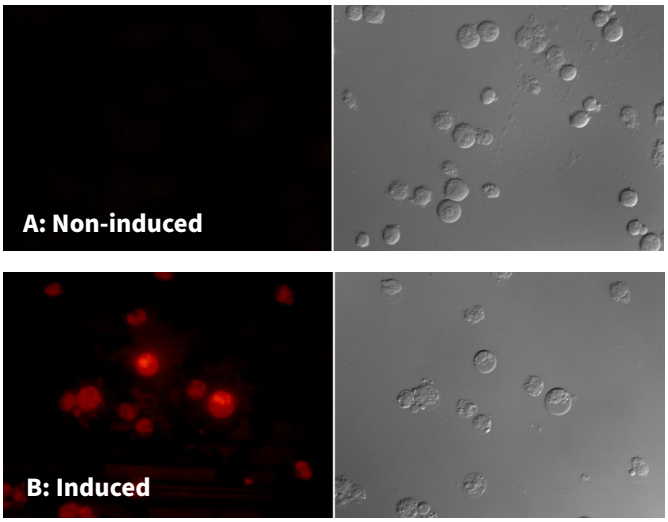
Is FLICA compatible with other reagents?

Once labeled with FLICA, cells can be fixed, embedded, or frozen for storage; protect cells from light during handling. You can even add other stains for dual labeling studies. To distinguish apoptotic from necrotic cells, add an additional stain like PI (catalog #638, included in the green kits) or 7-AAD (catalog #6163) to stain necrotic cells red. Hoechst 33342 is included in all kits for labeling DNA. Use our red or far red fluorescence FLICA kits with GFP-transfected cell lines.

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.

FIGURE 5: MICROSCOPY-BASED DETECTION OF CASPASE-9 IN JURKAT CELLS

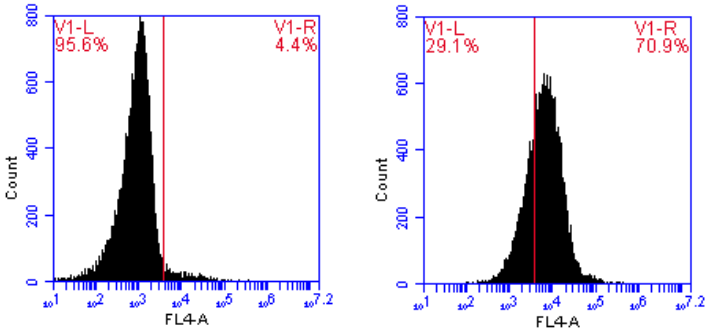
Jurkat cells were incubated with 1 µM staurosporine for 3 hours at 37°C to induce apoptosis. Cells were then labeled with ICT's red SR-FLICA caspase-9 inhibitor, SR-LEHD-FMK (catalog #961) for 60 minutes at 37°C. Cells were washed and slides were prepared. Samples were viewed through a fluorescence microscope using a broad band pass filter. On slide B, cells appear very bright red, indicating a high amount of active caspase-9; these cells were undergoing apoptosis at the time the reagent was added. Non-induced cells did not fluoresce (slide A).



FLICA® PRODUCTS	SIZE	CAT. #
FAM-FLICA Poly Caspase Assay	25 Tests	91
	100 Tests	92
SR-FLICA Poly Caspase Assay	25-50 Tests	916
	100-200 Tests	917
FLICA 660 Poly Caspase Assay	25-50 Tests	9120
FAM-FLICA Caspase-1 (YVAD) Assay	25 Tests	97
	100 Tests	98
FAM-FLICA Caspase-1 (WEHD) Assay	25 Tests	9161
	100 Tests	9162
FLICA 660 Caspase-1 Assay	25-50 Tests	9122
FAM-FLICA Caspase-2 Assay	25 Tests	918
	100 Tests	919
FAM-FLICA Caspase-3/7 Assay	25 Tests	93
	100 Tests	94
FLICA 660 Caspase-3/7 Assay	25-50 Tests	9125
FAM-FLICA Caspase-6 Assay	25 Tests	95
	100 Tests	96
FAM-FLICA Caspase-8 Assay	25 Tests	99
	100 Tests	910
SR-FLICA Caspase-8 Assay	25-50 Tests	9149
	100-20 Tests	9150
FAM-FLICA Caspase-9 Assay	25 Tests	912
	100 Tests	913
SR-FLICA Caspase-9 Assay	25-50 Tests	960
	100-200 Tests	961
FAM-FLICA Caspase-10 Assay	25 Tests	922
	100 Tests	923

FIGURE 6: FLOW CYTOMETRY-BASED DETECTION OF CASPASE-3/7 IN JURKAT CELLS

Jurkat cells were treated with a negative control (left) or staurosporine, an apoptosis-inducing agent (right), for 4 hours, then stained with ICT's far-red FLICA caspase-3/7 inhibitor probe, 660-DEVD-FMK (catalog #9125), for 1 hour. Cells were washed twice and read on an Accuri C6 flow cytometer. Treatment with the negative control induced caspase activity in only 4.4% of the cell population (left), whereas treatment with staurosporine induced caspase activity in 70.9% of the experimental cells (right). This is a ratio of 16:1.



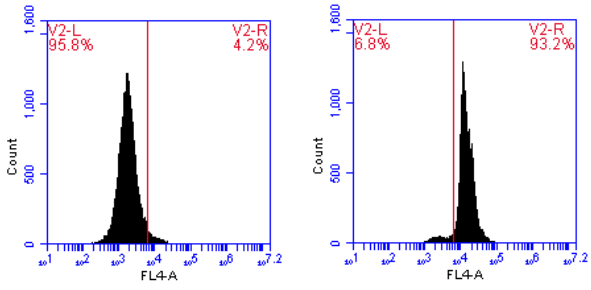
Magic Red® caspase-3/7 substrate to monitor apoptosis activity in vitro

- *ICT's Magic Red® Caspase-3/7 substrate contains a 4 amino acid sequence, Asp-Glu-Val-Asp (DEVD), which is the optimal target sequence for caspases-3 and -7.*
- *Two copies of the DEVD sequence are coupled to a photostable red fluorophore, cresyl violet, to create the Magic Red caspase-3/7 substrate MR-$(DEVD)_2$. In the intact MR-$(DEVD)_2$ substrate, the fluorescence has been quenched. Maximum fluorescence potential is achieved upon cleavage of both DEVD side chains by activated caspases-3 and -7.*
- *The red fluorescent signal can be monitored with a fluorescence microscope, plate reader, or flow cytometer. It has an optimal excitation of 592 nm and emission of 628 nm*

FIGURE 7: CASPASE-3/7 ACTIVITY IN APOPTOTIC CELLS

Jurkat cells were treated with a negative control (left) or staurosporine, an apoptosis-inducing agent (right), for 4 hours, then labeled with ICT's Magic Red caspase-3/7 substrate, MR-$(DEVD)_2$ (catalog #936), for 1 hour. Cells were read on an Accuri C6 flow cytometer using red laser excitation at 640 nm and a 675/25 emission filter pairing (FL4).

Treatment with the negative control induced caspase activity in only 4.2% of the cell population (left, V2-R), whereas treatment with staurosporine induced caspase activity in 93.2% of the experimental cells (right, V2-R). This is a ratio of 22:1.



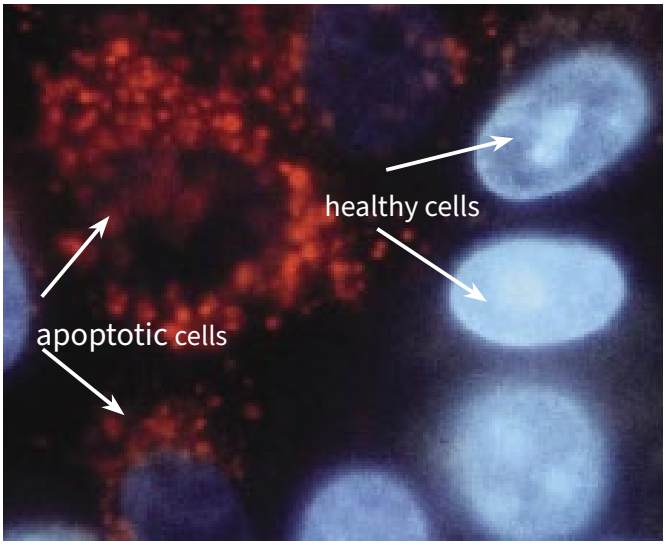
Negative Control, Non-Induced

Positive Control, Apoptosis-Induced

	V2-L MR-$(DEVD)_2$ Negative	V2-R MR-$(DEVD)_2$ Positive
Negative, Non-Induced (left)	95.8%	4.2%
Positive, Induced (right)	6.8%	93.2%
Ratio		22:1

FIGURE 8: MAGIC RED DETECTION OF APOPTOTIC CELLS

Apoptotic MCF-7 cells were dually stained using ICT's Magic Red MR-$(DEVD)_2$ fluorogenic caspase-3/7 substrate and Hoechst 33342 nuclear stain (catalog #936). MCF-7 cells were exposed to 0.15 µM camptothecin (catalog #6210) for 24 hours at 37°C, then stained with MR-$(DEVD)_2$ for 30 minutes at 37°C, washed twice in PBS, and supravitally stained with 1 µg/mL Hoechst stain for about 10 minutes. Using the Nikon Microphot-FXA system with multi-wavelength filter pairs (UV for Hoechst stain and green light for MR-$(DEVD)_2$), apoptotic cells bearing orange-red lysosomal bodies with less intense blue nuclei are intermixed with non-apoptotic cells bearing bright blue nuclei and absent or reduced orange-red lysosomal staining. Data provided by Dr. Zbigniew Darzynkiewicz at Brander Cancer Research Center Institute, New York, NY.



CASPASE-3/7 PRODUCTS	SIZE	CAT. #
Magic Red Caspase-3/7 Assay	25-50 Tests	935
	100-20 Tests	936

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Not for use in diagnostic procedures.

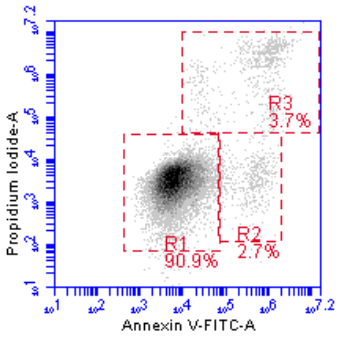
Additional Apoptosis Products

- ***Annexin V:*** *Detect apoptosis and membrane permability with ICT's Annexin V-FITC Apoptosis Assay. This in vitro apoptosis assay employs the green fluorescent Annexin V-FITC reagent to label exposed phosphatidylserine in cultured cell samples. Detect membrane compromised cells, a trademark of late apoptosis or cell necrosis, with the live/dead stain, propidium iodide. Analyze the fluorescent signals by flow cytometry. Available with recombinant fluorescein-conjugated bovine, canine, chicken, equine, human, and swine Annexin V.*
- ***Apoptosis inducing agents:*** *ICT offers staurosporine and camptothecin in a highly purified form ideal for creating positive controls in apoptosis detection experiments. Our apoptosis inducing agents are lyophilized to promote long-term stability.*
- ***OPH-based apoptosis detection reagents:*** *This family of cacspase inhibitor reagents employ an O-phenoxy (OPH) reactive group instead of an FMK group. In a manner analogous to the FMK class of cysteine reactive compounds, the OPH inhibitors form a stable covalent thioether adduct with the reactive SH-site of caspase enzymes present in apoptotic cells. These reagents are used as simple and reliable methods for screening apoptosis in live cells, and can be used with a fluorescence microscope, fluorescence plate reader, or flow cytometer.*
- ***Recombinant proteins:*** *ICT carries recombinant Granzyme B, Tumor Necrosis Factor-alpha, and Tumor Necrosis Factor-beta. Our recombinant proteins are sterile and are lyophilized to promote long-term stability.*

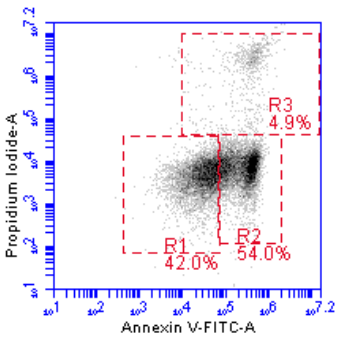
APOPTOSIS PRODUCTS	SIZE	CAT. #
Annexin V-FITC Apoptosis Assay	100 Tests	9124
Bovine Annexin V-Fluorescein Apoptosis Assay	500 Tests	9138
Canine Annexin V-Fluorescein Apoptosis Assay	500 Tests	9139
Chicken Annexin V-Fluorescein Apoptosis Assay	500 Tests	9140
Equine Annexin V-Fluorescein Apoptosis Assay	500 Tests	9141
Swine Annexin V-Fluorescein Apoptosis Assay	500 Tests	9142
Camptothecin	25 mg	6208
	100 mg	6209
	250 mg	6210
Staurosporine	1 mg	6212
FAM-VAD-OPH I in vitro Apoptosis Detection Reagent	4 x 62.3 µg vials	6354
FAM-VAD-OPH II in vitro Apoptosis Detection Reagent	4 x 58 µg vials	6355
SR-VAD-OPH in vitro Apoptosis Detection Reagent	4 x 146 µg vials	6357
FAM-DEVD-OPH in vitro Apoptosis Detection Reagent	4 x 37.5 µg vials	6356

FIGURE 9: ANNEXIN V DETECTION OF APOPTOTIC CELLS

Jurkat cells were treated with a negative control (left) or staurosporine, an apoptosis-inducing agent (right), for 4 hours, washed, and then stained with ICT's Annexin V-FITC Apoptosis Assay (catalog #9124) for 10 min. Cells were read on an Accuri C6 flow cytometer. Treatment with the negative control induced Annexin V-FITC labeling in only 2.7% of the cell population (R2, left), whereas treatment with staurosporine induced Annexin V-FITC labeling in 54% of the experimental cells (R2, right). This is a ratio of 20:1. Treatment with staurosporine did not significantly increase the proportion of late apoptotic/necrotic cells (R3) at 4 hours.



Negative Control, Non-induced



Positive Control, Apoptosis-induced

Pyroptosis

Pyroptosis is a form of inflammatory programmed cell death, triggered by various pathological stimuli such as stroke, heart attack, cancer, and microbial infections. Pyroptosis is fundamentally distinct from other cell death pathways, such as apoptosis and necroptosis, by its exclusive dependency on caspase-1. Because caspase-1 is a principle effector protease within the pyroptotic cell death pathway, it stands to reason that the intracellular detection of activated caspase-1 is a key indicator of pyroptosis activity.

Pyroptosis promotes the rapid clearance of various bacterial and viral infections by removing intracellular replication niches and by enhancing the host's defensive response. Cells can use a broad range of intracellular and extracellular mechanisms for detecting different "danger" signals generated or released during infection or injury. These detection mechanisms typically involve pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and Absent in melanoma 2-like receptors (ALRs). PRRs initiate a signaling cascade that leads to activation and production of inflammatory cytokines upon recognition of conserved microbial signatures or danger signals (commonly referred to as pathogen associated molecular patterns or PAMPs, or danger associated molecular patterns or DAMPS, respectively). Recognition of PAMPs, DAMPs, and some foreign toxins can lead to inflammasome activation, which triggers activation of caspase-1, and the initiation of pyroptosis. Cell surface-associated TLRs typically recognize microbial membrane components (such as the lipid A component from LPS, peptidoglycans, mannan, etc.), while intracellular TLRs recognize bacterial and viral nucleic acids, as well as some "self" nucleic acids present during particular disease states. In addition to promoting an immune response, activation of the inflammasome also leads to an increase in pyroptotic cell death. Some examples of pyroptosis include the death of salmonella-infected macrophages and T helper (Th) cells that die as a result of abortive HIV infection. In addition, pyroptosis is stimulated by non-infectious stimuli, including host factors produced during myocardial infarction.

Characteristic features of pyroptosis cell death pathway:

Pyroptotic cells undergo swelling which leads to lysis. Caspase-1-regulated cellular membrane pores dissipate cellular ionic gradients, producing a net increase in osmotic pressure, water influx, cell swelling and, eventually, osmotic lysis and release of inflammatory intracellular contents.

DNA cleavage occurs during pyroptosis, but lacks the nuclear integrity and oligonucleosomal DNA fragmentation patterns characteristic of apoptosis.

Formation of the inflammasome occurs. The inflammasome is a cytosolic multimeric signaling complex that coordinates the activation of an immune response against invading pathogens. Activation of the inflammasome subsequently leads to processing and activation of caspase-1. Once activated, caspase-1 cleaves pro-IL-1β and pro-IL-18 into their mature forms, and it cleaves Gasdermin D (encoded by GSDMD) to induce pore opening and pyroptosis. The cytosolic pathogen recognition receptors capable of forming inflammasome complexes include NLRs, ALRs, and the tripartite motif (TRIM) family member Pyrin.

The inflammatory cytokines IL-1β and IL-18 undergo caspase-1-dependent activation and secretion during pyroptosis. IL-1β is a potent, endogenous pyrogen that stimulates fever, leukocyte tissue migration, and the expression of a diverse array of cytokines and chemokines. IL-18 induces Interferon gamma (IFNγ) production and is important for the activation of T cells, macrophages and other cell types. Both IL-1β and IL-18 play crucial parts in the pathogenesis of a range of inflammatory and autoimmune diseases. Although neither cytokine is required for the process of cell death, their production contributes to the inflammatory response elicited by cells undergoing pyroptosis.

The signaling cascades:

TLR activation signals through the recruitment of specific adaptor molecules. Upon PAMP or DAMP recognition, TLRs recruit TIR-domain-containing adaptor proteins such as MyD88 and TRIF. MyD88 and TRIF then initiate signal transduction pathways that lead to the activation of NF-κB, IRFs, or MAP kinases, which subsequently regulate the expression of cytokines, chemokines, and type I IFN.

Cytosolic scaffolds can detect the presence of danger signals by directly detecting PAMPs or DAMPs, or indirectly detecting a secondary messenger. The scaffolds serve to recruit and oligomerize procaspase-1. Procaspase-1 recruitment can occur either directly or indirectly through the involvement of apoptosis-associated speck-like protein (ASC), which contains a caspase recruitment domain (CARD). Procaspase-1 is oligomerized into filaments that allow for its subsequent autoactivation. Active caspase-1 then functions to cleave pro-IL-1β and pro-IL-18, thus triggering their release into the extracellular space where they can exert an effect on immune cells, promoting both local and systemic immune responses.

Clinical aspects:

Pyroptosis acts as a defense mechanism against infection by inducing inflammation. The formation of inflammasomes and the activity of caspase-1 determine the balance between eradication of the pathogen-associated disease state versus coping with a protracted infection. In a healthy cell, caspase-1 activation helps to fight infection. However, pyroptosis can and does function as a double edge sword. Inflammasome activation results in an increase in cytokine levels, which will augment the consequences of inflammation. This in turn, contributes to the development of the adaptive immune response as infection progresses. The ultimate resolution will clear pathogens. In contrast, persistent inflammation has also been linked to a variety of autoimmune and autoinflammatory diseases such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, atherosclerosis, type 2 diabetes, and obesity. Since pyroptosis is involved in many beneficial and detrimental inflammatory processes, this conserved cell death process remains an important area of continued study.

Detect pyroptosis with FLICA®

- *ICT's Pyroptosis/Caspase-1 Assay Kit utilizes our popular FLICA® technology to detect caspase-1 activation.*
- *These kits contains the caspase-1 inhibitor reagents, which have the preferred binding sequence for caspase-1, Tyr-Val-Ala-Asp (YVAD).*
- *These kits also contains Nigericin, a potent microbial toxin derived from Streptomyces hygroscopicus, that acts as a potassium ionophore, capable of inducing a net decrease in intracellular levels of potassium which is crucial for oligomerization of the NLRP3 inflammasome and activation of caspase-1. Nigericin is included in this kit as a positive control. It has been shown to generate a robust caspase-1 activation response in various cell lines.*

PYROPTOSIS PRODUCTS	SIZE	CAT. #
Pyroptosis/Caspase-1 Assay, Green	25-50 Tests	9145
	100-200 Tests	9146
Pyroptosis/Caspase-1 Assay, Far Red	25-50 Tests	9158
FAM-FLICA Caspase-1 Assay	25 Tests	97
	100 Tests	98
FLICA 660 Caspase-1 Assay	25-50 Tests	9122
Nigericin	0.5 µmoles	6698

FIGURE 10: FLUORESCENCE MICROSCOPY IMAGING OF EPITHELIAL CELLS UNDERGOING PYROPTOSIS

C2BBe1 human colorectal adenocarcinoma cells were grown in polarized monolayers and infected with wild type Salmonella constitutively expressing mCherry (red cells, below). After 9 hours, live cells were incubated with ICT's active caspase-1 reagent, FAM-YVAD-FMK (green cell, below) for 1 hour in growth medium, washed, and fixed. The confocal image below reveals an extruding cell that is infected; many red mCherry-labeled *Salmonella* are visible. The infected cell is undergoing pyroptosis, as evidenced by the positive staining for active caspase-1 visible as increased green fluorescence compared to background levels of fluorescence in the surrounding caspase-1 negative cells. Data courtesy Knodler, Leigh A., et al. Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. PNAS. 107:41, 17733-17738 (2010).

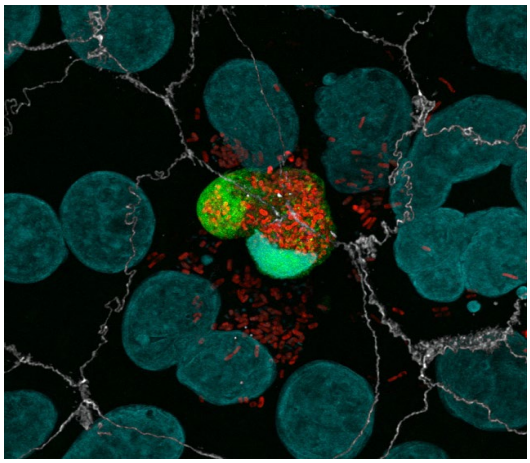
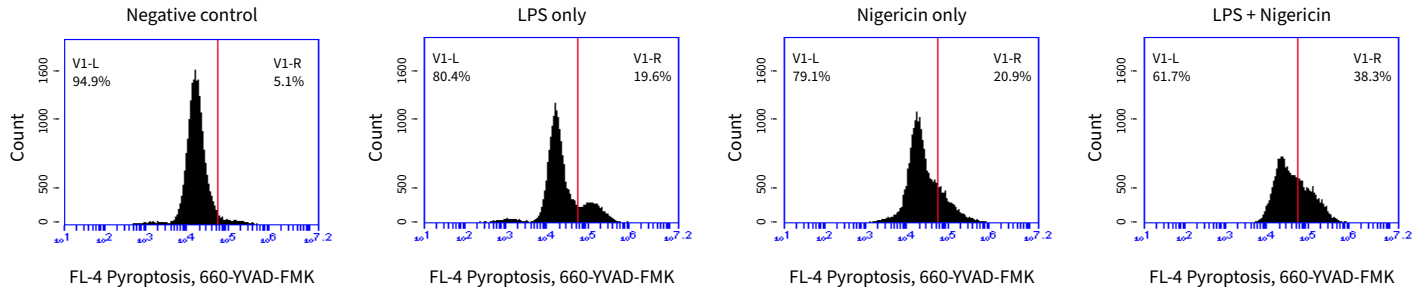


FIGURE 11: CASPASE-1 ACTIVITY IN THP-1 MONOCYTES

ICT's pyroptosis detection reagent, 660-YVAD-FMK was used to monitor the caspase-1 induction response in THP-1 cells treated with LPS (1 µg/mL for 3 hours), Nigericin (20 µM for 30 minutes), or LPS + Nigericin (1 µg/mL LPS for 3 hours, followed by 20 µM Nigericin for 30 minutes). A common cell pool was spiked with 660-YVAD-FMK and divided into the following treatment groups: 1.) Negative control, 2.) LPS only, 3.) Nigericin only, and 4.) LPS + Nigericin. LPS was added to "LPS only" and "LPS + Nigericin" samples at 1 µg/mL and the samples were incubated at 37°C in a cell culture incubator. After 3 hours, Nigericin was added to "Nigericin only" and "LPS + Nigericin" treatment groups. Samples were returned to the 37°C cell culture incubator for 30 additional minutes. Following their respective treatments, cells were washed and analyzed on an Accuri C6 flow cytometer. Samples treated with either LPS or Nigericin alone had approximately 4 times the level of caspase-1 activity compared to the negative control sample. The sample treated with both LPS and Nigericin had nearly twice the level of caspase-1 activity as either treatment alone.



Cathepsin Substrates

Cathepsins are a group of protease enzymes that were originally identified in lysosomes. Cathepsins are classified based on the key catalytic group present in their active site, and are categorized as aspartic, serine, or cysteine proteases. Cathepsins D and E are aspartic proteases, cathepsins A and G are serine proteases, and cathepsins B, C, F, H, K, L, O, S, V, W, and X are cysteine proteases. Initially synthesized as inactive zymogens, they are post-translationally processed into their active configurations after passing through the endoplasmic reticulum and subsequent incorporation into the acidic environment of the lysosomes. These enzymes exist in their processed form as disulfide-linked heavy and light chain subunits with molecular weights ranging from 20-35 kDa. Cathepsin C is the noted exception, existing as an oligomeric enzyme with a MW ~200 kDa.

Cathepsin B

Cathepsin B is thought to play an important role in intracellular proteolysis. Overexpression of cathepsin B is often correlated with invasive and metastatic cancers. It plays a specific role in pathologies of the central nervous system particularly in relation to Alzheimer's disease. Other disease pathologies known to involve some level of cathepsin B participation include ischemia and neuronal dysfunction.

Cathepsin K

Cathepsin K is essential for osteoclast-mediated bone resorption. The enzyme's ability to catabolize elastin, collagen, and gelatin is instrumental in the breakdown of bone and cartilage. This catabolic activity is also partially responsible for the loss of lung elasticity and recoil in emphysema. Cathepsin K inhibitors show great potential in the treatment of osteoporosis. Cathepsin K enzyme expression is reported in prostate and breast cancer, and is linked to increased metastasis of breast cancer to the bone. Cathepsin K inhibitors are promising therapeutic-candidates for treating breast cancer metastasis.

Cathepsin L

Cathepsin L is a lysosomal endopeptidase involved in antigen processing, bone resorption, tumor invasion and metastasis, and protein turnover during growth regulation. Cathepsin L can also be secreted, allowing for its involvement in the degradation of extracellular proteins such as collagen, elastin, and other basement membrane proteins. This degradation of the basement membrane and interstitial matrix may promote tumor cell invasion and metastasis by allowing cancer cells to breach the basement membrane barrier and invade local and distant tissue sites. Increases in the production of cathepsin L were found to correlate with elevated migration and invasiveness in human glioma U251 cells undergoing X-ray treatment compared to control populations. Activation of cathepsin L contributes to the irreversible depolarization induced by oxygen and glucose deprivation in rat hippocampal CA1 neurons.

Detect and monitor cathepsin activity in vitro

- *ICT's Magic Red® Assays and Green Cathepsin B Assay enable researchers to quantitate and monitor intracellular cathepsin activity over time in vitro.*
- *Magic Red detection substrates utilize the photostable red fluorophore, cresyl violet. When bi-substituted via amide linkage to two cathepsin target peptide sequences, cresyl violet is nonfluorescent. Following enzymatic cleavage at one or both arginine (R) amide linkage sites, the mono and non-substituted cresyl violet fluorophores generate red fluorescence when excited at 550-590 nm.*
- *The Magic Red cathepsin B substrate, MR-$(RR)_2$, is comprised of cresyl violet coupled to two pairs of the amino acid sequence, arginine-arginine (RR), which is the preferential target sequence for cathepsin B. In ICT's cathepsin K substrate, MR-$(LR)_2$, cresyl violet is coupled to two pairs of leucine-arginine (LR). ICT's MR cathepsin L substrate, MR-$(FR)_2$, contains two pairs of phenylalanine-arginine (FR) coupled to cresyl violet.*
- *The Green Cathepsin B Assay includes the Rhodamine 110 Cathepsin B substrate, which is comprised of rhodamine 110 coupled to two copies of the amino acid sequence, arginine-arginine (RR), which is the preferential target sequence for cathepsin B. When bi-substituted via amide linkage to two cathepsin B target peptide sequences, rhodamine 110 is nonfluorescent. Following enzymatic cleavage at one or both arginine (R) amide linkage sites, the mono and non-substituted rhodamine 110 fluorophores generate green fluorescence when excited at 500 nm.*

CATHEPSIN PRODUCTS	SIZE	CAT. #
Magic Red Cathepsin B Assay	25 Tests	937
	100 Tests	938
Magic Red Cathepsin K Assay	25 Tests	939
	100 Tests	940
Magic Red Cathepsin L Assay	25 Tests	941
	100 Tests	942
Green Cathepsin B Assay	25 Tests	9151
	100 Tests	9152
Cathepsin B Enzyme	25 µg	6202
Cathepsin D Enzyme	25 µg	6203

FIGURE 12: CATHEPSIN B IN THP-1 CELLS
Intracellular cathepsin B activity was detected in THP-1 cells using ICT's MR-$(RR)_2$ cathepsin B fluorogenic substrate. Intracellular localization of the hydrolyzed (fluorescent) Magic Red product was detected using a Nikon Eclipse E800 photomicroscope equipped with a 510 – 560 nm excitation filter and a 570 – 620 nm emission/barrier filter at 400X (A). Photo at right (B) shows the corresponding DIC image of the cells.

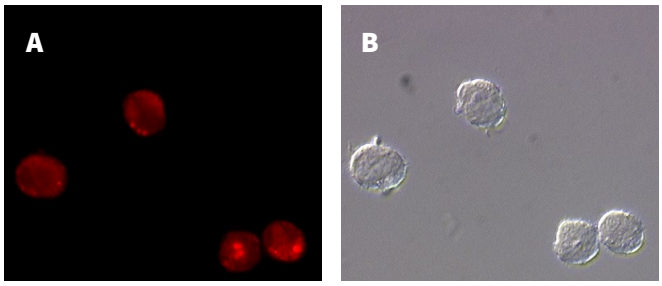


FIGURE 13: CATHEPSIN L IN JURKAT CELLS
Intracellular cathepsin L activity was detected in Jurkat cells using ICT's MR-$(FR)_2$ cathepsin L fluorogenic substrate. Intracellular localization of the hydrolyzed (fluorescent) Magic Red product was detected using a Nikon Eclipse E800 photomicroscope equipped with a 510 – 560 nm excitation filter and a 570 – 620 nm emission/barrier filter at 400X (A). Photo at right (B) shows the corresponding DIC image of the cells.

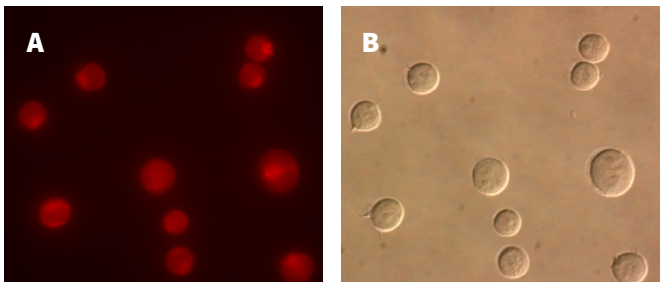


FIGURE 14: FLUORESCENCE MICROSCOPY IMAGING OF CATHEPSIN ACTIVITY IN THP-1 CELLS
Intracellular cathepsin activity was detected in THP-1 cells using ICT's R110-$(RR)_2$ cathepsin B substrate. THP-1 cells were seeded onto chamber slides and were then treated with PMA (5 ng/mL for 48 hours) to become macrophage-like. After 48 hours, media containing PMA was replaced with fresh media, and cells were allowed to recover for 4 days, and then were exposed to the cathepsin inhibitor, CA-074 Me for 3 hours (lower row of images), or were untreated (upper row of images). Following treatment, samples were stained with R110-$(RR)_2$ for 1 hour at 37°C and Hoechst 33342 for 15 minutes at room temperature, and then were imaged. Intracellular localization of the hydrolyzed (fluorescent) R110 product was detected using a Logos iRiS Digital Cell Imaging System equipped with a EGFP (Ex 470/30, Em 530/50) and a DAPI (Ex 375/28, Em 460/50) LED filter cubes at 20X. The images below show untreated cells (upper row of images) and inhibitor-treated cells (lower row of images) stained with R110-$(RR)_2$ (leftmost images), Hoechst 33342 (middle images), or an overlay of both the EGFP and DAPI channels (rightmost images).

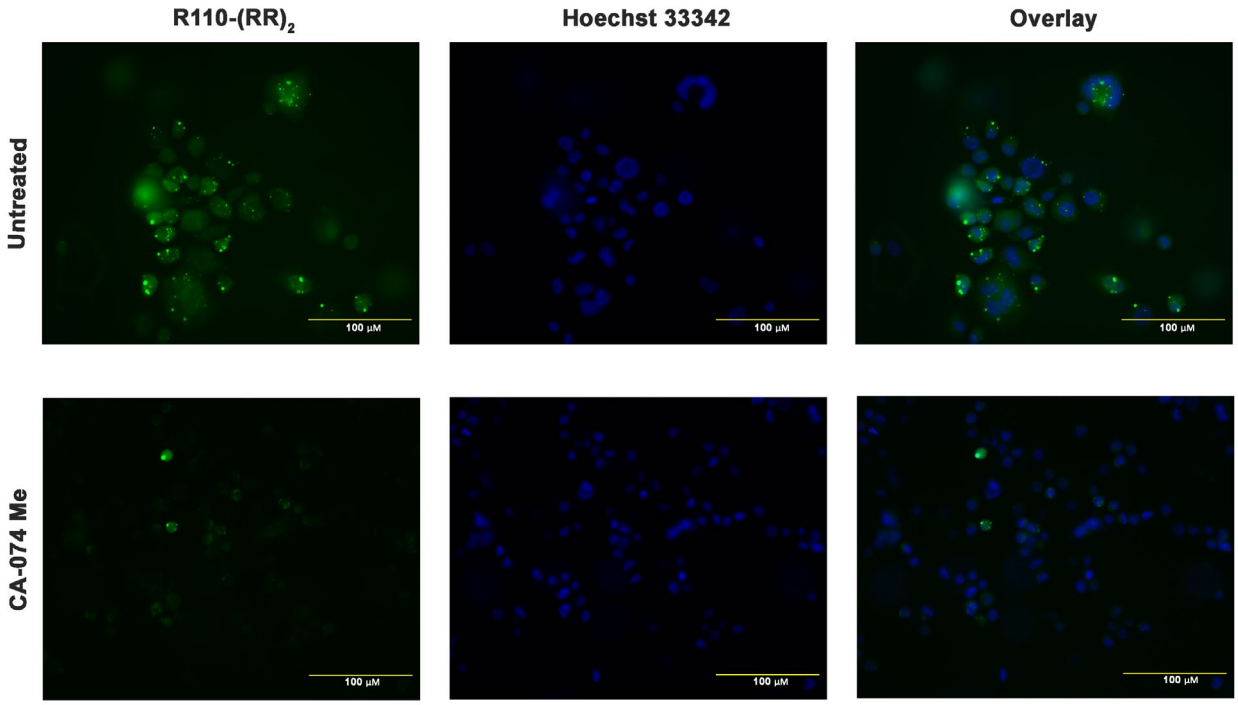
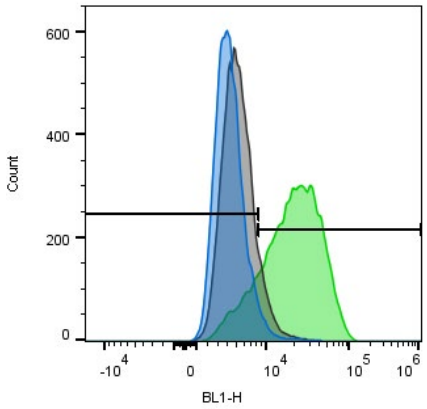


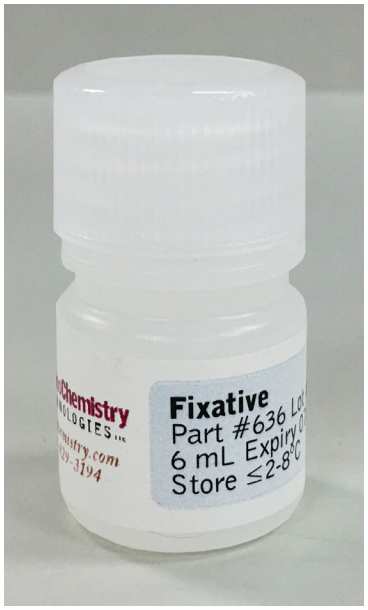
FIGURE 15: CATHEPSIN B ACTIVITY IN JURKAT CELLS
Intracellular cathepsin activity was detected in Jurkat cells using ICT's Green Cathepsin B substrate, R110-$(RR)_2$. Jurkat cells were treated with the cathepsin inhibitors CA-074 Me (blue histogram) and E-64d (gray histogram) for 3 hours and 1 hour, respectively, or were untreated (green histogram) and then were stained for 1 hour with R110-$(RR)_2$. After incubation with the R110-$(RR)_2$ substrate, the samples were analyzed by flow cytometry using a ThermoScientific Attune NxT flow cytometer equipped with a blue laser (488 nm excitation and a 530/30 emission filter). An overlay of the histograms and a table displaying the median fluorescence signal, % negative, and % positive cells are shown below. Treatment with CA-074 Me or E-64d decreased the fluorescence signal detected compared to the untreated control, which displayed a baseline level of cathepsin activity.



Treatment	Median (BL1-H)	% Negative	% Positive
Untreated	19,620	15.0	85.0
CA-074 Me	4,086	95.6	4.40
E-64d	5,094	89.5	10.5

Cellular Imaging

- *ICT carries a variety of cellular imaging stains and ancillary reagents such as nuclear stains, acridine orange, gel mounting media, fixative, etc.*

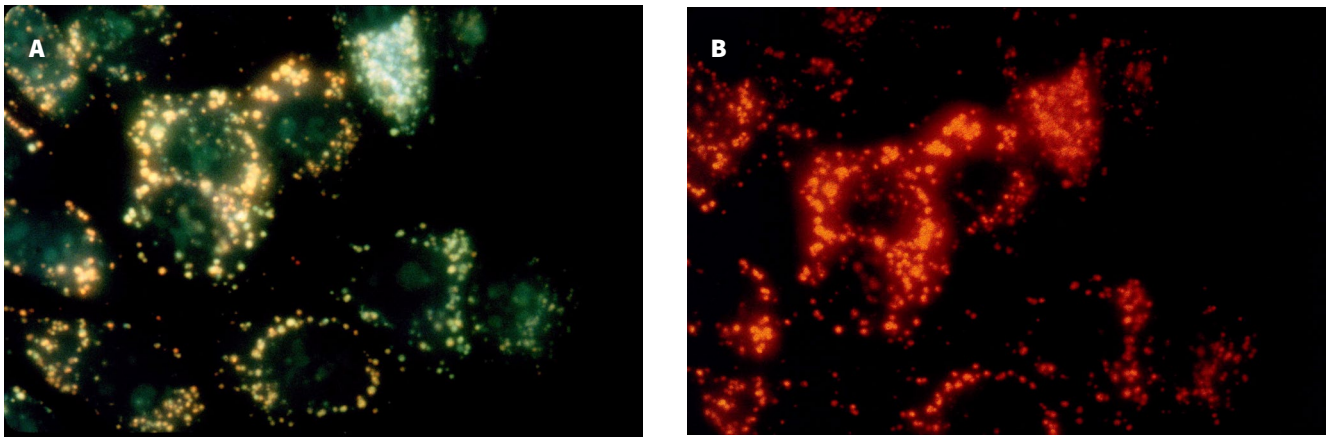


CELLULAR IMAGING PRODUCTS	SIZE	CAT. #
Acridine Orange Staining Solution	0.5 mL	6130
CFSE Fluorescent Cellular Stain	50 µg	6162
DAPI Nuclear Stain	10 mg	6244
Hoechst 33342 Fluorescent Nucleic Acid Stain	1 mL	639
Fixative	6 mL	636

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.

FIGURE 16: ACRIDINE ORANGE STAINING

MCF-7 cells were stained with acridine orange (AO) in PBS for 30 minutes, then washed twice in PBS (cells were not stained with Magic Red). Cells were photographed with a Nikon Microphot-FXA epifluorescence microscope at 400X using either a blue light excitation (492 nm) with a 540-550 nm emission filter (A, lysosomes appear yellowish green), or green light excitation (540 nm) with a long pass >640 nm barrier filter (B, lysosomes appear red). Experiment performed in the laboratory of Dr. Zbigniew Darzynkiewicz (Brander Cancer Research Center Institute, New York City, NY).



Cytotoxicity

Assessment of cell viability is a critical step during the evaluation of novel drug treatments and therapies for potential cytotoxic properties. With cell viability assessment playing a central role in countless research and environmental safety studies, there is an ever present need for simple, straightforward analysis methods capable of distinguishing between live and dead cells.

ICT carries several cell membrane impermeant dyes capable of differentiating live and dead cells. Our assortment of vital dyes include our Green Live/Dead stain, Propidium Iodide (PI), 7-aminoactinomycinD (7-AAD), and DRAQ7™. Healthy cells with intact membranes are able to exclude these dyes. In the presence of cells exhibiting compromised membrane integrity, our vital stains/dyes penetrate the cell and nuclear membrane barriers and intercalates tightly to DNA.

In addition to our membrane impermeant dyes, ICT also offers cell viability kits designed to detect live cells. Our basic Calcein AM Cell Viability Kit (catalog #9153) utilizes calcein AM to differentiate between live and dead cells. Calcein AM's overall hydrophobic nature allows it to readily traverse the lipid bilayer structure of the cell membrane in a concentration gradient-dependent manner. Once inside the cell, the hydrophobic and non-fluorescent Calcein AM is quickly hydrolyzed by intracellular esterases that are active in live cells. This leads to the cleavage and removal of two non-polar acetoxymethyl ester (AM) groups. Once the AM groups have been cleaved, the resulting polar (hydrophilic) and now fluorescence-capable Calcein dye molecule is efficiently retained within the confines of the cell membrane. Dead cells lack active esterases and do not cleave Calcein AM. Our Advanced Calcein AM Cell Viability Kit (catalog #9154) allows for the simultaneous labeling of live, membrane compromised, and dead cells within a single sample by pairing the live cell stain calcein AM with the dead cell stain 7-AAD.

Lastly, ICT carries cytotoxicity kits designed to allow for monitoring targeted cell killing. These kits are meant to be used to detect cytolytic activity within a target cell population. Effector cells such as Natural Killer (NK) cells and Cytotoxic T lymphocytes (CTL) are innate and adaptive (respectively) immune cells responsible for carrying out cytolyic activity. NK and CTL cells are capable of recognizing a stressed, infected, or cancerous cell (hereafter referred to as the Target cell), and upon recognition, trigger apoptosis in the target cell.

ICT's Basic Cytotoxicity Kit (catalogs #969 and #970) uses CFSE to label target cells and 7-AAD to detect cell membrane permeability. Target cells are labeled with CFSE prior to exposure to the Effector cells (which remain unlabeled, allowing for these two populations to be easily distinguished by flow cytometry). As all the target cells are initially labeled with green fluorescing CFSE, and the effector cells are not, these two populations can be easily distinguished. Cytolytic activity in the Target cell population is then detected by adding 7-AAD. If the Effector cells are triggering apoptosis in the Target cell population, there will be a detectable increase in 7-AAD-positive stained CFSE-labeled cells.

ICT also carries a Total Cytotoxicity & Apoptosis assay (catalogs #971 and #972) designed to more accurately quantify cell death. Similar to the Basic Cytotoxicity Assay, CFSE is first used to label the target cell population green. The unstained effector cells are then added and incubated with the target cells. Apoptotic target cells can then be identified by labeling with the second reagent, SR-FLICA. SR-FLICA is an orange/red fluorescent poly caspase inhibitor, SR-VAD-FMK, which binds to active caspase enzymes up-regulated for apoptosis. After the Effector and Target cells have been incubated together, 7-AAD is added to stain all membrane-compromised cells red. Collectively, this assay can be used to determine total cytotoxicity in the form of apoptosis and necrosis. It will quantify 4 populations of cells: live; early apoptotic; late apoptotic; and necrotic cells within a single sample tube.

CYTOTOXICITY PRODUCTS	SIZE	CAT. #
Green Live/Dead Stain	50 µL	6342
7-AAD Red Fluorescent Live/Dead Stain	0.26 mg	6163
Propidium Iodide	1 mL	638
Basic Calcein AM Cell Viability Kit	25-250 Tests	9153
Advanced Calcein AM Cell Viability Kit	25-250 Tests	9154
Basic Cytotoxicity Assay (Cell-mediated)	125 Tests	969
	250 Tests	970
Total Cytotoxicity & Apoptosis Assay (Cell-mediated)	125 Tests	971
	250 Tests	972

FIGURE 17: MONITORING NECROSIS IN RESPONSE TO STRESSFUL CELL CULTURE CONDITIONS
Jurkat cells were transferred to a suboptimal culture environment (serum-free DMEM at room temperature and 0.03% CO_2). Cell populations were monitored for >7 hours, and were stained with a panel of ICT's Cell Viability dyes, and analyzed by flow cytometry. Green Live/Dead Stain is represented by the green line, PI is shown as the blue line, 7-AAD is shown as the red line, and DRAQ7 is represented by the yellow line. Cells that stained positive with each vital dye were considered to be necrotic.

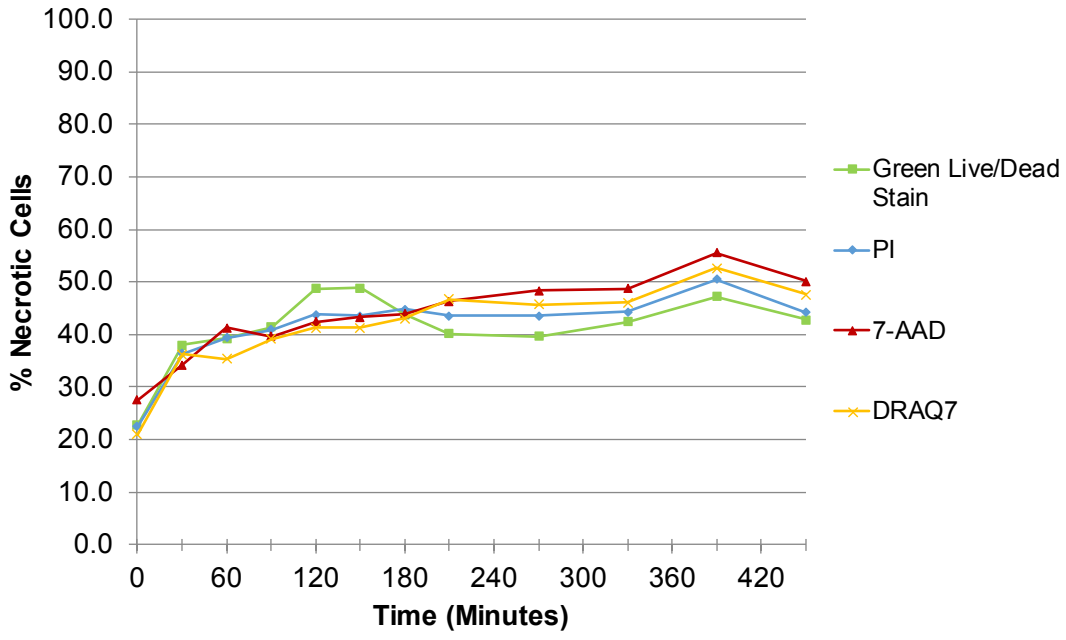


FIGURE 18: MICROSCOPY AND FLOW CYTOMETRY ANALYSIS OF APOPTOTIC CELLS STAINED WITH CALCEIN AM AND 7-AAD

Jurkat cells were treated with placebo (non-induced treatment with DMSO; A and B) or treated with 1 µM staurosporine for 4 hours to induce apoptosis via caspase activity (C and D). Cells were then dually stained with 1 µM Calcein AM for 60 minutes at 37°C (to detect live cells) and 4 µM 7-AAD for 10 minutes on ice (to detect membrane-compromised or dead cells).

Fluorescence microscopy revealed that all of the non-induced cells fluoresced green only, indicating they were live with intact cell membranes (A). Many staurosporine-treated cells were live (fluoresced green), although many cells fluoresced both green and red indicating they had compromised membranes C). Some of the staurosporine-treated cells fluoresced red only, indicating they were dead (C). Microscope images were obtained using a Nikon Eclipse 90i microscope with a Hamamatsu Flash 4.0 camera.

Flow cytometry was to quantitate populations of live, membrane-compromised, and dead cells. Live cells fluoresce green due to the ability of intracellular esterases to cleave Calcein AM into the green fluorescent molecule, Calcein (lower right quadrant of density plot B). Dually stained green and red fluorescing cells represent the population of membrane compromised Jurkat cells (most likely in mid to late stage apoptosis); these cells have active intracellular esterases and compromised cell membranes (upper right quadrant of density plot D). Necrotic cells fluoresce red (upper left, B and D). Cells were analyzed using an Accuri C6 flow cytometer. Calcein AM was analyzed using a 99% attenuated FL-1 channel, and 7-AAD was analyzed on the FL-3 channel.

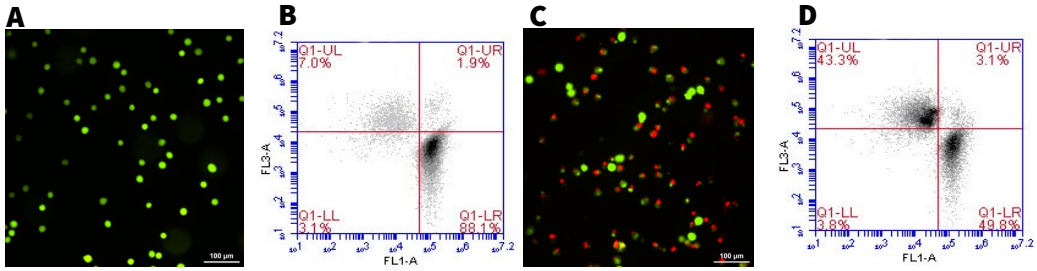


FIGURE 19: QUANTITATING CYTOLYTIC ACTIVITY USING THE TOTAL CYTOTOXICITY & APOPTOSIS KIT

Samples were prepared following the experimental setup depicted in (A.). Then a FSC vs. SSC plot or CFSE (FL-1) vs. SSC was derived to identify the target cells. Created a plot of CFSE (FL-1) vs. 7-AAD (FL-3) to further distinguish the green target cells from the unstained effector cells. Identified all green target cells and gated on them as R3 (R1+R2=R3). Collected events within R3.

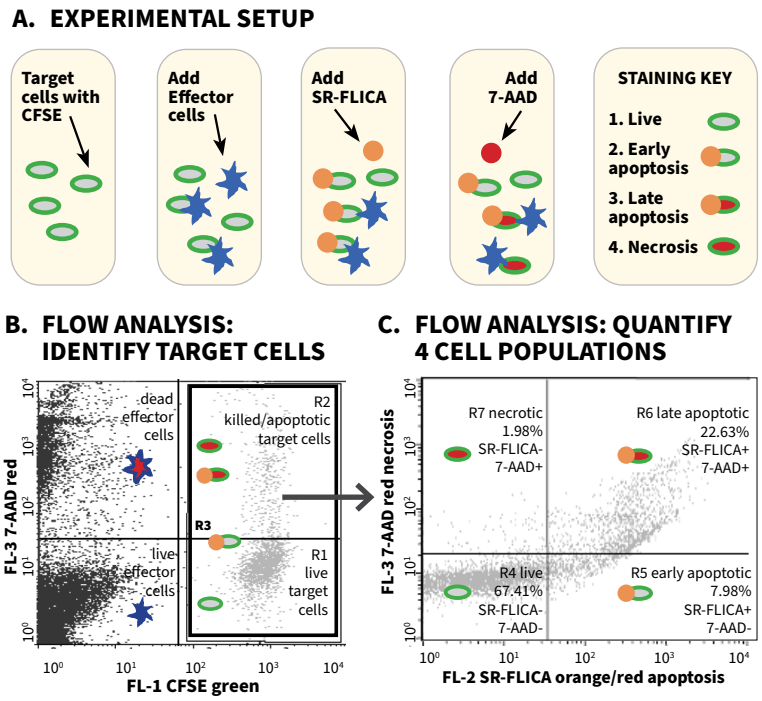
Once the green target cells were gated (R3, B.), a plot of the SR-FLICA poly caspase inhibitor in FL-2 vs. the 7-AAD live/dead stain in FL-3 was derived. This plot revealed 4 populations of cells: live cells and 3 populations of cells in the death process, including cells in early apoptosis which are not detectable by any other method.

Live Cells: R4 Lower left: Viable live cells are SR-FLICA apoptosis negative and 7-AAD live/dead negative (67.41%).

Early Apoptosis: R5 Lower right: Cells in early apoptosis are SR-FLICA apoptosis positive (they have active caspase enzymes and are becoming apoptotic and dying), but are 7-AAD live/dead negative (they are alive but do not have compromised membranes yet); these cells are not detectable by other methods (7.98%).

Late Apoptosis: R6 Upper right: Late apoptotic cells are SR-FLICA apoptosis positive and 7-AAD live/dead positive (22.63%).

Necrosis: R7 Upper left: Necrotic cells are SR-FLICA apoptosis negative and 7-AAD live/dead positive (1.98%).



MitoPT®: Mitochondrial Membrane Potential

Loss of mitochondrial membrane potential is indicative of mitochondrial stress. ICT carries several cell membrane permeant dyes with the capability of being retained within an active (polarized) mitochondrial membrane, and subsequently released upon loss of mitochondrial membrane gradient potential. Potentiometric fluorescent dyes can allow researchers to obtain a macroscopic picture of the general health status of different cell populations exposed to varying environmental, immunological, or pharmaceutical treatment regimens.

ICT carries three assays capable of detecting mitochondrial membrane depolarization: MitoPT JC-1 Assay, MitoPT TMRE, and MitoPT TMRM.

Mechanism behind JC-1 detection of mitochondrial depolarization

JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolcarbocyanine iodide) is a fluorescent lipophilic cation with the ability to form J-Aggregate structures when concentrated within polarized mitochondrial membrane structure. Its ability to perform as a potentiometric fluorescent probe for mitochondrial polarization status assessment can be directly attributed to these physical properties. Its tendency to form J-Aggregate structure when concentrated within polarized mitochondria gives it the ability to fluoresce at two different emission wavelengths, 527 nm and 590 nm, depending upon its localized concentration. At low cell staining concentrations, JC-1 would exist as the monomeric (green fluorescing) form. When concentrated within inner membrane regions of healthy = polarized mitochondria, JC-1 would exist as the aggregated (J-Aggregate) orange fluorescing form.

JC-1 has a delocalized positive charge dispersed throughout its molecular structure. In addition, its lipophilic solubility enables it to be readily membrane permeant and penetrate living cells. Weakly positive charged JC-1 dye enters the negatively charged inner mitochondrial membrane matrix regions. As the dye accumulates, a critical concentration point is reached, leading to J-Aggregate formation. In contrast to the monomeric form of JC-1 which fluoresces green (527 nm) following blue light excitation (490 nm), JC-1 dye in the aggregated state (J-Aggregates) fluoresce orange (590 nm) upon blue light (490 nm) excitation. Healthy JC-1 stained cells, bearing proton-pump-functional mitochondria, concentrate JC-1 dye leading to J-Aggregate structure formation. The presence of J-Aggregate-form-JC-1 within polarized mitochondria confers the ability to fluoresce orange upon blue light excitation. Within cells bearing diminished or collapsed mitochondrial membrane potentials, which can occur during apoptotic or oxidative stress events, absence of a conducive environment for concentration and retention of positively charged JC-1 dye (J-Aggregate structure) leads to its conversion to the monomeric form of the dye. Monomeric forms of the JC-1 dye quickly equilibrate via simple concentration gradient action throughout the cytosol and into the extracellular media. This depolarization event is conveniently illustrated via a rapid drop in orange fluorescence staining properties of the target/experimental cell population. This leads to the default green fluorescence associated with remaining JC-1 monomer retained within the confines of the plasma membrane.

Alternative Potentiometric MitoPT Dye Options

ICT also offers two rhodamine-based mitochondrial depolarization detection probes. They include: TetraMethylRhodamineEthylester TMRE (Excitation/Emission: 549 nm / 574 nm) and TetraMethylRhodamineMethylester TMRM (Excitation/Emission: 548 nm / 573 nm). As was the case with JC-1, these rhodamine based dyes also fluoresce within the orange wavelength spectra. Their mechanism of action is synonymous to that observed with JC-1. TMRM and TMRE share physical properties common to all potentiometric fluorescent dyes. They possess a lipophilic molecular structure enabling them to penetrate both cell and mitochondrial membrane(s) barriers as well as a weak positive charge to facilitate their concentration within healthy polarized mitochondria. In contrast to the dual fluorescence properties of JC-1, TMRM and TMRE only emit at a single fixed wavelength within the orange spectrum (573 nm or 574 nm). Their selective concentration within healthy cell polarized mitochondria provides the biochemical basis for their utility as mitochondrial health status monitoring probes.

FIGURE 20: FLOW CYTOMETRY ANALYSIS OF APOPTOTIC CELLS STAINED WITH TMRE

Jurkat cells were treated with 1 µM staurosporine, an apoptosis-inducing agent (left, white, open histogram), or DMSO, a negative control (right, orange, solid histogram), for 3 hours at 37°C, then stained with 30 nM MitoPT TMRE for 15-20 minutes. Cells were analyzed with an BD FACSCalibur™ flow cytometer.

Apoptotic cells (left), bearing depolarized mitochondria, exhibit significantly less orange fluorescence intensity compared to healthy, negative control cells (right). Cells in the negative control population contain mitochondria with polarized inner membranes; these cells concentrate MitoPT TMRE and fluoresce bright orange.

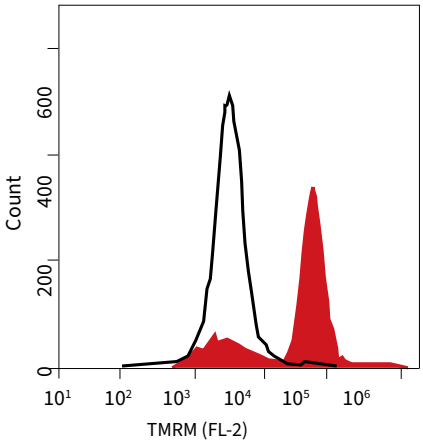


FIGURE 21: MICROSCOPY ANALYSIS OF APOPTOTIC CELLS STAINED WITH JC-1

Jurkat cells were treated with 1 µM staurosporine for 2 hours to induce apoptosis, or with DMSO as the negative control. Cells were stained with MitoPT JC-1 for 20 minutes at 37°C, then washed twice. Normal healthy cells (two cells, upper and lower left), containing mitochondria with polarized inner membranes, concentrate MitoPT JC-1 and fluoresce bright orange. Apoptotic cells (three cells, right), bearing mitochondria of various stages of permeability, exhibit a reduced orange fluorescence relative to the healthy cell population and increased green fluorescence, as the reagent becomes dispersed throughout the cells.

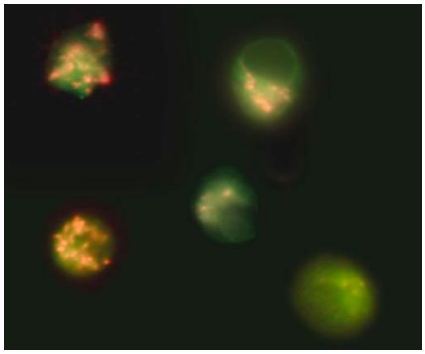
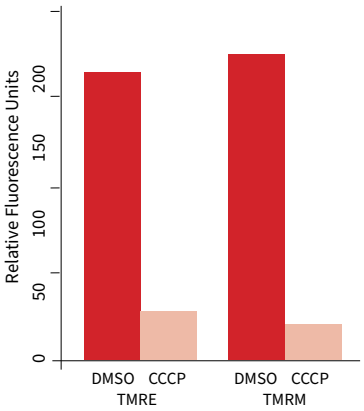


FIGURE 22: FLUORESCENCE PLATE READER ANALYSIS

Jurkat cells were exposed to DMSO as the negative control (left, dark orange bars) or 50 µM CCCP depolarizing agent (right, light orange bars) for 15 minutes at 37°C. Samples were subsequently incubated with MitoPT TMRE or TMRM for 20 minutes at 37°C and washed. Aliquots (100 µL) were analyzed in triplicate in a black 96-well plate using a Molecular Devices Gemini XS fluorescence plate reader set at 550 nm excitation and 580 nm emission using a 570 nm cut-off filter. The amount of orange fluorescence was measured by the plate reader. Healthy cells in the DMSO control populations exhibited a high level of orange fluorescence; metabolically stressed cells in the CCCP-stimulated samples exhibited a reduced level of orange fluorescence after the mitochondria became depolarized. As the membrane potential gradient collapses, TMRE and TMRM equilibrate out of the mitochondria and into the cytosol, causing cells to lose their orange fluorescence.



MITOCHONDRIAL MEMBRANE POTENTIAL PRODUCTS	SIZE	CAT. #
MitoPT JC-1 Assay	100 Tests	924
	400 Tests	911
MitoPT TMRE Assay	500 Tests	9103
MitoPT TMRM Assay	500 Tests	9105

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Autophagy

Autophagy is a conserved lysosomal recycling process by which cells break down their own components such as proteins, lipids, and carbohydrates. Autophagy plays a critical role in maintaining homeostasis by preventing the accumulation of damaged organelles by disassembling unnecessary or dysfunctional cells and cellular components. Autophagy occurs at low levels in the cell under normal conditions and can be rapidly upregulated during times of starvation or stress. Such degradation activities serve to provide nutrients (amino acids, nucleotides, fatty acids, etc.) and energy during periods of elevated bioenergetic demands. Another function of autophagy is to assist with the detection and destruction of intracellular pathogens (viruses, bacteria, and parasites). Dysregulation of autophagy has been associated with many disease states including cancer, infection, and degenerative diseases.

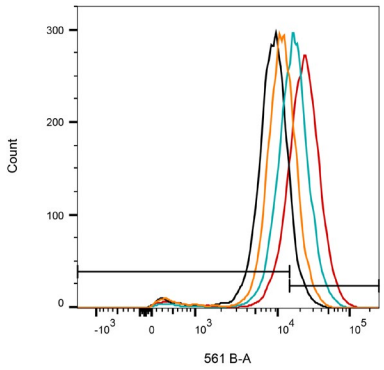
Autophagy is a dynamic process typically divided into three stages. During stage one, cytoplasmic components targeted for degradation are sequestered within a double-membrane phagophore (also called the isolation membrane). This results in the formation of double-membrane vesicle called the autophagosome. During stage two, the autophagosome fuses with the lysosome to form the autophagolysosome or autolysosome. Degradation of the autophagosomal contents occurs during stage three.

ICT's Autophagy Assay, Red enables researchers to detect and monitor the in vitro development of autophagy in living cells. Autophagy Probe, Red is a cell-permeant aliphatic molecule that fluoresces brightly when inserted in the lipid membranes of autophagosomes and autolysosomes. Autophagy Probe, Red can be readily detected by flow cytometry with optimal excitation at 590 nm and peak emission at 620 nm.

AUTOPHAGY PRODUCTS	SIZE	CAT. #
Autophagy Assay, Red	50 Tests	9156
	200 Tests	9157

FIGURE 24: DETECTION OF AUTOPHAGIC CELLS

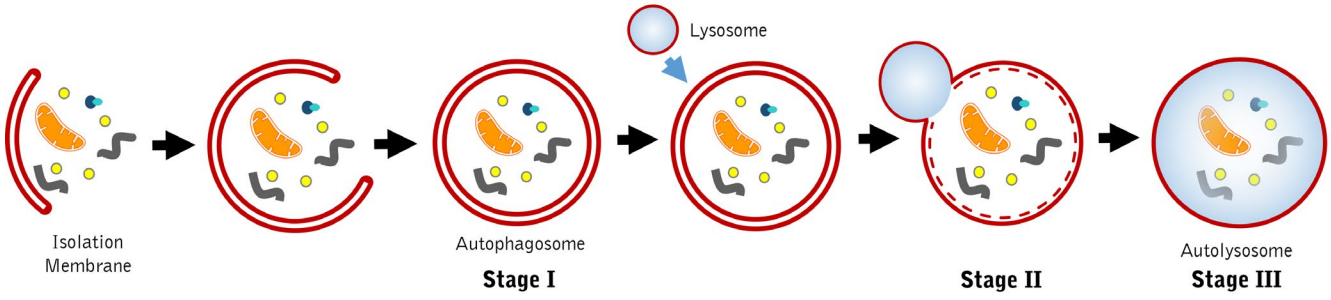
ICT's Autophagy Assay Kit, Red was used to assess the induction of autophagy in Jurkat cells. Cells were either untreated (Black) or treated with 0.5 µM Rapamycin (Orange), 10 µM Chloroquine (Blue), or both 0.5 µM Rapamycin and 10 µM Chloroquine (Red) for 18 hours. After staining with Autophagy Probe, Red for 60 minutes, cells were washed and analyzed by flow cytometry (BD LSRFortessa Special Order flow cytometer equipped with a green/yellow laser (561 nm excitation) and a 610/20 emission filter). An overlay of the histograms is shown on the right. A table displaying the median fluorescence signal, % negative, and % positive cells is shown below. Treatment with Rapamycin or chloroquine increased the fluorescence signal detected compared to the untreated control. Combined treatment of rapamycin and chloroquine further increased the fluorescence signal detected.



Treatment	Median (561 B-A)	% Negative	% Positive
Untreated (DMSO)	8658	86.0	14.0
Rapamycin	11146	67.8	32.2
Chloroquine	16004	36.5	63.5
Rapamycin + Chloroquine	22237	17.9	82.1

FIGURE 23: CELLULAR PROCESS OF AUTOPHAGY

Autophagy is an intracellular degradation process during which cytosolic organelles and materials are enclosed within an isolation membrane to form an autophagosome. The outer membrane of the autophagosome fuses with the lysosome. The sequestered material is subsequently degraded within the autolysosome.



Oxidative Stress

Oxidative stress reflects an imbalance between the production of reactive oxygen and nitrogen species and the body's ability to detoxify reactive intermediates. Under normal physiological conditions, cells control ROS levels by balancing the generation of ROS with their elimination by scavenging molecules such as antioxidants. However, under conditions of oxidative stress, excessive ROS can build up and cause damage to various cellular components (such as DNA, lipids, and proteins).

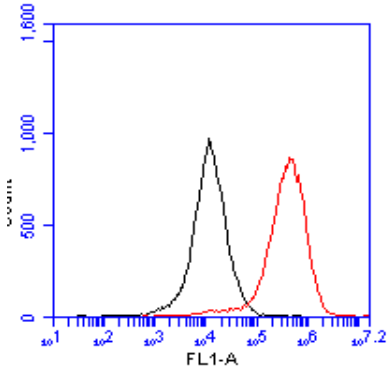
Some of the most harmful effects of reactive oxygen species exposure are the damage of DNA, the oxidation of polyunsaturated fatty acids in lipids (which is also known as lipid peroxidation), oxidation of the amino acids found in proteins, and the oxidative deactivation of specific enzymes. Ultimately, oxidative stress can trigger cell death. Moderate levels of oxidative stress can cause a cell to initiate apoptosis, while severe levels can trigger necrosis.

ICT carries products for detecting oxidative or nitrosative stress. One of our most popular oxidative stress kits is the Intracellular Total ROS Activity Assay. The Total ROS kit utilizes a proprietary oxidation-sensitive probe called Total ROS Green. On its own, this cell-permeant dye is nonfluorescent, but when in the presence of intracellular reactive oxygen species, this dye becomes oxidized to its green fluorescence capable form making it easy to detect by common methods such as flow cytometry. ICT also carries a Nitric Oxide Synthase Assay designed to detect intracellular nitric oxide and nitric oxide synthase activity. Our previously described mitochondrial membrane potential assays can also be used to monitor depolarization of the inner mitochondrial membrane because of oxidative stress.

Lastly, ICT carries an Intracellular GSH Assay designed to monitor the activity of glutathione. Glutathione peroxidase is an antioxidant, and its activity is important for controlling oxidative stress.

FIGURE 25: FLOW CYTOMETRY DETECTION OF INTRACELLULAR ROS ACTIVITY

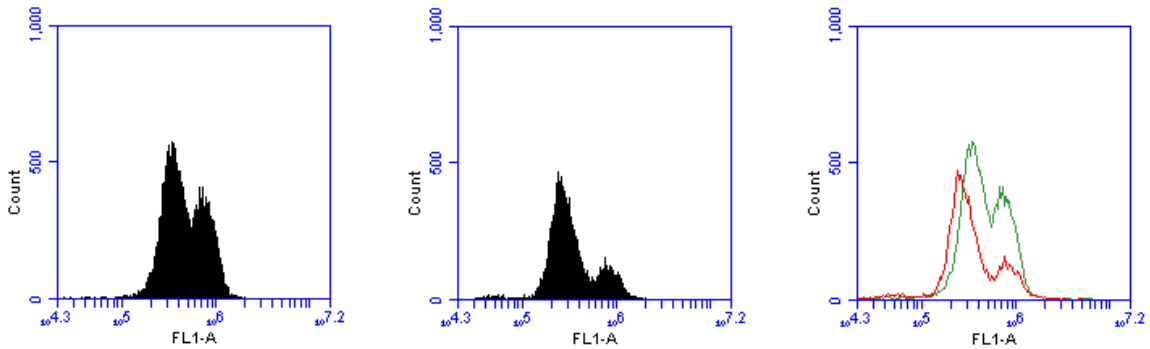
Jurkat cells were stained with ICT's Intracellular Total ROS Activity Assay (catalog #9144) for 1 hour, and then treated with a negative control (black histogram) or 100 µM tert-Butyl hydroperoxide, a reactive oxygen-inducing agent used to create the positive control (red histogram), for 30 minutes at 37°C. Cells were read on the FL-1 channel of an Accuri C6 flow cytometer. Treatment with tert-Butyl hydroperoxide resulted in a greater than 35 fold increase in median fluorescence intensity, which is easily visible when the samples are overlaid in a single plot.



OXIDATIVE STRESS PRODUCTS	SIZE	CAT. #
Intracellular Total ROS Activity Assay	100 Tests	9144
Nitric Oxide Synthase Assay	50-100 Tests	9155
Intracellular GSH Assay	100 Tests	9137
MitoPT JC-1 Assay	100 Tests	924
	400 Tests	911
MitoPT TMRE Assay	500 Tests	9103
MitoPT TMRM Assay	500 Tests	9105

FIGURE 26: EFFECT OF APOPTOSIS ON INTRACELLULAR GSH LEVELS

Jurkat cells were treated with a negative control (left histogram) or staurosporine, an apoptosis-inducing agent (middle histogram), for 4 hours at 37°C and then stained with ICT's Intracellular GSH Assay (catalog #9137) for 30 min. Cells were read on an Accuri C6 flow cytometer using an FL1 99% (2 log) attenuation filter. The median fluorescence intensity (MFI) of stained cells in the negative control was 425,971 in FL1-A (left: Negative, Non-Induced), whereas the treated population had a value of 289,169 (middle: Positive, Apoptosis-Induced), which is a decrease of more than 30%. The effect of staurosporine on intracellular GSH is easily visible when the samples are overlaid in a single plot (right: Overlay, green: Negative, red: Positive).



Negative, Non-Induced | Positive, Apoptosis-Induced | Overlay

FLISP® Serine Protease Detection

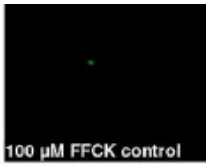
Serine proteases are a family of proteolytic enzymes defined by the presence of a serine residue at the active center of the enzyme, which participates in the formation of an intermediate ester to transiently form an acyl-enzyme complex. The most characterized enzymes of this type are trypsin and chymotrypsin. All living cells have a base level of chymotrypsin-like enzymatic activity which will vary with the physiological state of the cell as well as by cell type. Activated serine proteases play major roles in several different functions including: apoptosis; markers of tumor malignancy; diagnostic and prognostic indicators of breast carcinomas and neck and head carcinomas. Serine protease activity is also altered in a variety of other cell-mediated diseases related to transplant rejection and infections.

ICT's FLISP® serine protease assays enable researchers to detect intracellular chymotrypsin-like serine protease activity *in vitro* without lysing the cell. FLISP (Fluorescent Labeled Inhibitors of Serine Proteases) reagents are cell-permeant, non-cytotoxic green or red fluorescent inhibitors that covalently bind with active serine protease enzymes. If there is an active chymotrypsin-like enzyme inside the cell, it will covalently bind with the FLISP inhibitor and retain the green or red fluorescent signal within the cell. If the treatment is activating chymotrypsin-like serine proteases, positive cells will fluoresce brighter than the normal baseline negative cells, thus enabling researchers to clearly differentiate the populations.

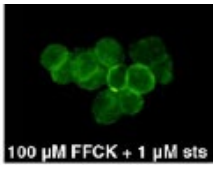
FLISP® PRODUCTS	SIZE	CAT. #
FAM-FLISP FFCK Serine Protease Assay	25 Tests	945
	100 Tests	946
FAM-FLISP FLCK Serine Protease Assay	25 Tests	949
	100 Tests	950
FAM-FLISP FSFCK Serine Protease Assay	25 Tests	963
	100 Tests	964
FAM-FLISP FSLCK Serine Protease Assay	25 Tests	965
	100 Tests	966
FAM-FLISP FLDAP Serine Protease Assay	25 Tests	967
	100 Tests	968
FAM-FLISP FFDAP Serine Protease Assay	25 Tests	984
	100 Tests	985
SR101-FLISP SFCK Serine Protease Assay	25 Tests	951
	100 Tests	952
SR101-FLISP SLCK Serine Protease Assay	25 Tests	955
	100 Tests	956

FIGURE 27: STAUROSPORINE ACTIVATES PARALLEL CASCADES OF CASPASE AND SERINE PROTEASE APOPTOSIS

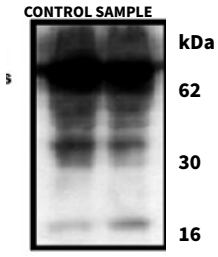
Staurosporine can activate apoptosis even when caspases are inhibited, indicating that other proteases may be involved. ICT's FAM-FLISP FFCK kit (catalog #946) was used to assess serine protease activity triggered by staurosporine. HL-60 cells were treated with FFCK for 1 hour, then treated with staurosporine for an additional 2 hours, and later stained with trypan blue. Cells treated with FFCK and staurosporine had a higher level of green fluorescence (B) than untreated cells (A). 97% of FFCK-treated cells were negative for trypan blue (data not shown), indicating that serine protease activity precedes the loss of plasma-membrane integrity. Cell lysates were analyzed via Western blotting using anti-fluorescein antibody indicating a 62 kDa protein (C). Staurosporine activates two independent yet parallel cell death programs in HL-60 cells: a caspase system and a cascade modulated by chymotrypsin-like serine proteases. Data courtesy of Dr. Catherine Stenson-Cox, National University of Ireland, Galway.



A: FFCK Control



B: FFCK + Staurosporine



C: FFCK Binding Proteins in Western Blot

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FIGURE 28: DETECTION OF SERINE PROTEASE ACTIVITY DURING 4 HOUR STAUROSPORINE TREATMENT

Intracellular serine protease activity was monitored over time in Jurkat cells exposed to 1 µM staurosporine using ICT's FAM-FLISP FFCK Serine Protease Assay. Cells were treated with staurosporine for 1, 2, 3, and 4 hours to induce apoptosis and increase serine protease activity (green histograms), or were untreated (gray histograms). Samples were stained with FAM-Phe-CMK (FFCK, catalog #946) for 1 hour at 37°C prior to being washed to remove unbound FLISP and then analyzed using an Attune NxT flow cytometer. The amount of serine protease detected correlated to the duration of the exposure period; the longer the cells were exposed to staurosporine, the larger the portion of serine protease positive cells found in the sample.

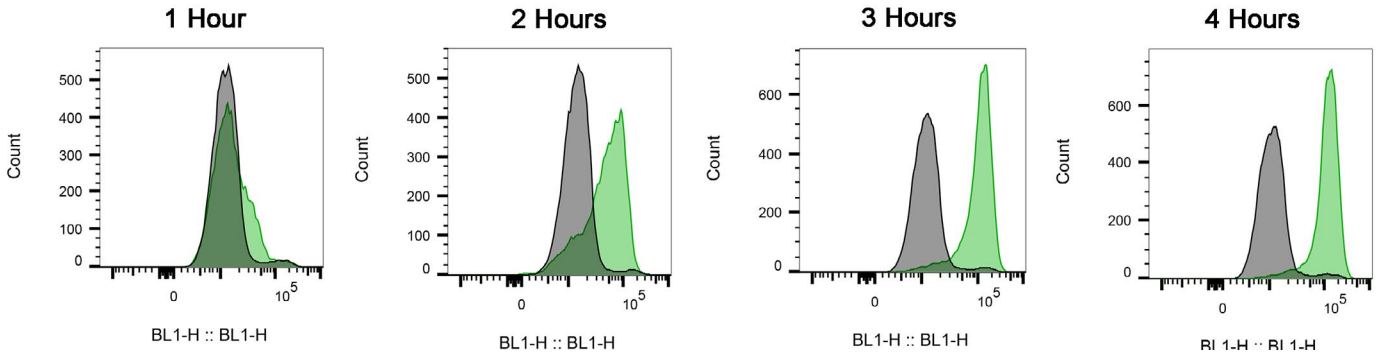
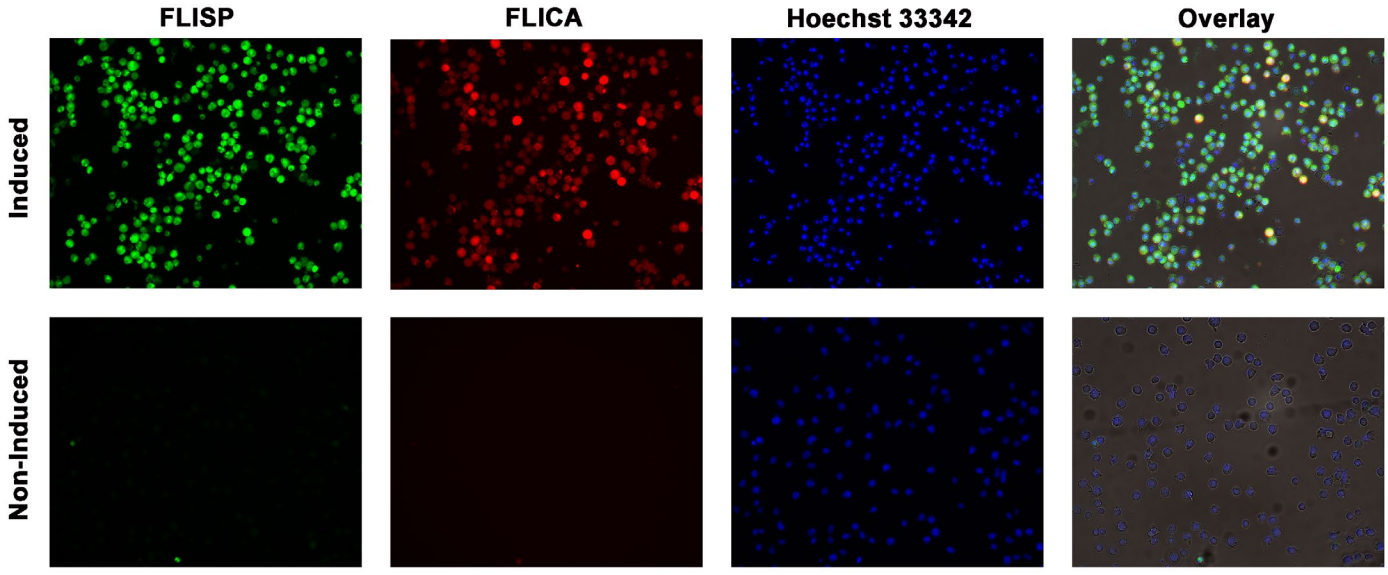


FIGURE 29: DUAL STAINING WITH FAM-FLISP AND FLICA 660

Intracellular serine protease activity was detected in Jurkat cells using ICT's green FAM-FLISP serine protease inhibitor (Kit #946). ICT's far red FLICA 660 poly-caspase inhibitor (Kit #9120) was used to detect caspase activation. Cells were exposed to 1 µM staurosporine for 4 hours to induce apoptosis and increase serine protease activity (upper row of images), or were untreated (lower row of images). During the final hour of treatment, samples were stained with FAM-Phe-CMK and 660-VAD-FMK for 1 hour at 37°C and Hoechst 33342 for 15 minutes at room temperature, and then were imaged. Intracellular localization of the FLISP and FLICA inhibitors, and Hoechst 33342 were detected using a Logos iRiS Digital Cell Imaging System equipped with EGFP (Ex 470/30, Em 530/50), Cy5 (Ex 620/60, Em 700/75), and DAPI (Ex 375/28, Em 460/50) LED filter cubes at 20X. Treatment with staurosporine triggered an increase in the intracellular levels of both serine proteases and caspases compared to non-induced controls.



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