1. INTRODUCTION

FLICA™ is a powerful method to assess cell death by detecting apoptosis *in vitro*. FLICA probes are non-cytotoxic Fluorescent Labeled Inhibitors of CAspases that covalently bind with 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 both Annexin V and TUNEL staining.

To use FLICA, add it directly to the cell culture media, incubate, and wash. FLICA is cell-permeant and will efficiently diffuse in and out of all cells. If there is an active caspase enzyme inside the cell, it will covalently bind with FLICA and retain the green fluorescent signal within the cell. Since non-apoptotic cells lack active caspases, these cells quickly return to their non-fluorescent status after the wash step. Therefore, apoptotic cells will retain a higher concentration of FLICA and fluoresce brighter than non-apoptotic cells. There is no interference from pro-caspases or inactive forms of the enzymes. If the treatment is causing cell death via apoptosis, apoptotic cells will have an elevated level of caspase activity relative to non-apoptotic cells or negative control cells and fluoresce with FLICA.

Apoptosis is an evolutionarily conserved process of programmed cell suicide. It is centered on a cascade of proteolytic enzymes called caspases that are triggered in response to pro-apoptotic signals. Once activated, caspases cleave protein substrates leading to the eventual disassembly of the cell[25]. Caspases have been identified in organisms ranging from *C. elegans* to humans. Mammalian caspases play distinct roles in both apoptosis and inflammation. In apoptosis, effector caspases (-3, -6, and -7) are responsible for proteolytic cleavages that lead to cell disassembly. Initiator caspases (-8, -9, and -10) regulate apoptosis upstream. Caspase-1 is associated with inflammasomes and takes on the role of a key house keeping enzyme in its conversion of pro-IL-1ß protein into the active IL-1ß cytokine.

Like the majority of other proteases, caspases are synthesized as pro-form precursors that undergo proteolytic maturation, either autocatalytically or in a cascade by enzymes with similar specificity[15]. Active caspase enzymes consist of two large (~20 kD) and two small (~10 kD) subunits that non-covalently associate to form a two heterodimer, tetrameric active caspase[29, 31, 24].

Caspase enzymes cleave proteins. They specifically recognize a 3 or 4 amino acid sequence that must include an aspartic acid (D) residue in the P1 position. This C–terminal residue is the target for the cleavage reaction at the carbonyl end[28]. Each of ImmunoChemistry Technologies' (ICT) FLICA probes contains a 3 or 4 amino acid sequence that is targeted by different activated caspases. This target sequence is sandwiched between a green fluorescent label, carboxyfluorescein (FAM), and a fluoromethyl ketone (FMK). A caspase enzyme cannot cleave the FLICA inhibitor probe; instead, it forms an irreversible covalent bond with the target sequence and becomes inhibited from further enzymatic activity. ICT's poly-caspase FLICA probe, FAM-VAD-FMK, may be used as a general reagent to detect apoptosis as it is recognized by all different types of activated caspases. To detect specific caspases, ICT also offers eight other FLICA reagents that contain different amino acid target sequences preferred by each caspase: caspase-1 (YVAD), -2 (VDVAD), -3 & -7 (DEVD), -6 (VEID), -8 (LETD), -9 (LEHD), -10 (AEVD), and -13 (LEED).

FLICA has been known to detect caspase activity in human, rabbit, rat, mice, drosophila, squid, paramecium, and yeast cell lines, among others. FLICA can be used to label suspension or adherent cells, thin tissue sections, and frozen 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 green FAM dye will be quenched during the paraffin embedding process).

Cells labeled with FAM-FLICA can be counter-stained with other reagents such as the red vital stains Propidium Iodide (included in FAM-FLICA kits) and 7-AAD (catalog # 6163) to distinguish apoptosis from necrosis. Nuclear morphology may be concurrently observed using Hoechst, a blue DNA binding dye (included in FLICA kits). Cells can be viewed directly through a fluorescence microscope (Figures 1-3, 9, and 10), or the fluorescence intensity can be quantified using a flow cytometer (Figures 4-7) or fluorescence plate reader (Figure 8). FAM-FLICA excites at 488-492 nm and emits at 515-535 nm. FLICA is for research use only. Not for use in diagnostic procedures.

3. KIT CONTENTS

Small kits contain:

- 1 vial of FAM-FLICA caspase inhibitor reagent
- 1 bottle of Fixative (5 mL) #636
- 1 bottle of 10X Apoptosis Wash Buffer (15 mL) #635
- 1 vial of Hoechst 33342, 200 µg/mL (1 mL) #639
- 1 vial of Propidium Iodide, 250 µg/mL (1 mL) #638

Large kits contain:

- 4 vials of FAM-FLICA caspase inhibitor reagent
- 1 bottle of Fixative (5 mL) #636
- 1 bottle of 10X Apoptosis Wash Buffer (60 mL) #634
- 1 vial of Hoechst 33342, 200 µg/mL (1 mL) #639
- 1 vial of Propidium Iodide, 250 µg/mL (1 mL) #638

4. STORAGE

Store the unopened kit and each unopened component at 2-8°C until the expiration date. Once reconstituted with DMSO, use FLICA immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.

5. MSDS

MSDS are available at www.immunochemistry.com.

6. RECOMMENDED MATERIALS

- DMSO, 50 µL per vial to reconstitute FLICA
- DiH_20, 135-540 mL to dilute Apoptosis Wash Buffer
- Phosphate buffered saline (PBS) pH 7.4, up to 100 mL, to dilute FLICA™ and handle cells
- Cultured cells or tissues treated with the experimental conditions ready to be labeled
- Reagents to induce apoptosis and create controls, such as staurosporine (cat. #6212) or camptothecin (cat. #6210)
- 90% ETOH (in 10% PBS or 1X Apoptosis Wash Buffer) to create live/dead controls for Propidium Iodide staining
- Hemocytometer
- Centrifuge at <400g
- 15 mL polystyrene centrifuge tubes (1 per sample)
- DAPI (catalog #6244)

7. DETECTION EQUIPMENT

FAM-FLICA excites at 488-492 nm and emits at 515-535 nm. View Propidium Iodide (PI) under a long pass filter with the excitation at 490 nm, emission >520 nm; nuclei-bound PI has a maximum emission at 617 nm (Section 13). Hoechst stain can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm (Section 14). Use filter pairings that best approximate these settings.

- Fluorescence microscope
- Fluorescence plate reader
- Flow cytometer

8. EXPERIMENTAL PREPARATION

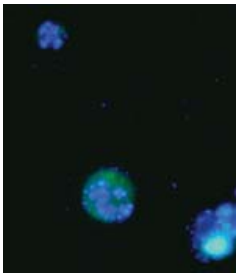
Staining apoptotic cells with FLICA can be completed within a few hours. However, FLICA is used with living cells, which require periodic maintenance and cultivation several days in advance. In addition, once the proper number of cells has been cultivated, time must be allotted for the experimental treatment or apoptosis induction process which typically requires a 2-6 hour incubation at 37°C based on the cell line and concentration. Create cell populations, such as:

a. Cells that were exposed to the experimental condition or treatment

b. A placebo population of cells that received a blank treatment instead of the experimental treatment

As FLICA detects caspase-mediated apoptosis, plan the experiment so that FLICA will be diluted and administered at the time when caspases are expected to be activated in the cells. The recommended volume of 30X FLICA is 10 µL per 300 µL of cells at 5 x 10^5 cells/mL, but the amount may vary based on the experimental conditions and the instrument used for analysis. Each investigator should adjust the amount of FLICA to accommodate the particular cell line and research conditions.

Culture cells to a density optimal for the specific experiment or apoptosis induction protocol. Cell density should not exceed 10^6 cells/mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. An initial experiment may be necessary to determine when and how much FLICA to use as the resulting positive signal is a direct measurement of caspase activity occurring during the incubation period.



ImmunoChemistry TECHNOLOGIES LLC

9. CONTROLS

At least two sets of controls should be run:

a. Positive control cells induced to undergo apoptosis

b. Negative control cells not induced to undergo apoptosis

The induced positive cell population and negative control cell population tubes should come from a common pool of cells and contain similar quantities of cells. Create negative controls by culturing an equal volume of non-induced cells for every labeling condition. For example, if labeling with FLICA and Hoechst stain, make eight populations:

1&2. Unlabeled: induced and non-induced

3&4. FLICA-labeled: induced and non-induced

5&6. FLICA-labeled and Hoechst-labeled: induced and non-induced

7&8. Hoechst-labeled: induced and non-induced

If analyzing cells with a flow cytometer, create Propidium Iodide (PI) instrument controls using ETOH to compensate for bleed-over of the PI signal into FL-1 (Section 19).

10. APOPTOSIS INDUCTION

Prior to commencing the experiment, determine a reproducible method for obtaining a positive control by triggering caspase activity. This process varies significantly with each cell line. For example, apoptosis may be induced with 2-4 μg/ml camptothecin for >4 hours; or 1-2 μM staurosporine for >4 hours.

11. PREPARATION OF FLICA

FLICA is supplied as a lyophilized powder that may be slightly visible as an iridescent sheen inside the vial. Protect from light and use gloves when handling. Because the 30X FLICA solution must be used immediately, prepare it just before staining.

1. Reconstitute each vial of FLICA with 50 μL DMSO to form the 150X stock. The stock solution should be colorless or light yellow. Once reconstituted, it may be stored at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.
2. Immediately prior to addition to the samples and controls, dilute FLICA 1:5 by adding 200 μL PBS to each vial to form the 30X FLICA solution. Use 30X FLICA within 30 minutes of dilution into aqueous buffers.

12. PREPARATION OF 1X APOPTOSIS WASH BUFFER

ICT's Apoptosis Wash Buffer (catalog #634 and #635) is used to wash cells. It contains mammalian proteins to stabilize cells stained with FLICA and sodium azide to retard contamination (1X Apoptosis Wash Buffer contains 0.01% w/v sodium azide). Cell media may be used instead of Apoptosis Wash Buffer to wash cells.

1. 10X Apoptosis Wash Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
2. Dilute 10X Apoptosis Wash Buffer 1:10 in di$H_2$0. For example, add 15 mL 10X Apoptosis Wash Buffer to 135 mL di$H_2$0 for a total of 150 mL. 1X Apoptosis Wash Buffer may be stored at 2-8°C and used within 1 week or frozen and used within 6 months.

13. PROPIDIUM IODIDE

Warning: Propidium Iodide (PI) is a mutagen. It may cause serious eye irritation. Gloves, protective clothing, and eyewear are strongly recommended. When disposing, flush sink with copious amounts of water; see MSDS for further information.

Propidium Iodide (catalog #638) is used to distinguish between living and dead cells by staining necrotic, dead, and membrane-compromised cells red. PI is an intercalating fluorescent reagent that binds between the bases of DNA. PI is membrane impermeant, which prevents it from reaching DNA in viable cells, allowing the identification of dead cells in a population.

One molecule of PI stoichiometrically binds every four to five base pairs of DNA. Upon binding to DNA, the fluorescence of PI is enhanced 20-30 fold. PI excites at 488-492 nm and exhibits an emission maximum at 635 nm. The maximum fluorescence excitation is shifted about 35 nm towards red, and the maximum emis-

FIGURE 2: CASPASE ACTIVITY IN JURKAT CELLS

Jurkat cells (T lymphocytes) were labeled with ICT's poly-caspase inhibitor FLICA kit #92 and viewed under a fluorescence microscope. The grey DIC image (B) reveals five cells in the field of view, but only four of them fluoresce green (A). Four out of five cells are apoptotic and have active caspases present (green). Data courtesy of Dr. Brian W. Lee, ICT.

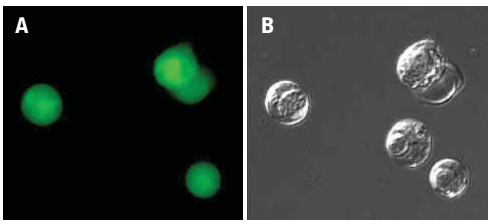
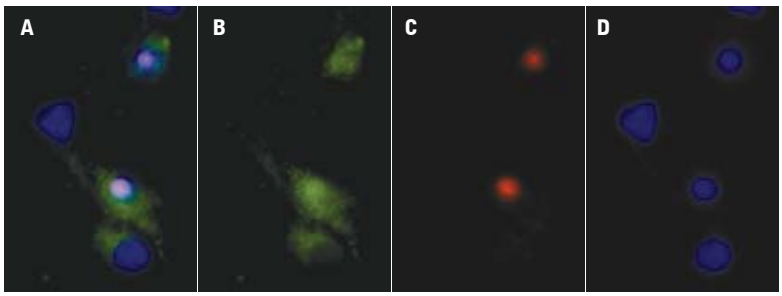


FIGURE 3: CELL DEATH IN PRIMARY RAT HIPPOCAMPAL NEURONS

ICT's FAM-FLICA caspase-3&7 inhibitor kit (catalog #94) was used to assess cell death in primay rat hippocampal neurons. Subjects were first-generation descendants of Sprague–Dawley albino rats. Hippocampi from PND 0 male pups were used for primary cultures of hippocampal neurons. Cells were plated on 25-mm poly-l-lysine-coated coverslips at 300,000 cells per coverslip, and cells were used at 4 or 8 days *in vitro*. In the composite image (A), three out of four cells are apoptotic (green). Apoptotic cells are shown in B: three caspase-positive cells fluoresce green with FAM-DEVD-FMK FLICA. No cells are necrotic as both of the PI-positive cells (C) are also FLICA-positive (B); they have compromised membranes and are in the late stages of apoptosis rather than necrosis. All four cells (D) are revealed by labeling DNA blue with Hoechst. Data courtesy of Dr. Z. Kahraman Akozer, University of Maryland.



RESEARCH USE ONLY
NOT FOR DIAGNOSTIC PROCEDURES

sion is shifted about 15 nm towards blue. When bound to nucleic acids, the maximum absorption is 535 nm and the maximum emission is 617 nm. Cells may be viewed through a fluorescence microscope or analyzed on a flow cytometer.

PI is provided ready-to-use at 250 µg/mL. It can be used with FAM-FLICA to identify four populations of cells: living; early apoptotic; late apoptotic; and necrotic (Figures 3, 5, 6).

If analyzing with a flow cytometer (Section 19), create PI instrument controls using ETOH to kill cells instead of an apoptosis-induction procedure using staurosporine or camptothecin (as outlined in Section 10). An induction method is not as effective to create PI instrument controls because induced samples will not have enough late-stage apoptotic cells that have become membrane-compromised to stain positive for PI. It is more effective to compensate using controls that are essentially either all PI-positive (>90%) or all PI-negative. Set up PI instrument controls:

1. Label 2 centrifuge tubes:
   a) PI-negative
   b) PI-positive
2. Add 1-5 x 10^5 non-induced live healthy cells to each tube.
3. Centrifuge gently to pellet cells; remove supernatants.
4. To create the PI-negative control and keep the cells alive, resuspend these cells in 300 µL PBS or AWB.
5. To create the PI-positive control and kill most of the cells, resuspend these cells in 300 µL 90% ETOH (in 10% PBS or 1X Apoptosis Wash Buffer).
6. Gently vortex each tube for 30 seconds.
7. Add 1 mL PBS or 1X Apoptosis Wash Buffer.
8. Centrifuge gently to pellet cells; remove supernatants.
9. Resuspend in 600 µL PBS or 1X Apoptosis Wash Buffer.
10. Add 3 µL PI to both tubes. If different volumes were used, add PI at 0.5% v/v. Incubate 5-10 minutes.
11. Read immediately on the flow cytometer and compensate bleed-over of the red PI signal from FL-2 into FL-1.

14. HOECHST 33342 STAIN

Warning: Hoechst 33342 is a mutagen. It may be irritating to respiratory system and skin. Gloves, protective clothing, and eyewear are strongly recommended. When disposing, flush sink with copious amounts of water; see MSDS for further information.

Hoechst 33342 (catalog #639) is a cell-permeant nuclear stain that emits blue fluorescence when bound to double stranded DNA. It is used to stain the nuclei of living or fixed cells blue and is often used to distinguish condensed, pyknotic nuclei in apoptotic cells. When bound to nucleic acids, the maximum absorption is 350 nm, and its maximum emission is 480 nm. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm. Hoechst stain is provided ready-to-use at 200 µg/mL. Hoechst stain can be used with FAM-FLICA to label the nuclei of live, dying, and apoptotic cells.

15. FIXATIVE

Warning: Fixative is toxic: danger exists of very serious irreversible effects through inhalation, contact with skin, or if swallowed. Gloves, protective clothing, and eyewear are strongly recommended. When disposing, flush sink with copious amounts of water; see MSDS for further information.

ICT's Fixative (catalog #636) is a formaldehyde solution designed to cross-link intracellular components. It will not interfere with the carboxyfluorescein (FAM) label, unlike the use of absolute ethanol- or methanol-based fixatives, which will inactivate the FLICA label.

If the stained cell populations cannot be evaluated immediately after labeling with FLICA, add Fixative at a 1:10 ratio. For example, add 100 µL Fixative to 900 µL cells and incubate for least 15 minutes. Never add Fixative until the staining and final wash steps have been completed. Fixed cells may be stored on ice or at 4°C up to 24 hours.

16. STAINING PROTOCOL FOR SUSPENSION CELLS

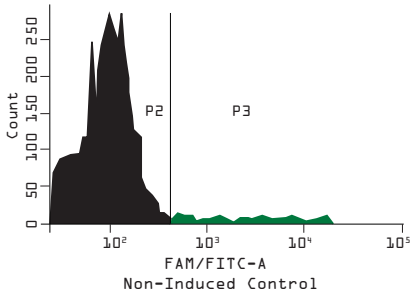
Prepare experimental cell and control cell populations. Cell concentration should be 3-5 x 10^5 cells/mL but should not exceed 10^6 cells/mL, as cells cultivated in excess of this concentration may begin to naturally enter apoptosis. Just prior to staining with FLICA, cells may need to be concentrated to 2-5 x 10^6 cells/mL as both microscopy and plate reader analysis methods (Sections 17 and 20) require high cell concentrations. Start with a larger volume of cells at 3-5 x 10^5 cells/mL (which is a typical density for cell culture) and then concentrate cells and resuspend to 300 µL when ready for FLICA staining.

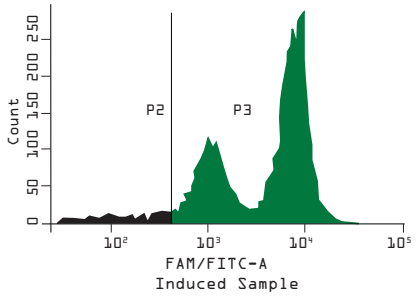
1. Expose cells to the experimental or control condition. If analyzing with a flow cytometer, set aside four populations to create instrument controls with PI-positive and PI-negative cells, and FLICA-induced and FLICA-non-induced cells (Section 19).
2. If analyzing with a fluorescence microscope or plate reader, concentrate cells to 2-5 x 10^6 cells/mL just prior to FLICA staining. Fluorescence microscopy requires an excess of 2 x 10^6 cells/mL to obtain 5 - 20 cells per image field. Flow cytometry can analzye samples at 3-5 x 10^5 cells/mL.
3. Transfer 290 µL cells into fresh tubes.

FIGURE 4: SINGLE COLOR ANALYSIS VIA FLOW CYTOMETRY

Jurkat cells were treated with DMSO, a negative control (left), or staurosporine, an apoptosis-inducing agent (right), for 4 hours, then stained with ICT's green poly caspase inhibitor probe, FAM-VAD-FMK (kit #92), for 1 hour. Cells were washed twice and read on a flow cytometer. Treatment with staurosporine induced caspase activity in 94.8% of the experimental cells (P3, right), whereas the negative control treatment exhibited caspase activity in only 3.3% of the cell population (P2, left). This is a ratio of 28:1. Data courtesy of Ms. Tracy Hanson, ICT, 10G5.

% of cells	P2-Negative	P3-Positive
Non-induced	87.5	3.3
Induced	4.8	94.8
Ratio	28:1	





ImmunoChemistry TECHNOLOGIES LLC

4. Add 10 µL 30X FLICA solution, forming a final volume of 300 µL. If different cell volumes were used, add 30X FLICA at a ratio of 1:30. Mix by slightly flicking the tubes. The amount of FLICA should be optimized for each cell line and experimental condition.
5. Incubate cells at 37°C protected from light. The incubation period may range from 30 minutes to several hours and should be optimized for each cell line and experimental condition. As cells may settle on the bottom of the tubes, gently resuspend them by swirling cells every 20 minutes to ensure an even distribution of FLICA.
6. If cells are to be analyzed with a microscope, cells may be dually stained with Hoechst. Add Hoechst at 0.5% v/v and incubate 5 minutes at 37°C. For example, if the cell suspension is at 300 µL, add 1.5 µL Hoechst.
7. Add 2 mL 1X Apoptosis Wash Buffer and gently mix.
8. Centrifuge at <400 x g for 5 minutes at RT.
9. Carefully remove and discard supernatants. Gently vortex the pellets to disrupt clumping. Resuspend in 1 mL 1X Apoptosis Wash Buffer and gently mix.
10. Centrifuge cells at <400 x g for 5 minutes at RT.
11. Carefully remove and discard supernatants. Gently vortex pellets to disrupt clumping.

17. MICROSCOPY ANALYSIS OF SUSPENSION CELLS

Follow Section 16, Steps 1-11. See Figures 1-2.

12. Resuspend cells in 300-500 µL 1X Apoptosis Wash Buffer and place on ice. At this point, the cells may be stained with Propidium Iodide (PI) for bicolor analysis (Step 13), fixed for future viewing (Step 14), or observed immediately (Step 15).
13. To identify dead cells by staining with PI, add 1.5 µL PI to the 300 µL cell suspension. If different volumes were used, add it at 0.5% v/v. Incubate 5 minutes at 37°C.
    a. Wash cells to remove excess PI from the media. Centrifuge at <400 x g for 5 minutes at RT.
    b. Carefully remove and discard supernatants. Gently vortex pellets to disrupt clumping.
    c. Resuspend cells in 300 µL 1X Apoptosis Wash Buffer and gently mix. Go to Step 14 or 15.
14. If not viewing immediately, cells may be fixed for viewing up to 24 hours later. Add 30 µL Fixative. If cells were resuspended in a different volume, add it at a ratio of 1:10.
    a. Incubate 15 minutes at RT in the dark.
    b. Place cells onto a microscope slide and allow to dry.
    c. Briefly wash cells with PBS.
    d. Cover cells with mounting media and coverslip.
    e. Store slides at 2-8°C up to 24 hours.
15. To view cells immediately, place 1 drop of cell suspension onto a microscope slide and cover with a coverslip.
16. Observe cells under a fluorescence microscope using a bandpass filter (excitation 490 nm, emission >520 nm) to view green fluorescence. Cells bearing active caspase enzymes covalently coupled to FLICA appear green. Hoechst stain can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm. View PI under a long pass filter with the excitation at 490 nm, emission >520 nm; nuclei-bound PI has a maximum emission at 617 nm (Figures 1-3 and 12).

FIGURE 5: BICOLOR STAINING OF RABBIT CELLS

ICT's poly-caspase inhibitor FLICA reagent, FAM-VAD-FMK (catalog #92), was used to assess apoptosis in rabbit cells (both suspension and adherent cells). Cells were grown to 3 x 10^6 cells per sample and treated with a placebo (left) or a condition which induced apoptosis (right). Cells were stained with FAM-VAD-FMK, washed, stained with Propidium Iodide (PI), a red vital stain, and analyzed using two-color flow cytometry. Dot plots were set up to detect caspase activity (green, FL-1) on the X-axis and necrosis (red, FL-2) on the Y-axis (compare with Figure 6). Four populations of cells were detected: (A) unstained live cells do not fluoresce; (D) cells in early apoptosis fluoresce green with FAM-FLICA™; (C) cells in late apoptosis are dually stained with FAM-FLICA™and PI: they fluoresce green (they have active caspases) and red (the cell membrane has permeabilized); and (B) necrotic cells fluoresce red. Cells became necrotic when treated with the placebo (left, B) but not apoptotic (left, C and D), while many cells entered early and late apoptosis when treated with the inducer (right, C and D), but were not fully necrotic (right, B).

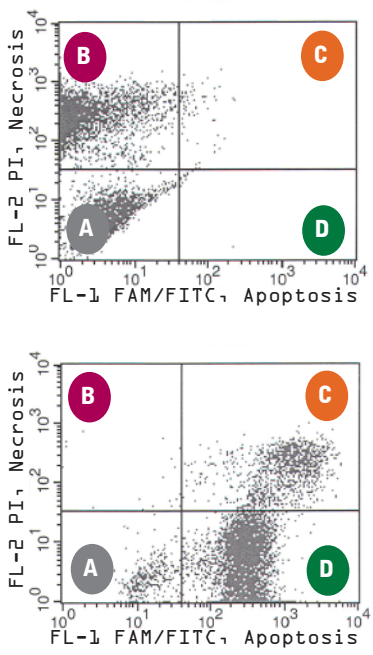
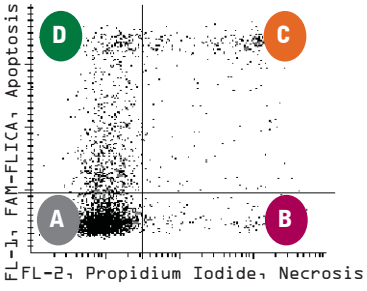
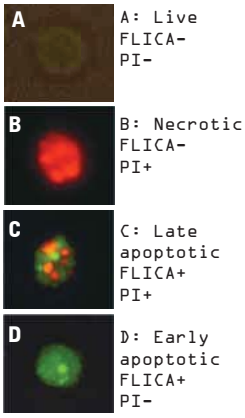


FIGURE 6: FOUR POPULATIONS OF HL-60 CELLS

HL-60 cells (human promyelocytic leukemia) were treated with a drug, then stained with ICT's FAM-VAD-FMK poly-caspase inhibitor FLICA reagent and a red vital stain, Propidium Iodide (PI), from kit #92. Cells were analyzed on a scanning laser cytometer to detect red on the X-axis (necrosis) and green on the Y-axis (apoptosis; compare with Figure 5). Four populations of cells were detected: (A) unstained live cells do not fluoresce; (D) cells in early apoptosis fluoresce green with FAM-FLICA; (C) cells in late apoptosis are dually stained with FAM-FLICA and PI: they fluoresce green (they have active caspases) and red (the cell membrane has permeabilized); and (B) necrotic cells fluoresce red. In this experiment, the drug triggered the caspase cascade rather than being overtly toxic to the cells. Data courtesy of Dr. Zbigniew Darzynkiewicz, Brander Cancer Center, NY.





RESEARCH USE ONLY
NOT FOR DIAGNOSTIC PROCEDURES

18. SINGLE COLOR FLOW CYTOMETRY ANALYSIS OF SUSPENSION CELLS

Follow Section 16, Steps 1-11, but omit Hoechst staining in Step 16.6. See Figure 4.

12. Resuspend cells in 300 µL 1X Apoptosis Wash Buffer and place on ice.
13. For single-color analysis, use a 15 mW argon ion laser at 488 nm. Measure fluorescein on the FL1 channel. Generate a histogram with the log FL1 on the X-axis versus the number of cells on the Y-axis. Caspase negative (FLICA-) cells will occur in the lower log fluorescence output decades of the FL1 (X) axis, whereas caspase-positive (FLICA+) cells will appear as a shoulder on the right side (brighter) or separate peak on the right side of the negative peak histogram (Figure 4).

19. MULTICOLOR FLOW CYTOMETRY ANALYSIS OF SUSPENSION CELLS

To address compensation issues and set up the flow cytometer, prepare two sets of instrument controls: live and killed cells that are only stained with PI (Section 13); and induced and non-induced cells that are only stained with FLICA. These controls are needed to adjust the instrument PMT's to separate PI-positive and PI-negative samples and compensate for bleed-over of the red PI signal from FL-2 into FL-1. They will also help to clearly differentiate the induced FLICA-positive population from the induced FLICA-negative population and compensate bleed-over of the green FAM-FLICA signal from FL-1 into FL-2. If using an instrument that does not have the option to adjust PMT's, like the Accuri C6, these control populations can help set the positive gate for the PI-positive and FLICA-positive cells prior to reading dual-labeled samples.

While setting up the PI controls (Section 13), continue working with the test samples and experimental controls as prepared in Section 16, Steps 1-11, but omit Hoechst staining in Step 16.6. See Figures 5-7.

12. Resuspend cells in 400 µL 1X Apoptosis Wash Buffer.
13. Set aside a control of FLICA-stained induced cells that does not contain PI. FLICA-stained cells may be stored at 2-8°C up to 4 hours protected from light, depending upon the cell line.
14. Stain cells with 2 µL PI, mix, and put samples on ice for up to 1 hour and analyze (after 1 hour, PI-stained cells may have toxicity issues). If not analyzing PI-stained cells within 1 hour, then wash and fix:
    a. Wash cells to remove the PI from the media to prevent false-positives as any excess PI could go into all cells after fixation. Centrifuge at <400 x g for 5 minutes at RT.
    b. Carefully remove and discard supernatants. Gently vortex the pellets to disrupt clumping.
    c. Resuspend cells in 300 µL 1X Apoptosis Wash Buffer and gently mix.
    d. Add 30 µL Fixative. If cells were resuspended in a different volume, add Fixative at a ratio of 1:10.
    e. Incubate 15 minutes at RT in the dark. Store fixed cells at 2-8°C up to 24 hours protected from light.
15. Set up the instrument compensation. Read the PI-positive and PI-negative controls (Section 13) to compensate bleed-over of the red PI signal from FL-2 into FL-1. Read the FLICA-only-positive and FLICA-only-negative controls to compensate bleed-over of the green FAM-FLICA signal from FL-1 into FL-2.
16. When ready to read the samples for bicolor analysis, measure carboxyfluorescein (FAM) on the FL1 channel and red fluorescence (PI) on the FL2 channel. Generate a log FL1 versus log FL2 dot plot (Figures 5 and 6). This will reveal four populations of cells:
    a. Cells in early apoptosis fluoresce green with FAM-FLICA.
    b. Cells in late apoptosis are dually stained with FAM-FLICA and PI; they fluoresce green (they have active caspases) and red (the cell membrane has permeabilized).
    c. Necrotic cells fluoresce red.
    d. Unstained live cells do not fluoresce.

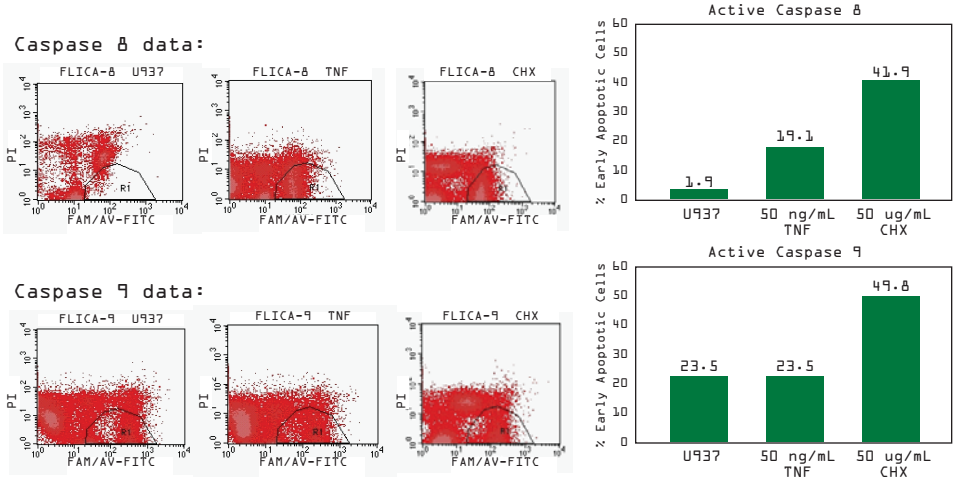
20. FLUORESCENCE PLATE READER ANALYSIS OF SUSPENSION CELLS

Follow Section 16, Steps 1-11, but omit Hoechst staining in Step 16.6. See Figure 8.

12. Resuspend cells in 500 µL PBS.
13. Determine the concentration and compare the cell density of each sample. The non-induced population may have more cells than the induced population, as some apoptotic cells in the induced samples may be lost during the wash steps. Adjust the volume of the cell suspensions to equalize the cell density. When ready to read, cells should be >3 x 10^6 cells/mL.

FIGURE 7: FLOW CYTOMETRY ANALYSIS

FAM-FLICA kits were used to detect early apoptosis in U937 cells, a histiocytic lymphoma cell line, upon treatment with TNF or cycloheximide for 24 hours. 200 µL of U937 cells at 10^6 cells/mL were pipetted into a 96-well U-bottom plate. Apoptosis was induced by treating cells with either 50 ng/mL TNF or 50 µg/mL cycloheximide (CHX) for 24 hours. Cells were labeled with 6 µL 30X FLICA caspase-8 inhibitor reagent (FAM-LETD-FMK, catalog #910) or FLICA caspase-9 inhibitor reagent (FAM-LEHD-FMK, catalog #913) for 1 hour. Cells were washed three times, stained with propidium idodide (PI), a red vital stain, and analyzed with a BD FACS set at 465 FL1 and 359 FL2. Caspase-9 was more active in these cells than caspase-8. Very few of the cells became necrotic (PI-positive).



14. Pipette 100 µL per well into a black microtiter plate. Analyze at least 2 aliquots per sample. Do not use clear plates. Avoid bubbles.
15. Perform an endpoint read. Set the excitation wavelength at 488 nm and the emission wavelength to 530 nm; use a cut-off filter of 515 nm. FAM-FLICA has an optimal excitation range of 488 - 492 nm and emission range of 515 - 535 nm (Figure 8).

21. STAINING PROTOCOL FOR ADHERENT CELLS

Adherent cells need to be carefully washed to avoid the loss of any cells that round up and come off the plate surface. Loose cells may be harvested from the plate or slide surface and treated as suspension cells, while those remaining adherent to the surface should be washed as adherent cells. If the adherent cells are trypsinized, the loose cells can be recombined with the trypsinzed pool; alternatively, the loose cells can be recombined with the adherent portion when the analysis is performed. If growing adherent cells in a plate, the entire plate may be gently spun as part of the wash process to sediment any loose floating cells. Avoid trypsinizing cells prior to labeling with a vital dye, like PI. Cell membranes exposed to trypsin could be transiently permeant to vital dyes for a variable time depending upon the cell line. Cells may be labeled with FLICA before or after trypsinization (Figures 3 and 9).

1. Culture cells in T25 flasks and expose to the experimental conditions.
2. Apoptotic cells may detach and begin to float into the media. Save and spin to pellet and include these cells in the analysis.
3. Trypsinize adherent cells; neutralize with trypsin inhibitor present in cell culture media with 20% FBS; pool cells with any pellets created in Step 2; add 2-5 mL media.
4. Centrifuge at 200 x g for 5 minutes.
5. Remove all but ~100 µL supernatant. Resuspend cells in 300-500 µL in cell culture media containing 10-20% FBS. If necessary, count cells and adjust the volume of cell suspension to fit the experiment. Transfer cells into a 15 mL tube.
6. Add 30X FLICA at 1:30.
7. Incubate 30-60 minutes at 37°C, mixing gently every 10 minutes.
8. Wash by adding 2 mL 1X Apoptosis Wash Buffer.
9. Centrifuge at 200 x g for 5 minutes.
10. Aspirate supernatant and resuspend cells in 2 mL 1X Apoptosis Wash Buffer. Incubate 10 minutes at 37°C to allow any unbound FLICA to diffuse out of the cells.
11. Centrifuge at 200 x g for 5 minutes.
12. Aspirate supernatant and resuspend cells in 1X Apoptosis Wash Buffer. Store cells on ice and protect from light; read within 4 hours. At this point, cells may be analyzed with a fluorescence microscope, flow cytometer, or plate reader.
    a. If viewing under a microscope, cells may be stained with PI or analyzed following Section 17 (omit fixation steps; Figures 3 and 9).
    b. If analyzing via flow cytometry, cells do not need to be counted. Follow Section 18 or 19 (omit fixation steps).
    c. If analyzing with a fluorescence plate reader, follow Section 20.

FIGURE 10: FROZEN TUMOR SECTIONS

A human tumor xenograph was grown subcutaneously in nude mice. Unfixed frozen 10 µm sections were prepared and stained with ICT's green FLICA poly-caspase inhibitor reagent (FAM-VAD-FMK, catalog #92) and counterstained with DAPI (blue); total magnification is 400X. A low level of caspase activity was detected in photo A compared with photo B, which reveals extensive green staining in cells throughout the tissue section. Data courtesy of Dr. Rolf Brekken, UTSW.

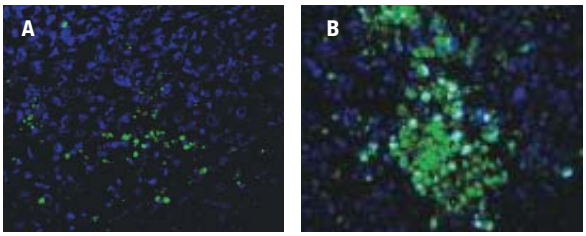


FIGURE 8: CASPASE ACTIVITY ANALYZED WITH A FLUORESCENCE PLATE READER

Jurkat cells were treated with either DMSO (negative, non-induced cells; left bar) or staurosporine (apoptotic, induced cells; right bar) for 2 hours at 37°C. Cells were labeled with ICT's poly-caspase inhibitor reagent, FAM-VAD-FMK (cat. #92), for 60 minutes at 37°C. Samples were read on a Molecular Devices Gemini XS 96-well fluorescence plate reader. In the induced population, the relative fluorescence units (RFU) of the green fluorescent signal was five times greater than the RFU of the non-induced population (34.1 vs. 6.4). Staurosporine induced poly-caspase activity in Jurkat cells.

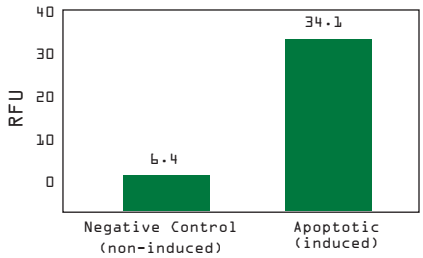
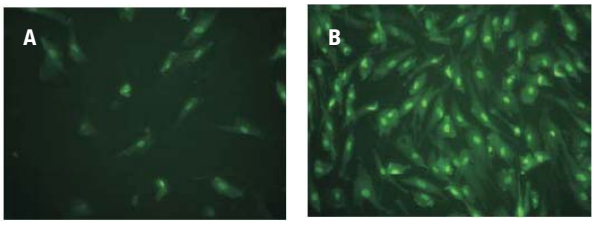


FIGURE 9: ADHERENT CORNEAL FIBROBLASTS

Normal (A) and keratoconus (B) corneal fibroblasts were treated with 200 µM H_2O_2 for 1 hour, washed, and allowed to recover for 1-3 hours. The culture media was removed and replaced with ICT's FAM-FLICA caspase 3&7 inhibitor reagent, FAM-DEVD-FMK (catalog #94) in cell culture media at 300 µL/well for 1 hour. The cell layer was washed 3 times with 1X Apoptosis Wash Buffer; 300 µL was used to keep the cells from drying. Keratoconus corneal fibroblasts treated with H_2O_2 (B) show a significant increase in caspase 3&7 activity compared to normal cells (A). Non-apoptotic cells are dark in background. Data courtesy of Dr. Cristina Kenney, MD, PhD, Dept. of Ophthalmology, University of California, Irvine.



RESEARCH USE ONLY
NOT FOR DIAGNOSTIC PROCEDURES

22. FLUORESCENCE PLATE READER ANALYSIS OF ADHERENT CELLS

1. Adherent cells can be grown in a microtiter plate. Use plates with clear bottoms and black walls. Culture cells to approximately 90% confluency.
2. Add 30X FLICA at 1:30.
3. Incubate 30-60 minutes at 37°C; mix gently every 10 minutes.
4. Wash by adding ~400 µL media to each well and incubate 60 minutes at 37°C to allow any unbound FLICA to diffuse out of the cells.
5. Gently centrifuge the entire plate to sediment any loose floating cells.
6. Aspirate the media. Resuspend with fresh media or PBS.
7. Read plates using a bottom-reading instrument.

23. STAINING PROTOCOL FOR TISSUE SECTIONS

See Figure 10.

1. Prepare frozen tissues according to the experiment.
2. Dry slides and fix with acetone for 1 minute.
3. Rehydrate slides by washing in TBS-Tween (TBSt) or PBS-Tween (PBSt) for 5 minutes.
4. Wash again in TBSt or PBSt for 5 minutes.
5. Block slides for 20 minutes. Use a blocker such as 20% Aqua-block in media with 0.2% Tween.
6. Dilute 150X FLICA stock 1:50 in PBS to form the tissue section staining solution (TSSS). For example, add 50 µL 150X stock to 2450 µL PBS (2.5 mL total).
7. Add 50 µL TSSS and incubate >1hr protected from light.
8. Wash in TBSt or PBSt for 5 minutes.
9. Wash again in TBSt or PBSt for 5 minutes.
10. Set slides in a dish containing 1X Apoptosis Wash Buffer.
11. Stain nuclei with DAPI (catalog #6244) and apply a coverslip.
12. Store samples at 2-8°C for short-term storage or -20°C for long-term storage (Figure 10).

24. REFERENCES & CITATIONS

1. Amstad, P. A., et al. Detection of caspase activation *in situ* by fluorochrome-labeled caspase inhibitors. *Biotechniques.* 31, 608-614 (2001).
2. Bedner, E., et al. Activation of caspases measured *in situ* by binding of fluorochrome-labeled inhibitors of caspases (FLICA): correlation with DNA fragmentation. *Exp Cell Res.* 259(1):308-313 (2000).
3. Cascao, R., et al. Caspase-1 is active since the early phase of rheumatoid arthritis. *Ann Rheum Dis.* 70:A2 (2011).
4. Chwa, M., et al. Increased stress-induced generation of reactive oxygen species and apoptosis in human keratoconus fibroblasts. *Invest Ophthalmol Vis Sci.* 47(5): 1902-1910 (2006).
5. Cuevas-Ramos, G., et al. *Escherichia coli* induces DNA damage *in vivo* and triggers genomic instability in mammalian cells. *PNAS.* 107:11537-11542 (2010).
6. Darzynkiewicz, Z., et al. Detection of caspases activation *in situ* by fluorochrome-labeled inhibitors of caspases (FLICA). *Methods Mol Biol.* 203:289-299 (2002).
7. Dunne, A., et al. Inflammasome activation by adenylate cyclase toxin directs Th17 responses and protection against *Bordetella pertussis*. *J Immunol.* 185:1711-1719 (2010).
8. Ekert, P. G., et al. Caspase inhibitors. *Cell Death Differ.* 6:1081-1086 (1999).
9. Evangelisti, C., et al. Preclinical testing of the Akt inhibitor triciribine in T-cell acute lymphoblastic leukemia. *J Cell Physiol.* 226(3):822-31 (2011).
10. Fang, C., et al. The capsid proteins of Aleutian mink disease virus activate caspases and are specifically cleaved during infection. *J Virol.* 84(6):2687-2696 (2010).
11. Fiala, M., et al. HIV-1 induces cardiomyopathy by cardiomyocyte invasion and gp120, Tat and cytokine apoptotic signaling. *Cardiovasc Toxicol.* 4(2):97-107 (2004).
12. Freishtat, R. J., et al. Asthmatic airway epithelium is intrinsically inflammatory and mitotically dyssynchronous. *Am J Respir Cell Mol Biol.* 44:863-869 (2011).
13. Grabarek, J., et al. Use of fluorescently labeled caspase inhibitors as affinity labels to detect activated caspases. *Human Cell.* 15(1):1-12 (2002).
14. Grunewald, S., et al. Caspase activity in human spermatozoa in response to physiological and pathological stimuli. *Fertil Steril* 83:1106-1112 (2005).
15. Kumar, S. Mechanisms mediating caspase activation in cell death. *Cell Death Differ.* 6:1060-1066 (1999).
16. Li, D., et al. Enzymatic dysfunction of mitochondrial complex I of the *Candida albicans goa1* mutant is associated with increased reactive oxidants and cell death. *Eukaryotic Cell.* 10(5):672 (2011).
17. Lopez-Marure, R., et al. Effects of dehydroepiandrosterone on proliferation, migration, and death of breast cancer cells. *Eur J Pharmacol.* 660(2-3):268-74 (2011).
18. Luthra, S. et al. Activation of caspase-8 and caspase-12 pathways by 7-ketocholesterol in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 47:5569-5575 (2006).
19. Mansoor, S. et al. Inhibition of apoptosis in human retinal pigment epithelial cells treated with benzo(e)pyrene, a toxic component of cigarette smoke. *Invest Ophthalmol Vis Sci.* 51:2601-2607 (2010).
20. Olivares-Zavaleta, N., et al. *Chlamydia pheumoniae* inhibits activated human T lymphocyte proliferation by the induction of apoptotic and pyroptotic pathways. *J Immunol.* 186:7120-7126 (2011).
21. Osuka, A., et al. A protective role for inflammasome activation following injury. *Shock.* (2011, Sep 3 Epub ahead of print).
22. Pozarowska, D., et al. Cytometric assessment of cytostatic and cytotoxic effects of topical glaucoma medications on human epithelial corneal line cells. *Cytometry B Clin Cytom.* 78(2):130-7 (2010).
23. Rausch, V., et al. Synergistic activity of sorafenib and sulforaphane abolishes pancreatic cancer stem cell characteristics. *Cancer Res.* 70:5004-5013 (2010).
24. Rotonda, J., et al. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. *Nature Struct. Biol.* 3(7):619-625 (1996).
25. Slee, E. A., et al. Serial Killers: ordering caspase activation events in apoptosis. *Cell Death Differ.* 6:1067-1074 (1999).
26. Smoleski, P., et al. Assay of caspase activation *in situ* combined with probing plasma membrane integrity to detect three distinct stages of apoptosis. *J Immunol Methods.* 265(1-2):111-121 (2002).
27. Thomson, L., et al. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod.* 24:2061-2070 (2009).
28. Thornberry, N. A., et al. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J Biol Chem.* 272(29): 17907-17911 (1997).
29. Walker, N. P., et al. Crystal structure of the cysteine protease interleukin-1ß-converting enzyme: a (p20/p10)2 homodimer. *Cell.* 78:343-352 (1994).
30. Wei, J., et al. Temporal regulation of intracellular organelle homeostasis in T lymphocytes by autophagy. *J Immunol.* 186:5313-5322 (2011).
31. Wilson, K. P., et al. Structure and mechanism of interleukin-1 beta converting enzyme. *Nature.* 370:270-275 (1994).
32. Wolbers, F., et al. Apoptotic cell death kinetics *in vitro* depend on the cell types and the inducers used. *Apoptosis.* 9:385-392 (2004).
33. Yaacob, N. S., et al. Anticancer activity of a sub-fraction of dichloromethane extract of *Strobilanthes crispus* on human breast and prostate cancer cells *in vitro*. *BMC Complement Altern Med.* 10:42 (2010).

Thank you for using FLICA™! If you have any questions, or would like to share your data, please contact us at 1-800-829-3194 or 952-888-8788, or send an

GENTAUR

Gentaur Molecular Products
Voortstraat 49
1910 Kampenhout, BELGIUM

Tel 0032 16 58 90 45 | Fax 0032 16 50 90 45
www.gentaur-worldwide.com
info@gentaur.com