

1. INTRODUCTION

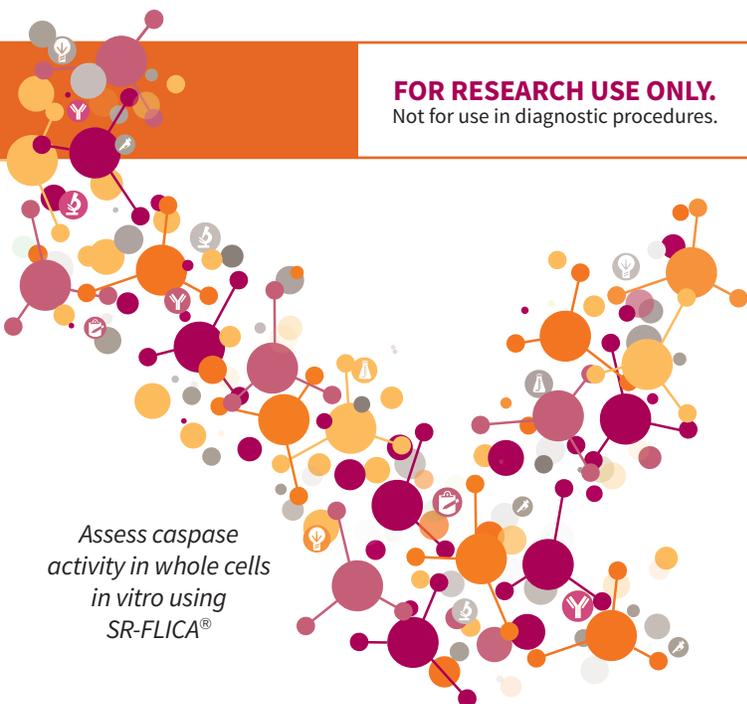
FLICA® is a powerful method to assess cell death by detecting apoptosis *in vitro*. ImmunoChemistry Technologies' (ICT) FLICA probes are non-cytotoxic Fluorescent Labeled Inhibitors of CASpases that covalently bind to active caspase enzymes^{1,2}. FLICA measures the intracellular process of apoptosis 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.

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 red fluorescent signal within the cell. Unbound FLICA will diffuse out of the cell during the wash steps. 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 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 cell³. 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 inflammasome activity and takes on the role of a key housekeeping enzyme in its conversion of pro-IL-1 β protein into the active IL-1 β cytokine. (Use FLICA kits #98, #9122, and #9145 to detect caspase-1). Please note that macrophages and monocytes have been shown to rapidly secrete caspase-1 upon activation^{4,5}.

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 specificity⁶. 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 caspase⁷⁻⁹.

Activated caspase enzymes cleave proteins by recognizing 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 end¹⁰. Each FLICA probe contains a 3 or 4 amino acid sequence that is targeted by different activated caspases. This target sequence is sandwiched between a red fluorescent label, sulforhodamine B (SR), and a fluoromethyl ketone (FMK). A caspase enzyme cannot cleave the FLICA inhibitor



Assess caspase activity in whole cells *in vitro* using SR-FLICA®

probe; instead, it forms an irreversible covalent bond with the FMK on the target sequence and becomes inhibited from further enzymatic activity.

ICT offers four red SR-FLICA inhibitors: SR-VAD-FMK, a poly-caspase probe, which may be used as a general reagent to detect apoptosis as it is recognized by all different types of activated caspases; SR-DEVD-FMK, which is preferred by active caspase-3/7; SR-LETD-FMK, which is preferred by active caspase-8; and SR-LEHD-FMK, which is preferred by active caspase-9. ICT also offers FLICA inhibitor reagents with a green or far-red label (see our website for more details). Caspases, like most other crucial cell survival enzymes, are somewhat permissive in the target amino acid sequence they will recognize and cleave. Although FLICA reagents contain the different amino acid target sequences preferred by each caspase, they can also recognize other active caspases when they are present. ICT encourages validation of caspase activity by an orthogonal technique.

FLICA can be used to label suspension or adherent cells and thin tissue sections. After labeling with SR-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 SR-FLICA can be counter-stained with reagents such as the live/dead stain 7-AAD (catalog # 6163, Figures 7 and 8) to distinguish apoptosis from necrosis. Nuclear morphology can be concurrently observed using Hoechst 33342, a blue DNA binding dye (included in FLICA kits). Cells can be viewed directly through a fluorescence microscope (Figures 1-3, 6, 9, and 10) or the fluorescence intensity can be quantified using a fluorescence plate reader (Figures 4 and 5) or flow cytometer (Figures 1, 7, and 8). For best results with flow cytometry, use a yellow or green laser. SR-FLICA excites at 550-580 nm and emits at 590-600 nm.

2. SR-FLICA KITS

Caspase	Inhibitor Reagent (part#)	Kit Catalog #	
		Trial	Standard
Poly Caspase	SR-VAD-FMK (679)	916	917
Caspase-3/7	SR-DEVD-FMK (678)	931	932
Caspase-8	SR-LETD-FMK (6693)	9149	9150
Caspase-9	SR-LEHD-FMK (6145)	960	961

3. KIT CONTENTS

Trial size kits contain:

- 1 vial of SR-FLICA caspase inhibitor reagent
- 1 bottle of 10X Apoptosis Wash Buffer (15 mL), #635
- 1 bottle of Fixative (6 mL), #636
- 1 vial of Hoechst 33342, 200 µg/mL (1 mL), #639

Standard size kits contain:

- 4 vials of SR-FLICA caspase inhibitor reagent
- 1 bottle of 10X Apoptosis Wash Buffer (60 mL), #634
- 1 bottle of Fixative (6 mL), #636
- 1 vial of Hoechst 33342, 200 µg/mL (1 mL), #639

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. SAFETY DATA SHEETS (SDS)

Safety data sheets are available online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

6. RECOMMENDED MATERIALS

- DMSO, 50 µL per vial to reconstitute FLICA
- DiH₂O, 135-540 mL to dilute 10X Apoptosis Wash Buffer
- Phosphate buffered saline (PBS) pH 7.4, up to 100 mL, to dilute FLICA and handle cells
- FBS and/or BSA to add to the buffer when handling cells
- Cultured cells or tissues treated with the experimental conditions ready to be labeled
- Reagents to induce apoptosis and create controls, such as staurosporine (catalog #6212) or camptothecin (catalog #6210)
- Hemocytometer
- Black 96-well microtiter plate, flat bottom, non-treated, non-sterile (ICT catalog #266). If using a bottom reading instrument, use a plate with black walls and a clear bottom. If culturing cells in the plate, use a sterile black tissue culture plate.
- Centrifuge at 200 x g
- 15 mL polypropylene centrifuge tubes (1 per sample)

7. DETECTION EQUIPMENT

The assay can be analyzed with a:

- Fluorescence microscope
- Fluorescence plate reader
- Flow cytometer

Use filter pairings that best approximate these settings:

- SR-FLICA excites at 550-580 nm (excitation peak is at 570 nm) and emits at 590-600 nm (peak emission is at 590 nm).
- Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm (Section 13).

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:

- Cells that were exposed to the experimental condition or treatment
- A placebo population of cells that received a blank treatment instead of the experimental treatment

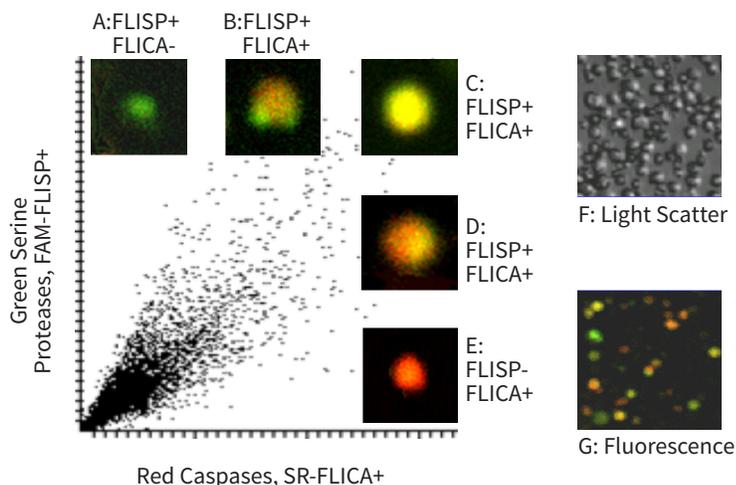
As FLICA detects the presence of catalytically active forms of caspase enzymes, plan the experiment so that FLICA will be diluted and administered at the time when caspases are expected to be activated in the cells.

The recommended volume of 30-60X FLICA is 5-10 µL per 300 µL of cells at 3-5 x 10⁵ 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

Figure 1: Dual staining with SR-FLICA® and FAM-FLISP®

HL-60 cells were treated with camptothecin (catalog #6210), an apoptosis inducer, for 3 hours then stained with ICT's red SR-FLICA poly-caspase inhibitor SR-VAD-FMK (catalog #917) and ICT's green FAM-FLISP serine protease inhibitor FFCK (catalog #946) for 1 hour. Cells were washed, then analyzed on a scanning laser cytometer. Cells with active caspases stain red with SR-VAD-FMK along the X-axis and cells with active serine proteases stain green with FFCK along the Y-axis. Co-localization of caspase activity versus serine protease activity is evident in dually stained cells (B, C, D, and G). The light-scatter image (F) reveals many negative cells. In this experiment, the treatment triggered both caspase activity and serine protease activation and the time between was relatively short as very few cells are just red (G). Data courtesy of Dr. Jerzy Grabarek, Brander Cancer Center, NY, and Pomeranian School of Medicine, Szczecin, Poland.





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.

9. CONTROLS

Create experimental samples and control cell populations:

- Treated experimental population(s): cells exposed to the experimental condition(s).
- Negative control: non-treated cells grown in a normal culture environment.
- Positive control: cells induced to undergo apoptosis using a known caspase induction protocol.

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 33342 (which is optional), 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

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 $\mu\text{g}/\text{mL}$ camptothecin (catalog #6210) or 1-2 μM staurosporine (catalog #6212) 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 30-60X FLICA solution must be used immediately, prepare it just before staining.

- Reconstitute each vial of FLICA with 50 μL DMSO to form the 150-300X stock concentrate. The stock solution should be pink to red in color. Once reconstituted, it may be stored at $\leq -20^\circ\text{C}$ for 6 months protected from light and thawed no more than twice during that time.
- Immediately prior to addition to the samples and controls, dilute FLICA 1:5 by adding 200 μL PBS to each vial to form the 30-60X FLICA solution. Use 30-60X FLICA within 30 minutes of dilution into aqueous buffers.

12. PREPARATION OF 1X APOPTOSIS WASH BUFFER

ICT's 10X Apoptosis Wash Buffer (catalog #634 and #635, AWB) is an isotonic solution used to wash cells following exposure to FLICA. It contains mammalian proteins to stabilize cells stained with FLICA and sodium azide to retard bacterial growth (1X Apoptosis Wash Buffer contains 0.01% w/v sodium azide). Cell culture media containing FBS and other additives may be used instead of Apoptosis Wash Buffer.

- 10X Apoptosis Wash Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
- Dilute 10X Apoptosis Wash Buffer 1:10 in dH_2O . For example, add 15 mL 10X Apoptosis Wash Buffer to 135 mL dH_2O for a total of 150 mL.
 - 1X Apoptosis Wash Buffer may be stored at 2-8 $^\circ\text{C}$ and used within 1 week or frozen and used within 6 months.

13. HOECHST 33342

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, to distinguish condensed pyknotic nuclei in apoptotic cells, and for cell cycle studies.

Hoechst 33342 is provided ready-to-use at 200 $\mu\text{g}/\text{mL}$. Hoechst 33342 can be used with FLICA to label the nuclei of live, dying, and apoptotic cells (Figure 2). When bound to nucleic acids, it has a maximum absorbance at 350 nm and a maximum emission at 480 nm. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm.

- Warning:** Hoechst 33342 contains a low concentration of Bis benzimide H 33342 trihydrochloride (CAS 23491-52-3) which is below the threshold for reporting on the safety data sheet (SDS). It is a suspected mutagen at high concentrations. Prolonged skin contact may cause redness and irritation. Because of the small quantity of product, the health hazard is small. See SDS for further information.

14. FIXATIVE

ICT's Fixative (catalog #636) is a formaldehyde solution designed to cross-link and aggregate intracellular components. If the stained cell populations cannot be evaluated immediately after labeling with FLICA, add Fixative at a ratio of 1:5-1:10. For example, to use Fixative at 1:10, add 100 μL Fixative to 900 μL cells. Never add Fixative until all the staining and final wash steps have been completed. Fixed cells may be stored on ice or at 4 $^\circ\text{C}$ for up to 16 hours, protected from light.

ICT's Fixative will not interfere with the sulforhodamine (SR) label. Do not use absolute ethanol- or methanol-based fixatives, as they may inactivate the SR-FLICA label.

- Danger:** Fixative contains formaldehyde <10% and methanol <5% and is harmful. Avoid contact with skin, eyes, and clothing by wearing lab coat, gloves, and safety glasses. In case of exposure, immediately flush eyes or skin with water. See SDS for further information.

15. STAINING PROTOCOL FOR SUSPENSION CELLS

Prepare experimental and control cell populations. Ideally, cell concentration should be 3-5 $\times 10^5$ cells/mL. The concentration 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 $\times 10^6$ cells/mL as both microscopy and plate reader analysis methods (Sections 17 and 18) require high cell concentrations. Start with a larger volume of cells at 3-5 $\times 10^5$ cells/mL (which is a typical density for cell culture) and then concentrate cells and resuspend to 300 μL per sample when ready for FLICA staining.

- Expose cells to the experimental and control conditions. Include the following controls:
 - Positive control cells induced to undergo apoptosis.
 - Negative control cells not induced to undergo apoptosis.
- If analyzing with a fluorescence microscope or plate reader, concentrate cells to 2-5 $\times 10^6$ cells/mL just prior to FLICA staining. Fluorescence microscopy requires an excess of 2 $\times 10^6$ cells/mL to obtain 5-20 cells per image field. Flow cytometry can efficiently analyze samples at 3-5 $\times 10^5$ cells/mL.

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

3. Transfer 290-295 μL cells into fresh tubes.
4. Add 5-10 μL 30-60X FLICA solution, forming a final volume of 300 μL . If different cell volumes were used, add FLICA at a ratio of 1:30-1:60. Mix by gently flicking the tubes. The amount of FLICA should be optimized for each cell line and experimental condition. However, 1:60 is generally sufficient for flow cytometry applications, and 1:30 is recommended for fluorescence microscopy or plate reader applications.
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 by swirling cells every 10-20 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. Do not stain with Hoechst if using a plate reader (Section 18) or flow cytometer (Section 19). 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 200 x g for 5-10 minutes at room temperature (RT).
9. Carefully remove and discard supernatants. Gently vortex pellets to disrupt clumping. Resuspend in 1 mL 1X Apoptosis Wash Buffer and gently mix.
10. Centrifuge cells at 200 x g for 5-10 minutes at RT.
11. Carefully remove and discard supernatants. If analyzing by fluorescence microscopy or fluorescence plate reader, repeat wash process a third time. If using a flow cytometer, two wash steps are generally sufficient.
12. Gently vortex pellets to disrupt clumping.
 - If analyzing with a fluorescence microscope, go to Section 17.
 - If using a fluorescence plate reader, go to Section 18.
 - If using a flow cytometer, go to Section 19.

16. STAINING PROTOCOL FOR ADHERENT CELLS

Adherent cells need to be handled carefully to avoid the loss of any cells that round up and come off the culture surface. In microscopy or plate reader

applications where trypsinization is not required, adherent cells can be stained and washed directly on the chamber slide, well, or culture surface. To avoid losing cells that are no longer adherent during washing, spin down all overlay media and wash buffer and recombine washed cell pellets with the adherent samples prior to analysis.

Cells may be trypsinized to create suspensions, which may be labeled with FLICA before or after trypsinization. Avoid trypsinizing cells prior to labeling with a live/dead DNA dye, like 7-AAD. Cell membranes exposed to trypsin could be transiently permeable to live/dead dyes for a variable time depending upon the cell line.

1. Culture cells in T25 flasks, culture dishes, or chamber slides and expose to the experimental or control conditions.
2. If staining cells while adherent, go to Step 4. If suspension cells are required for the final analysis:
 - a. Remove overlay media. Spin to pellet any loose cells.
 - b. Trypsinize adherent cells. Alternatively, FLICA labeling can be performed first, followed by washing and trypsinization steps.
 - c. Neutralize with trypsin inhibitor, as found in cell culture media with 20% FBS.
 - d. Add 2-5 mL media.
3. Prepare trypsinized cells for staining:
 - a. Centrifuge at 200 x g for 5-10 minutes at RT.
 - b. Remove all but ~100 μL supernatant.
 - c. Resuspend cells in 300-500 μL cell culture media containing 10-20% FBS.
 - d. If necessary, count cells and adjust the concentration and volume of cell suspension to fit the experiment.
4. Add the SR-FLICA working solution to samples at a v/v ratio of 1:30-1:60 and mix the cell suspension to disperse the SR-FLICA reagent. If staining adherent cells, add SR-FLICA directly to the overlay media. The concentration of SR-FLICA should be optimized for each cell line, experimental condition, and method of analysis:
 - Flow cytometry analysis may provide the sensitivity to detect SR-FLICA when used at 1:60.
 - For analysis by fluorescence microscopy, fluorescence plate reader, or applications where a higher staining concentration is needed, it is recommended that the SR-FLICA working solution be used at 1:30. For example, if staining cells at 1:30, add 10 μL SR-FLICA working solution to 290 μL cells, forming a final volume of 300 μL .
5. Incubate 30-60 minutes at 37°C, mixing gently every 10-20 minutes to disperse the reagent.

Figure 2: Caspases detected in Paramecium

During the process of autogamy in *Paramecium*, a new macronucleus is formed after its predecessor has been destroyed. *Paramecium* were starved for 12-24 hours in 5 mM KCl to induce autogamy. ICT's poly caspase reagent, SR-VAD-FMK (kit catalog #917), was added to the buffer with live *Paramecium* for 30 minutes in the dark. Cells were then washed and resuspended in 10 mL of buffer. Hoechst 33342 (Section 13) was added and cells were incubated in the dark for 10 minutes, washed, and applied to coverslips. Images were captured using a Nikon TE-300 Epi-fluorescent microscope with a SPOT-RT digital camera and merged using SPOT software. Data courtesy of Dr. Wade E. Bell, Virginia Military Institute.

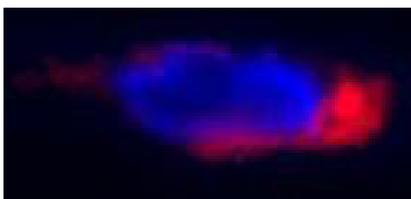
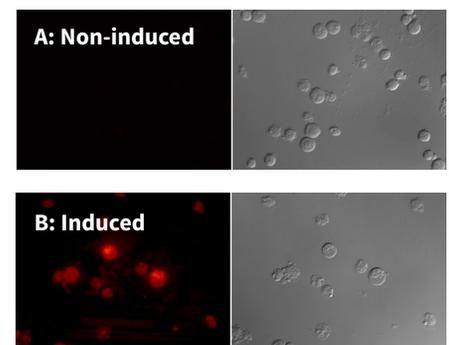


Figure 3: Microscopy analysis of Jurkat suspension cells

Jurkat cells, grown in suspension, 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 (kit 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). Data courtesy of Dr. Brian W. Lee, ICT.



6. Wash cells. For trypsinized adherent (suspension) cells, go to Step 7. For adherent cells, go to Step 8.
7. Wash trypsinized adherent (suspension) cells:
 - a. Add 2 mL 1X Apoptosis Wash Buffer and gently mix.
 - b. Centrifuge at 200 x g for 5-10 minutes at RT.
 - c. Carefully aspirate supernatant.
 - d. Resuspend samples in 1-2 mL wash buffer and gently mix.
 - e. Centrifuge a second time at 200 x g for 5-10 minutes at RT.
 - f. Carefully aspirate supernatant.
 - g. For flow cytometry analysis, two wash steps are generally sufficient. For microscopy or fluorescence plate reader analysis, repeat wash procedure a third time (resuspend samples, gently pellet by centrifugation, and carefully remove supernatants). Go to Step 9.
8. Wash adherent cells.
 - To avoid losing cells that are no longer adherent during washing, spin down all overlay media and wash buffer and recombine washed cell pellets with the adherent samples prior to analysis.
 - a. Carefully remove overlay media containing SR-FLICA and replace with 1X Apoptosis Wash Buffer.
 - b. Incubate 10 minutes at 37°C to allow any unbound SR-FLICA to diffuse out of cells.
 - c. Carefully remove and replace wash buffer with fresh wash buffer and incubate another 10 minutes at 37°C.
 - d. Gently remove overlay buffer and replace for a third wash step. Incubate 10 minutes at 37°C.
9. If desired and using a microscope, cells may be counter-stained with ancillary dyes like the nuclear stain Hoechst 33342 or other compatible fluorescent dye.
 - Gently remove overlay buffer. Go to Step 9.
 - Resuspend cells or replace overlay buffer, add Hoechst 33342 at 0.5% v/v, and incubate 5 minutes at 37°C. For example, if the cell suspension or overlay volume is at 1 mL, add 5 µL Hoechst 33342.
 - Live/dead cell stains should not be used after trypsinization. Cell membranes exposed to trypsin could be transiently permeable to live/dead dyes for a variable time depending upon the cell line. To identify dead cells with a live/dead stain, perform the FLICA and ancillary dye staining and wash steps prior to using trypsin. To avoid false positives, include another wash step to remove excess live/dead dye prior to trypsinization.
 - If using any compatible ancillary dyes, follow the manufacturer's specific instructions for staining samples.
10. Read cells within 4 hours or fix.
 - If analyzing with a fluorescence microscope, go to Section 17.
 - If using a fluorescence plate reader, go to Section 18.
 - If using a flow cytometer, go to Section 19.

17. MICROSCOPY ANALYSIS

Follow Section 15 or Section 16.

1. Resuspend cells or replace overlay media in 300-500 µL 1X Apoptosis Wash Buffer and place on ice. At this point, the cells may be stained with other dyes, fixed for future viewing (Step 2), or observed immediately (Step 3).
2. If not viewing immediately, cells may be fixed for viewing up to 16 hours later.

Figure 4: Quantification of caspase-9 activity using a fluorescence plate reader

Jurkat cells were exposed to two experimental conditions (A, left; and B, right) to compare the activity level of caspase-9 under each condition. Each condition was split in two populations and each sub-population was subsequently treated with either DMSO (negative, non-induced cells) or staurosporine (apoptotic, induced cells) for 4 hours at 37°C. All cell populations were labeled with ICT's red caspase-9 inhibitor, SR-LEHD-FMK (kit catalog #961), for 60 minutes at 37°C. Samples were washed 3X to remove any unbound SR-LEHD-FMK. Aliquots were pipetted (100 µL/well) into a black microtiter plate and read on a Molecular Devices Spectramax Gemini XPS 96-well fluorescence plate reader set at 550 nm excitation and 590 nm emission using a 570 nm cut-off filter. In the induced populations, the relative fluorescence units (RFU) of the red fluorescent signal was greater than the RFU of the non-induced populations. As caspase-9 was activated in the induced cell populations, the amount of red fluorescence increased 3.3X in the cells treated with condition A (11.0 to 36.5) and 3.3X in the cells treated with condition B (12.7 to 41.8). Both conditions had the same effect on caspase-9 activity. Data courtesy of Mrs. Tracy Murphy, ICT 8Y18.

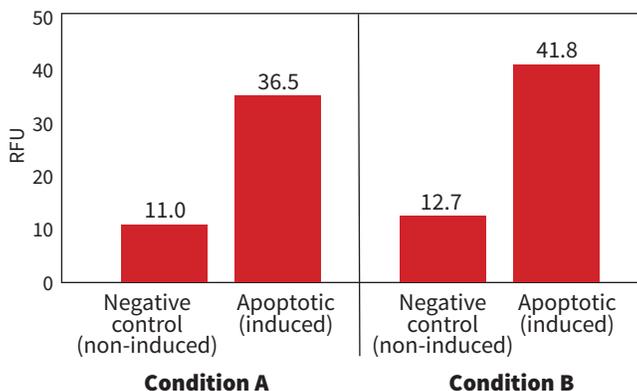
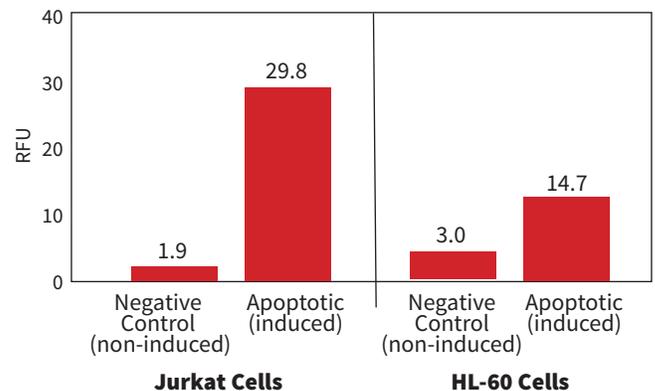


Figure 5: Comparison of caspase activity in cell lines using a fluorescence plate reader

Jurkat cells (left) and HL-60 cells (right) were treated with either DMSO (negative, non-induced cells) or staurosporine (apoptotic, induced cells) for 3 hours at 37°C. Cells were labeled with ICT's red poly-caspase inhibitor reagent, SR-VAD-FMK (catalog #917), for 60 minutes at 37°C to measure apoptosis. Samples were read on a Molecular Devices Gemini XS 96-well fluorescence plate reader set at 550 nm excitation and 595 nm emission using a 570 nm cut-off filter. In the induced populations, the relative fluorescence units (RFU) of the red fluorescent signal was greater than the RFU of the non-induced populations. As caspases became more active, indicating apoptosis, the amount of red fluorescence increased 15X in the Jurkat cells (1.9 to 29.8) but only 5X in HL-60 cells (3.0 to 14.7). Staurosporine induced caspase activity in both cell lines, but the largest effect was observed in Jurkat cells. Data courtesy of Dr. Brian W. Lee, ICT.



- a. Add Fixative at a v/v ratio of 1:5-1:10.
 - b. Incubate 15 minutes at RT in the dark.
 - c. Place cells on a microscope slide and allow to dry.
 - d. Briefly rinse cells with PBS.
 - e. Cover with mounting media and coverslip.
 - f. Store slides at 2-8°C for up to 16 hours.
3. To view cells immediately, place 1 drop of cell suspension onto a microscope slide and cover with a coverslip.
 4. Observe cells with a fluorescence microscope using a bandpass filter (excitation 550 nm, emission >580 nm) to view red fluorescence. Cells bearing active caspase enzymes covalently coupled to FLICA appear red. Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

18. FLUORESCENCE PLATE READER ANALYSIS

Follow Section 15 or Section 16, but omit optional nuclear staining with Hoechst 33342.

1. 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 $>3 \times 10^6$ cells/mL. Adherent cells should be cultured to 80-90% confluency. Please note that some cell lines will not tolerate confluency levels $>60\%$; adjust as necessary for the particular cells being used.
2. If using suspension cells, pipette 100 μ L stained and washed cells per well into a black microtiter plate. Do not use clear plates. If using a bottom-reading instrument, use a plate with black walls and a clear bottom. Analyze at least 2 aliquots per sample. Avoid bubbles.
3. Perform an endpoint read. Set the excitation wavelength at 550 nm and the emission wavelength at 595 nm; if possible, use a 570 nm cut-off filter. SR-FLICA is excited at 550-580 nm (excitation peak is 570 nm) and the emission optima is at 590-600 nm (emission peak is 590 nm).

19. FLOW CYTOMETRY ANALYSIS

Flow cytometry with SR-FLICA is best performed using a laser that can efficiently excite the fluorophore, such as a yellow-green 561 nm laser. A green 532 nm laser with a 610/20 filter pairing has also been shown to be effective. If yellow-green or green laser options are unavailable, a standard 488 nm blue excitation laser paired with an appropriate emission filter set, such as 585/40 (often FL-2/PE channel), can be used.

When using SR-FLICA in combination with other fluorochromes for bi-color analyses, compensation may be necessary. Correcting for fluorescence spill over is especially important when using a sub-optimal excitation laser to excite SR-FLICA in combination with a fluorochrome with significant spectral overlap with the SR-FLICA detector. For instance, some green dyes, like FITC or Green Live/Dead Stain (catalog #6342), can spill over from FL-1 into FL-2. If using such a dye to co-label SR-FLICA stained cells, consider reading the SR-FLICA reagent in an alternate channel with reduced spectral overlap, such as 670LP (often FL-3/PerCP), or similar.

Because of its orangy-red profile, SR-FLICA can be measured in flow cytometry with the red live/dead stain, 7-AAD (catalog #6163), to detect necrosis concurrently (Figure 7). Careful compensation and gating of SR-FLICA and 7-AAD fluorophores (using the FL-2 and FL-3 channels, respectively) will distinguish the red 7-AAD live/dead fluorescence signal from the orangy-red SR-FLICA caspase positive signal within each sample. This will reveal four populations of cells:

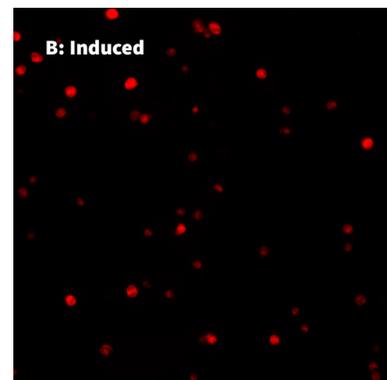
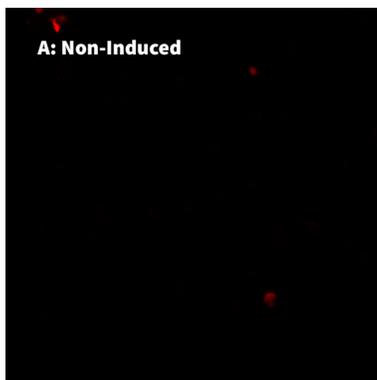
- Live unstained cells display only normal background or autofluorescence (revealed in lower left quadrant).
- Early apoptotic cells fluoresce orangy-red with SR-FLICA (revealed in lower right quadrant).
- Late apoptotic cells are dually stained with SR-FLICA and 7-AAD: they fluoresce orangy-red (they have active caspases) and red (the cell membrane has permeabilized). This population will be revealed in the upper right quadrant.
- Necrotic cells fluoresce red with 7-AAD (revealed in the upper left quadrant).

Follow Section 15 or Section 16, but omit optional nuclear staining with Hoechst 33342.

1. Resuspend cells in 300 μ L 1X Apoptosis Wash Buffer and place on ice.
2. Cells may be fixed for analysis up to 16 hours later. Add Fixative at a v/v ratio of 1:5-1:10. Store samples at 2-8°C and protected from light.
3. Run the unstained control. If possible, adjust voltages to place the unstained sample in the first decade of the FL dot plots.
4. For single-color analysis, a 488 nm blue argon laser or comparable can be used with the emission filter pairing that best approximates 585/40 (often FL-2/PE channel). If other options are available, use the laser/filter pairing that most closely resembles the excitation and emission optima of sulforhodamine B, i.e. 570 nm and 590-600 nm, respectively.
5. Generate a histogram with the log FL-2 on the X-axis versus the number of cells on the Y-axis. Caspase negative (SR-FLICA -) cells will fall within the lower log fluorescence output decades of the FL-2 X-axis, whereas caspase-positive (SR-FLICA +) cells will appear as a shoulder or as a separate peak on the right side of the negative peak histogram.

Figure 6: Microscopy analysis of caspase-8 activity in Jurkat cells

Jurkat cells were grown in suspension to 4×10^5 cells/mL, then divided into two separate TC-flasks. One population, “Non-Induced”, received a DMSO vehicle control (A). The other population, “Induced”, was spiked with 1 μ M staurosporine (B). Cells were incubated for 4 hours at 37°C, then stained with ICT’s red SR-FLICA caspase-8 inhibitor, SR-LETD-FMK (kit catalog #9150) for 1 hour at 37°C. After labeling, samples were washed three times and slides were prepared. Fluorescence images were acquired using a Nikon Eclipse 90i microscope equipped with a Hamamatsu Flash 4.0 camera. In the treated sample, cells appear bright red, indicating a high level of caspase-8 activity (B, Induced, right). In the non-induced sample, few red positive cells are visible, indicating minimal caspase-8 activity (A, Non-Induced, left). Data courtesy of Mrs. Tracy Murphy, ICT (220:68, 121815).



6. For bi-color analyses, run each single color control. Adjust compensation to remove spectral overlap from interfering FL channels. When the data have been correctly compensated, the median fluorescence intensity (MFI) values in non-primary detectors of any given single-stained control sample should be the same as an unstained control sample (e.g. an SR-FLICA stained sample being read in FL-2 should have the same MFI in FL-3 as an unstained sample). For example, if reading SR-FLICA in FL-2 and 7-AAD in FL-3:
 - a. Subtract a percentage of the fluorescence in the SR-FLICA channel from the fluorescent channel used for 7-AAD (e.g. FL-3 - %FL-2).
 - b. Subtract a percentage of the fluorescence in the channel used for 7-AAD from the fluorescence in the SR-FLICA channel (e.g. FL-2 - %FL-3).
7. Run the bi-color experimental samples and analyze.

Figure 7: Apoptosis vs necrosis: bi-color staining of K562 cells

ICT's poly-caspase SR-FLICA reagent, SR-VAD-FMK (catalog #917), was used with 7-AAD (catalog #6163), a red live/dead stain, to simultaneously assess apoptosis and necrosis in K-562 human erythroleukemia cells. Cells were grown to 3×10^6 cells per sample and treated with a condition which induced apoptosis. Cells were stained with SR-VAD-FMK, washed, stained with 7-AAD, and analyzed using bi-color flow cytometry. A dot plot was set up to detect caspase activity (orangy-red, FL-2) on the X-axis and necrosis (red, FL-3) on the Y-axis, and carefully gated to distinguish SR-FLICA and 7-AAD fluorophores within a single sample tube. Four populations of cells were detected: unstained live cells have minimal fluorescence (67%, lower left); cells in early apoptosis fluoresce orangy-red with SR-FLICA (8%; lower right); cells in late apoptosis are dually stained with SR-FLICA and 7-AAD: they fluoresce orangy-red (they have active caspases) and red (the cell membrane has permeabilized) (23%; upper right); and necrotic cells fluoresce red with 7-AAD (2%; upper left). Data courtesy of Dr. Michael Olin, University of Minnesota.

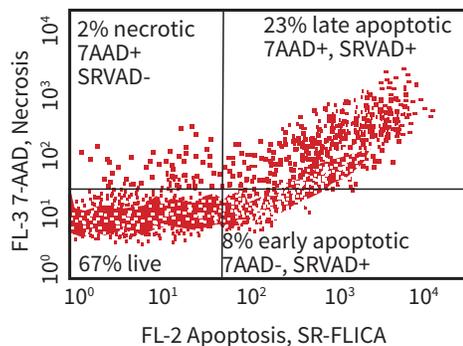


Figure 8: Positive vs. negative cells in FL-2 vs. FL-3

ICT's SR-FLICA poly-caspase inhibitor reagent, SR-VAD-FMK (catalog #917), along with 7-AAD, a red fluorescing live/dead stain (catalog # 6163) were used to simultaneously assess apoptosis and necrosis in K-562 human erythroleukemia cells. Cells were grown to 3×10^6 cells per sample and treated with staurosporine (catalog #6212) to induce apoptosis and potentially late apoptosis-associated necrosis, or a control (DMSO) for 4 hours. Cells were stained with SR-VAD-FMK for 1 hour at 37°C, washed in 1X Apoptosis Wash Buffer (3X), and stained with 7-AAD (10 minutes in an ice bath) and analyzed on a flow cytometer previously compensated for respective FL-2 and FL-3 emissions spillover. A dot plot was set up to detect caspase activity (orangy-red fluorescence, FL-2) on the X-axis and necrosis (red fluorescence, FL-3) on the Y-axis. Careful gating of SR-FLICA and other red fluorophores, such as 7-AAD, using the FL-2 and FL-3 channels respectively, distinguishes the red fluorescence signal (7-AAD positive necrotic cells) from the orangy-red fluorescence signal (SR-FLICA caspase positive cells) within a single sample tube. Apoptotic cells fluoresce orangy-red with SR-FLICA and shift to the right on the X-axis. The 4 hour treatment did not significantly increase necrosis in the K562 cell population but it did increase apoptosis. Data courtesy of Dr. Michael Olin, University of Minnesota.

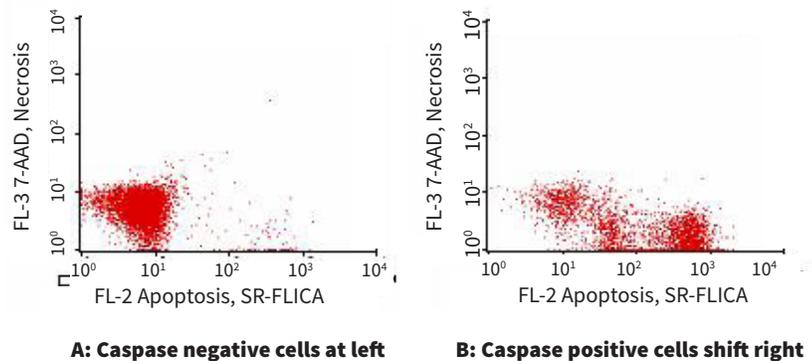


Figure 9: Dual staining of SR-DEVD-FMK (red) and Aβ-FITC (green) in macrophages from Alzheimer's patients

Alzheimer's disease (AD) patients exhibit plaques of amyloid beta (Aβ) in the brain. In healthy people, Aβ is phagocytosed and cleared from the brain by macrophages. It is proposed that AD may be caused by a malfunction of the macrophage, which prevents them from properly clearing Aβ. Curcumin, a substance found in tumeric, is thought to enhance the function of macrophages. SR-FLICA was used to test this theory in AD macrophages. Untreated control AD macrophages and curcumin-treated AD macrophages were exposed to FITC-labeled Aβ and stained with ICT's red SR-DEVD-FMK Caspase-3/7 SR-FLICA kit (#932). Macrophages that engulf FITC-Aβ will fluoresce green (right), and caspase-positive cells undergoing apoptosis will fluoresce red (left).

In this experiment, essentially all of the untreated AD macrophages engulfed FITC-Aβ (green, top right) and became apoptotic (red, top left). They are dually stained green and red. Curcumin-treated AD macrophages engulfed FITC-Aβ (green, bottom right) but did not become apoptotic, as evidenced by the lack of red fluorescence (bottom left). It appears that in some AD patients, macrophages engulf Aβ but undergo apoptosis before Aβ is cleared from the brain. Curcumin may protect macrophages from apoptosis; more studies are needed to validate this conclusion. Data courtesy of Dr. Milan Fiala, UCLA 072205.

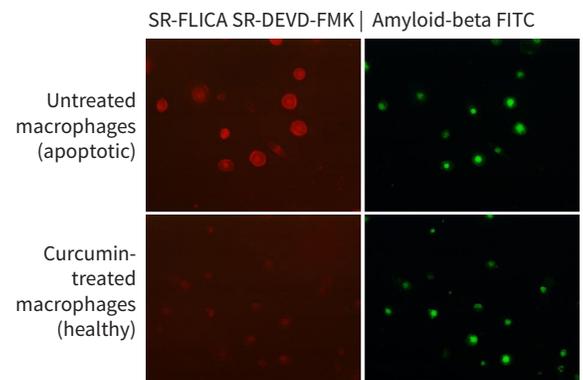
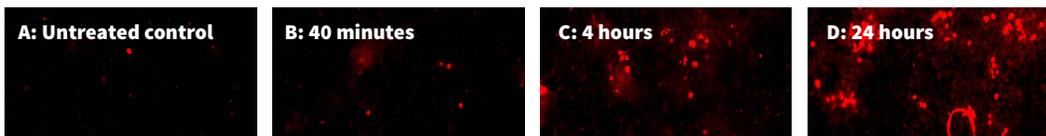


Figure 10: Apoptosis in adherent rat brain astrocytes

Frozen rat brain astrocytes were revived and grown in culture. They were treated with (A) a control or (B) kainic acid for 40 minutes, (C) 4 hours, and (D) 24 hours. Cells were then labeled with ICT's red poly-caspase SR-FLICA inhibitor, SR-VAD-FMK (catalog #917), for 1 hour to detect apoptosis. Rat brain cortical cells show increasing levels of apoptosis with increased exposure to kainic acid compared with the untreated control cells (A) which exhibit little caspase activity. Data courtesy of Dr. Babben Tinner-Staines, QBM Cell Sciences¹¹.



20. REFERENCES

1. Amstad, P. A. et al. Detection of caspase activation in situ by fluorochrome-labeled caspase inhibitors. *Biotechniques* 31, 608-610, 612, 614, passim (2001).
2. Bedner, E., Smolewski, P., Amstad, P. & Darzynkiewicz, Z. Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA): correlation with DNA fragmentation. *Exp Cell Res* 259, 308-313, doi:10.1006/excr.2000.4955 (2000).
3. Slee, E. A., Adrain, C. & Martin, S. J. Serial killers: ordering caspase activation events in apoptosis. *Cell Death Differ* 6, 1067-1074, doi:10.1038/sj.cdd.4400601 (1999).
4. Shamaa, O. R., Mitra, S., Gavrilin, M. A. & Wewers, M. D. Monocyte Caspase-1 Is Released in a Stable, Active High Molecular Weight Complex Distinct from the Unstable Cell Lysate-Activated Caspase-1. *PLoS One* 10, e0142203, doi:10.1371/journal.pone.0142203 (2015).
5. Mariathasan, S. et al. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430, 213-218, doi:10.1038/nature02664 (2004).
6. Kumar, S. Mechanisms mediating caspase activation in cell death. *Cell Death Differ* 6, 1060-1066, doi:10.1038/sj.cdd.4400600 (1999).
7. Rotonda, J. et al. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. *Nat Struct Biol* 3, 619-625 (1996).
8. Walker, N. P. et al. Crystal structure of the cysteine protease interleukin-1 beta-converting enzyme: a (p20/p10)₂ homodimer. *Cell* 78, 343-352 (1994).
9. Wilson, K. P. et al. Structure and mechanism of interleukin-1 beta converting enzyme. *Nature* 370, 270-275, doi:10.1038/370270a0 (1994).
10. 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, 17907-17911 (1997).
11. Tinner-Staines, B., et al. Using Cryopreserved Neuronal Cells for Multi-well Neurotoxicity Assays. Presentation number 922.4 from the Society for Neuroscience 34th Annual Meeting (2004).

Thank you for using SR-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 email to help@immunochemistry.com.