FAM-FLICA® Caspase Assays

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

1. INTRODUCTION

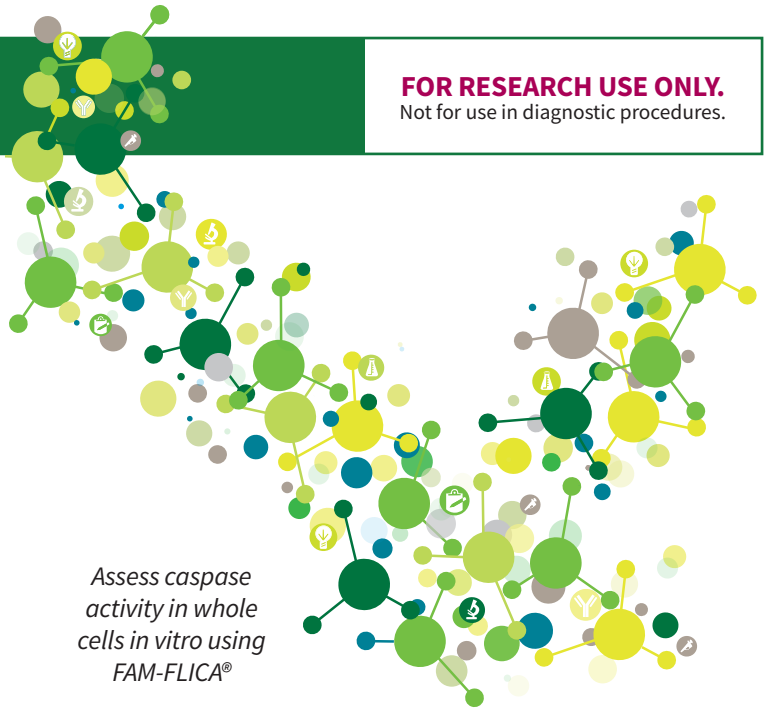
FLICA® is a powerful method to assess cell death by detecting apoptosis *in vitro*. ImmunoChemistry Technologies' (ICT) FLICA probes are non-cytotoxic **F**luorescent **L**abeled **I**nhibitors of **CA**spases that covalently bind to active caspase enzymes[1,2]. FLICA measures the intracellular process 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. FLICA can also be used to measure pyroptosis, a highly inflammatory form of programmed cell death.

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. Unbound FLICA will diffuse out of the cell during the wash steps. Apoptotic and pyroptotic cells will retain a higher concentration of FLICA and fluoresce brighter than healthy cells. There is no interference from pro-caspases or inactive forms of the enzymes. If the treatment is causing cell death via apoptosis and/or pyroptosis, the cells will contain an elevated level of caspase activity relative to 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[3]. 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 pyroptosis and inflammasome activity and takes on the role of a key housekeeping enzyme in its conversion of pro-IL-1ß protein into the active IL-1ß cytokine. (Use FLICA kits #98, #9122, and #9162 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 specificity[6]. 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[7-9].

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 end[10]. 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 green fluorescent label, carboxyfluorescein (FAM), and a fluoromethyl ketone (FMK).



Assess caspase activity in whole cells in vitro using FAM-FLICA®

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. ICT's poly caspase FLICA probe, FAM-VAD-FMK, can be used as a general reagent to detect apoptosis as it is recognized by many types of activated caspases. To more specifically target a particular caspase enzyme, use one of ICT's specialized FLICA reagents. ICT has kits for the detection of: caspase-1 (YVAD or WEHD) (also recognizes caspases 4 and 5), -2 (VDVAD), -3/7 (DEVD), -6 (VEID), -8 (LETD), -9 (LEHD), and -10 (AEVD). FLICA kits are also available with a red or far red fluorescent label. 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 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.

Cells labeled with FAM-FLICA can be counter-stained with reagents such as the red live/dead stains Propidium Iodide (included in FAM-FLICA kits) and 7-AAD (catalog # 6163) 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 1-3,7, and 9-10), or the fluorescence intensity can be quantified using a flow cytometer (Figures 4-6) or fluorescence plate reader (Figure 8). FAM-FLICA optimally excites at 488-492 nm and has a peak emission at 515-535 nm.

2. FAM-FLICA KITS:

Caspase	Inhibitor Reagent (part#)	Kit Catalog # Trial	Standard
Poly Caspase	FAM-VAD-FMK (637)	91	92
Caspase-1, 4, & 5	FAM-YVAD-FMK (655)	97	98
Caspase-1, 4, & 5	FAM-WEHD-FMK (6708)	9161	9162
Caspase-2	FAM-VDVAD-FMK (680)	918	919
Caspase-3/7	FAM-DEVD-FMK (653)	93	94
Caspase-6	FAM-VEID-FMK (654)	95	96
Caspase-8	FAM-LETD-FMK (656)	99	910
Caspase-9	FAM-LEHD-FMK (677)	912	913
Caspase-10	FAM-AEVD-FMK (682)	922	923

3. KIT CONTENTS

Trial size kits contain:

- 1 vial of FAM-FLICA caspase inhibitor reagent
- 1 bottle of 10X Apoptosis Wash Buffer (15 mL), #635
- 1 bottle of Fixative (6 mL), #636
- 1 vial of Propidium Iodide, 250 µg/mL (1 mL), #638
- 1 vial of Hoechst 33342, 200 µg/mL (1 mL), #639

Standard size kits contain:

- 4 vials of FAM-FLICA caspase inhibitor reagent
- 1 bottle of 10X Apoptosis Wash Buffer (60 mL), #634
- 1 bottle of Fixative (6 mL), #636
- 1 vial of Propidium Iodide, 250 µg/mL (1 mL), #638
- 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 immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

6. RECOMMENDED MATERIALS

- DMSO, 50 µL per vial to reconstitute FLICA
- DiH_20, 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 caspase activity and create controls, such as staurosporine (catalog #6212), camptothecin (catalog #6210), or nigericin (catalog #6698)
- 90% EtOH or 3% formaldehyde to create live/dead controls for Propidium Iodide staining
- 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.
- Hemocytometer
- 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:

- FAM-FLICA optimally excites at 488-492 nm and has a peak emission at 515-535 nm.
- View Propidium Iodide (PI) under a long pass filter with the excitation at 488-492 nm, emission >610 nm; nuclei-bound PI has a maximum emission at 617 nm (Section 14).
- Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm (Section 15).

8. EXPERIMENTAL PREPARATION

Staining 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 caspase activation process. Create cell populations, such as:

a. Cells that were exposed to the experimental treatment
b. A negative control population of cells that received a placebo treatment

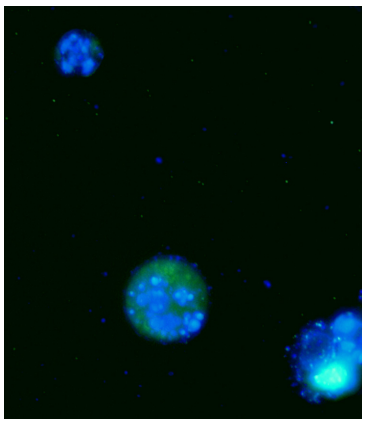
As FLICA detects the presence of catalytically active forms of caspase enzymes, plan the experiment so that FLICA will be diluted and administered at the time when caspases are expected to be activated in the cells.

The recommended volume of 30X FLICA is 10 µL per 300 µL of cells at 3-5 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 experimental conditions 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.

FIGURE 1: MICROSCOPY ANALYSIS OF THP-1 SUSPENSION CELLS

Human monocytic leukemia THP-1 cells were dually stained with ICT's green FAM-FLICA poly caspase inhibitor reagent, FAM-VAD-FMK (kit #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 right cell indicate it is alive (not apoptotic). The upper left cell is necrotic (no green, scattered blue). Data courtesy of Dr. Brian W. Lee, ICT.



9. CONTROLS

Create experimental samples and control cell populations:

a. Treated experimental population(s): cells exposed to the experimental condition(s).

b. Negative control: non-treated cells grown in a normal cell culture environment.

c. Positive control: cells induced to activate caspases using a known caspase activation 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 8 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 using FAM-FLICA with Propidium Iodide (PI) vital dye for dual staining, create PI instrument controls using formaldehyde or EtOH (Section 14) to compensate for bleed-over of the PI signal into the FAM-FLICA channel (Section 20).

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 or 1-2 µM staurosporine for >4 hours.

11. PYROPTOSIS INDUCTION

The optimal pyroptosis/caspase-1 induction protocol will vary significantly among cell lines. Determine a reproducible method for obtaining a caspase-1 positive control prior to commencing the experiment. For example, caspase-1 activation may be induced in THP-1 cells using 5-10 ng/mL Phorbol myristate acetate (PMA) in cell culture media for 12-24 hours (until cells become adherent), followed by exposure to 100 ng/mL Lipopolysaccharide (LPS) and 5 mM Adenosine triphosphate (ATP) for 24 hours.

Alternatively, pyroptosis may be induced in cells using Nigericin in cell culture media at 1-20 µM for 2-24 hours at 37°C. Each investigator should adjust the concentration of Nigericin and treatment period to accommodate the particular cell line and research conditions.

12. 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 to yellow or orange. 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.

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13. PREPARATION OF 1X APOPTOSIS WASH BUFFER

ICT's 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 to wash cells instead of Apoptosis Wash Buffer.

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 diH_2O. For example, add 15 mL 10X Apoptosis Wash Buffer to 135 mL diH_2O 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.

FIGURE 2: CASPASE ACTIVITY IN JURKAT CELLS

Jurkat cells (T lymphocytes) were labeled with ICT's green FAM-FLICA poly caspase inhibitor FAM-VAD-FMK (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.

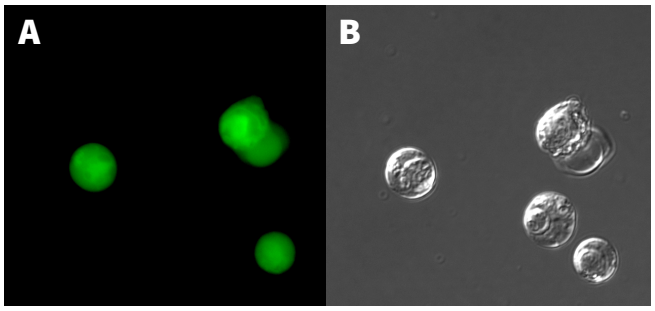
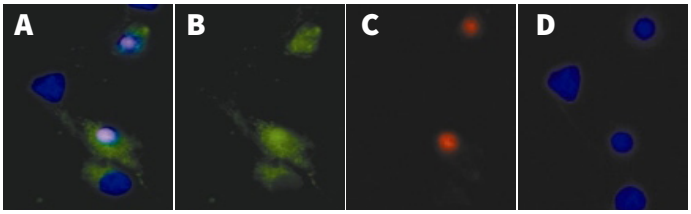


FIGURE 3: CELL DEATH IN PRIMARY RAT HIPPOCAMPAL NEURONS

ICT's FAM-FLICA caspase-3/7 inhibitor reagent, FAM-DEVD-FMK (kit #94), was used to assess cell death in primary rat hippocampal neurons. Subjects were first-generation descendants of Sprague–Dawley albino rats. Hippocampi from postnatal day 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. Image A is a composite of FLICA (B), PI (C), and Hoechst (D). In A, four cells are revealed by labeling DNA with Hoechst (blue, D), three out of the four cells are caspase-positive (green, B), and two of those cells are also membrane-compromised (red, C). Three of the cells fluoresce green with FLICA FAM-DEVD-FMK (B); they are caspase-positive and undergoing apoptosis. One of the cells is in early apoptosis, as it is green but not red. Two of the FLICA-positive cells are also PI-positive (red, C) meaning they are becoming membrane compromised and are in the late stages of apoptosis rather than necrosis. Data courtesy of Dr. Z. Kahraman Akozer, University of Maryland.



14. PROPIDIUM IODIDE

Propidium Iodide (PI, 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 stoichiometrically binds every four to five base pairs of DNA. PI is membrane impermeant, which prevents it from reaching the DNA in viable cells, thus allowing the identification of dead cells in a population.

Upon binding to DNA, the fluorescence intensity potential of PI is enhanced 20-30 fold. Nucleic acid-bound PI has a red-shifted absorbance/excitation maximum of 535 nm and an emission maximum of 617 nm. PI efficiently excites at 488-492 nm. Its excitation and emission spectra allow for efficient analysis using fluorescence microscopy or flow cytometry.

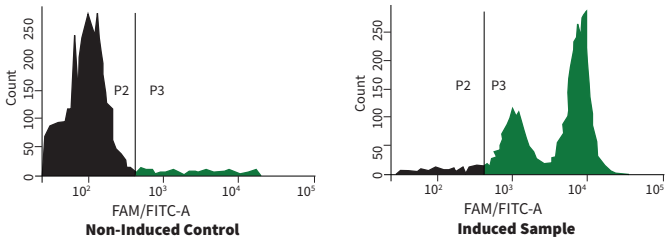
- PI contains a low concentration of Phenanthridinium, 3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6-phenyl-, diiodide (CAS 25535-16-4) which is below the threshold for reporting. PI is a suspected mutagen at high concentrations. Prolonged skin contact may cause redness and irritation. Because of the small quantity of product, the health hazard is small. See SDS for further information.

PI is included in a soluble, ready-to-use formulation (1 mL at 250 µg/mL). If analyzing cells by flow cytometry, PI can be used with FAM-FLICA to identify four populations of cells: living; early apoptotic; late apoptotic; and necrotic (Figures 3, 5, and 6). If analyzing PI-labeled cells using flow cytometry (Section 20), create PI instrument controls using formaldehyde or EtOH to kill cells. An apoptosis-induction method (Section 10) is not as effective as a solvent treatment at creating PI-positive cell controls for instrument calibration because apoptosis-induced samples may not have enough late-stage apoptotic cells that have become membrane-compromised to stain positive for PI. To set up PI instrument controls:

1. Label 2 centrifuge tubes:
   a. PI-negative (live)
   b. PI-positive (dead)
2. Add 1-5 x 10^5 non-induced, healthy cells to each tube.
3. Centrifuge at 200 x g for 5-10 minutes at room temperature (RT) to pellet cells; remove supernatants.
4. To create the PI-negative control (live cells), resuspend the cells in 300 µL 1X AWB or PBS+1% BSA to maintain the integrity of the cell membrane.
5. To create the PI-positive control (membrane-compromised dying cells):
   - Use formaldehyde: resuspend cells in 300 µL 3% v/v formaldehyde (in 97% PBS or 1X AWB). Incubate 30 minutes on ice and then wash cells (Steps 6-8).
   - Or use EtOH: resuspend cells in 300 µL 90% EtOH (in 10% PBS or 1X AWB). Gently vortex for 30 seconds then wash cells (Steps 6-8).
6. Add 1 mL 1X AWB or PBS+1% BSA.
7. Centrifuge at 200 x g for 5-10 minutes; remove supernatants.
8. Resuspend in 500 µL 1X AWB or PBS+1% BSA.
9. If EtOH was used, add 500 µL non-induced, healthy cells to the tube of EtOH-killed cells and mix; use roughly the same number of cells as originally included in Step 2. This will create a sample with distinct positive and negative peaks.
10. Add PI at 0.5% v/v. For example, add 2.5 µL PI to a 500 µL sample. Incubate 5 minutes, protected from light at RT.
11. Read immediately on the flow cytometer (Section 20) and use to compensate bleed over of the PI signal into the FAM-FLICA channel.

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. The negative control exhibited caspase activity in only 3.3% of the cell population (P3, left histogram), whereas treatment with staurosporine induced caspase activity in 94.8% of the experimental cells (P3, right histogram). This is a ratio of 28:1. Data courtesy of Mrs. Tracy Murphy, ICT, 10G5.



% of Cells	P2 Negative	P3 Positive
Negative, Non-Induced (left)	87.5%	3.3%
Positive, Induced (right)	4.8%	94.8%
Ratio		28:1

15. 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 µg/mL. Hoechst 33342 can be used with FAM-FLICA and PI to label the nuclei of live, dying, and apoptotic cells (Figures 1 and 7).

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.

- Hoechst 33342 contains a low concentration of Bis benzimide H 33342 trihydrochloride (CAS 23491-52-3) which is below the threshold for reporting. Hoechst 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.

16. 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°C for up to 16 hours, protected from light.

ICT's Fixative will not interfere with the carboxyfluorescein (FAM) label. Do not use absolute ethanol- or methanol-based fixatives as they will inactivate the FAM-FLICA label. Do not fix cells that will be stained later with Propidium Iodide.

- **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.

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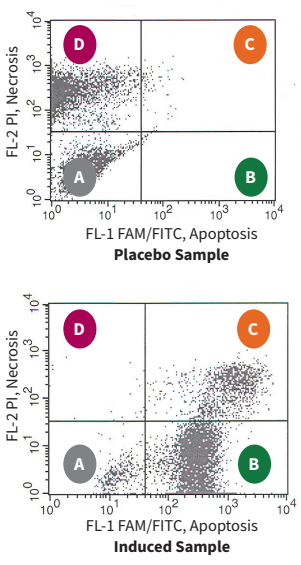
17. STAINING PROTOCOL FOR SUSPENSION CELLS

Prepare experimental and control cell populations. Ideally, cell concentration should be 3-5 x 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 x 10^6 cells/mL as both microscopy and plate reader analysis methods (Sections 19 and 21) 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 per sample 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
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 analyze samples at 3-5 x 10^5 cells/mL.
3. Transfer 290 µL cells into fresh tubes.
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 gently 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 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. Add Hoechst at 0.5% v/v and incubate 10-20 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 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 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 19.
    - If using a flow cytometer, go to Section 20.
    - If using a fluorescence plate reader, go to Section 21.

FIGURE 5: BICOLOR STAINING OF RABBIT CELLS



ICT's poly caspase inhibitor FLICA reagent, FAM-VAD-FMK (kit #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 (top), or a condition that induced apoptosis (bottom). Cells were stained with FAM-VAD-FMK, washed, stained with the red live/dead stain Propidium Iodide (PI), 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. Four populations of cells were detected: (A) unstained live cells; (B) 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 (active caspases) and red (the cell membrane has been permeabilized); and (D) necrotic cells fluoresce red. Cells became necrotic when treated with the placebo (top, D) but not apoptotic (top, B and C), while many cells entered early and late apoptosis when treated with the inducer (bottom, B and C). Compare data with Figure 6. Data courtesy of JH at HMC.

18. STAINING PROTOCOL FOR ADHERENT CELLS

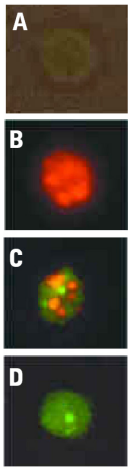
Adherent cells need to be handled carefully to avoid the loss of any cells that round up and come off the culture surface. In microscopy or plate reader applications where trypsinization is not required, adherent cells can be stained and washed directly on the chamber slide, well, or culture surface. 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 stain, like PI or 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 tissue culture flasks, 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:

FIGURE 6: FOUR POPULATIONS OF HL-60 CELLS

HL-60 cells (human promyelocytic leukemia) were treated with a drug, then stained with ICT's poly caspase inhibitor reagent, FLICA FAM-VAD-FMK (kit #92), and a red live/dead stain, Propidium Iodide (PI). Cells were analyzed on a scanning laser cytometer to detect red on the X-axis (necrosis) and green on the Y-axis (apoptosis). Four populations of cells were detected: (A) unstained live cells; (B) necrotic cells fluoresce red; (C) cells in late apoptosis are dually stained with FAM-FLICA and PI: they fluoresce green (active caspases) and red (the cell membrane is compromised); and (D) cells in early apoptosis fluoresce green with FAM-FLICA. In this experiment, the drug triggered the caspase cascade rather than being overtly toxic to the cells. Compare data with Figure 5. Data courtesy of Dr. Zbigniew Darzynkiewicz, Brander Cancer Center, NY.

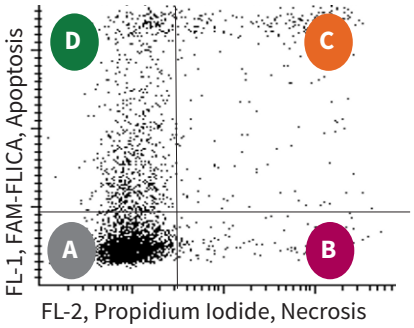


A: Live FLICA- PI-

B: Necrotic FLICA- PI+

C: Late apoptotic FLICA+ PI+

D: Early apoptotic FLICA+ PI-



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 30X FAM-FLICA working solution to samples at a v/v ratio of 1:30 and mix the cell suspension to disperse the FAM-FLICA reagent. If staining adherent cells, add FAM-FLICA directly to the overlay media. The concentration of FAM-FLICA should be optimized for each cell line, experimental condition, and method of analysis.
5. Incubate the cells for 30-60 minutes at 37°C, mixing gently every 10-20 minutes to disperse the reagent.
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 FAM-FLICA and replace with 1X Apoptosis Wash Buffer.
   b. Incubate 10 minutes at 37°C to allow any unbound FAM-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 wash buffer and replace for a third wash step. Incubate 10 minutes at 37°C.
   e. Gently remove overlay wash buffer. Go to Step 9.
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.
   - Resuspend cells or replace overlay wash buffer, add Hoechst 33342 at 0.5% v/v, and incubate 10-20 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 19.
    - If using a flow cytometer, go to Section 20.
    - If using a fluorescence plate reader, go to Section 21.

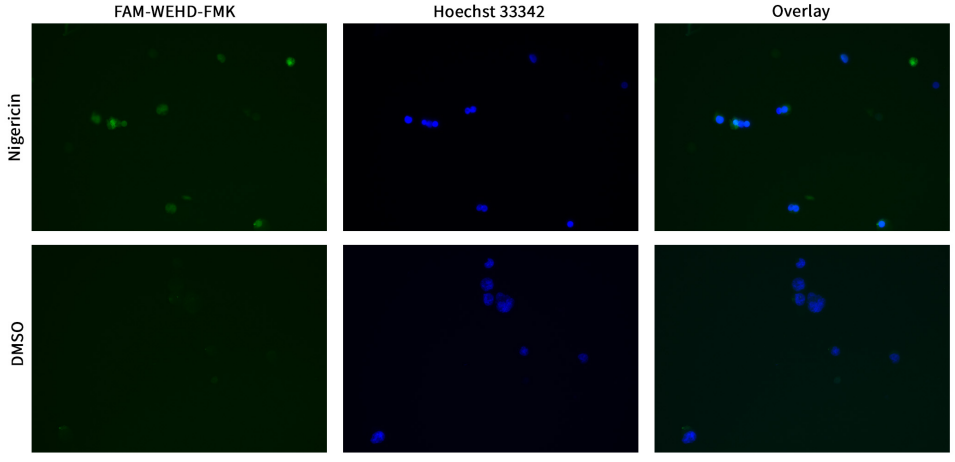
19. MICROSCOPY ANALYSIS

Follow Section 17 or 18.

1. Resuspend cells or replace overlay wash buffer 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 2), fixed for future viewing (Step 3), or observed immediately (Step 4).
2. 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.

FIGURE 7: CASPASE-1 ACTIVITY DETECTED IN THP-1 MONOCYTES USING FAM-WEHD-FMK

ICT's FAM-FLICA Caspase-1 (WEHD) kit (#9162) was used to detect pyroptosis in THP-1 monocytes. To create a common pool of cells labeled with FAM-WEHD-FMK, 125 µL of 30X FAM-FLICA stock was added to 3.625 mL of cells. Monocytes are capable of secreting active caspase-1[4,5], therefore cells were labeled with FAM-FLICA prior to treatment to ensure FAM-FLICA was present when caspase-1 activation began. After labeling with FLICA, cells were treated with a negative control (DMSO), or 10 µM nigericin (#6698) for 2 hours to induce pyroptosis. Following treatment, cells were washed 3 times with 1X Apoptosis Wash Buffer, and then were resuspended in 0.45 mL 1X Apoptosis Wash Buffer. Cells were then stained with Hoechst 33342, fixed (using ICT's Fixative, #636), and viewed 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. Increased levels of green FAM-FLICA staining (leftmost images) were seen in the nigericin-treated cells (upper row of images) compared to DMSO mock-treated cells (lower row of images). Hoechst 33342 staining (middle images) and overlays of both the EGFP and DAPI channels (rightmost images) are also shown. Data courtesy of Dr. Kristi Strandberg, ICT (235:60).



a. Wash cells to remove excess PI from the media. Centrifuge at 200 x g for 5-10 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 3 or 4.

3. If not viewing immediately, cells may be fixed for viewing up to 16 hours later.
   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 onto a microscope slide and allow to dry.
   d. Briefly wash cells with PBS.
   e. Cover cells with mounting media and coverslip.
   f. Store slides at 2-8°C for up to 16 hours.
4. To view cells immediately, place 1 drop of cell suspension onto a microscope slide and cover with a coverslip.
5. 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 (Figures 1-3, 6-7, and 9-10). Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm (Figures 1, 3, and 7). View nucleic-acid-bound PI under a long pass filter with the excitation at 490 nm, emission >610 nm.; Nuclei-bound PI has a maximum emission at 617 nm (Figures 3 and 6).

20. FLOW CYTOMETRY ANALYSIS

Follow Section 17 or 18, but omit optional nuclear staining with Hoechst 33342.

1. Resuspend cells in 300 µL 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. Generate a FSC vs SSC dot or density plot and gate on the population of interest. Adjust the voltages, if necessary, so that the cell population is easily distinguished.
4. For single-color analysis of FAM-FLICA, a 488 nm blue argon laser or comparable can be used with the emission filter pairing that best approximates 530/30 (often FL-1/FITC channel).
5. Run single color controls. Generate a histogram with the log FL channel on the X-axis versus the number of cells on the Y-axis. Caspase negative (FLICA-) cells will fall within the lower log fluorescence output decades of the 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). Adjust the voltage on the fluorescence channel, if necessary, to ensure fluorescence is on scale and caspase positive and negative populations are distinguished.
6. For dual-color analyses, run each single-color control. Adjust compensation to remove spectral overlap from interfering FL channels.
7. Run experimental samples and analyze.

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21. FLUORESCENCE PLATE READER ANALYSIS

Follow Section 17 or 18, 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 x 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 µL 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 488 nm and the emission wavelength at 530 nm; if possible, use a 515 nm cut-off filter. FAM-FLICA excites at 488-492 nm and emits at 515-535 nm.

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 (kit #92), for 60 minutes at 37°C. Samples were read on a Molecular Devices Gemini XS 96-well fluorescence plate reader set at 490 nm excitation and 520 nm emission using a 495 nm cut-off filter. 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 (34.11 vs. 6.35). Staurosporine induced poly caspase activity in Jurkat cells. Data courtesy of Dr. Brian Lee, ICT.

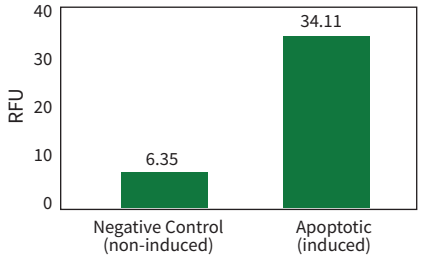


FIGURE 9: ADHERENT CORNEAL FIBROBLASTS

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 (kit #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, University of California, Irvine.

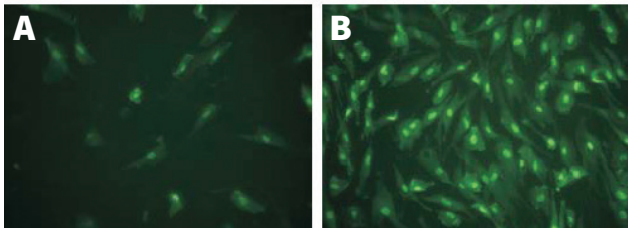
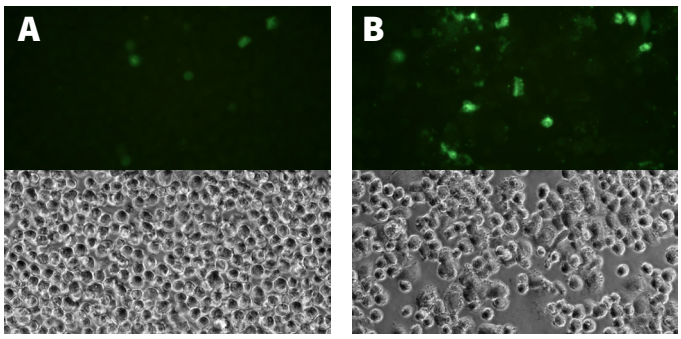


FIGURE 10: MICROSCOPY ANALYSIS OF CASPASE-1 ACTIVITY

THP-1 cells were treated with either a negative control (Non-Induced, A), or PMA at 5 ng/mL to induce differentiation into macrophages (Induced, B). After 48 hours, PMA was removed from the induced population and replaced with fresh medium containing LPS at 10 ng/mL to induce caspase-1 activation. After 2 hours, cells were stained FAM-YVAD-FMK (kit #98) for 1 hour, washed, and examined under an Olympus IX-70 inverted photomicroscope equipped with phase contrast and fluorescence optics. In the treated sample, many cells appear bright green, indicating an increased level of caspase-1 activity (B, Induced, right). In the non-induced sample, few green cells are visible, indicating a low level of caspase-1 activity (A, Non-Induced, left). Data courtesy of Dr. Brian Lee, ICT (207:11).



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